



Mechanisms of Spontaneous Melanoma Regression in MeLiM Pigs

Florian Rambow

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Florian RAMBOW

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Mechanisms of Spontaneous Melanoma Regression in MeLiM Pigs

Thesis Director: P. CHARDON

Jury:	Referee:	Prof Dr M. Groenen
	Referee:	Dr A. Prévost-Blondel
	Examiner:	Prof Dr B. Mignotte
	Examiner:	Prof Dr F. Velten
	Examiner:	Dr P. Chardon



**"It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change."
(Charles Darwin)**

Dedicated to my parents

Abstract

Cutaneous melanoma, the most aggressive form of skin cancer, is a tumor originating from melanocytes with rapidly increasing incidence. Patients with advanced disease have a poor prognosis since melanoma is mostly resistant to current therapies. Therefore, the development of novel strategies for preventing and treating melanoma is important. To explore novel therapies we need to find appropriate targets and for that knowledge about the biology of melanoma is important. One elegant way to do so, is to study the natural but rare phenomenon of spontaneous melanoma regression. Unfortunately, complete spontaneous regression of advanced melanoma is extremely rare, and therefore hard to investigate.

The Melanoblastoma-bearing Libechov Minipig (MeLiM) is a great opportunity to study spontaneous tumor regression. MeLiM pigs, suffering from hereditary melanoma, develop tumors naturally and regress them completely without external influence. Mechanisms leading to spontaneous melanoma regression are poorly understood at present.

Therefore the overall objective of this PhD was to investigate mechanisms of spontaneous melanoma regression in MeLiM pigs.

In an initial study we gained a global overview of differentially expressed genes between a growing and regressing tumor by conducting Subtractive Suppression Hybridization (SSH). We also performed SSH analysis of cell cultures isolated from growing and regressing melanoma to be able to distinguish between expressional changes induced by tumor microenvironment. Only a few genes were in common between these two SSH analyses. Thus we focused on the SSH results obtained from tumor tissue. We were able to identify a gene signature for growing melanoma that demonstrated common genes with human progressive melanoma (TYR, MITF, MLANA, SDCBP, SILV, TYRP and ZFP106). Genes overexpressed at the beginning of regression were mainly involved in functional classes such as differentiation, immune system, cell cycle arrest, and tumor suppression. Two genes, CD9 and RARRES1, showed a strong upregulation during early melanoma regression on the mRNA and protein level whereas CD9 is a motility-related protein and RARRES1 a putative tumor suppressor. Since spontaneous regression is a dynamic process, we performed time dependent gene expression profiling using DNA chips in a second study. We identified significant gene signatures at different stages of regression that were able to explain the corresponding phenotype. Identified gene signatures were mainly involved in the immune response, the cell cycle, and melanocyte

differentiation/pigmentation processes. Interestingly, we showed an early downregulation of cell cycle related genes that could play a role in regression. Also, we identified different immune gene signatures, suggesting a major role of the host's immune system in eradicating melanoma cells. By using immunohistology and flow cytometry we characterized tumor infiltrating cells of the innate and adaptive immune system. During regression the infiltration occurred in two phases: an early phase consisting of antigen presenting cells (SWC3+), followed by a later infiltration of mainly cytotoxic T lymphocytes (CD8+). Furthermore the regression process is accompanied by the presence of big highly pigmented cells that we began to characterize.

This work allowed us to point out similarities between the pig and human melanoma on the transcriptomic level along with the already observed parallels on the clinical and genetic level. These findings underline the utility for this model to study human melanoma. This work helped also to decipher the process of spontaneous regression on a cellular and molecular level. In this way we observed an early mitotic arrest of tumor cells, linked to regression, that has not been described so far, and an implication of the immune system. If the immune response is the real inducer of regression needs to be verified. Already, this work contributes to human anti-melanoma therapy as it provides potential targets that could be used for developing new strategies.

Résumé :

Le mélanome est une tumeur originaire des mélanocytes; c'est la forme la plus agressive des cancers cutanés. Son incidence s'accroît régulièrement. De plus sa résistance aux traitements actuels (chimiothérapie, radiothérapie et immunothérapie) ne laisse qu'une faible espérance de survie aux patients présentant un stade avancé de la maladie. Aujourd'hui il est nécessaire de développer de nouveaux traitements ciblés sur la cellule tumorale. Une stratégie originale consisterait à étudier le phénomène naturel de régression spontanée des mélanomes. Malheureusement chez l'homme la régression n'est que partielle et l'extrême rareté des cas de régression totale à un stade avancé de la maladie rend impossible son étude.

Cependant le modèle porcin de mélanome cutané (MeLiM) présente une opportunité unique d'étudier ces mécanismes complexes de régression spontanée. En effet, les minis porcs MeLiM développent des mélanomes héréditaires qui régressent totalement et spontanément, indépendamment de tous facteurs externes.

Actuellement, les mécanismes responsables de cette régression spontanée sont peu ou mal connus. C'est pourquoi l'objectif principal de cette thèse a été l'étude des mécanismes de la régression spontanée du mélanome dans ce modèle animal.

Dans un premier temps, grâce à la technique d'hybridation suppressive soustractive (HSS) nous avons comparé le transcriptome d'une tumeur en croissance et d'une tumeur en tout début de régression. Dans le but de distinguer les signaux provenant du microenvironnement tumoral de ceux propres à la cellule tumorale, nous avons également réalisé une HSS à partir des cellules isolées d'une tumeur en progression et d'une tumeur au début de la régression. Nous avons trouvé très peu de gènes en commun entre les deux HSS. Nous avons donc focalisé notre étude sur les résultats issus de la HSS à partir des tumeurs. Des gènes surexprimés chez le porc pendant la phase de prolifération tumorale sont également retrouvés dans les études d'expression conduites dans le mélanome chez l'homme (TYR, MITF, MLANA, SDCBP, SILV, TYRP and ZFP106). Au tout début du phénomène de régression, on observe une surexpression des gènes impliqués dans les fonctions de la différenciation, le système immunitaire, l'arrêt du cycle cellulaire et la suppression des tumeurs.

Deux gènes CD9 et RARRES1 montrent une très forte surexpression pendant la régression tant au niveau transcriptomique que protéique. Le CD9 est lié à la motilité cellulaire et RARRES1 est un gène potentiel suppresseur de tumeur.

Dans un second temps, la dynamique du processus de régression a été étudiée grâce à la technologie des puces à ADN qui a permis d'établir un profil cinétique de l'expression génique. Ainsi, pour chacun des stades de la régression une corrélation entre la signature génique et son expression phénotypique a pu être établie. La signature de la régression comprend entre autres, des gènes impliqués dans la réponse immunitaire, le cycle cellulaire et la différenciation ainsi que la pigmentation des mélanocytes. Ce travail révèle que la régression des mélanomes semblerait être liée à une sous régulation précoce du cycle cellulaire. De plus le système immunitaire semble jouer un rôle majeur dans l'éradication des cellules tumorales. Par immunohistologie et cytométrie en flux, nous avons caractérisé le phénotype des cellules infiltrant les tumeurs : les cellules de l'immunité innée et acquise. On observe deux phases pendant le processus de la régression : une phase précoce consistant principalement à une infiltration par des cellules présentatrice d'antigènes (SWC3+) puis une phase d'infiltration tardive par des lymphocytes cytotoxiques (CD8+). La régression s'accompagne également de la présence de grosses cellules hyperpigmentées que nous avons commencés à caractériser.

Ce travail a permis de montrer qu'il existe des similarités entre le transcriptome du mélanome chez le porc et chez l'homme en plus de celles déjà observées au niveau clinique et génétique ce qui permet de confirmer l'utilité de ce modèle pour l'étude du mélanome chez l'homme. Il a surtout permis de disséquer le processus de régression au niveau cellulaire et moléculaire. Ainsi on a observé un signal précoce d'arrêt en mitose des cellules tumorales, phénomène inconnue jusqu'alors, puis une implication du système immunitaire. Il reste à démontrer si ce dernier est le réel inducteur de la régression. D'ores et déjà, ce travail apporte des cibles à utiliser pour développer des traitements contre le mélanome chez l'homme.

Abstrakt

Das Melanom der Haut (cutane melanom), die aggressivste Form des Hautkrebses, ist ein Tumor melanocytärem Ursprungs mit steigender Häufigkeit. In späteren Stadien, wenn der Tumor bereits Metastasen gebildet hat, ist die Chance auf eine Heilung gering, da das Melanom eine hohe Resistenz gegen gängige Therapien zeigt. Daher ist die Entwicklung von neuen Strategien zur Heilung vom cutanem Melanom von Priorität. Um alternative Anti-Melanom Therapien zu entwickeln, müssen erst geeignete Ziele identifiziert werden mittels Erforschung der Melanombiologie. Ein eleganter Ansatz besteht in der Studie des natürlichen aber raren Phänomens der spontanen Tumorregression, bei der sich Tumore ohne externer Hilfe zurückbilden. Leider ist die komplette und spontane Regression des avancierten Melanoms beim Menschen sehr selten und damit nur schwer zu analysieren.

Das Melanoma-bearing Libechov Minipig (MeLiM) ist ein porcines Melanom Model, welches die Erforschung der spontanen Tumorregression ermöglicht. Die Miniaturschweine leiden am familiären (vererbaren) Melanom und zeigen spontanes Tumorstadium mit erstaunlicherweise folgender kompletten und spontanen Regression. Die zugrundeliegenden Mechanismen der spontanen Regression sind bis heute unklar.

Das Gesamtziel der Doktorarbeit bestand daher in der Untersuchung der Mechanismen, welche zur spontanen Regression im Melanomschwein führen.

In einer anfänglichen Studie verschafften wir uns einen globalen Überblick über differentiell exprimierte Gene zwischen wachsendem und regressierendem Tumorgewebe mittels Subtraktiver Suppressions Hybridisierung (SSH). Um unterscheiden zu können welcher Einfluss die Tumormikroumgebung auf das Genexpressionsverhalten hat, wurde ebenso eine SSH Analyse von Melanomzellkulturen, isoliert aus primären Melanomen, durchgeführt. Die beiden SSH Analysen zeigten nur wenige Gene gemein. Daher konzentrierten wir uns auf die Resultate der Tumorgewebsstudie. Für das wachsende porcine Melanom konnten Gene identifiziert werden, die auch beim menschlichen Melanomwachstum eine Rolle spielen (TYR, MITF, MLANA, SDCBP, SILV, TYRP and ZFP106). Gene, überexprimiert im regressierenden Melanom, waren hauptsächlich involviert in funktionelle Klassen wie Differentiation, Immunantwort, Zellzyklusarrest und Tumorsuppression. Zwei Gene, CD9 und RARRES1, zeigten auf mRNA Ebene eine verstärkte Expression während frühzeitiger Regression. Die erhöhte Expression des zellmotilitätsverwandtem Gens CD9 und des vermeintlichem Tumorsuppressors RARRES1,

welcher zum erstenmal im Melanom beschrieben wurde, wurden auf Proteinniveau bestätigt. Da die spontane Regression ein dynamischer Prozess ist, wurde fortführend eine zeitabhängige Genexpressionsstudie mittels Genchips ausgeführt. Wir identifizierten signifikante Gensignaturen für verschiedene Stadien der Regression, welche Aufschluss über den jeweiligen Phenotyp gaben. Zugehörige biologische Prozesse der identifizierten Gensignaturen beinhalteten hauptsächlich Immunantwort, Zellzyklus, Melanocytdifferentiation und Pigmentation. Interessanterweise, konnte eine frühe Herunterregulation der Zellzyklusgene gezeigt werden, die möglicherweise eine Rolle im Regressionsmechanismus spielen könnten. Weiterhin wurden verschiedene Immunsignaturen identifiziert, welche auf eine wichtige Rolle des Immunsystems bei der Eradikation von Tumorzellen hindeuten. Mittels Durchflusszytometrie und Immunohistologie analysierten wir tumorinfiltrierende Zellen des angeborenen und adaptiven Immunsystems. Die Infiltrierung des Tumorgewebes während der Regression geschieht in zwei Phasen: eine frühzeitige Infiltrierung durch antigenpräsentierender Zellen (SWC3+) und darauffolgender Infiltration durch hauptsächlich zytotoxischen T Lymphozyten (CD8+). Außerdem ist der Regressionsprozess begleitet von der Präsenz extrem grosser und pigmentierter Zellen, die wir ebenso charakterisierten.

Diese Arbeit ermöglichte die Identifizierung von Gemeinsamkeiten auf Transkriptionsniveau zwischen porzinem und menschlichem Melanom, welche im Einklang mit den bereits beobachteten klinischen und genetischen Parallelen stehen. Die Resultate unterstreichen damit die Nützlichkeit des porzinen Models zur Untersuchung vom menschlichen Melanom. Wir konnten weiterhin auf zellulärer und molekularer Ebene Prozesse identifizieren die zur spontanen Regression führen. Auf diese Art und Weise wurde ein frühzeitiger Mitosearrest von Melanomzellen beobachtet, der bis dato als Regressionsmechanismus unbeschrieben blieb, und eine Implikation des Immunsystems. Ob das Immunsystem der einzige Regressionsinduktor darstellt, bleibt zu verifizieren. Die Arbeit trägt aber jetzt schon zur humanen Anti Melanomtherapie bei, da sie potentielle molekulare Ziele enthält, welche für die Entwicklung alternativer Strategien verwendet werden könnten.

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CHAPTER 1

Introduction

This study is focussing on transcriptomic and cellular changes involved in spontaneous melanoma regression using a porcine animal model. Melanoma, the most aggressive form of skin cancer, arises from melanocytes, pigment-producing cells that are found predominantly in the basal layer of the human epidermis. Genetic and environmental factors can lead to the transformation of melanocytes that finally give rise to melanoma. The incidence of melanoma is rising steadily in western populations whereas the number of cases worldwide has doubled in the past 20 years. Melanoma is one of the most deadly human cancers with no effective cure for metastatic disease since the tumors are highly resistant to chemo-, radio-, and immunotherapy.

Complete tumor regression is the ideal of any anti cancer therapy. In a very few cases of human melanoma, the tumor regresses spontaneously without any treatment. Mechanisms leading to this favourable phenomenon remain elusive but are of enormous interest.

Melanocytes

Melanocyte precursor cells (melanoblasts), which are derived from the neural crest, migrate to the skin and hair follicles during embryonic development (Bennett, 1993). Some melanoblasts end up as melanocyte stem cells that are found in the bulge region of the hair follicle in mice and humans (Nishimura et al., 2002; Gleason et al., 2008). Unlike differentiated melanocytes that are of highly dendritic morphology, melanocyte stem cells are round and do not express markers of the melanocytic lineage (Osawa et al., 2005). The fact that stem cells may have a limited replicative potential is strikingly illustrated by the fact that in humans age-related hair greying is accompanied by depletion of the stem cell population. Melanocytes are also found in a number of other locations including the choroid of the eye, the heart, and brain where their function is obscure (Goding, 2007). The most obvious function of melanocytes is the production of melanin in specialised organelles termed melanosomes which are endosomes containing melanosomal proteins such as TYR, DCT and SILV amongst others. Several melanin monomers exist, and the predominant monomer in a melanin polymer depends on its location within an organism. In skin and hair, melanin differs in eumelanin (brownish-black) or pheomelanin (yellow-reddish). Eumelanin and pheomelanin differ not only in color but also in size, shape and packaging of their granules. Both melanins derive from a common tyrosinase-dependent pathway with the same precursor, tyrosine (Lin

and Fisher, 2007). Eumelanin in the skin ensures the only natural UV protection by eliminating the generated free radicals/ROS whereas pheomelanin is more photolabile and can produce, among its by-products, radicals which can cause DNA damage (Land and Riley, 2000). Melanosomes are transferred from melanocytes via their dendrites to the surrounding keratinocytes, where they cap the nucleus and in this way are considered to protect the DNA of dividing cells from UV radiation (Kobayashi et al., 1998). There is little doubt that melanin provides the skin with protection against UV radiation as supported by *in vitro* and *in vivo* studies (Smit et al., 2001). Rather than simply acting as a UV block, melanin within the melanosomes is likely to act as a sink for highly reactive oxygen species that would otherwise lead to DNA damage. In addition to their photoprotective role, melanocytes are essential for hearing as they are present in the stria vascularis where they are required for the generation of endolymph-mediated action potentials. Genetic mutations that lead to loss of melanocytes in the inner ear are a major cause of deafness (Steel and Barkway, 1989). Other melanocyte associated pathologies besides melanoma include vitiligo and albinism for example. Vitiligo is characterized by a localized and progressive loss of pigment cells. The precise mechanisms underlying the onset and progression of vitiligo are not entirely clear, though three contributing factors are likely to play a role either independently or synergistically: the neural hypothesis implies that nerve endings release toxins that accumulate and damage melanocytes resulting in decreased melanin productions; the biochemical hypothesis suggests that there might be a selfdestruction process of the melanocytes due to accumulation of toxic melanin-synthesis intermediates; the autoimmune etiology includes the existence of autoantibodies and autoreactive T lymphocytes against melanocyte antigens (Ram and Shoenfeld, 2007). Albinism is characterized by the presence of melanocytes that either do not produce pigment or have other defects in pigment production or melanosome function. In its severest form, individuals with inactivating mutations in the TYR gene that encodes the rate limiting enzyme for the production of melanin are completely depigmented. Although mutations in the TYR gene can lead to the most severe form of albinism, mutations affecting at least 12 different genes encoding additional melanosomal proteins can also lead to various forms of albinism (Gronskov et al., 2007).

Melanoma

Although the majority of melanoma patients are cured after surgical excision of a poorly invasive primary tumor, metastatic melanoma is refractory to all current forms of therapy and has a median survival rate of 6 months (Miller and Mihm, Jr., 2006). While melanoma is relatively rare (<5%) compared with other skin cancers such as basal and squamous cell carcinoma, it is responsible for 80% of all skin cancer related deaths (Miller and Mihm, Jr., 2006). Unresponsiveness of melanoma cells to therapy is mostly caused by apoptosis resistance mechanisms rising from mutations in pro-apoptotic signalling pathways (Eberle et al., 2007). Unlike most other cancers, melanoma is frequent among young and middle aged adults (Lange et al., 2007; Tsao et al., 2004), but rare in the pediatric population. It accounts only for 1.3% of all childhood malignancies (<20 years) (Pappo, 2003). Congenital and infantile melanomas arising from primary cutaneous lesions as well as transplacentally are rare, with only 23 cases reported in the English medical literature since 1925 (Richardson et al., 2002).

As in most types of cancers, there are two sets of factors that present significant risk for melanoma in humans: genetic and environmental factors.

Epidemiological studies have identified host factors important for risk of melanoma. These include family history of melanoma, alterations in melanoma susceptibility genes, number and type of nevi, skin type and pigmentation (Tucker and Goldstein, 2003). Melanoma is more common in individuals with fair skin, blue or green eyes, red or blond hair, many freckles, and in individuals who tend to get sunburn easily. The main environmental risk factor is ultraviolet radiation (UV) exposure leading to mutations in melanocytes and thus melanoma (Tucker and Goldstein, 2003). However incidence of melanoma among people who work outdoors is lower compared with people who work indoors. One possible explanation may be the protective effect of the tanning response including stimulation of melanin production. It is well recognized that dark-skinned individuals tolerate sunlight better than those with lighter skin and red hair, who are less capable of producing pigment due to genetic variants in the melanocortin-1 receptor (Valverde et al., 1995).

The initiation and progression of melanoma include a series of histological changes such as 1) nevus, a benign lesion characterized by an agglomeration of nested melanocytes; 2) dysplastic nevus (atypical mole), characterized by random and discontiguous cytologic atypia; 3) radial growth phase (RGP) melanoma, where the cells acquire the ability to proliferate intraepidermally; 4) vertical growth phase (VGP) melanoma, where the cells acquire the

ability to penetrate through the basal membrane into the underlying dermis and subcutaneous tissue (Fig. 1); and 5) metastatic melanoma, characterized by the spread of cells to other areas of the skin and organs (Clark, Jr., 1991).

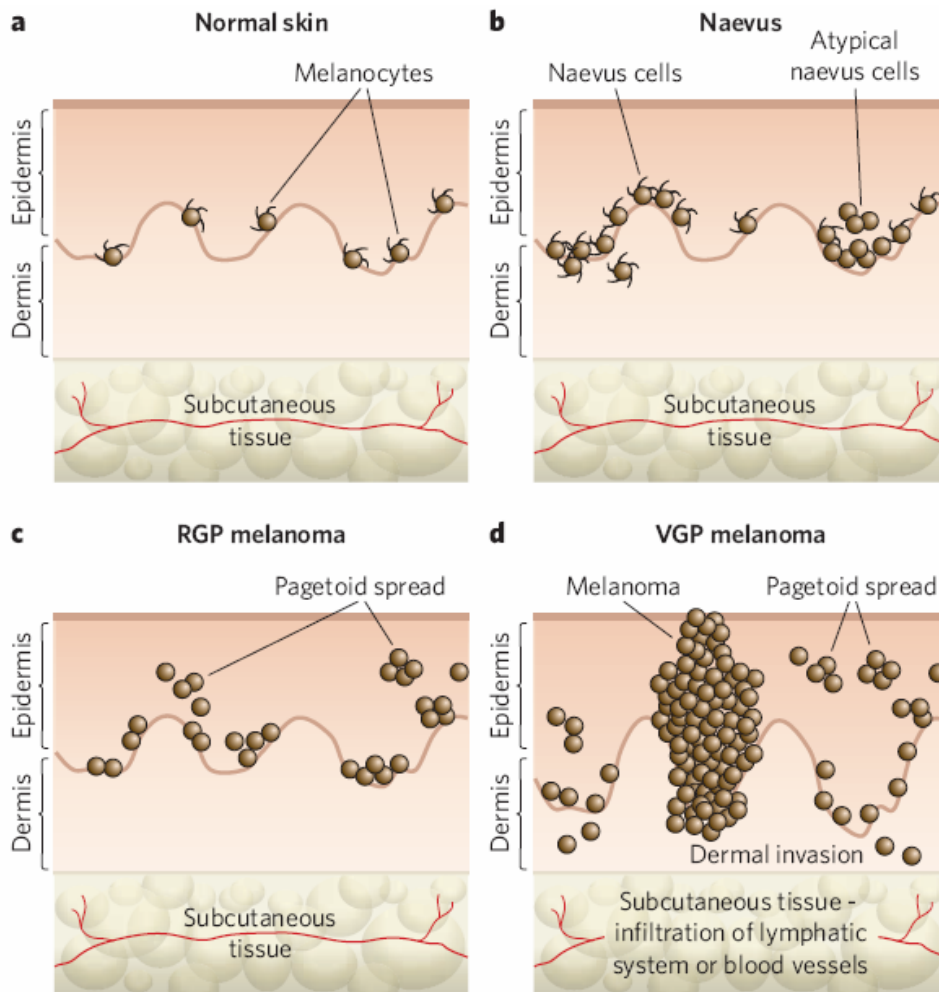


Fig. 1: Progression of melanocyte transformation.

There are various stages of melanocytic lesion, each of which is marked by a new clone of cells with growth advantages over the surrounding tissues. a, Normal skin. This shows an even distribution of dendritic melanocytes within the basal layer of the epidermis. b, Naevus. In the early stages, benign melanocytic naevus cells proliferate. According to their localization, naevi are termed either junctional, dermal or compound. Some naevi are dysplastic, with morphologically atypical naevus cells. c, Radial-growth-phase (RGP) melanoma. This is considered to be the primary malignant stage. d, Vertical-growth-phase (VGP) melanoma. This is the first stage that is considered to have malignant potential and leads directly to metastatic malignant melanoma, the most deadly stage, by infiltration of the vascular and lymphatic systems. Pagetoid spread describes the upward migration or vertical stacking of melanocytes that is a histological characteristic of melanoma (adopted from Gray-Schopfer et al., 2007).

The most critical event along this progression is probably the transition from RGP to VGP, which includes the escape from keratinocyte-mediated growth control (Tsao et al., 2004) as melanoma cells acquire specific changes in gene expression that facilitates escape and subsequent invasion (Gaggioli and Sahai, 2007). Since keratinocytes usually help to control the behaviour of normal melanocytes, melanoma cells reduce these interactions by downregulation of E-cadherin and upregulation of N-cadherin that may enable interaction

with stromal fibroblasts and therefore survival outside the epidermis (Haass et al., 2005). The cell adhesion molecule MCAM is also overexpressed as melanomas become more invasive and can promote melanoma cell interactions with endothelial cells and thereby facilitate entry into the vasculature (Bogenrieder and Herlyn, 2002). There are different subtypes of melanoma. Superficial spreading melanoma (SSM) is by far the most common form of melanoma (~70% of all melanoma cases). It is characterized by a first intra-epidermal component, followed by an invasive component after several months and is linked to episodes of severe sunburn at early age. Nodular melanoma (~15% of all melanoma cases) consists of raised nodules without a significant flat portion and is characterized by a concomitant intraepidermal and dermal invasion. However nodular melanoma accounts for up to 70% of thick melanoma (>3mm). Acral lentiginous melanoma (~5% of all melanoma cases) which is also a SSM, is mostly found on the palms of the hands, the soles of the feet and is not linked to UV exposure. Lentigo maligna (~10% of all melanoma cases), also a SSM, is generally flat in appearance and occurs on sun-exposed regions in elderly (Clark, Jr. et al., 1984).

Melanoma susceptibility and altered signalling

Melanoma is a disease of complex genetic background where acquired (somatic) and/or inherited (germline) mutations lead to altered intracellular signalling. Inherited (familial) melanoma accounts for 8%-12% of cases (Fountain et al., 1990). The 2 main types of genes playing a role in cancer are oncogenes and tumor suppressor genes. Oncogenes are mutation-activated forms of proto-oncogenes which in turn normally control cell division or differentiation (growth factors, growth factor receptors, signal transducers, transcription factors, anti-apoptotic regulators). Tumor suppressor genes are normally involved in cell division control, DNA repair mechanisms, and proapoptotic genes. The difference between oncogenes and tumor suppressor genes to induce cancer is that oncogenes result from activation (gain of function) whereas tumor suppressor genes result from inactivation (loss of function). In melanoma different proto-oncogenes and tumor suppressor genes have been described (Table 1).

Table 1: Selected genes altered by mutation, deletion or amplification in malignant melanoma
(adapted and modified from Dahl and Guldberg, 2007)

Gene Type	Gene	Most frequent type of alteration	Human chromosome
Oncogenes	NRAS	Mutation	1p13.2
	BRAF	Mutation	7q34
	AKT3	Amplification	1q43-q44
	CDK4	Mutation, amplification	12q14
Tumor suppressor genes	p16INK4A (CDKN2A locus)	Deletion, mutation	9p21
	p14ARF (CDKN2A locus)	Deletion, mutation	9p21
	PTEN	Deletion, mutation	10q23-24
	TP53	Mutation	17p13.1
Others	MC1R	Mutation	16q24.3
	MITF	Amplification	3p14.2
	CCND1	Amplification	11q13

Mutations in two high-risk genes encoding cell-cycle regulatory proteins have been shown to cause familial melanoma, including the cyclin-dependent kinase inhibitor 2A (CDKN2A), and the cyclin dependent kinase 4 (CDK4). Both of these genes are important in controlling cell division. The CDKN2A locus encodes for 2 proteins such as p16INK4A and p14ARF (Fig. 2).

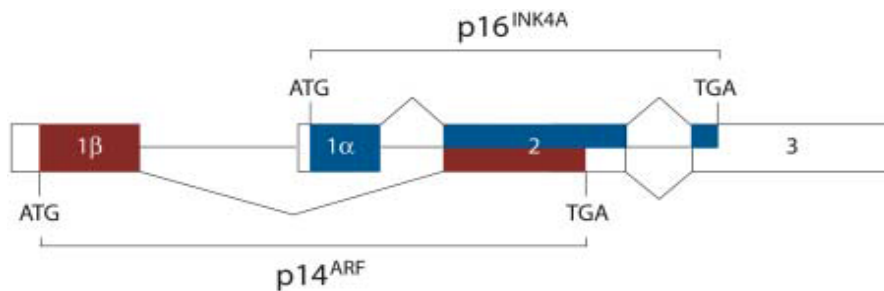


Fig. 2: The CDKN2A locus. The CDKN2A locus encodes two unrelated tumor suppressors in different reading frames. Exons 2 and 3 are spliced with exon 1a to produce mRNA for p16INK4A, and with exon 1b to produce mRNA for p14ARF. Although most melanoma-associated mutations and deletions at this locus affect both genes, both p16INK4A-specific and p14ARF-specific alterations have been reported, suggesting that these genes encode independent melanoma-suppressive activities (adopted from Dahl and Guldberg, 2007).

Several studies have shown that replicative senescence in melanocytes is accompanied by elevated expression levels of p16INK4A (Bennett, 2003), a tumor suppressor that activates the tumor-suppressive effects of the retinoblastoma protein (RB) by binding and inhibiting the cyclin dependent kinases CDK4 and CDK6 (Fig. 3).

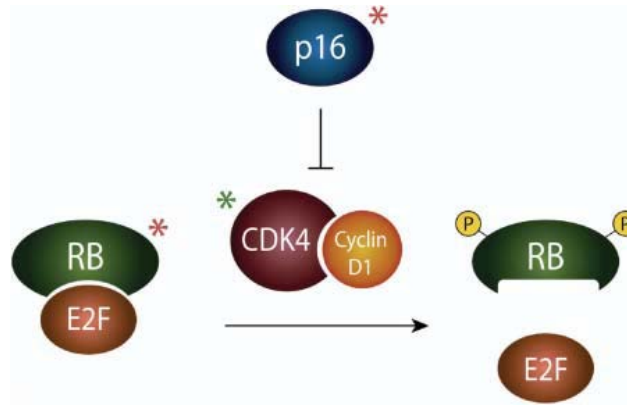


Fig. 3: The p16INK4A-CDK4/6-RB pathway. p16INK4A is an inhibitor of the G1 cyclin-dependent kinases CDK4 and CDK6, and keeps the tumor suppressor RB in an activated, hypophosphorylated state. Inactivation of p16INK4A leads to unopposed CDK activity, hyperphosphorylation of RB and release of E2F transcription factors, allowing for S-phase entry. While p16INK4A is the preferred target in melanoma, rare mutations have also been found in *CDK4* and *RB1* (adopted from Dahl and Guldberg, 2007).

Somatic inactivation of p16INK4A by point mutation, deletion or promoter methylation is found in the majority of sporadic melanomas (Bartkova et al., 1996) and has also been identified in about 30% of melanoma families (Hussussian et al., 1994). The second gene of the CDKN2A locus, p14ARF has also a tumor suppressor activity by positively regulating p53 in response to oncogenic signalling or aberrant growth by binding and inactivating MDM2, a negative regulator of p53 (Kamijo et al., 1998) (Fig. 4). CDK4 mutations leading to insensitivity to inhibition by p16INK4A, are rather rare but lead then to the hyperphosphorylation of RB and release of E2F transcription factors, allowing S-phase entry (Ceha et al., 1998). However, germline CDK4 mutations have been identified only in some melanoma kindreds (Soufir et al., 1998).

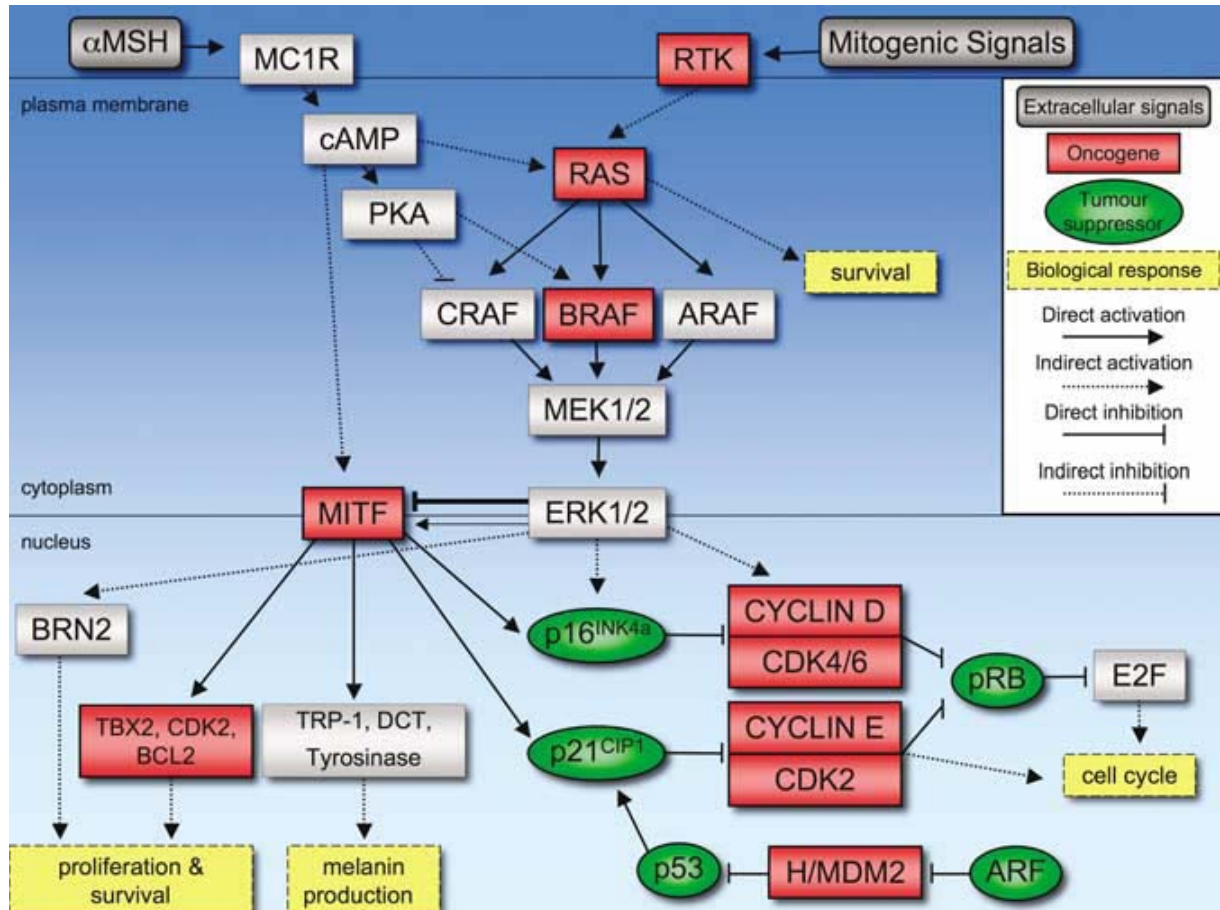


Fig. 4: Prominent molecular pathways involved in development and regulation of melanoma. Schematic representation of the RAS/RAF/MEK/ERK signalling pathway, which feeds into various effector processes, including those governing cell proliferation and survival. In melanocytes, the microphthalmia-associated transcription factor (MITF) is under (both positive and negative) control of BRAF-(and cAMP-) dependent signals to regulate melanin production in response to αMSH. Whereas in non-malignant cells BRAF activity is modulated as a function of extracellular signals through RTKs, the cancer-derived BRAF^{V600E} mutant functions autonomously. A central cell cycle pathway downstream of BRAF corresponds to the p16^{INK4A}/CDK4/pRB/E2F route, which in melanocytes is also under control of MITF. The CDK inhibitor p21^{CIP1} acts as a nodal point connecting the pRB pathway to the p53 tumour suppressor and MITF. Proteins are colour-coded as explained in the insert (adopted from Michaloglou et al., 2008).

A low penetrance gene in familial melanoma is the melanocortin 1 receptor (MC1R). MC1R encodes for a seven-pass membrane G-protein receptor that regulates the production of melanin (Fig. 4). Variations in the MC1R gene sequence were described first in animals, in which they contribute to the multitude of coat colours (Cone et al., 1996). In humans, pale skin and light hair colour have been linked to variations within the MC1R gene (Valverde et al., 1995). Regarding melanoma, MC1R variations seem to modify the impact of other melanoma-associated genes as carriers with CDKN2A mutations (Box et al., 2001). Furthermore, an important link between genetic and environmental factors was identified since MC1R variants were associated with melanoma risk in patients who had melanoma arising on intermittently sun exposed skin and that this risk was associated with BRAF

mutations (Landi et al., 2006). This suggested that germline mutations can influence later genetic events, leading later to tumorigenesis in response to environmental exposures like UV light (High and Robinson, 2007). About 50% of melanoma predisposed families do not have an identifiable mutation in CDKNA2, CDK4, suggesting that other as yet undiscovered mutations are involved in familial melanoma development.

The occurrence of somatic NRAS or BRAF mutations in 80-90% of all melanoma cases suggests that activation of the RAS-RAF-MEK-ERK pathway may constitute an obligatory event in the transformation of melanocytes (Fig. 4). The RAS-RAF-MEK-ERK pathway regulates cell fate decisions downstream of receptor tyrosine kinases, cytokines and heterotrimeric G-protein-coupled receptors (Wellbrock et al., 2004). In normal melanocytes, this pathway is activated by growth factors such as stem cell factor (SCF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) (Bohm et al., 1995). Since these growth factors induce individually only a weak transient ERK activation, leading to a modest mitogenic effect, the interplay of several growth factors is required to stimulate strong ERK activity in melanocytes (Wellbrock et al., 2002). Activated ERK translocates to the nucleus where it activates transcription factors important for cell proliferation. In human melanoma, ERK hyperactivity is gained in about 90% of the cases, while NRAS and BRAF are the most commonly mutated components of this pathway (Cohen et al., 2002). NRAS belongs to the family of small guanine-nucleotide binding proteins, which also includes HRAS and KRAS. NRAS mutations involve the substitution of glutamine at amino acid residue 61 which impairs GTP hydrolysis and therefore maintains the protein in a state of constitutive activation. Mutations of KRAS and HRAS are only rarely found in melanoma.

BRAF, a downstream effector of RAS, is a serine-threonine-specific protein kinase that activates MEK, which in turn activates ERK (Fig. 4). More than 90% of BRAF mutations show a substitution of valine with glutamic acid at residue 600 (BRAF^{V600E}) which leads to constitutive activation of the protein with a large increase in the basal kinase activity (Michaloglou et al., 2008; Wan et al., 2004). Mutations of BRAF rarely coincide with NRAS mutations suggesting that both mutant forms are efficient in activating the MEK-ERK pathway (Goel et al., 2006).

Another signalling pathway involved in melanoma development is the phosphoinositide-3-OH kinase (PI3K) pathway (Fig. 5). Phosphoinositides are membrane lipids that are converted to

second messengers through hyper-phosphorylation by PI3K family members (Bohm et al., 1995).

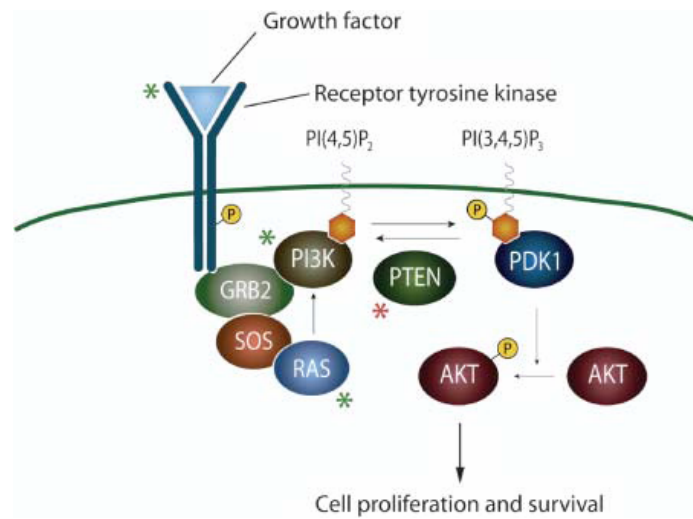


Fig. 5: The PI3K-AKT pathway. Activation of RTKs by growth factors leads to activation of PI3K, which then converts the plasma membrane lipid PIP₂ into the second messenger, PIP₃. This activation step leads to phosphorylation and activation of AKT kinase, which in turn phosphorylates several proteins affecting cell growth and survival. PTEN is an inhibitor of this pathway, which acts by dephosphorylating PIP₃. Increased signaling through the PI3K-AKT pathway can also be achieved by activation of RAS proteins. The most frequent melanoma-associated genetic events in this pathway are inactivating PTEN mutations and activating NRAS mutations (adopted from Dahl and Guldberg, 2007).

The lipid second messengers activate numerous downstream effector pathways such as AKT (protein kinase B) (Cully et al., 2006). Activated AKT regulates a network of factors that control cell proliferation and survival. Signalling is terminated by the lipid phosphatase PTEN (phosphate and tensin homologue) which antagonizes PI3K activity. PTEN function was shown to be lost in late stage melanomas (Wu et al., 2003) favouring hyperactivation of the PI3K-AKT pathway and finally progression of melanoma (Stahl et al., 2004). Furthermore, mutation in the catalytic subunit of PI3K (PI3KCA) leading to constitutive AKT activation, occur only at low frequencies in melanoma (Omholt et al., 2006; Samuels et al., 2005). Instead, it has been proposed that a common mechanism of increased AKT activity is a DNA copy gain at the AKT3 locus, which is found in 40-60% of melanomas (Stahl et al., 2004). Another gene of interest is the putative melanoma oncogene MITF, a basic helix-loop-helix leucine zipper transcription factor that is considered to be the master regulator of melanocyte biology (Fig. 4). MITF regulates the expression of melanogenic proteins such as tyrosinase (TYR), silver homologue (gp100), melanoma associated antigen recognized by T cells-1 (MART1 or MLANA) (Levy et al., 2006) (Fig. 6).

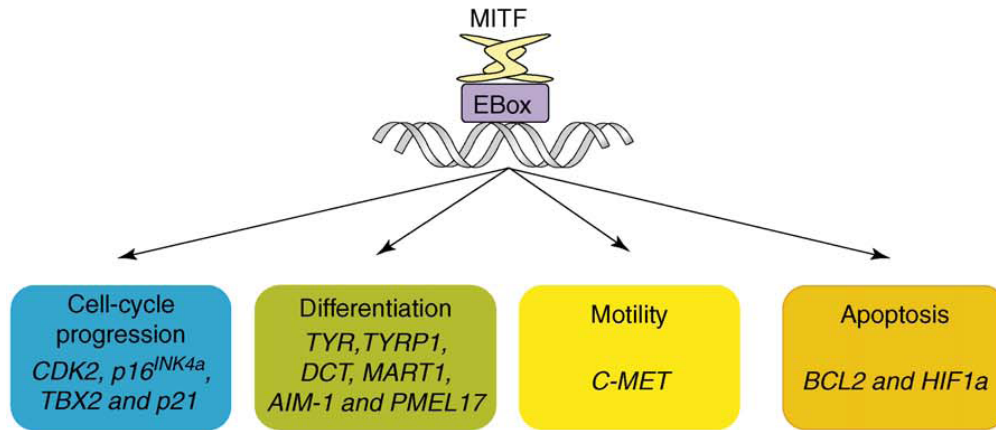


Fig. 6: MITF target genes. MITF-M regulates the transcription of multiple genes by binding specific sequences, as subset of E-boxes, present in promoter or enhancer elements containing the consensus CATGTG, CACATG or CACGTG. MITF regulates multiple targets in melanocytes and melanoma cells involved in various cellular processes such as cell-cycle control, survival, motility, invasion and differentiation and/or pigmentation (adapted from Levy et al., 2006).

Amplification of the MITF gene was found in 10-20% of primary melanoma cases, with a higher incidence among advanced melanomas (Garraway et al., 2005). In metastatic cases MITF amplification was associated with a decreased 5-year survival. Overexpression of MITF in human melanocytes in vitro has been correlated with malignant transformation (Garraway et al., 2005) and it is possible that MITF may function as an oncogene, similar to CDK4 (Miller and Mihm, 2006).

Although studies aimed already to assess the oncogenic activity of melanoma associated mutations in human tissue (Chudnovsky et al., 2005), further studies are needed to establish how many and which are the genetic events required for autonomous growth and unlimited life span of melanocytes, and to unravel the interactions between genetic and environmental factors in the tumorigenic process.

Conventional anti-melanoma therapy

Whereas surgical removal of early detected thin melanoma leads to cure in >97% of patients (Balch et al., 2003), successful treatment of metastasized melanoma by chemo-, radiation-, and immunotherapy remains marginal (Atallah and Flaherty, 2005). This resistance to treatment is thought to be linked to the origin of melanocytes, since they derive from highly motile neural crest progenitors that have enhanced survival properties. Just recently, malignant melanoma initiating cells were shown to express high levels of ABCB5, an ATP-binding cassette (ABC) transporter which acts as a melanoma chemoresistance mediator (Schatton et al., 2008). Various types of ABC transporters including the multidrug resistant

MDR gene product 1 (MDR1), contribute to drug resistance in many cancers by removing cytotoxic drugs from the cell (Gottesman and Ambudkar, 2001). MDR1 as well, has been recently shown to be suitable marker for melanoma stem cells (Keshet et al., 2008). Also, melanoma cells have low levels of spontaneous apoptosis *in vivo* compared to other tumor cell types and are relatively resistant to drug-induced apoptosis *in vitro* (Soengas and Lowe, 2003).

Chemotherapy:

Dacarbazine (DTIC), an alkylating agent, is for now the reference chemotherapeutic agent approved for the treatment of advanced melanoma besides others such as carmustin, paclitaxel, temozolomide and cisplatin (Tarhini and Agarwala, 2006). Even when combining dacarbazine treatment with high doses of interleukin 2 (IL2), a pleiotropic cytokine needed mainly for T cell activation, response rates were below 20% with only rare long term responders and severe side effects (Stoter et al., 1991).

Another class of anti-melanoma drugs target directly hyperactivated oncogenes and their molecular pathways such as Sorafenib. This multikinase inhibitor targets BRAF, CRAF, PDGF and VEGF. However as a monotherapy, sorafenib showed only modest activity against melanoma. For now, it is not clear why sorafenib rests inefficient. A hypothesis is that melanoma cells might circumvent blocked RAF/MEK/ERK signalling by using alternative pathways for proliferation (Eisen et al., 2006).

Radiotherapy:

The role of radiotherapy is well established in the management of most locally advanced and metastatic cancers. However there has been reluctance to extend this role to melanoma due resistance to radiation. Human melanomas have a wide range of radiation sensitivities, and should not be regarded as uniformly radioresistant. The best radiotherapy schedule for melanoma remains uncertain, although hypofractionation seems reasonable, as suggested by the average response of melanomas in experimental and clinical studies (Stevens and McKay, 2006). Radiation therapy that gives larger doses (fractions) of radiation in fewer treatment sessions and over a shorter period of time than standard radiation therapy is defined as hypofractionated radiation therapy.

Anti-melanoma immunotherapy

Immunotherapy against melanoma attracted a lot of attention over the last 30 years after the identification of melanoma associated antigens (MAA). Extensive evidence suggests that the immune system is able to recognize and destroy cancer cells even though they do not

fit neatly into the self/non self paradigm as cancer is not an exogenous pathogen. In this regard, cancer antigens recognized by the immune system are self or mutated self molecules (Houghton and Guevara-Patino, 2004). Why even self proteins get recognized leads back to the phenomenon of autoreactive T and B cells. Melanoma is known to be one of the most immunogenic malignancies (Enk et al., 2006) and the rationale therefore is the existence of various melanoma antigens. MAAs can be divided into different classes such as differentiation antigens, unique antigens and germline antigens (Table 2).

The melanocyte lineage proteins, or differentiation antigens, are involved in melanin production and are present in both melanoma cells and normal melanocytes (TYR, TYRP1, TYRP2, gp100, MLANA). Cancer testis antigens (MAGE, GAGE, BAGE, NY-ESO-1) are normally expressed in male germ cells or placenta and silenced in healthy somatic cells but reexpressed in a variety of cancers such as melanoma (Van den Eynde et al., 1995). The third class consists of tumor specific/unique antigens that arise from mutations in oncogenes or tumor suppressor genes (CDK4, β -catenin). Some of these mutations create new antigenic epitopes which become immunogenic (Demunter et al., 2002; Wolfel et al., 1995).

Table 2: Melanoma associated antigens (adapted from Hodi, 2006)

- Melanocyte lineage/differentiation antigens: abundant proteins that function in melanin production.
 - Tyrosinase (TYR)
 - Tyrosinase related protein 1 (TYRP1) or gp75
 - Human homologue of the silver locus (SILV) or gp100
 - Melan-A (MLANA) or MART1
 - Dopachrome tautomerase (DCT) or TYRP2
- Cancer testis antigens: normally expressed in testis and placenta.
 - Melanoma antigen (MAGE) family
 - B melanoma antigen (BAGE) family
 - G antigen (GAGE) family
 - New York esophageal squamous cell carcinoma 1 (NY-ESO-1)
- Tumor specific antigens: subtle mutations of normal cellular proteins.
 - CDK4
 - β -catenin

To enhance anti-tumor immunity, active and passive strategies have been developed. One of the first clinical trials of active immunization used a modified immunodominant peptide of the

gp100 antigen to stimulate a tumor-specific T cell response. Even though the majority of the vaccinated stage IV melanoma patients mounted an immunologic response to the peptide, all patients went on to develop progressive disease (Rosenberg et al., 1998). In addition to peptide-based vaccines, recombinant viruses, DNA, whole tumor cell lysates, heat shock protein complexes, irradiated autologous or allogeneic tumor cells, and dendritic cell-based vaccines have been administered, alone or in combination with adjuvants as extensively reviewed by Wilcox (Wilcox and Markovic, 2007). While a trend towards improved median time to disease progression and overall survival was observed in the vaccinated group, results failed to reach statistical significance. In contrast to active strategies, passive immunotherapy aims to stimulate and expand autologous, tumor specific T cells *ex vivo* followed by infusion of these reactive T cells back into the same patient, usually accompanied with high dose IL2. However, the initial studies using cloned melanoma-antigen specific T cells, with or without IL2, seemed to be ineffective in inducing an objective antitumor response (Dudley et al., 2001). Another approach is the use of tumor infiltrating lymphocytes (TIL), containing a diverse effector population composed of CD4+ T helper and CD8+ cytotoxic T cells. In clinical studies using TILs in conjunction with high dose IL2, objective tumor regressions were seen in 33% of the patient treated, unfortunately, clinical responses were of short duration with the persistence of the transferred TILs transient (Rosenberg et al., 1994). To enhance the efficacy of adoptive cell transfer (ACT) using TILs, another study applied preconditional lymphodepletion by chemotherapy before transferring lymphocytes and high dose IL2 (Dudley et al., 2001). Lymphodepletion by cyclophosphamide and fludarabine aimed to eliminate counteracting regulatory T cells that could act as IL2 cytokine sink. 6 out of 13 patients treated, achieved an objective clinical response. In a recent follow up study, 18 of 35 patients showed clinical responses (Rosenberg and Dudley, 2004). The next step in ACT was the administration of genetically modified cells. Hence autologous peripheral blood monocytes (PBMC) were transduced with a retrovirus encoding a T cell receptor (TCR) for the MART1 antigen *ex vivo* in order to increase antigen specific recognition. 15 patients were treated with these engineered PBMCs in conjunction with high dose IL2 and lymphodepletion (Rosenberg et al., 2006). 2 of 15 patients achieved objective clinical responses that are ongoing for 1 year, demonstrating for the first time the efficacy of gene therapy in combination with IL2 and lymphodepletion in the treatment of late stage melanoma.

Other novel approaches include anti CTLA4 antibody treatment and antisense oligonucleotides against BCL2. CTLA4 is a negative regulatory receptor expressed on T cells. To reduce the sensitivity of activated T cells to negative regulatory signals, inhibition by an anti CTLA

antibody (ipilimumab) was developed. In a recent Phase I/II trial, 36 pretreated patients with late stage melanoma received ipilimumab and high dose IL2 of which 22% experienced an objective tumor response (Maker et al., 2005).

Antisense oligonucleotides bind to specific complementary sequences and prevent therefore mRNA translation, with resultant abrogation of protein synthesis. The antiapoptotic BCL2 is a suitable antisense target because overexpression of BCL2 in melanoma is linked to a higher resistance against chemotherapy. A randomized Phase III trial compared dacarbazine alone against dacarbazine plus oblimersen (BCL2 antisense oligonucleotide) (Bedikian et al., 2006). This randomized trial included 771 patients but could only reveal a trend towards improved survival at 24 months of follow up.

Since recent efforts to develop novel therapies against advanced melanoma yielded in minimal clinical outcome, it is clear that monotherapy will not be an option. Accumulating evidence indicates that conventional chemotherapy mediated antitumor effects have an influence on the immune system and vice versa (Zitvogel et al., 2008). So, anti-cancer treatment and resulting effects should always be regarded in its complexity. For example, treatment with DNA damaging agents stimulates a complex response that involves the activation of tumor suppressor genes such as ATM, CHK, and p53 and in turn induces the expression of NKG2D ligands on the tumor cells surface in a CHK1 and ATM dependent manner (Gasser et al., 2005). NKG2D is an activating receptor involved in tumor surveillance by NK cells, γ/δ T cells and activated CD8⁺ T cells.

The development of multimodality strategies to fight cancer and its stem cells is therefore a requirement (Riker et al., 2007). Studying molecular tumor-signatures using gene microarray analysis has come to the forefront of medical research identifying patients who may have an aggressive form of melanoma and its implication to therapy.

Gene expression profiling in human melanoma

Gene expression profiling in melanoma has become a powerful tool to study the complexity of molecular biology useful for formulating hypotheses to explain disease characteristics. Amongst other transcriptomic techniques Subtractive Suppression Hybridization (SSH), Serial Analysis of Gene Expression (SAGE), and DNA microarray analysis aim to identify differentially expressed genes between different RNA populations. SSH is an “open ended” technique as it allows the discovery of novel transcripts. The SSH

approach uses hybridization of an excess of “driver” cDNA to remove sequences in the “tester” cDNA that are present in both populations in similar amounts. Suppression based PCR leads then to the amplification of target sequences that are unique for each RNA population and finally to the construction of subtracted SSH-clone libraries, ready for sequencing (Diatchenko et al., 1996). Weak points of the SSH procedure include: 1) only two samples can be compared in one SSH; 2) SSH is very elaborate in terms of clone library construction and sequencing; 3) SSH is prone for the detection of false positives (Huang et al., 2007). Another sequencing-based method to estimate transcript abundance is SAGE. This approach whose sole purpose is developing a statistically robust estimate of the relative amounts of expressed sequences in the genome of interest; no cDNA clones or libraries are created. Double stranded cDNA is digested with specific restriction enzymes, adapters are ligated to cDNA fragments, and the resulting fragments are concatenated and sequenced extensively to obtain thousands of 10-20bp sequence “tags”. These tags can then be mapped back to known cDNA sequences and the resulting data is analyzed for relative abundance. Due to their small size, mapping of these tags is quite extensive and a major drawback of this technique. Matsuzaki and colleagues were able to identify human melanoma antigens using SAGE (Matsuzaki et al., 2005). In contrast, DNA microarray analysis is a “closed” technique, meaning one can only detect what has been immobilized before. DNA microarrays are small platforms of glass or silica hosting tens of thousands of single stranded DNA sequences that are well characterized. Labelled RNA populations get hybridized on the genechips and complementary binding results in a detectable signal. Signal intensity gives an approximation of the relative proportion for each labelled sequence and therefore an estimate of gene expression. Disadvantages of the microarray technique comprise high costs and difficulties in detection of low abundance genes. However DNA microarray analysis has become the matter of choice studying differentially expressed genes in a high throughput manner. Between 1996 and 2006, at least 129 separate reports were published using DNA microarrays to investigate various aspects of gene expression in melanoma biology (Hoek, 2007). Biological questions that have been addressed by microarray analyses yielded to understand the transcriptomic changes that lead to the transformation from a melanocyte to a melanoma cell (Talantov et al., 2005; Hoek et al., 2006), as well as, the progression of primary melanoma to distal metastasis (Winnepenninckx et al., 2006; Smith et al., 2005; Jaeger et al., 2007). Closely related issues of disease progression are patient survival and response to treatment. DNA microarray studies have been used as prognostic tools resulting in gene signatures linked to metastasis free survival (Winnepenninckx et al., 2006; Mandruzzato et al., 2006). To characterize

transcriptional changes of metastasis that either responded to immunotherapy or not, microarray analysis was performed. Even though the treatment was unsuccessful a small number of genes were identified that changed significantly upon treatment (Wang et al., 2002). Furthermore transcriptomic analysis of the microenvironment of melanoma metastases revealed an unique immunological signature characterized by the expression of genes associated with natural killer cell and CTL activation (Wang et al., 2004). This immunological signature was not shared by renal cell cancers even though renal carcinomas also show immune-responsiveness, neither other non-immune-responsive cancers, suggesting that this NK, T cell signature might be unique to melanoma.

Spontaneous melanoma regression

Spontaneous regression (SR) is defined as 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) (Everson and Cole, 1966). The phenomenon of spontaneous regression has been observed for more than hundreds of years. An early example, reported in 1742, was a case study of a 35 years old patient with inoperable breast cancer that regressed spontaneously after ulceration and subsequent inflammation. Throughout history the diagnosis of spontaneous regression was not homogenous. Everson and Cole reviewed cases of SR between 1900 and 1964 and identified 176 in total which met the following criteria such as observed and documented histological regression of biopsy proven metastases, radiological documentation of presumptive malignant neoplastic disease and advanced neoplastic disease regression following a therapeutical approach generally deemed ineffective. Challis and Stam reviewed 504 cases of spontaneous regression between 1960 and 1987 which also included lymphomas and leukemias (Challis and Stam, 1990). In 1993, O'Reagan and Hirshberg published 3500 well reported cases of SR which met the criteria of complete biopsy, histological confirmation and adequate disease follow up (O'Regan and Hirshberg, 1993). According to this review the five most common types of neoplasms undergoing SR were renal cell carcinomas, lymphomas and leukemias, neuroblastomas, breast carcinoma and melanoma whereas melanoma demonstrates regression 6 times more often than other malignant neoplasms (Ceballos and Barnhill, 1993). However complete spontaneous regression of advanced melanoma, including metastasis, is extremely rare with only 38 well documented cases (High et al., 2005).

Regression could be more common than reported since it is prone to escape detection (Barnetson and Halliday, 1997). Nevertheless, partial regression is observed more frequently

with 7% to 61% in thin melanoma (Abramova et al., 2002). Clinically, partial regression is characterized by a heterogeneous pigmentation of the tumor site, while on a histopathological level, the process starts with a dense infiltrate of lymphocytes, and ends with fibrosis and/or melanosis within a thickened papillary dermis (Paredes, 2007). 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 (Papac, 1998).

In the case of melanoma, strong evidence exists for an implication of the immune response as mediator for regression. Even though spontaneous regression is rare in humans, larger numbers of CD4⁺ T cells were found in infiltrates of regressing melanomas besides high percentages of Langerhans cells, macrophages and other MHCII expressing cells (Halliday et al., 1995; Byrne and Halliday, 2003). Also, increased T helper 1-cytokine mRNA has been associated with spontaneously regressing primary melanomas (Lowe et al., 1997).

Even patients with metastatic melanoma revealed the presence of spontaneously generated tumor antigen specific (MLANA) CD8⁺ T cells in the tumor microenvironment (Dudley et al., 2002). It has been repeatedly observed that during the rejection phase of immune responsive melanoma patients, NK cell and activated CTL function seem to predominate (Wang et al., 2008). However, the presence of these effector cells does not lead automatically to melanoma regression. Reasons therefore are intrinsic mechanisms that lead to oncogenesis such as autocrine growth signalling, ignorance of growth inhibitory signals, avoidance of cell death, replication without limits, angiogenesis, and avoidance of tumor surveillance (Zitvogel et al., 2006).

Immune escape mechanisms

Cancer cells have the ability to escape innate and adaptive immune responses (cancer immunosurveillance) by either immunoselection (selection of non immunogenic tumor cell variants) or immunosubversion (active suppression of the immune response) (Dunn et al., 2004). Mechanisms of immunosubversion that render the melanoma tumor microenvironment tolerogenic include:

- 1) Loss of major histocompatibility complex (MHC) class I antigens during progression of melanoma cells (Paschen et al., 2003).

- 2) Downregulation of specific molecules, e.g. Fas/CD95 that protect melanomas from T cell mediated apoptosis (Dong et al., 2002).
- 3) Induction of several membrane receptors on the melanoma cell surface that engage inhibitory receptors on activated T cells (PD1/PD1L) (Zha et al., 2004).
- 4) Expression of indoleamine 2, 3-dioxygenase (IDO) by melanoma cells. IDO plays a role in T cell suppression by depleting tryptophan which is needed for T cell development (Fallarino et al., 2002).
- 5) Production of immunosuppressive cytokines (IL10, TGF β 1) by melanoma cells that interfere with T cell activity by induction of regulatory T cells and DC differentiation (Yang and Carbone, 2004).
- 6) Induction of T cell anergy through incomplete activation of T cells (loss of costimulatory molecules such as B7-1 and B7-2). T cell anergy is defined as a reversible state of unresponsiveness in regards to cytolytic activity and the capacity of cytokine production (Denfeld et al., 1995).

Despite these diverse and complex immune escape mechanisms a few patients succeed to regress melanoma spontaneously by overcoming immunosubversion and therefore favouring proper immunosurveillance. Up to date, evidence is missing that could explain a reversal of immune escape rendering in tumor regression.

Animal models of melanoma

Today, several animal models exist to study various aspects of melanoma such as tumorigenesis, genetics, immunology, and therapy (Dooley, 1994). Although neither of these models perfectly matches the genetic, biochemical, and pathological characteristics of human melanoma, each model harbours some value in specific research areas for comparative studies relative to human melanoma.

Animal models of spontaneous melanoma include pigs (see *Pigs in biomedical research*), horses, dogs, fish, and opossum (Ha et al., 2005). In contrast, mice do not show spontaneous melanoma.

Up to 80% of grey horses develop melanomas spontaneously especially during the second half of their life (>15years) without influence of UV radiation. In contrast to humans, equine melanomas are encapsulated and metastasis is retarded or inhibited by yet unknown factors. Equine dermal melanomas in grey horses mainly develop under the tail root, in the anal, perianal and genital regions, perineum, lips and eyelids (Seltenhammer et al., 2004). The high

incidence of late onset melanoma in grey horses might be related with age-related pigment changes (Rieder et al., 2001; Fleury et al., 2000). Melanoma accounts for 3% of all neoplasms in dogs, whereas the majority (56%) of canine melanomas affect the oral cavity only 11% account for cutaneous melanoma, 23% affect the lip, the digits (8%) and 2% other sites like the eye (Goldschmidt, 1994). However cutaneous melanoma occurs more commonly in dogs with heavily pigmented skin such as the miniature Schnautzer and Scottish terrier (Goldschmidt, 1985). The Xiphophorus fish model has been used to study the photobiology and genetics of melanoma (Meierjohann and Schartl, 2006). Although the Xiphophorus fish develops melanomas spontaneously or as a result of UV radiation (Walter and Kazianis, 2001), the tumor histology differs substantially from human melanomas. The opossum *Monodelphis domestica* responds also to UV radiation to induce melanoma (Kusewitt et al., 1991), but the inability to derive inbred strains and few experimental tools limit the utility of this model for genetic and immunological investigations.

Spontaneous development of melanoma in rodents is rare and difficult to initiate. *In vivo* models are either achieved by grafting human skin or reconstructed human skin to nude mice or generating genetically modified mice. Nevertheless, *in vivo* models, especially transgenic models, are relatively expensive and time-consuming, and effects of a gene when overexpressed or downregulated might be compensated by other mechanisms (Beermann, 2006). Regarding cutaneous melanoma, the mouse skin is different from human skin since melanocytes are confined to hair follicles within the dermis. However, the mouse is the most widely used model organism in biomedical research as it is genetically well characterized, has a high availability of genetic, transcriptomic and proteomic tools, and is easily accessible for genetic manipulations (Larue and Beermann, 2007). Over the past 15 years, a number of genetically modified murine models have been generated successfully to study different aspects of melanoma. Especially knock out/in of genes implicated in melanoma signalling (CDK4, H-ras, N-ras, β -catenin) are of great help to understand the molecular complexity of the disease (Larue and Beermann, 2007).

A murine model that mimics metastatic familial human melanoma very closely is the Tyr::N-ras p16INK4A^{-/-} mouse (Ackermann et al., 2005). Transgenic mice develop melanoma at 6 months whereas primary melanomas are melanotic and multifocal, they microinvade the epidermis or epithelium of hair follicles, and disseminate as metastases to lymph nodes, lung, and liver. These mice were generated by injecting a vector that included a human mutant N-RASQ61K sequence under the control of a Tyr promoter, into murine oocytes. Tyr::N-RASQ61K transgenic mice were then mated to p16INK4A deficient mice resulting in N-

RASQ61K overexpression on a p16INK4 negative background. The mutant N-RASQ61K was chosen because 95% of patients with germ line p16INK4A mutations show also mutated N-RAS at codon 61 (Q-Glutamine to K-lysine) (Eskandarpour et al., 2003). To simulate the effects of UV radiation on melanoma development, hepatocyte growth factor/scatter factor (HGF/SF) mice were genetically engineered (Noonan et al., 2000). HGF/SF mice is a multifunctional cytokine that can elicit mitogenic, motogenic and morphogenic responses in a variety of cells, including melanocytes that express the receptor tyrosinase kinase c-Met. The skin of these transgenic mice (MT::HGF), in which a metallothionein-gene promoter forces the overexpression of HGF/SF, has melanocytes in the dermis, epidermis and dermal-epidermal junction, and is thus more akin to human skin (Noonan et al., 2001). In the UV-irradiated HGF/SF mouse model most cutaneous melanomas, arising in HGF/SF mice as a consequence of neonatal UV irradiation, possess a junctional as well as a dermal component, with epidermal interaction, demonstrating a remarkably similarity to a variety of lesions found in human melanoma patients (Noonan et al., 2003). The latest generation of transgenic animals are conditional knockout mice. Conditional knockout mice are obtained by mating mice with floxed (loxP site in target vector) with mice expressing Cre recombinase specifically in melanocytes. In consequence, a knockout is only obtained in melanocytes, whereas all other cell types remain wildtype for expression of the gene of interest. In this way, transgenic mice were produced bearing a tamoxifen-dependent Cre recombinase (Tyr::Cre-ERT2) expression under the control of a murine tyrosinase promoter in order to facilitate targeted spatiotemporally controlled somatic recombination in melanoblasts/melanocytes (Yajima et al., 2006). Cre-ERT2 production was detected in tissues containing melanocytes. After tamoxifen induction at various times during embryogenesis and adulthood in a Cre-responsive reporter mouse strain, genetic recombination was detected in the melanoblasts and melanocytes of the skin. Even though transgenic mouse models are elaborate in their generation and strongly dependent on an induction of melanomagenesis, they have been instrumental in the experimental demonstration of the role of mutations activating the MAPK pathway or affecting the pRb and p53 pathways. They, furthermore, allowed investigations of the role of UV irradiation in animals carrying predetermined genetic alterations. The near future will provide insight into whether genetically modified animals can also be useful for testing novel anti-melanoma therapies, particularly immunogenic treatments, and to what extent they remain valuable and necessary tools for the elucidation of melanomagenesis.

Pigs in biomedical research

Pigs were domesticated from wild boars (*sus scrofa*) approximately 9000 years ago while last shared ancestry with man is dated back to something between 79 and 87 million years (Kumar and Hedges, 1998). It has coevolved with humans for thousands of years and today has economic importance as meat source. The pig is also being used in biomedical research for studies of human disease that may be modelled less well in rodents, including obesity, arthritis, cardiovascular disease and skin and eye conditions (Lunney, 2007). The similarity in size (minipig), physiology, organ development, and disease progression as well as the possibility to obtain large litters make the swine an ideal model (Lunney, 2007). Furthermore comparative analysis of mammalian genomes showed that the pig is genetically closer to human than the mouse (Wernersson et al., 2005). With the swine genome sequence, being now well advanced with ~1.3 million pig entries in the public domain and a 4x genome coverage coming up in early 2009 by the Swine Genome Sequencing Consortium (SGSC), genetic, transcriptomic and proteomic tools are rapidly improving in pig research (Jiang and Rothschild, 2007; Tuggle et al., 2007). With the genetic induction of tumors in pigs, clinical cancer research has now an attractive option to the mouse since porcine organs and tumors allow imaging, radiation and photodynamic therapy (Adam et al., 2007).

In regards to melanoma, pig skin and its melanocyte biology is closer to humans than the mouse. Advantages of the pig for human melanoma research are therefore similar melanocyte biology that results in a similar microenvironment for melanocyte and melanoma development, comparable melanoma pathology, immunological response, and high genetic homology (Dooley, 1994). Worldwide 3 pig breeds (Sinclair, MeLiM, Munich Trolls) have been shown to be a suitable model to study non UV induced melanoma since they develop skin lesions naturally (Millikan et al., 1974; Muller et al., 1995; Horak et al., 1999). The Munich Miniature Swine (MMS) Troll breed, characterized by a high rate of spontaneously occurring congenital or after-birth melanocytic lesions including invasive cutaneous melanomas and metastases, was established at the University of Munich (Germany) and maintained as closed colony since 1986. Founder animals were derived from stock MMS Trolls originally developed from Hanford and Columbian miniature swine (Sambraus, 1987). Studies of the inheritance of melanocytic lesions suggested different modes for nevi and melanomas. For melanoma, a major gene model did not fit and therefore a two or three locus model was supposed (Muller et al., 1995). Melanomas of MMS Troll resemble histologically various types of cutaneous melanomas in humans but with a less aggressive course. MMS Trolls represent a suitable animal model to study melanogenesis, metastasis and melanoma

regression, associated with vitiligo. Also, MMS Trolls provide the opportunity to study a possible participation of endogenous retrovirus in tumor development as recently demonstrated (Dieckhoff et al., 2007). Porcine endogenous retrovirus (PERV) expression was found to be elevated in MMS Troll melanomas when compared to normal skin suggesting an active role in malignant transformation by insertional mutagenesis. Cutaneous malignant melanoma of the Sinclair Swine is highly heritable with about 85% of the pigs showing lesions either shortly after or at birth (Hook, Jr. et al., 1979; Tissot et al., 1987). Tumors and metastases are histopathologically well-characterized and close to the human pathology (Greene et al., 1994a). Some animals die due to widespread disease but the majority regress completely without treatment. Regarding spontaneous regression, the Sinclair swine is the most extensively studied pig model with different hypothesized mechanisms that will be discussed later. Modeling of melanoma inheritance with DNA markers in Sinclair swine, revealed a QTL affecting number of tumors at birth located on *sus scrofa* chromosome 8 (SSC8), whereas initiator loci for melanoma could not be identified (Gomez-Raya et al., 2007). The fact that MeLiM and Sinclair and probably the MMS Troll swine share common ancestors like the Hormel strain might explain their similar behaviour in developing and regressing melanoma (Horak et al., 1999; Millikan et al., 1974; Porter, 1993).

Melanoblastoma bearing Libechov Minipig (MeLiM)

The Melanoblastoma bearing Libechov Minipig (MeLiM) pig strain was created and maintained at the Institute of Animal Physiology and Genetics in Libechov (Czech Republic) and was derived from crossings with several breeds (Hormel, Gottingen, Canadian Landrace, Cornwall, and Vietnamese). MeLiM animals were imported from Czech Republic to France in 1998 where a herd was bred at LREG (CEA-INRA) in Jouy-en-Josas, France.

Within this strain, melanomas appear spontaneously and selective breeding led to a closed stock in which melanoma is inherited and occurs, both *in utero* and postnatally. Porcine melanomas can be classified according human melanoma criteria with an excess of SSM over NM like in human melanoma. The skin lesions are classified into three morphological types. Type 1 lesions are basically flat lesions with about 11.4% of cases in MeLiM, whereas type 2 (15.9%) and type 3 (72.7%) are raised lesions. The macular lesions are variable in size and their color varied from brown to black. Type 2 lesions (Clark level I-IV) (Clark, Jr. et al., 1969) consist of small, heavily pigmented, dome shaped or sharply defined plateau-shaped lesions showing slow growth and absence of bleeding and ulceration. These tumors have

usually no mitotic activity, don't show vascular invasion and never metastasize. Lesions of type 3 (Clark level III-V) are heavily pigmented, large nodular or exophytic tumors. They can be distinguished furthermore by their fast or slow growth rate. Only these lesions metastasize. All pigmented lesions previously described occur without predilection for any anatomical site. Distant metastases are normally not observed in pigs with only lesions of type 1 and/or type 2. However pigs with fast growing type 3 lesions show metastases in lymph nodes and also visceral metastases (11%) located in the lungs, the liver, spleen, gastrointestinal tract, the pancreas and kidneys. Type 3 lesions with a slow growth rate do also show metastatic lymph nodes but less visceral metastases (Vincent-Naulleau et al., 2004).

The development of melanomas in MeLiMs is genetically determined by a multigenic dominant trait with incomplete penetrance as in humans. In a preliminary study, MeLiMs were used to search for melanoma predisposing genes by conducting a genome wide scan on 79 backcross animals (MeLiM x Duroc). Linkage was shown to 3 chromosomal regions, two located on SSC1q25 and one located on SSC2p17 (Geffrotin et al., 2004). SSC1q25 corresponds to the human region HSA9p21 and SSC2p17 is homologous to HSA11p15 and 11q13. To locate additional regions of interest, a follow up study including 331 MeLiM backcross pigs was performed. Pigs were classified according to their clinical and histopathological characteristics. Quantitative trait loci (QTLs) corresponding to the development of melanoma, described in a synthetic trait which combined all phenotypic information were first detected on SSC1, SSC13, SSC15, and SSC17. Next, specific traits corresponding to precise tumor characteristics were studied and revealed QTLs on SSC10 (ulceration), on SSC12 (presence of melanoma at birth), on SSC13 (lesion type), and on SSC16 and SSC17 (number of aggressive melanomas). The recently identified regions are homologous to human chromosomal regions that contain melanoma associated genes such as MITF and CCND1 amongst others, and are now target for a fine mapping study (Du et al., 2007; High and Robinson, 2007). Interestingly, the high penetrance gene CDKN2A was mapped to the SSC1q25 region (Lechalony et al., 2000) but was excluded as major susceptibility gene in this MeLiM family by a haplotype study (Lechalony C. et al., 2003). CDK4 and BRAF did not either appear to be melanoma susceptibility genes in MeLiM but an association was observed with a MeLiM allele for a marker close to the MC1R gene on SSC6p1.5 (Geffrotin et al., 2004).

Spontaneous melanoma regression in MeLiM

Fascinatingly, melanomas do not only develop naturally but also disappear spontaneously in MeLiM pigs. Spontaneous complete tumor regression occurs in 96% of pigs and is characterized by tumor-flattening, -drying and depigmentation (Vincent-Naulleau et al., 2004). This regression process even appears in metastatic melanomas. However complications associated with metastatic disease can lead to up to 4% of morbidity.

Similar to human melanoma regression, the tumor site gets infiltrated by firstly melanophages and then lymphocytes. The biggest difference is yet the early onset of regression in MeLiM minipigs that occurs during childhood (3-6 months after birth) and its efficiency. Furthermore, the lymphocyte infiltration seems to be delayed in pigs compared to humans. Moreover, hair, skin and iris depigmentation is observed in swine melanoma regression (Vincent-Naulleau et al., 2004). This vitiligo-like phenomenon can be explained via the cross-antigenicity that occurs between melanoma cells and normal melanocytes. About 10% of melanoma patients develop vitiligo like patches known as melanoma-associated hypopigmentation (Merimsky et al., 1996). The presence of vitiligo in melanoma patients seems to improve the prognosis by means of effective immunity against the tumor (Oyarbide-Valencia et al., 2006).

Various approaches to identify key mechanisms of spontaneous regression were already performed in the 90's using the Sinclair swine. Terminal differentiation of melanoma cells (Greene et al., 1997) and the loss of telomerase activity resulting in abnormal chromosomal configuration and finally DNA fragmentation (Pathak et al., 2000) were associated with spontaneous regression. Besides these intrinsic mechanisms, evidence was also found for immune mediated mechanisms. Histopathological analysis revealed that regression occurs in two phases in the Sinclair model, whereas the first consists of massive infiltration of pigmented macrophages, the second is characterized by a heavy infiltration of lymphocytes accompanied by a progressive loss of tumor cells (Greene et al., 1994b). Higher percentages of CD8⁺ and MHC II⁺ cells were identified in TIL suspensions than in peripheral blood leukocytes (PBL) regardless of tumor status (Morgan et al., 1996). The percentages of T-lymphocytes co-expressing CD4 and CD8, a subset characteristic for swine, were generally consistent in all TIL and PBL suspensions suggesting a minor role in regression. Another T cell subset which is unique in its elevated levels in PBL, namely the γ/δ T cell population was isolated from peripheral blood of the Sinclair swine (Grimm et al., 1993). Additionally, antibodies were isolated from Sinclair swine sera that recognized human melanoma and melanocyte antigens. The rise in melanoma antibodies in swine preceded or appeared together with tumor regression and depigmentation. Even in a cross of Iberian and Duroc pigs in which

melanomas occurred and regressed spontaneously, an immune response was detected (Perez et al., 2002). However in these pigs, an infiltration of the tumor site by CD79+ B cells and plasma levels of IgG, IgM, IgA were low, whereas pigmented macrophages and CD3+ T cells were detected.

Spontaneous melanoma regression in porcine models seems to be associated with an immune response directed against the tumor. It remains questionable to which extent intrinsic mechanisms render tumor cells either vulnerable for immunodetection and destruction or directly induce apoptosis. Hence the aim of this work was to identify crucial events in early spontaneous regression and put them together with already proposed mechanisms to finally describe regression as a whole. We tackled this task by studying early regressing melanoma on a molecular and cellular level.

CHAPTER 2

Introduction of: “Identification of differentially expressed genes in spontaneously regressing melanoma using the MeLiM Swine Model”

In this initial study, we wanted to gain an overview about genes differentially expressed between growing and early regressing melanoma using Subtractive Suppression Hybridization (SSH). SSH selectively amplifies differentially expressed genes by suppression PCR. Subsequent cloning of SSH products lead to the construction of cDNA libraries whose clones were then sequenced and annotated using BLAST search against mammalian species. SSH is a very elaborate technique to detect differentially expressed genes and therefore often limited by number of replicates. Anyhow, at the time this method was chosen, as porcine DNA chips were not commercially available and SSH allows also the detection of unknown genes. As we were interested in the detection of initial transcriptomic changes that could lead to regression, we chose a tumor at the beginning of regression which was identified by clinical aspects and excised at day 52 after birth (d+52) and compared its transcriptome to a growing tumor (d+8). The 2 animals that served as tumor donors were not of the same family. Furthermore to distinguish between melanoma cell specific and tumor microenvironment related transcriptional changes, we also performed SSH analysis of cell cultures isolated from the used melanoma tissue. Because SSH analysis does not provide an expression signal, we introduced the criteria of redundancy comparable to a cut off in fold change. That means we focused our further analysis only on repetitive genes that we were at least present twice per SSH library. Following this strategy we were able to identify 25 unique genes that were present at least twice in regressing melanoma tissue and 74 unique genes that were present at least twice in cell cultures from regressing melanoma. In addition, we identified 57 unique genes present at least twice in growing melanoma tissue and 31 unique genes present at least twice in cell cultures from growing melanoma. Only little overlap was detected between genes expressed in melanoma tissue and its corresponding cell culture for the growing and for the regressing stage. Explanations therefore could be the culture conditions that changed the expression profiles of melanoma cells or simply that melanoma cells isolated from regressive tumor tissue were lost or transformed into a different cells during culturing. Therefore we concentrated our functional analysis only on genes identified in growing and regressing melanoma tissue. Overall, we were able to assign functional classes to genes which were up- or downregulated during early regression and compared them to human melanoma expression

studies. Finally, after verification of differential gene expression by qRT-PCR we focused on 2 genes and confirmed their regulation even on a protein level.

Comment on Suppression Subtractive Hybridization (SSH) method

Since the method section in the following article is really condensed for word count reason only, a detailed description of SSH is presented below:

cDNAs were generated using SMART PCR cDNA synthesis kit (Clontech). 2µg of total RNA was used as a template in first-strand cDNA synthesis according to manufacturer's protocol (Fig. 7). Double-strand cDNA was further amplified by PCR before processing to cDNA subtraction. SSH was performed using Clontech PCR-Select cDNA Subtraction Kit following the manufacturer's protocol. Briefly, tester and driver cDNAs were digested with the restriction enzyme RsaI. The tester fragments were then divided into two portions and each ligated with a different adaptor, which resulted in two populations of tester-adaptor cDNA. An excess of the driver cDNA was added to each ligated tester cDNA for the first hybridization. The mixed tester-driver samples were heat-denatured and allowed to reanneal. The two hybridization samples were then mixed together with new driver cDNA. Second round of hybridization was performed followed by two rounds of nested PCR reactions resulting in exponential amplification of the differentially expressed sequences. The PCR products were analysed on agarose gel electrophoresis. Subtraction efficiency was tested using gene-specific primers and PCR by comparing of abundance the cDNAs before and after SSH. The subtracted cDNAs were cloned into the pGEM-T Easy vector system (Promega) to construct the 4 subtractive cDNA libraries:

1. SCAW: represents genes over expressed in early regressive melanoma tissue since it was used as a tester and progressive melanoma tissue as a driver.
2. SCBE: represents genes over expressed in growing melanoma, as in this case regressive melanoma tissue served as driver.
3. SCBF: genes over expressed in cell cultures isolated from regressive melanoma (forward subtraction).
4. SCAX library, genes over expressed in cell cultures isolated from progressive melanoma (reverse subtraction).

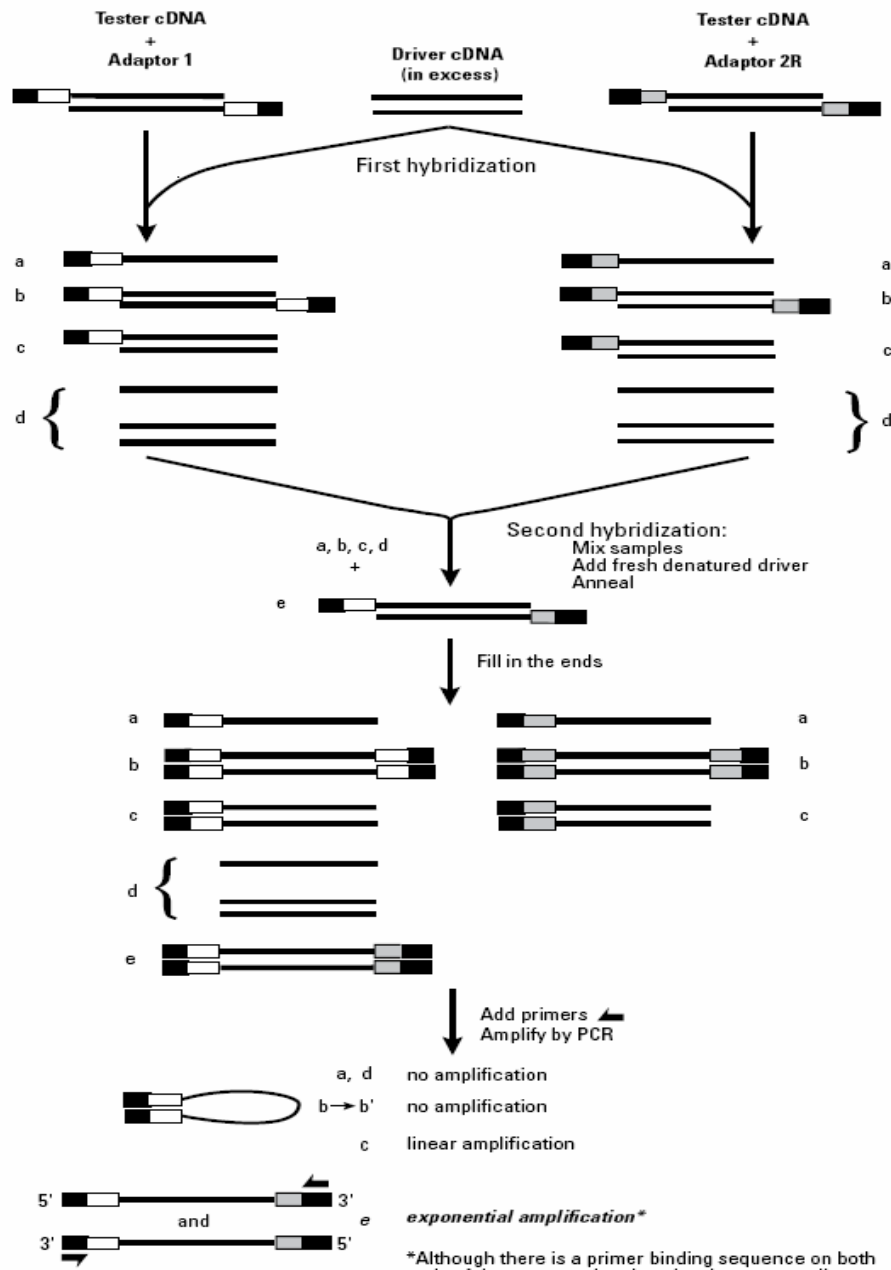


Fig. 7: SSH method (adapted from PCR-Select™ cDNA Subtraction Kit User Manual, Clontech).

cDNA from two populations (tester and driver) get digested with RSA1. The tester cDNA is then divided in 2 portions of which each is then ligated with an certain cDNA adaptor. In the first hybridization, the driver is added in excess to each tester population. Annealing generates molecules such as a, b, c, d whereas molecule (a) is a differentially expressed sequence, since no homologous sequence of the tester population annealed. During the second hybridization, the two primary hybridization samples are mixed together. Now, only the remaining equalized and subtracted single strand tester cDNA can reassociate and for new hybrids (e). These new hybrids are double strand molecules with different ends- After filling the ends by DNA polymerase the type e molecules the differentially expressed tester sequences- have different annealing sites for nested primers on their 5' and 3' ends. The entire population of molecules is then subjected to PCR to amplify the desired differentially expressed sequences.

Paper I: Identification of differentially expressed genes in spontaneously regressing melanoma using the MeLiM Swine Model

Rambow, F.^{1*}, Malek, O.^{1,2*}, Geffrotin C.¹, Leplat J.-J.¹, Bouet S.¹, Hugot K.³, Bevilacqua C.⁴, Horak V.², Vincent-Naulleau S.¹

¹CEA, DSV, IRCM, SREIT, Laboratoire de Radiobiologie et d'Etude du Génome, Jouy-en-Josas, F-78352 France; INRA, DGA, Laboratoire de Radiobiologie et d'Etude du Génome, Jouy-en-Josas, F-78352 France.

²Institute of Animal Physiology and Genetics, 27721 Libechov, Czech Republic.

³INRA, DGA, Centre de Ressources Biologiques Gadie, Jouy en Josas, F-78352 France.

⁴INRA, DGA, Plateau d'instrumentation et de compétences en transcriptomique, Jouy-en-Josas, F-78352 France.

* These authors contributed equally to this work.

Short title: Differential gene expression in swine melanoma

Corresponding author: F. Rambow

Telephone: +33-1-34-65-28-05

Fax: +33-1-34-65-29-64

E-mail: florian.rambow@jouy.inra.fr

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Summary

Partial and some few cases of complete spontaneous regression have been observed in cutaneous melanoma patients but little is known about the molecular mechanisms involved. The Melanoblastoma bearing Libechov Minipig (MeLiM) is a suitable animal model to study the phenomenon of spontaneous regression since MeLiM pigs exhibit naturally occurring melanomas which regress completely 6 months after birth. In this study, we used suppressive subtractive hybridization (SSH) to identify molecular determinants of melanoma regression within swine melanoma tissues and melanoma cell cultures. Several markers involved in cell - adhesion, -communication, -motility, signal transduction, negative regulation of cell proliferation, transport and immune response were identified that correlated with melanoma regression whereas the main genes involved in melanin synthesis showed a strong downregulation. For the most differentially expressed genes, we validated the results obtained by SSH with qRT-PCR and with immunohistochemistry for some of them (CD9, MITF, RARRES1). Most notable, for the first time in melanoma, we identified the Retinoic Acid Responder 1 gene (RARRES1) as a main actor of the regression process in melanoma. This first gene expression study in swine melanoma regression, may contribute to the finding of new therapeutic targets for human melanoma treatment.

Key words: Swine Melanoma, Suppression Subtractive Hybridization, Differential gene expression, Real-time PCR, Tumour regression

A) INTRODUCTION

Cutaneous melanoma is the most severe form of skin cancer due to its aggressive clinical behaviour, therapeutic resistance and predisposition for late metastasis (Soengas and Lowe, 2003). Spontaneous partial regression is a common finding within human primary cutaneous melanoma, and it occurs in 10-35% of cases (Blessing and McLaren, 1992). Spontaneous regression refers to the complete or partial disappearance of malignant tumour mass without treatment or as a consequence of an inadequate one (Everson and Cole, 1966). Clinically, partial regression is mainly characterized by a heterogeneous pigmentation of the tumour site. On a histopathological level, the process starts with a dense infiltrate of lymphocytes, and ends with fibrosis and/or melanosis within a thickened papillary dermis (Paredes, 2007). Contrastingly, complete spontaneous regression of cutaneous melanoma is a rarely occurring phenomenon, with only 38 well-documented cases in the MEDLINE-cited English based literature, whereas 37 of 38 showed metastasis (High et al., 2005). However the phenomenon of complete regression of advanced melanoma seems to be underestimated (Barnetson and Halliday, 1997). The diagnosis of complete regression remains difficult since different phenomena play together such as aggregation of melanin and melanophages, complete loss of neoplastic melanocytes and fibrosis (Paredes, 2007). Different mechanisms such as immune recognition, virus infection of tumour cells, cytokine induced apoptosis, high levels of stress-induced steroids, hypoxic conditions, telomeric breaks, and gene mutations are being discussed as mediators of regression (Papac, 1998). The elucidation of mechanisms involved in melanoma regression is of valuable interest in order to find more specific therapeutical ways to treat the disease. The MeLiM Minipigs have been described as a suitable animal model to study melanoma regression besides the Sinclair Swine and the Munich Miniature Troll Swine (Greene, Jr. et al., 1997; Pathak et al., 2000; Horak et al., 1999). Spontaneous complete tumour regression occurs in 94% of pigs and is characterized by tumour-flattening, -drying and depigmentation and appearance of a dense infiltrate of highly pigmented cells on histological levels (Vincent-Naulleau et al., 2004). Similar to human melanoma regression, the tumour site gets infiltrated by firstly melanophages and then lymphocytes. The biggest difference yet is the early onset of regression in MeLiM minipigs that occurs during childhood and its efficiency. Furthermore, the lymphocyte infiltration seems to be delayed in pigs compared to human. The molecular analysis of tumours, such as melanoma, has benefited significantly from various differential gene expression techniques. Different developmental stages of human cutaneous melanoma have been characterized by their expression profiles (Haqq et al., 2005; Smith et al., 2005).

The objective of this work is to characterize molecular events involved in the phenomenon of regression. Therefore we used a highly effective method for differential gene expression analysis, namely subtractive suppression hybridization (SSH), which has been developed for the generation of subtracted cDNA libraries (Diatchenko et al., 1996). We constructed 4 subtractive cDNA libraries of growing and early regressing melanoma tissues as well as cultured cells isolated from growing and early regressing melanoma tissues. After cloning and subsequent sequencing, the identified genes were clustered by Gene Ontology means. To confirm differential expression of SSH identified genes which play a central role in gene networks, we performed quantitative real time RT-PCR and immunohistochemistry.

B) RESULTS

1) Characterization of the subtracted libraries

SSH was used to screen for differentially expressed genes at the very beginning of melanoma regression in tumour cells and the surrounding stroma. Consequently, two SSH experiments were performed which resulted in four subtracted libraries such as SCAW and SCBE representing respectively up and downregulated genes in regressive melanoma tissues and SCBF and SCAX representing up and downregulated genes in cells isolated from regressive tumours. To characterize each tissue derived transcriptomic profile, 1152 (3 x 384 well plates) clones from each tissue library were sequenced, 1853 of which resulted in exploitable gene sequences. These sequences were aligned systematically against databases as described in materials and methods. The remaining sequences were either empty clones or unreadable sequences. 486 different genes could be identified and were classified according to their redundancy. This demonstrated that a majority of genes (74.27%) were present only once in the complete set of sequenced clones, while 17.07% were present at least twice. These proportions reflect the normalizing effect of the SSH procedure. 8.66% of transcripts were found in both tissue libraries and were discarded since they reflect false positives (Table Ia). Regarding the cell derived libraries (SCAX, SCBF), 2016 clones were sequenced, 923 of which resulted in exploitable gene sequences. About 70.97% of the genes were present once in each library, whereas 15.97% were found at least twice and 13.06% of false positives were discarded (Table Ib). To minimize the possibility of false positives, we focused our work on transcripts found at least twice in each library, knowing that this consideration does not replace false positive control for this high throughput technique. Thus, our SSH-gene lists underly no FDR control and are limited in their exploitation. The construction of multiple SSH libraries with biological replicates for each population was technically impossible, therefore the level of biological variation could not be addressed on the SSH level. As a consequence we tested differentially expressed genes on $n = 8$ progressive and $n = 8$ regressive melanomas by qRT-PCR.

Table I a. Up and downregulated genes in regressive melanoma tissue.

Upregulated genes (SCAW)				Downregulated genes (SCBE)			
Swiss-Prot	Gene symbol	Redundancy	Protein Name	Swiss-Prot	Gene symbol	Redundancy	Protein Name
P60709	ACTB	3	beta cytoskeletal actin	P68137	ACTA1	7	Actin, alpha skeletal muscle
Q69DK8	C1S	2	complement component 1, s subcomponent	Q9GKJ6	BCHE	2	Butyrylcholinesterase
Q6RVA9	CAV1	2	caveolin 1	P62158	CALM1	2	Calmodulin
Q8WMQ3	CD9	7	CD9 antigen	P09803	CDH1	2	Epithelial-cadherin
Q29549	CLU	2	Clusterin	P63167	DNCL1	2	Dynein light chain 1, cytoplasmic
O46392	COL1A2	8	Collagen alpha-2(I) chain	P68103	EEF1A1	6	Elongation factor 1-alpha 1
P00450	CP	2	Ceruloplasmin	P23588	EIF4B	3	Eukaryotic translation initiation factor 4B
Q9GLE3	CTSK	2	Cathepsin K	P14324	FDPS	3	Farnesyl pyrophosphate synthetase
O15523	DDX3Y	2	ATP-dependent RNA helicase	Q96M96	FGD4	2	FYVE, RhoGEF and PH domain-containing protein 4
Q6GWX0	DDX4	2	Probable ATP-dependent RNA helicase	P08059	GPI	2	Glucose-6-phosphate isomerase
Q2NL08	DDX55	2	ATP-dependent RNA helicase	Q14956	GNPMB	2	Transmembrane glycoprotein NMB
Q9GKQ8	DSG1	2	Desmoglein-1	Q8MJ14	GPX1	2	Glutathione peroxidase 1
P54852	EMP3	2	Epithelial membrane protein 3	O75367	H2AFY	2	Core histone macro-H2A.1
P19130	FTH1	6	Ferritin heavy chain	P02067	HBB	15	Hemoglobin subunit beta
Q07666	KHDRBS1	2	KH domain-containing, RNA-binding, signal transduction-associated protein 1	P55209	NAP1L1	3	Nucleosome assembly protein 1-like 1
P53715	LCN1	2	Lipocalin-1	P07910	HNRPC	3	Heterogeneous nuclear ribonucleoproteins C1/C2
Q14766	LTBP1	2	Latent-transforming growth factor beta-binding protein,	Q5E9H7	HSD17B12	2	Estradiol 17-beta-dehydrogenase 12

			isoform 1L				
P21692	MMP1	3	Matrix metalloproteinase-1	Q5EEL9	HUMML C2B	10	Skeletal muscle myosin regulatory light chain 2
Q96M98	PACRG	2	Parkin coregulated gene protein	P26889	IL1B	2	Interleukin-1 beta
P97570	PLA2G6	2	85 kDa calcium-independent phospholipase A2	P08548	LIN1	2	LINE-1 reverse transcriptase homolog
P49792	RANBP2	2	E3 SUMO-protein ligase	Q5U5L8	LIN9	2	Lin-9 homolog
P49788	RARRES1	4	Retinoic acid receptor responder protein 1	Q9UNF1	MAGED2	3	Melanoma-associated antigen D2
Q28999	SAT1	2	Diamine acetyltransferase 1	Q86YT6	MIB1	2	E3 ubiquitin-protein ligase
Q95274	TMSB4	2	Thymosin beta-4	O75030	MITF	3	Microphthalmia-associated transcription factor
P19320	VCAM1	3	Vascular cell adhesion protein 1	Q16655	MLANA	3	Melanoma antigen recognized by T-cells 1
				Q8MHY0	MLC2V	2	Myosin regulatory light chain ventricular isoform
				Q5EFJ2	MLC3F	6	Alkali myosin light chain 3 A2 catalytic
				P02602	MYL1	2	Myosin light chain 1, skeletal muscle isoform
				P60662	MYL6	4	Myosin light polypeptide 6
				P29269	MYL9	13	Myosin regulatory light chain 2, smooth muscle isoform
				P02547	NEFL	2	Neurofilament light polypeptide
				Q96E58	NUSAP1	2	Nucleolar and spindle associated protein 1
				Q92576	PHF3	2	PHD finger protein 3
				O15460	P4HA2	2	Prolyl 4-hydroxylase subunit alpha-2
				Q64L94	PSME1	2	Proteasome activator complex subunit 1

Q12923	PTPN13	4	Tyrosine-protein phosphatase non-receptor type 13
Q8TEU7	RAPGEF6	2	Rap guanine nucleotide exchange factor 6
P84100	RPL19	3	60S ribosomal protein L19
P62831	RPL23	2	60S ribosomal protein L23
Q5R8K6	RPL35A	3	60S ribosomal protein L35a
Q29375	RPL7A	3	60S ribosomal protein L7a
P32969	RPL9	4	60S ribosomal protein L9
P62280	RPS11	3	40S ribosomal protein S11
Q99P72	RTN4	2	Reticulon-4
Q8NBX0	SCCPDH	2	Probable saccharopine dehydrogenase
Q3T0M8	SDBCAG84	2	Endoplasmic reticulum-Golgi intermediate compartment protein 3
O00560	SDCBP	3	Syndecan-binding protein 1
Q06154	SILV	6	Melanocyte protein Pmel 17
Q8TCJ2	SIMP	2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B
Q71RS6	Slc24a5	4	Sodium/potassium/calcium exchanger 5
P16949	STMN1	2	Stathmin
Q9ULW0	TPX2	2	Targeting protein for Xklp2
Q4R1H3	TYR	3	Tyrosinase
Q4R1H2	TYRP1	6	Tyrosinase related protein 1
Q9H2Y7	ZFP106	2	Zinc finger protein 106 homolog

Q7TMA2	Zfp503	2	Zinc-finger protein NOLZ1
Q9H582	ZNF644	2	Zinc finger prot 644

Table I b. Up and downregulated genes in regressive melanoma cell culture.

Upregulated genes (SCBF)				Downregulated genes (SCAX)			
Swiss-Prot	Gene symbol	Redun dancy	Protein Name	Swiss-Prot	Gene symbol	Redun dancy	Protein Name
O95573	ACSL3	3	Long-chain-fatty-acid-CoA ligase 3	Q99541	ADRP	2	Adipophilin
P08243	ASNS	2	Asparagine synthetase	O43687	AKAP7	2	A-kinase anchor protein 7 isoforms alpha and beta
O75882	ATRN	2	Attractin	P36543	ATP6V1E1	2	Vacuolar ATP synthase subunit E 1
Q96RK4	BBS4	3	Bardet-Biedl syndrome 4 protein	P48643	CCT5	2	T-complex protein 1 subunit epsilon
P54687	BCAT1	2	Branched-chain-amino-acid aminotransferase, cytosolic	P50990	CCT8	2	T-complex protein 1 subunit theta
Q9NX08	COMMD8	2	COMM domain-containing protein 8	Q9NY35	CLDND1	3	Claudin domain-containing protein 1
O14548	COX7A2L	2	Cytochrome c oxidase subunit VIIa-related protein, mitochondrial	Q9Y678	COPG	2	Coatomer subunit gamma
Q80TP8	Csde1	2	MKIAA0885 protein	Q3B8N6	CPEB2	2	Cytoplasmic polyadenylation element-binding protein 2
P43235	CTSK	6	Cathepsin K	P55060	CSE1L	2	Exportin-2
P61803	DAD1	2	Defender against cell death 1	O00571	DDX3X	2	ATP-dependent RNA helicase
O15075	DCAMKL1	13	Serine/threonine-protein kinase DCLK1	P06744	GPI	2	Glucose-6-phosphate isomerase
P35638	DDIT3	5	DNA damage-inducible transcript 3	Q5BJF3	MAGED2	2	Melanoma-associated antigen D2
P09622	DLD	2	Dihydrolipoyl dehydrogenase, mitochondrial	Q9UBU8	MORF4L1	2	Mortality factor 4-like protein 1
P24534	EEF1B	2	Elongation factor 1-beta	P19404	NDUFV2	2	NADH dehydrogenase [ubiquinone] flavoprotein 2,

							mitochondrial
P78344	EIF4G2	2	Eukaryotic translation initiation factor 4 gamma 2	Q92570	NR4A3	2	Orphan nuclear receptor
O43432	EIF4G3	2	Eukaryotic translation initiation factor 4 gamma 3	Q8NDX5	PHC3	2	Polyhomeotic-like protein 3
P21415	ENV	2	Envelope glycoprotein	Q4J6C3	PREPL	3	Prolyl endopeptidase-like variant D
P02792	FTL	2	Ferritin light chain	P34925	RYK	2	Tyrosine-protein kinase
Q9H3K2	GHITM	3	Growth hormone-inducible transmembrane protein	P49591	SARS	2	Seryl-tRNA synthetase, cytoplasmic
Q14956	GPNMB	3	Transmembrane glycoprotein NMB	P40967	SILV	2	Melanocyte protein Pmel 17
O60381	HBP1	3	HMG box-containing protein 1	Q71RS6	SLC24A5	3	Sodium/potassium/calcium exchanger 5
P61978	HNRPK	3	Heterogeneous nuclear ribonucleoprotein K	O43808	SLC25A17	2	Peroxisomal membrane protein PMP34
P07900	HSPCA	2	Heat shock protein HSP 90-alpha	Q96QE2	SLC2A13	2	Proton myo-inositol cotransporter
P41252	IARS	3	Isoleucyl-tRNA synthetase, cytoplasmic	Q13573	SNW1	2	SNW domain-containing protein 1
P10145	IL8	6	Interleukin-8	P46977	Stt3a	3	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 3A
P05106	ITGB3	2	Integrin beta-3	Q9BXU0	TEX12	2	Testis-expressed sequence 12 protein
P31025	LCN1	2	Lipocalin-1	P62328	Tmsb4x	2	Thymosin beta-4
Q9Y5X9	LIPG	2	Endothelial lipase	O95801	TTC4	3	Tetratricopeptide repeat protein 4
P46821	MAP1B	3	Microtubule-associated protein 1B	P61088	UBE2N	3	Ubiquitin-conjugating enzyme E2 N
P58340	MLF1	2	Myeloid leukemia factor 1	P18206	Vcl	2	Vinculin
Q8KRT7	MRX1	2	Site-specific recombinase	P08670	VIM	2	Vimentin
P13995	MTDC	2	Bifunctional methylenetetrahydrofolate dehydrogenase/cyclo				

			hydrolase, mitochondrial
Q9P2K5	MYEF2	4	Myelin expression factor 2
Q9U6A0	Nckx30C	4	Sodium/potassium/cal cium exchanger
Q9NPE3	Nola3	2	H/ACA ribonucleoprotein complex subunit 3
P55809	OXCT1	2	Succinyl-CoA:3- ketoacid-coenzyme A transferase 1, mitochondrial
P62937	PPIA	5	Peptidyl-prolyl cis- trans isomerase A
Q5RBU7	PRCP	2	Lysosomal Pro-X carboxypeptidase
Q9Y617	PSAT1	21	Phosphoserine aminotransferase
O75884	Rbbp9	2	Retinoblastoma- binding protein 9
Q9Y3B8	REXO2	2	Oligoribonuclease, mitochondrial
P18621	RPL17	5	60S ribosomal protein L17
P84098	Rpl19	2	60S ribosomal protein L19
P62750	Rpl23a	2	60S ribosomal protein L23a
P61254	Rpl26	2	60S ribosomal protein L26
Q02878	RPL6	3	60S ribosomal protein L6
P32969	RPL9	2	60S ribosomal protein L9
P05388	RPLP0	4	60S acidic ribosomal protein P0
P82912	RPS11	7	28S ribosomal protein S11, mitochondrial
P25398	RPS12	2	40S ribosomal protein S12
P42677	Rps27	2	40S ribosomal protein S27
P23396	RPS3	4	40S ribosomal protein S3
P62753	Rps6	6	40S ribosomal protein

			S6
O83241	rpsK	2	30S ribosomal protein S11
P06703	S100A6	2	Protein S100-A6
P02735	SAA2	2	Serum amyloid A protein
P13521	SCG2	2	Secretogranin-2
O00560	SDCBP	6	Syndecan-binding protein 1
P43005	SLC1A1	2	Excitatory amino acid transporter 3
Q99624	SLC38A3	2	System N amino acid transporter 1
P42765	THIM	2	3-ketoacyl-CoA thiolase, mitochondrial
O60220	TIMM8A	2	Mitochondrial import inner membrane translocase subunit Tim8 A
O75674	TOM1L1	2	TOM1-like protein 1
Q92547	TOPBP1	2	DNA topoisomerase 2-binding protein 1
P13693	TPT1	2	Translationally-controlled tumour protein
P10155	TROVE2	2	60 kDa SS-A/Ro ribonucleoprotein
P41732	TSPAN7	3	Tetraspanin-7
P17643	TYRP1	10	Tyrosinase-related protein 1
P22314	UBE1	2	Ubiquitin-activating enzyme E1
Q13404	Ube2v1	2	Ubiquitin-conjugating enzyme E2 variant 1
Q01831	XPC	2	DNA-repair protein complementing XP-C cells
Q9NQW7	XPNPEP1	2	Xaa-Pro aminopeptidase 1
P67809	Ybx1	2	Nuclease sensitive element-binding protein 1
O95218	ZNF265	2	Zinc finger Ran-binding domain-containing protein 2

2) Analysis of sequenced cDNAs

Subtracted cDNAs were divided into four groups after sequencing (Figure 1). The first class contains sequences which have a high homology with swine, rat, bovine, mouse or human ESTs. More ESTs were identified in the tissue library representing overexpressed sequences (30.70%) and cell library representing downregulated sequences at early regression (20.64%) than in the tissue library representing downregulated sequences and cell library representing overexpressed sequences at early regression (6.62 and 6.93%), respectively. The second class consists of cDNAs similar to mitochondrial genes, which were highly present in the library of downregulated sequences in regressive tissue (12.28%) compared to its counterpart (1.69%) but in equal quantities in both cells libraries (2.06% in SCAX and 4.04% in SCBF). The third class comprises ribosomal RNAs which were over represented in the library containing upregulated sequences in regressive tissue (15.36%) compared to its counterpart (1.32%) and nearly absent in cells libraries. The last class were cDNAs showing high homology towards known genes. They represented 52.70 to 87.60% in our four libraries and were classified by their number of occurrence.

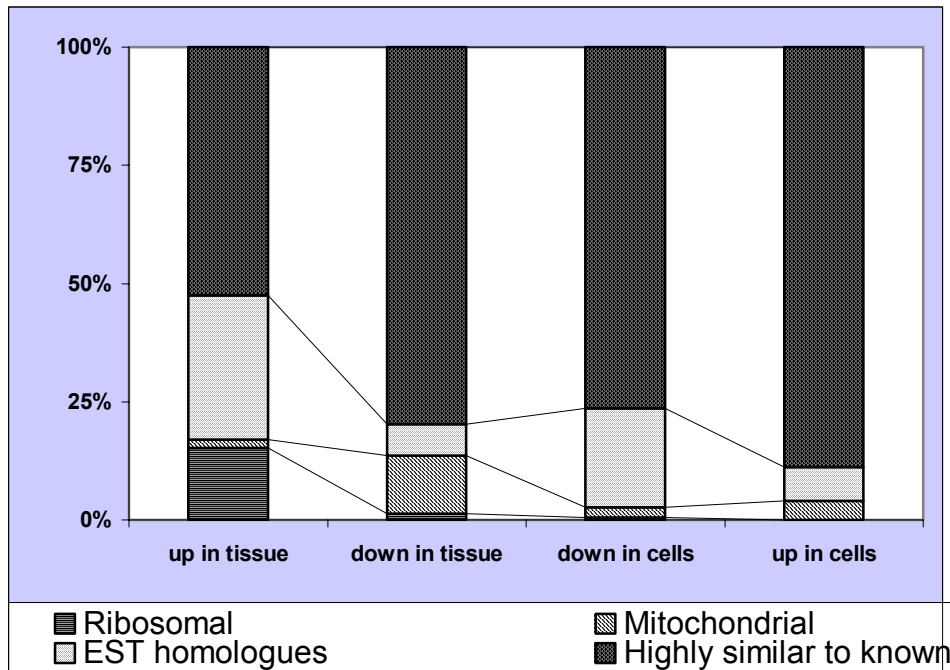


Fig.1. Classification of differentially expressed cDNAs

The figure represents the percentage of known and unknown genes found in the four subtracted cDNA libraries: genes upregulated in melanoma tissue at the beginning of regression (SCAW); genes downregulated in melanoma tissue at the beginning of regression (SCBE); genes downregulated in melanoma cells isolated from regressive tissue (SCAX); genes upregulated in melanoma cells isolated from regressive tissue (SCBF).

3) Analysis of redundant transcripts in each library

The list of redundant genes is shown in Table I a,b. They were classified by Gene Ontology means, taking into account their predicted protein function. The functional category «biological process» was subdivided into 15 subclasses, whereas molecular function and cellular component were split into 3 subclasses (Figure 2). Upregulated genes in regression were overrepresented in classes such as cell -adhesion, -communication, signal transduction, cell -differentiation, -death, -motility, negative regulation of cell proliferation, transport and immune response. Genes involved in cellular and melanin biosynthesis were found to be downregulated. Furthermore, genes with unknown biological process were also overrepresented in the phase of early tumour regression. In the cell libraries, genes upregulated genes at early regression (SCBF) were enriched in the classes of cell –adhesion, -differentiation, -cycle, cellular metabolism and biosynthesis and negative regulation of cell proliferation. Nearly no difference between the cell libraries was detectable regarding classes such as response to stimulus, cell motility and signal transduction. The GO category «molecular function», showed an enrichment of genes upregulated in regressive tissue regarding the subclass catalytic activity. Genes identified from the library of downregulated genes in regressive tissue showed a similar «molecular function» profile. In the «cellular component» category, we observed a stronger expression of genes involved in extracellular space in the library of upregulated tissue genes but a downregulation of those involved in intracellular region.

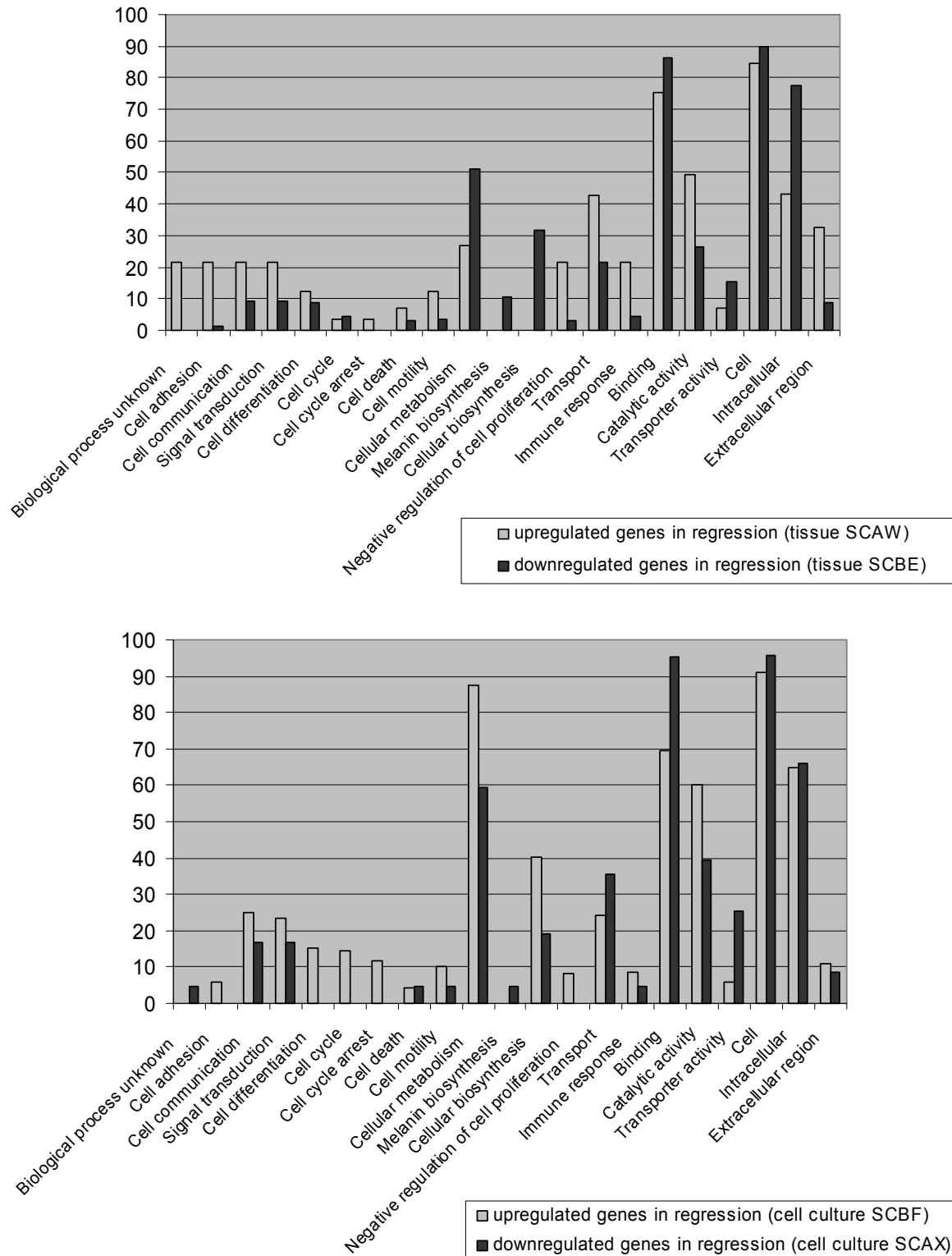
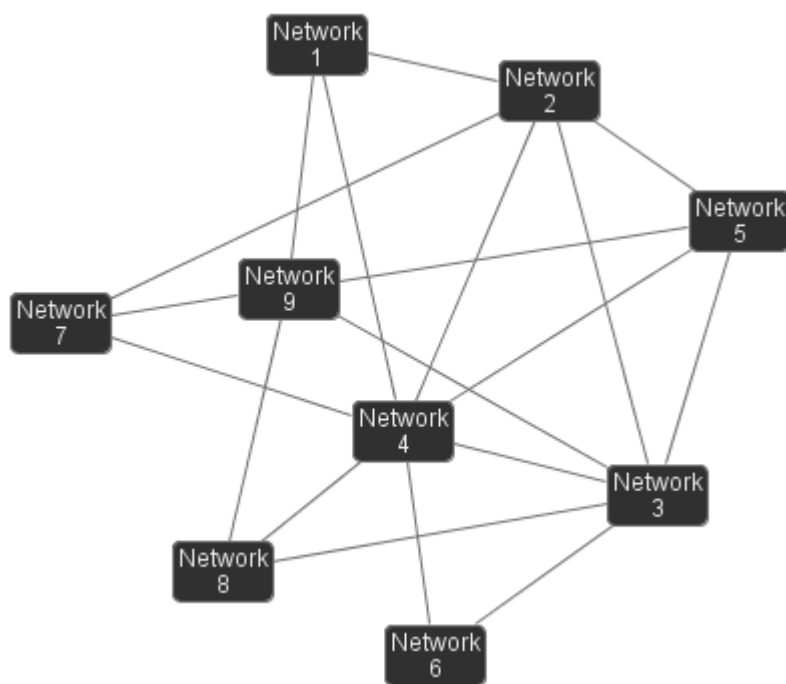


Fig. 2. Functional comparison of differentially expressed genes identified by SSH in melanoma regression. The 4 SSH libraries were functionally analyzed such as up/downregulated genes in regressive melanoma tissue (SCAW and SCBE) and up/downregulated genes in cell cultures derived from regressive melanoma (SCBF and SCAX). Genes were classified in different classes by Gene Ontology analysis (X axis) and the percentage of expression (Y axis) is calculated by taking into account the redundancy of each hit in each class on the number of total hits in the Gene Ontology category.

4) Gene Network Analysis

To demonstrate biological interaction of SSH identified genes in spontaneously regressing melanoma, we carried out Ingenuity™ pathway analysis (IPA). 135 out of 188 uploaded genes were eligible for network analysis and clustered into 9 interacting score-significant networks (Figure 3). The number of interacting partners for each network ranged from 2 (network 6) up to 7 (network 4). Network 4 which harbours genes of functions such as cancer, cell death, and reproductive system disease interacts with 7 different networks and plays therefore a central role. Among all networks, network 1, showing top gene functions involved in Cancer, Cellular Movement, and Cellular Assembly and Organization, reached the highest significance level (score 67 with 35 identified genes). To demonstrate distinct gene interactions we zoomed into the overall network with focus on highly redundant genes and highlighted direct and indirect gene interactions (Figure 4). This analysis revealed genes relevant to melanocytic biology such as microphthalmia-associated transcription factor (MITF) as a central gene, melan-A (MLANA), silver homolog (SILV), tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), and solute carrier family 24 member 5 (SLC24A5). These 6 genes as well as cathepsin K (CTSK), integrin beta 3 (ITGB3), and E-cadherin (CDH1) were up regulated in progressive melanoma. Other SSH identified genes in the network were up regulated in regressive melanoma samples comprised CD9 molecule (CD9), matrix-metalloproteinase 1 (MMP1), retinoic acid receptor responder 1 (RARRES1), collagen type I alpha 2 (COL1A2), and beta actin (ACTB). These genes are mainly involved in cell migration, -adhesion and -invasion processes. Three genes which showed up in the network as uncoloured symbols were not identified by SSH but linked to our expression data and introduced by the IPA algorithm such as transcription factor binding to IGHM enhancer 3 (TFE3), tumour protein p53 (TP53), and interferon gamma (IFNG).



	<i>Top functions</i>	<i>score</i>	<i>N° involved genes</i>
Network 1	Cancer, Cellular Movement, Cellular Assembly and Organisation	67	35
Network 2	Protein Synthesis, Cancer, Cell Death	22	17
Network 3	DNA Replication, Recombination, and Repair, Small Molecule Biochemistry, Cellular Function and Maintenance	20	16
Network 4	Cancer, Cell Death, Reproductive System Disease	17	14
Network 5	Cell Cycle, Cell to Cell Signalling and Interaction, Cellular Assembly and Organisation	17	14
Network 6	Cellular Development, Gene Expression, Cellular Growth and Proliferation	15	13
Network 7	Cellular Morphology, Cancer, Genetic Disorder	15	13
Network 8	Cell to Cell Signalling and Interaction, Haematological System Development and Function, Immune Response	14	12
Network 9	Protein Synthesis, Lipid Metabolism, Molecular Transport	5	6

Fig. 3. Ingenuity based gene network analysis of SSH subtracted genes

Functional clustering of differentially expressed genes in tissue libraries (SCBE, SCAW) and cell libraries (SCBF, SCAX) reveals 9 interrelated networks.

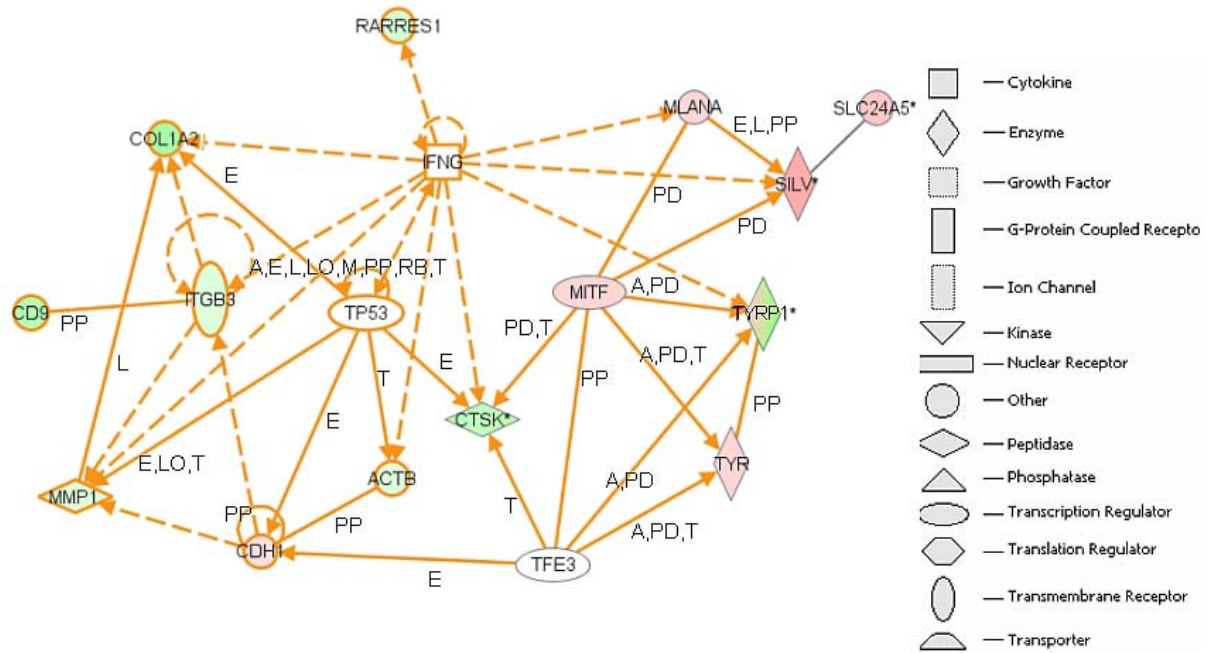


Fig. 4. Gene Network of differential gene expression between growing and regressing melanoma

Ingenuity™ pathway analysis of SSH identified genes revealed a network including melanoma and skin pigmentation relevant genes such as MITF, TYRP1, TYR, SILV, and SLC24A5 which were found in progressive melanoma SSH libraries. Whereas genes like CD9, MMP1, RARRES1, COL1A2, ACTB were over expressed in SSH libraries generated from regressive melanoma samples.

White symbols: genes which were not identified by SSH, Green symbols: genes identified in SSH libraries (SCAW and SCBF) generated from regressive melanoma samples, Red symbols: genes identified in SSH libraries (SCBE and SCAX) generated from growing melanoma samples. Colour intensity of symbols is proportional to number of gene occurrence in corresponding SSH library. A: Activation, E: Expression, T: Transcription, L: Proteolysis, PP: Protein-Protein binding, PD: Protein-DNA binding, LO: Localization, M: Biochemical Modification. Dashed line: indirect interaction, Bold line: direct interaction.

5) Confirmation of differential expression of selected genes by real time quantitative RT-PCR

We chose 14 genes for qRT-PCR confirmation (Table II) to conduct multiple comparisons of regressing (n=8) vs. progressing (n=8) melanomas to evaluate the confidence of our SSH results. 6 genes (CAV1, CLU, COL1A2, EMP3, RARRES1, CD9) upregulated in regressive melanoma tissue and 8 (BCHE, CDH1, MAGED2, NUSAP1, TYR, SLC24A5, TYRP1, MITF) downregulated in regressive melanoma tissue were chosen. qRT-PCR analysis and subsequent unpaired t test analysis including Welch's correction for different populations variances revealed 10 differentially expressed ($p < 0.05$) genes between progressive and regressive melanoma samples (Figure 5). 4 genes did not meet the criteria of significance, even though two of them (TYRP1, COL1A2) showed the same trend in SSH and qRT-PCR. CAV1 and EMP3 expression were almost equal between progressive and regressive melanomas. However, we confirmed the results for the original tumours used for SSH regarding EMP3 expression which seems to be in this case a particularity for these 2 tumours.

RARRES1, CD9, and CLU were all significantly upregulated during regression. CLU showed the most dramatic change with a 11.5 times overexpression during regression. RARRES1 was about 6.6 times higher in regressive versus progressive melanoma tissue and about 4 times higher when comparing regressive melanoma tissue and normal skin (data not shown). The pigmentation related genes such as MITF, TYR, SLC24A5 were all downregulated during early regression by a fold change (FC) of 2. Furthermore CDH1, NUSAP1 and BCHE which were reported to play a role in human tumour progression were downregulated during MeLiM regression. After applying the Benjamini Hochberg correction to control the false discovery rate (FDR=0.05) for multiple testing, 9 genes were significantly differentially expressed between progressive and regressive melanoma (BCHE, CDH1, CLU, MAGED2, NUSAP1, TYR, RARRES1, MITF, SLC24A5).

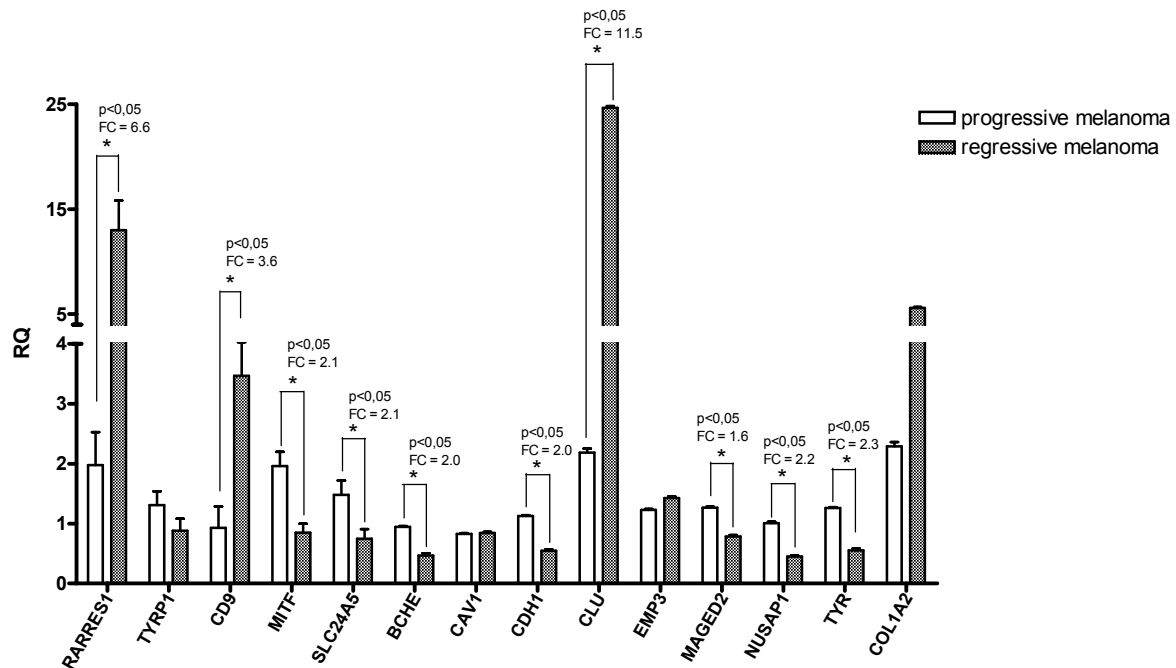


Fig. 5. qRT-PCR analysis of SSH identified genes.

Relative gene expression was calculated using the comparative $\Delta\Delta C_t$ method. RQ (Y-axis) represents the relative expression level compared to the reference sample. Histograms represent the expression-means of n=8 regressive and n=8 progressive tumour samples whereas error bars represent the standard deviation. Significance of differential gene expression was calculated using the t test including welch's correction p<0.05. Fold change (FC) represents average expression ratio of regression over progression or vice versa.

Table II. Selected genes for qRT-PCR

<i>Gene Symbol</i>	<i>Primers</i>	<i>Clone number</i>	<i>Exploitable Base pairs after sequencing</i>	<i>Homology e-value</i>	<i>Species</i>	<i>Library</i>	<i>Genbank accession</i>	<i>Human Swiss prot accession</i>
RARRES1	F: 5'-GCCCAGAAAAGCTAAATCCCAG-3' R: 5'-TGAAGCAACTTAGCACCCCCT-3'	scaw0005.d.04.c_5.1	285	0	Human	SCAW	BM659515	P49788
		scaw0005.i.09.c_5.1	287	0				
		scaw0005.m.06.c_5.1	553	0				
		scaw0006.a.14.c_5.1	275	4,00E-63				
SLC24A5	F: 5'-TTTATGGCAGCCGGTAGTTCAG-3' R: 5'-GCAGACCCAAGAATGGTGCTAA-3'	scbe0001.b.23.b_5.1	425	0	Cow	SCBE	XM_606255	Q71RS6
		scbe0001.p.16.b_5.1	474	0	Human		NM_205850	
		scbe0002.p.03.b_5.1	634	0	Human		NM_205850	
		scbe0003.e.22.b_5.1	286	4,00E-116	Cow		XM_606255	
TYRP1	F: 5'-GATGTCGCTCAGTGCTTGAA-3' R: 5'-ATTGTGAAGGCTTCGGACAGC-3'	scbe0002.p.12.b_5.1	431	0	Pig	SCBE	NM_001025226	P17643
		scbe0003.h.07.b_5.1	431	0				
		scbe0002.j.02.b_5.1	292	1E-158				
		scbe0003.f.21.b_5.1	366	4E-141				
CD9	F: 5'-TGCCATTGAAATAGCCGCG-3' R: 5'-TCCTTGCCCTTCAGCTTGTTG-3'	scaw0001.e.06.c_5.1	297	9,00E-156	Pig	SCAW	NM_214006	P21926
		scaw0002.c.15.c_5.1	449	0				
		scaw0002.f.10.c_5.1	445	0				
		scaw0002.j.02.c_5.1	297	4,00E-154				
		scaw0002.k.01.c_5.1	261	4,00E-149				
		scaw0002.m.08.c_5.1	452	0				
		scaw0002.n.15.c_5.1	285	9,00E-156				

MITF	F: 5'-GAACCTACCCTGGAGAACTGCA-3' R: 5'-TGTTGAAGGTGATGGTGCCA-3'	scbe0001.f.16.b_5.1 scbe0001.p.06.b_5.1 scbe0002.o.14.b_5.1	708 563 472	0 1E-130 6E-113	Human	SCBE	NM_198178.1, NM_198177.1, NM_006722.1, NM_198158.1, NM_198159.1, NM_000248.2	O75030
EMP3	F: 5'-AATGTCAGCGAGAATGGCTGG-3' R: 5'-CGCATGGTGACAGTTGGAACA-3'	scaw0001.f.05.c_5.1 scaw0002.m.14.c_5.1	104 104	5,00E-49 5,00E-49	Human	SCAW	CB469406 CB469406	P54852
CAV1	F: 5'-AGACTCGGAGGGACATCTCTACAC-3' R: 5'-CACTTGCTTCTCGTTCATTTCCCT-3'	scaw0005.d.06.c_5.1 scaw0002.c.18.c_5.1	426 242	1E-141 1E-57	Pig	SCAW	BM083169 BM083169	Q6RVA9
TYR	F: 5'-GCCTTGGCATCGACTCTTCTT-3' R: 5'-CACAAATTTTCTGCATCTCGCC-3'	scbe0003.a.23 scbe0001.b.06 scbe0003.o.24	533 639 136	0 0 1E-52	Pig	SCBE	NM_001025212 NM_001025212 NM_001025212	Q4R1H3
MAGED2	F: 5'-AACGACCAAGGACTCTCCCAAG-3' R: 5'-CGTCCCCAAAAAGTGAGTGATG-3'	scbe0001.f.13 scbe0002.i.01 scbe0003.e.08	408 324 120	0 2E-155 5E-54	Human	SCBE	CK465059 NM_014599 NM_014599	Q5BJF3
CLU	F: 5'-AAGATGCTTAACACGTCCTCCC-3' R: 5'-ACCGTGGTGACCTGGAGATAGT-3'	scaw0001.l.01.c_5.1 scaw0002.d.22.c_5.1	253 292	0 1,00E-162	Pig	SCAW	BM658671 BM658671	Q29549
COL1A2	F: 5'-CAATGGTGGTACCCAGTTTGAA-3' R: 5'-GCAATGGTAGGTGATGTTTTGG-3'	scaw0001.o.21.c_5.1 scaw0001.h.22.c_5.1 scaw0002.m.03.c_5.1 scaw0005.p.01.c_5.1 scaw0005.f.02.c_5.1 scaw0001.o.11.c_5.1 scaw0006.g.21.c_5.1	562 307 307 404 507 383 507	0 1,00E-154 1,00E-154 1,00E-151 1,00E-129 1,00E-92 2,00E-76	Dog	SCAW	BM190694 BM190694 BM190694 BM190694 BM190694 BM190694 J03464	O46392

		scaw0002.m.07.c_5.1	189	1,00E-74			BM190694	
CDH1	F: 5'-GTAATGATGTGGCACCAACCCT-3' R: 5'-GCTGCCCTCAGGTTTTCATCAA-3'	scbe0001.j.24 scbe0001.p.20	591 541	0 0	Mouse	SCBE	BM659150 BM659150	P09803
NUSAP1	F: 5'-TTCGCCATGATTGTGTCCTC-3' R: 5'-TTGTCTGCCCTGAGATTAGCC-3'	scbe0002.h.05 scbe0002.a.09	287 317	1E-146 1E-72	Pig Cow	SCBE	BQ603089 XM_869910	Q96E58
BCHE	F: 5'-TTGTCTTCGGTTTCCTCTGGAA-3' R: 5'-TTGAAGACCGGCCATTTGTACT-3'	scbe0003.n.19 scbe0002.f.19	406 571	0 0	Pig	SCBE	AF222914 CF179086	Q9GKJ6

6) Immunohistochemistry

We confirmed the differential gene expression of CD9, MITF, and RARRES1 on the protein level by immunohistochemistry. CD9 was over expressed in regressive melanoma samples as suggested by SSH and qRT-PCR. Mainly highly pigmented cells which infiltrate melanocytic tumours during the early regression phase were shown to be CD9 positive (Figure 6: B2 and B3). In progressive melanoma, small pigmented tumour cells were not at all or weakly stained whereas sparse highly pigmented cells showed high CD9 expression (Figure 6: A2 and A3). CD9 is also expressed by endothelial cells and keratinocytes. For MITF and RARRES1 detection we used antibodies directed against the human protein showing crossreactivity with their swine homologues. The MITF transcription factor is highly expressed in the nuclei of pigmented melanoma cells during tumour progression. Contrastingly, highly pigmented cells in regressive melanoma show a weak signal (Figure 7), suggesting a downregulation of MITF protein expression during swine melanoma regression. In swine, RARRES1 is expressed ubiquitously in skin; fibroblasts, keratinocytes and melanocytes show a nuclear or perinuclear staining (data not shown). In growing melanoma tissue, all tumour cells show a weak signal. However, upon regression we observed the highest expression of the protein by highly pigmented cells infiltrating regressive tumour area (Figure 7).

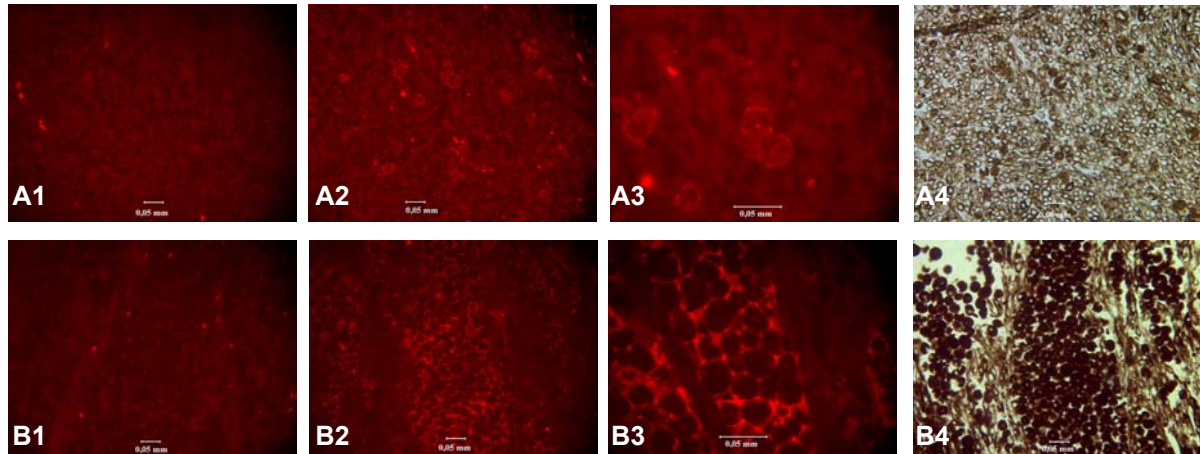


Fig.6. Comparison of the expression levels of CD9 antigen in swine melanoma tissue between growing stage (A) and regression (B).

Analysis was conducted on 5 growing and 5 regressive tumours, whereas one representative for each tumour stage is demonstrated. Paraffin sections of growing (A) and regressive (B) cutaneous melanomas were subjected to immunohistochemical analysis with anti-CD9 antibody. The immunoreactivity was visualised by Alexa Fluor® 555. Figures show negative control (1), CD9 staining at original magnification x400 (2), x1000 (3) and paraffin-embedded tissue sections without staining (4) (original magnification x400) (bar = 50µm). CD9 antigen is higher expressed in regressive than in growing melanomas.

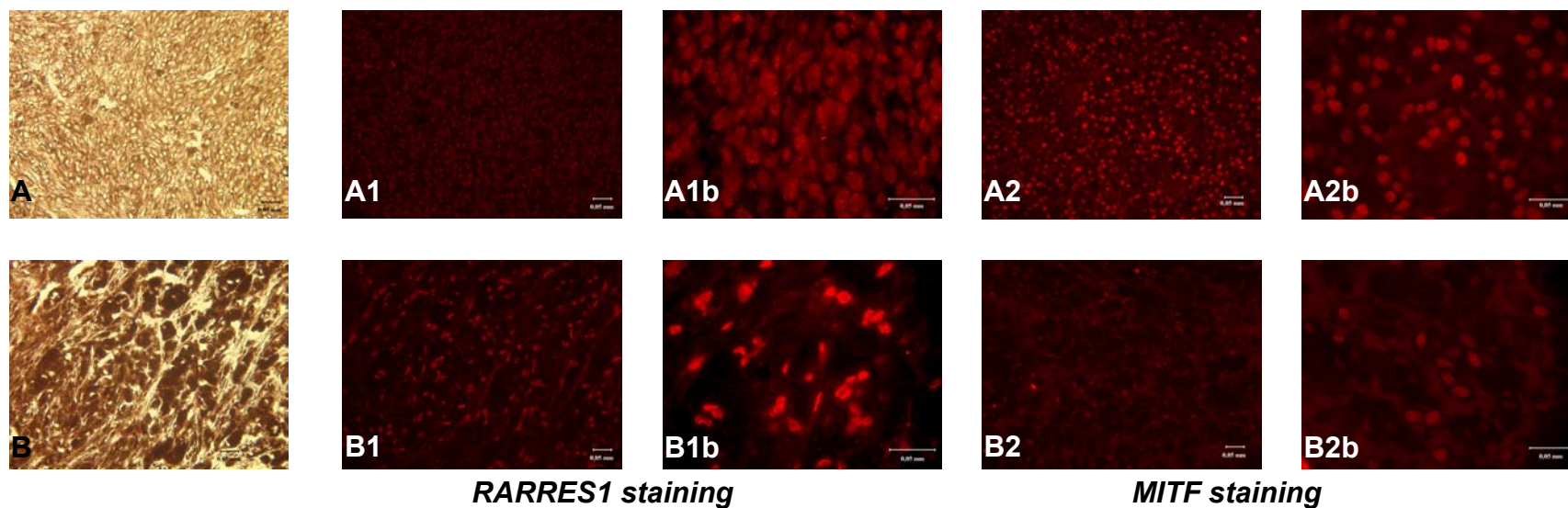


Fig.7. Comparison of the expression levels of RARRES1 and MITF antigens in swine melanoma tissue between growing stage (A) and regression (B).

Paraffin sections of growing (A) and regressive (B) cutaneous melanomas were subjected to immunohistochemical analysis with anti RARRES1 (1) or anti MITF (2) antibodies. The immunoreactivity was visualised by Alexa Fluor® 555. Figures show paraffin-embedded tissue sections without staining (original magnification x400) (A or B), RARRES1 staining at original magnification x400 (1a), x1000 (1b) or MITF staining at original magnification x400 (2a), x1000 (2b)) (bar = 50 µm). RARRES1 antigen is weakly expressed in growing tumours (A1a,b) but it is strongly expressed by highly pigmented cells infiltrating the tumour area during the regression process (B1a,b). MITF is strongly expressed in progressive swine melanoma (A2a,b) but downregulated at the regressive stage (B2 a,b).

7) Gene list comparison

To compare human melanoma expression profiles with those of pigs, we checked our SSH gene lists against the gene list of Hoek et al 2006, knowing that the statistical value of our list was compromised due to the lack of biological replicates for SSH analysis. The reference gene list contained 223 cohort specific genes that were the outcome of an intersection of 3 different microarray analyses dealing with tissue and cell cultures derived from metastatic and in situ melanomas. The reference gene list was shown to be tightly linked with a strong metastatic potential. Our SSH-SCBE library gene list, derived from progressive melanoma tissue, contained 57 genes that were overexpressed during melanoma progression and showed an overlap of 8 genes (GPNMB, MITF, MLANA, SDCBP, SILV, TYR, TYRP1, ZFP106) with the reference. The probability of at least 8 genes being selected by chance alone as well as their having appropriate expression patterns was calculated to be $<4.8 \times 10^{-8}$. Significant downregulation of MITF and TYR during regression was confirmed by qRT-PCR. When comparing our SSH gene list of upregulated genes in regressive melanoma tissue (SCAW), we found no overlap with the reference gene list. Furthermore only one gene, namely SILV was found in common between the reference gene list and downregulated genes in cell cultures derived from regressive melanoma (SCAX). The comparison of SSH-identified upregulated genes in cell cultures derived from regressive melanoma (SCBF) with the 223 reference genes revealed an overlap of 6 genes (GPNMB, IL8, MAP1B, MYEF2, SDCBP, TYRP1). The probability of at least 6 genes being selected by chance alone was calculated to be $<1.12 \times 10^{-4}$. We expected these genes actually being downregulated during regression since they are linked with a high metastatic potential. 3 of them (GPNMB, SDCBP, TYRP1) were shown to be downregulated in regressive melanoma tissue and contrastingly they were identified as upregulated in cell cultures derived from regressive melanoma tissue. A possible explanation could be that the cell cultures derived from a regressive tumour still contained highly metastatic melanoma cells that remained throughout culturing. Extracting melanoma cells for a culturing at a very early stage of regression and the fact that the SSH libraries were constructed from only one sample at only one time point could be the source of this contradictory result.

C) DISCUSSION

This work describes the first global analysis of differential gene expression in swine melanoma models. The precise objective was to investigate the molecular mechanisms inducing the tumour regression process. To further differentiate the transcripts, expressed by tumour cells or by the stroma, we compared the subtractive libraries generated from melanoma tissues and from isolated cell cultures. Confirmation of the results obtained from SSH was performed for some relevant genes by quantitative real time PCR and immunohistochemistry. A major limitation of SSH analysis is the fact that generally only two samples can be compared. Therefore our resulting SSH gene lists were compromised in terms of statistical confidence, since no biological replicates and false discovery rate control were considered. We focused our work on genes found multiple times within a library which indicates a higher native occurrence of those transcripts, knowing that this indication was only true for those original SSH-samples. To address issues of biological variance we tested 14 SSH-identified genes by qRT-PCR for differential expression in 8 growing and 8 regressing melanomas. The n=8 biological samples were derived from 8 different animals. After statistical analysis, we were able to validate 9 of 14 genes that were significantly differentially expressed between progressive and regressive melanomas.

In detail, comparing tissue and cell libraries we observed very few common genes. We found only 2 common transcripts at the beginning of regression (SCBF and SCAW): cathepsin K (CTSK), a proteinase involved in bone and extracellular matrix remodelling which is also expressed in human breast and prostate cancer, where it could contribute to tumour invasiveness (Rapa et al., 2006) and the lipocalin1 (LCN1) gene which functions as a physiologic protection factor of epithelia *in vivo* (Fluckinger et al., 2004). We found also four common genes in growing stage (SCAX and SCBE): GPI which has been already found in a wide spectrum of malignancies and associated with cancer progression and apoptosis resistance (Tsutsumi et al., 2003), the melanoma antigen D2 gene (MAGED2) previously identified to be up regulated in several cancer tissues (Kidd et al., 2006), and two genes involved in pigmentation SILV (Du et al., 2003) and SLC24A5 (Lamason et al., 2005). A hypothesis for the low number of common genes between tissue and cell libraries could be the cell culture conditions which significantly altered the gene expression. A study comparing gene expression profiles of 60 tumour cell lines with the corresponding *in vivo* tumours and normal tissues further supports the current findings (Sandberg and Ernberg, 2005). It demonstrated that gene expression was more similar between normal and tumour tissues, than

between tumour tissues and corresponding cell lines. Nevertheless, we found some common functions between cell culture and tumour tissue for the same stage. This was mainly true for the beginning of regression: the biological process groups of cell adhesion, cell communication, cell cycle arrest, cell differentiation, cell motility and negative regulation of cell proliferation and the molecular function group of catalytic activity are up regulated in the regressive tumour and the corresponding cell line. In growing melanoma, only the melanin synthesis process and the molecular function groups of binding and transporter activity are common between cells and tissue. Some biological functions appear to be more up regulated in tissue than in cell culture such as cell adhesion and immune response, but they could be supported mainly by the stroma. This fact could also explain the stronger expression of genes localised in the extracellular space. So the big difference in transcriptomic analysis between tissue and cell cultures could be explained by the loss of stroma associated cells within cell culture. Considering also the bias induced by the different culture conditions, we focused our work mainly on results obtained from tumour tissue libraries (SCAW and SCBE).

We found several transcripts that were expressed during swine growing melanoma but down regulated or lost in regression. Most of them were already described in human melanoma or other cancers and are involved in cell cycle or tumour growth (CALM1, FDPS, hNRP, NUSAP1, SDBCAG4, SDCBP, STMN1, TPX2), cell motility (ACTA1, DNCL1) and anti-apoptosis (GPI, GPX1, PTPN13). For example, we found 3 times SDCBP (mda-9/Syntenin) which is over expressed in multiple human cancers as melanomas and which functions as a positive regulator of melanoma metastasis (Boukerche et al., 2005); Cadherin 1 (CDH1) is also presented 2 times in SCBE library and was over expressed in primary melanomas and lost with melanoma invasion and metastasis potential (Bonitsis et al., 2006). We found also overexpression of genes involved in pigmentation and considered as major melanoma markers (MITF, MLANA, SILV, TYR, TYRP1, TYRP2, SLC24A5) (Lamason et al., 2005). Comparison of our 4 gene lists derived from SSH with of human melanoma expression studies identified interesting overlaps. Even though our SSH gene lists for up and downregulated genes in early regression of melanoma tissue and cell culture were derived only from one sample and time point each, we used them as an indicator for differential gene expression during melanoma regression. We found 8 genes in common with the 223 gene list of Hoek et al. These 223 genes were shown to be tightly associated with a strong metastatic potential. Interestingly, these 8 genes (GPNMB, MITF, MLANA, SDCBP, SILV, TYR, TYRP1, ZFP106) were downregulated at early melanoma regression in MeLiM pigs as indicated by SSH. At the same time that would mean that these common genes were

upregulated in porcine melanoma progression and therefore in coherence with human melanoma. In addition, upregulation of TYR and MITF in porcine progressive melanoma were validated by qRT-PCR analysis. The probability to select at least 8 genes by random chance alone was calculated to be $<4.8 \times 10^{-8}$, suggesting the MeLiM to be an accurate model to study melanoma biology. Furthermore, 5 (MITF, MLANA, SILV, TYR, TYRP1) of the 8 overlapping genes were according to Hoek et al cohort specific genes, characteristic for a high proliferative and weak metastatic potential. Genes overexpressed at early regression showed no overlap with the reference gene list as expected since no regressive melanoma samples were included in their studies. This suggests also that the SSH gene list of upregulated genes in melanoma regression was unique. When comparing the SSH-gene lists of our cell cultures we found 6 genes overlapping with the reference list. These 6 genes (GPNMB, IL8, MAP1B, MYEF2, SDCBP, TYRP1) were associated with high metastatic potential according to the reference but contrastingly were found to be upregulated in cell cultures derived from regressive melanoma. In detail, actually 3 genes were already identified to be downregulated in regressive melanoma tissue such as the glycoprotein GPNMB, which shows homology to the melanocyte specific protein PMEL17/SILV, syntenin (SDCBP) and tyrosinase-related protein 1 (TYRP1). A possible explanation could be that the cell cultures derived from an early stage regressive tumour still contained highly metastatic melanoma cells that remained throughout cell culture. Extracting melanoma cells for a culturing at a very early stage of regression and the fact that the SSH libraries were constructed from only sample at only one time point could be the source of this contradictory result. These results, obtained from growing swine melanoma, are in agreement with the corresponding human malignancy and confirm similarities on a molecular level between our swine model and human melanoma. This has already been suggested on a clinical, histological, and genetic level (Du et al., 2007; Vincent-Naulleau et al., 2004).

In our swine melanoma model, MITF appears to be a potential key player. This transcription factor is implicated in development and survival of melanocytes and in the regulation of the expression of major melanogenic proteins such as tyrosinase and tyrosinase-related proteins (Goding, 2000). The role of MITF in melanoma remains relatively controversial in literature; it could be amplified in 10-20% of melanoma and appears as a dominant oncogene involved in stimulation of tumour cells, growth, and survival (Garraway et al., 2005). However, Lekmine et coll. (Lekmine et al., 2007) demonstrated recently by transfection of aggressive melanoma cell lines with the melanocyte-specific MITF-M isoform, that MITF is involved in melanoma differentiation and induces a less aggressive phenotype. MITF-M regulates cell-

cycle progression by inducing p21 and p27 expression, two negative regulators of cyclin-dependent kinases. Interestingly, low levels of melanoma markers such as MLANA and MITF in human melanoma, correlate with poor prognosis whereas higher levels of these antigens represent a more differentiated state and better prognosis (Du et al., 2003). Swine melanomas are highly pigmented and we confirmed by qRT-PCR and immunohistochemistry a high expression of MITF in growing tumours. Furthermore, at the very beginning of regression we observed a drastic downregulation of the genes involved in pigmentation.

We identified earlier a genomic region on swine chromosome 13 which is linked to melanoma susceptibility and whose human counterpart, localised on HSA3p14, contains the MITF candidate gene (Du et al., 2007). Hypothetically, MITF could also play a dual role in our model while its upregulation might lead to regression by terminal differentiation but it appears also as a main candidate gene in swine melanoma induction.

Sequencing of SCAW clones lead to the identification of interesting genes in the early phase of regression. We identified less genes differentially expressed in regressive tissue than in growing melanoma probably because of lower RNA integrity. To detect preliminary events of melanoma regression, we chose an early regression stage. At this time, the tumour begins to dry, and histologically we observed nests of highly pigmented cells in the tumour area (Vincent-Naulleau et al., 2004). In parallel swine melanoma exhibits lymph node metastasis potential (Boisgard et al., 2003) at this time. This could explain why a mixture of genes involved in cell cycle progression (RANBP2, TMSB4), cell growth and proliferation (DDX3X, FTH1), tumour invasiveness (CLU, CP, CTSK, COL1A2, MMP1, VCAM1) was found in the SCAW library, as these functions are useful for proliferation, invasion and metastasis. On the contrary, we found also genes involved in tumour suppression (CAV1, EMP3), reduction of motility (CD9), inhibition of cell growth (SAT), cell cycle arrest and apoptosis (KHDRBS1, RARRES1) hence genes involved in suppressing malignant transformation. Additionally, some of these genes serve dual functions, depending on the cancer type. Their exact function in our model remains elusive. These dual genes comprise *CAV1* (Williams and Lisanti, 2005) which can act as tumour suppressor or oncogene, *CP* (Kwok and Richardson, 2002) which plays a role in anti tumour defense or metastasis development, and clusterin (CLU). Clusterin (CLU) showed the highest upregulation by qRT-PCR during melanoma regression. It is a pleiotrophic heterodimeric glycoprotein that is expressed in various tissue and tumor types (Trougakos and Gonos, 2002). A nuclear form of CLU protein (nCLU) was found to be pro-apoptotic, while a secretory form (sCLU) pro-survival (Shannan et al., 2006a). Both forms are implicated in various cell functions,

including DNA repair, cell cycle regulation, and apoptotic cell death. CLU expression has been associated with tumorigenesis and the progression of various malignancies. In response to DNA damage, cell survival can be enhanced by activation of DNA repair mechanisms, while simultaneously stimulating energy-expensive cell cycle checkpoints that delay the cell cycle progression to allow more time for DNA repair (Shannan et al., 2006b). It has been found in most physiological fluids and implicated to play a role in diverse physiological processes, such as sperm maturation, cholesterol transport, tissue remodelling, radiation response, cell-cell and cell-matrix interactions, and promotion or inhibition of apoptosis. Recently, CLU was shown to be expressed in primary and metastatic melanomas but only in a minority of them, whereas desmoplastic melanomas were more commonly represented (Busam et al., 2006). In our model a proapoptotic role of clusterin could have an effect on the regression process and could be an interested target to follow.

We also observed an upregulation of genes coding for ribosomal proteins at early regression. This phenomenon has already been described during tumour metastasis which requires activation of protein synthesis (Cui et al., 2005). Research on spontaneous melanoma regression in humans has focused mainly on immunological mechanisms such as T cell-mediated cytotoxicity (High et al., 2005). In contrary to human melanoma, tumour infiltrating lymphocytes (TILs) invade the swine tumour area at an advanced stage of regression and are therefore absent or in low rate at the beginning of regression when morphologically, tumours begin to dry (Vincent-Naulleau et al., 2004). In the regressive tissue, we observed an upregulation of genes involved in a non specific immune response: the complement component 1 (C1S), the ferritin, heavy polypeptide 1 (FTH1), LCN1 (Fluckinger et al., 2004). Some epidemiological studies have demonstrated also a reduced risk of human melanoma after infection or vaccination with BCG and variola (High et al., 2005). Hence, we can suspect that enhanced overall immunosurveillance may be helpful in aborting neoplasia. We are currently conducting studies to characterise the role of the immune system in porcine melanoma regression.

More interestingly, is the upregulation of genes involved in negative regulation of cell proliferation and cell cycle arrest very early in the regression process. Two major genes such as CD9 and RARRES1 seem to be highly relevant for the melanoma regression process in swine. CD9 belongs to the transmembrane 4 superfamily (TM4SF) so called tetraspanins. Although the biological functions of tetraspanins remain elusive, some experiments have suggested their involvement in the regulation of cell development, proliferation, activation and motility (Berditchevski and Odintsova, 1999; Funakoshi et al., 2003; Longo et al., 2001).

The expression of many members of this superfamily is altered in different types of human cancer. Generally, they get down regulated in metastatic tumours (Funakoshi et al., 2003; Longo et al., 2001; Mischiati et al., 2006; Si and Hersey, 1993). CD9, CD63 or CD82 are considered to be metastasis-inhibitory factors (Miyake et al., 2000). Recently, Mischiati and coll. (Mischiati et al., 2006) have shown that CD9 is expressed in all human nevi, but lost in 71.4% of primary melanomas and half of metastatic lesions. In our model, CD9 had a low expression in primary melanomas but was over expressed at the beginning of regression, mostly on the surface of highly pigmented cells. Consistent with a protective role of the tetraspanin CD9 molecule against invasion, its upregulation at the beginning of regression could contribute to reduced motility of tumour cells and also to the inhibition of their propensity to migrate outside their primary location.

Another gene that showed a significant overexpression in regression after qRT-PCR analysis is RARRES1. It is a retinoic acid receptor-responsive gene that was originally isolated by a subtraction hybridization approach from skin and whose expression is increased by stimulation with synthetic retinoid tazarotene (Nagpal et al., 1996). Relatively high levels of RARRES1 mRNA were found to be expressed in normal tissues as prostate, heart, lung, liver, colon and small intestine (Jing et al., 2002) but a loss or reduced expression was detected in several types of cancer (Jing et al., 2002; Mizuiri et al., 2005; Takai et al., 2005; Wu et al., 2006; Youssef et al., 2004; Zirn et al., 2005). In malignant tissues, expression of RARRES1 progressively declined with a concomitant decrease in tumour differentiation and increase in disease progression (Wu et al., 2006). This is the first time that this gene is reported in melanoma tissue and this observation was confirmed on the protein level. Youssef and coll. (Youssef et al., 2004) showed that RARRES1 silencing was correlated with RARRES1 promoter methylation in several types of malignant tissues. The restoration of RARRES1 expression strongly inhibits cellular proliferation and mediates differentiation of normal and cancer tissues (Takai et al., 2005; Wu et al., 2006; Youssef et al., 2004; Zirn et al., 2005), two events that may support a tumour suppressor role for RARRES1. It is a potential transmembrane protein which contains a putative hyaluronic acid binding motif and might function as a cell adhesion molecule whose expression on the cell surface could lead to a better cell to cell contact and reduced proliferation (Nagpal et al., 1996). Retinoids have been found to inhibit growth acute in promyelocytic leukaemia (APL) and to induce differentiation in a variety of cancer cell lines (Wang et al., 2004). Treatment of B16 melanoma cells with all-trans-retinoic acid results in inhibition of proliferation and induction of differentiation characterised by increased melanin production, arrest in G1 phase, loss of anchorage-

independent growth, and acquisition of cell surface nerve growth factor receptors (Desai et al., 2000). In our swine melanoma model, regression is linked to the appearance of highly pigmented cells which were suspected to be terminally differentiated melanoma cells in the Sinclair model (Greene, Jr. et al., 1997) rather than pigment laden histiocytes. We confirmed in this study the expression of MITF in the nuclei of these cells. The exact molecular mechanisms leading to the induction of regression in swine cutaneous melanoma are barely characterised, however this present study contributes to its understanding by introducing potential candidate genes which could be able to reverse melanoma cells into terminally differentiated and phenotypically benign cells. A consequence of differentiation into benign cells would be the downregulation of pigmentation genes such as MITF, SILV, SLC24A5, TYR, TYRP1 during regression, as we observed.

In conclusion, we have succeeded to unravel some molecular pathways related to the MeLiM regression process using SSH. Although the immediate significance of some sequences identified by SSH may not be readily apparent, we confirmed several genes that are differentially expressed dealing with differentiation, immune response, cell cycle arrest, and tumour suppression and some of unknown function. It is worthwhile to note that this gene expression study was conducted at one specific time point while tumour regression is a dynamic process. Indeed, one would expect progressive changes in the expression pattern of a gene over time. Future studies have been planned to address this issue.

D) METHODS

Tissue collection and cell culture

Tumour tissues were obtained surgically from MeLiM swines under complete anaesthesia. The growing tumours were excised between day 3 and 38 after birth (8 samples). The regressive tumours were taken between day 42 and 70 (8 samples), when the first clinical signs of regression such as drying aspect, tumour-flattening, and –greying appeared. One part of the tumour was quickly frozen in liquid nitrogen and stored at -80°C until RNA extraction. Specimens of excised tumour tissue were paraffin embedded and evaluated histologically by criteria such as presence of ulceration, vessels, inflammatory cells, hyperkeratosis, and fibrosis. Two nodular melanomas (Clark level V), one at growing stage (8 days after birth) and the other one at a regressive stage (52 days after birth), showing comparative histological criteria were therefore chosen for SSH analysis. Permission for animal experiments was obtained from the Animal Protection office of the French Ministry of Agriculture and Forestry (N°78-16). All melanoma cell cultures were established from fresh specimens obtained at the time of biopsy. Tumour tissue was collected in supplemented DMEM. Epidermis and hypodermis were carefully removed and single cell suspension was obtained by collagenase B (Roche) treatment. Cells were plated in supplemented MEM including cholera toxin (5nM/ml). TPA (200 nM/ml) was added at first day of culture. Cells used for SSH were extracted during the phase of tumour growth (day 3 after birth) and early regression (day 46). Both isolates were cultivated for 7 and 5 days, respectively.

RNA isolation

Total RNA was isolated using TRIzol™ Reagent (Invitrogen). Frozen tissues were cryopowdered, homogenized in TRIzol reagent, and then centrifuged to remove melanin. The manufacturer's instructions were followed throughout extraction protocol. The concentration of total RNA was quantified by measuring optical density at 260nm and its quality was evaluated by visualizing the 28S and 18S ribosomal bands after electrophoresis.

Suppression subtractive hybridization

SSH was performed between melanoma tissues, as well as melanoma cell cultures. At first, complementary DNAs were generated using BD SMART™ PCR cDNA synthesis kit (BD Biosciences Clontech) according to the manufacturer's protocol. Briefly, 2µg of total RNA were used as a template for first-strand cDNA synthesis and double-strand cDNA was further amplified by PCR before processing to cDNA subtraction. We performed SSH using the

PCR-Select Subtraction (Clontech) following the suppliers instructions. The subtracted cDNAs were cloned into the pGEM-T Easy vector system (Promega) to construct the 4 subtractive cDNA libraries:

1. SCAW: represents genes over expressed in early regressive melanoma tissue since it was used as a tester and progressive melanoma tissue as a driver.
2. SCBE: represents genes over expressed in growing melanoma, as in this case regressive melanoma tissue served as driver.
3. SCBF: genes over expressed in cell cultures isolated from regressive melanoma (forward subtraction).
4. SCAX library, genes over expressed in cell cultures isolated from progressive melanoma (reverse subtraction).

To simplify the reading, we consider genes in SCBE as being downregulated during regression and genes found in SCAW as being upregulated in regressive melanoma tissue. Same is true for SCAX (downregulated in regressive melanoma cell culture) and SCBF (upregulated in regressive melanoma cell culture).

Plasmid isolation and cDNA sequencing

Bacterial colonies were picked and inoculated individually into 96-deep well plates containing 2YT media including ampicillin (200µg/ml). After a preculture for 19 h at 38°C at 130rpm, the main culture was performed with 10µl of precultured bacteria solution for 19h under equal conditions. After centrifugation, the dry bacteria pellets were sealed and stored at -20°C. About 1000 clones for each cDNA library (1152 clones for SCBE and SCAW and 960 clones for SCAX and SCBF) were sent for sequencing to MilleGen (Labege, France).

SSH-sequence data analysis

The obtained SSH-sequences were cleaned from their vector sequence and compared for homology to different databases such as SWISS-PROT/TrEMBL, TIGR and ENSEMBL by the help of SIGENAE (Système d'Information du projet d'Analyse des GENomes des Animaux d'Elevage, INRA). The criteria for a valid homologous sequence was set to an e-value $< e^{-20}$ which allowed a sequence identity $> 90\%$ for at least 100bp. The identified sequences were annotated including the human SWISS-PROT identifier and the human gene symbol.

Quantitative RT-PCR

Total RNA was extracted from 16µm tissue cryosections. RNA extraction was performed according to the PicoPure™ (Arcturus) manufacturer protocol including DNase (Qiagen) treatment. RNA quantity and quality were assessed using the Agilent 2100 Bioanalyzer. Complementary DNA was synthesized using the Roche™ Transcriptor Reverse Transcriptase (Roche Applied Science) and 1.5 µg of total RNA. The cDNA was quantified with the Bioanalyzer. 1ng of cDNA was used per qRT-PCR reaction. The final volume of one reaction was 20µl including 15µl of SYBR® Green PCR Master Mix (Applied Biosystems), and 300nM of each primer. All samples (n=16) were run in triplicates. qRT-PCR primers were designed taking into account the identified SSH sequence and its human conserved homolog by using Primer Express® (V.2.0). Amplicons which were between 101-218bps in size were re-blasted to ensure sequence specificity. Amplifications were accomplished on an ABI®PRISM 7900. The thermal profile was as follows: 2min at 50°C, 10min at 95°C followed by 40 cycles of 15s at 95°C and 60s at 60°C. The specificity of each amplified RT-PCR product was checked by melt curve analysis. Relative expression was calculated using the comparative $\Delta\Delta C_t$ method. Briefly, this method compares the amounts of target gene expression relative to an endogenous control, in our case RPL32, within a sample to normalise the expression. We tested different endogenous controls such ACTB, GAPDH, HPRT, 18s, B2M, PBDG, RPL32, whereas RPL32 turned out to be the most stable one. Within a group of samples, one appropriate sample is chosen as a reference sample (in our case: progressive melanoma sample with highest RNA quality). Each sample (n=15) is then compared to the nominated reference to give the relative expression of the target gene compare to this reference sample.

Statistical Analysis

Expression data from the qRT-PCR analysis was analyzed using the unpaired t-test including Welch's correction, taking into account the different variances of the two populations (GraphPad InStat software). Results were considered to be significant when the corrected p-value $P < 0.05$. Since 14 t-test were performed simultaneously, we controlled the false discovery rate by Benjamini Hochberg correction (FDR=5%).

Gene List comparison

We compared our SSH retrieved gene lists to the one of Hoek et al 2006 (supplementary data) in order to identify common genes. Overlapping genes serve in this case as an indicator of biological relationship. In order to evaluate an eventual gene-overlap with a certain significance, we applied the hypergeometric distribution test (HDT) (Hoek et al., 2006). Briefly, the test calculates the probability of obtaining by chance a number k of annotated genes for a given term among a dataset of size n , knowing that the reference dataset contains m such annotated genes out of N genes. In our case, m was the number of genes in the target gene list, k = the number of genes in our gene list, n = the number of overlapping genes between m and k and N = the number of all possible genes. Since the HDT calculates the probability of having exactly n overlaps, we repeated the calculation up to 4 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.

Gene Ontology annotation and Ingenuity Pathway Analysis

Functional classifications from Gene Ontology (GO) Consortium were assigned to each identified gene within the different SSH-libraries using GoTree Machine (GOTM) (<http://bioinfo.vanderbilt.edu/gotm/>) (Ashburner et al., 2000; Zhang et al., 2004). Since gene classification in GOTM is comprehensive and complex, we adopted genes at the default level 4 and higher as the final export results.

SSH identified genes were also analysed by Ingenuity™ Pathway Analysis (www.ingenuity.com), leading to the creation of gene networks and functional clustering. The 135 genes were mapped to genetic networks available in the Ingenuity database and ranked by score. The score is the probability that a group of genes equal or greater than the focus gene number in a network could be achieved by chance alone. (Raponi et al., 2004).

Immunofluorescence analysis

Paraffin sections were dewaxed and rehydrated. Epitopes were retrieved with 10mM citrate buffer (pH = 6) at 95°C for 20min. After blocking non-specific binding sites by incubation with goat serum, melanoma sections were incubated overnight with rabbit anti-CD9 polyclonal antibody (gift from J. Garrido Pavon, Spain), mouse monoclonal anti human MITF (clone C5+D5, Zymed), and goat polyclonal anti human RARRES1 antibody (TIG1 (N-18), Santa Cruz Biotechnology). CD9, MITF, and RARRES1 protein stainings were revealed

respectively by: biotin goat anti rabbit IgG (Dako), Alexafluor® 555 labelled, goat anti mouse IgG1 (Molecular Probes) and biotin donkey anti goat IgG (Santa Cruz Biotechnology). For biotin conjugated secondary antibodies subsequent streptavidin, Alexafluor® 555 conjugate (Molecular Probes) treatment was performed. Negative controls were assessed by replacing the primary antibodies with the non immune goat serum at the same concentration. Staining patterns were assessed independently by two different investigators.

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CHAPTER 3

Introduction of: “Gene Expression Signature for Spontaneous Cancer Regression in Melanoma Pigs”

The aim of the 2nd study was to identify molecular mechanisms throughout the kinetics of melanoma progression and regression that could finally lead to the explanation of the phenotype. The initial SSH study allowed already the identification of differentially expressed genes between melanoma growth and early melanoma regression. However this study had major limits as SSH-cDNA libraries were constructed from one single tumor (animal) and one single timepoint only regarding melanoma progression and regression. Furthermore animals chosen for SSH analysis were not of the same family. To overcome these limitations, we performed time dependent gene expression profiling using porcine DNA chips (Affymetrix). We chose the same starting point (d+8) for our microarray study like for SSH analysis. Since SSH analysis showed already genes clearly associated with regression processes such as differentiation and apoptosis at d+52, we chose an intermediate time d+28 as 2nd timepoint in our kinetic to allow the detection of precocious expressional changes. The tumor sampling was then performed in 3 week intervals till d+91. We used whole tumor tissue since SSH analysis demonstrated a heavy influence of culture conditions on gene expression of melanoma cells. Only animals of the same litter were chosen as biological replicates. The siblings were all homozygous for chromosomal regions on SSC1 and SSC6 that were associated to specific QTLs such as “number of melanoma at birth” (NAM), “ulceration” (ULC), and “extreme phenotype” (EP) (Du et al., 2007). We identified 1411 genes regulated during regression after statistical analysis and reannotation of the 25 porcine microarrays. Clustering and subsequent functional interpretation of clustered genes via Ingenuity Pathway Analysis revealed significant pathways playing a role in melanoma regression. The comparison of human melanoma expression profiles with ours, revealed significant overlaps. Briefly, some genes overexpressed in highly aggressive human melanoma, were found to be downregulated in regressing porcine melanoma. These common inversely regulated genes were mainly cell cycle related. Furthermore genes involved in melanocyte-differentiation and pigmentation as well as genes of the immune response seem to be potential mediators of melanoma regression.

A limitation of this study was the use of porcine microarrays (Affymetrix). Less than 10% of the claimed ~23k transcripts representing ~20k *sus scrofa* genes were described with gene names. Reannotation of this microarray resulted in ~11k unique genes. Nevertheless, the

choice of Affymetrix microarrays was the most complex at the time and could be reanalysed once the whole genome sequence for the pig will be available.

Comment on microarray processing method

Since the method section in the following article is really condensed for word count reason only, a detailed description of the microarray experiment is presented below:

Target Production and Hybridization

Microarray experiments and part of data analysis were performed in collaboration with ParterChip (Evry, France) by following the procedure recommended by Affymetrix (Fig. 4). Before target production, the quality of RNA was assessed by capillary electrophoresis using a 2100 BioAnalyzer (Agilent). The quantity as well as the 260/230nm ratio (detection of organic contamination), 260/280nm (detection of protein contamination) and 405/415nm (detection of melanin contamination) was determined by spectrometry using Nanodrop. Target was prepared and hybridized according to the Affymetrix 1-cycle amplification technical manual. Between 2.5 and 5µg of total RNA was converted into cDNA using Reverse Transcriptase (Superscript II) and a modified oligo (dT)₂₄ primer that contains T7 promoter sequences (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)₂₄-3'). After first strand synthesis, residual RNA was degraded by the addition of RNase H and double strand cDNA was generated using DNA Polymerase I and DNA Ligase. The cDNA was then purified using the GeneChip Sample Cleanup Module. The cDNA products were incubated with T7 RNA polymerase and biotinylated ribonucleotides using an In Vitro Transcription kit (BioArray HighYield RNA Transcript Labeling Kit). The cRNA product was then quantified with a spectrophotometer (nanodrop). The cRNA profile was equally assessed using a 2100 Bioanalyzer (Agilent). The cRNA target (20µg) was incubated at 94°C for 35min in a fragmentation buffer (200mM Tris-acetate, pH 8.1, 500mM KOAc, 150mM MgOAc). The fragmented cRNA was then diluted in hybridization buffer (MES, NaCl, EDTA, Tween20, Herring Sperm DNA, Acetylated BSA) containing biotin-labeled Oligo B2 and eukaryotic controls (Affymetrix). The hybridization cocktail was denatured at 99°C for 5 min, incubated at 45°C for 5min and then injected into a GeneChip cartridge. The GeneChip array was incubated at 45°C for at least 16h in a rotating oven at 60rpm. GeneChips were washed with a series of nonstringent (25°C) and stringent (50°C) solutions containing variable amounts of MES, Tween 20 and SSPE. The microarrays were then stained with Streptavidin Phycoerythrin and the fluorescent signal was amplified using a biotinylated

antibody solution. Fluorescent images were detected in a GeneChip® Scanner 3000 and raw expression data (CEL files) were generated using GCOS software (Affymetrix). Quality control was assessed based on 3'/5' ratios of GAPDH and β -actin control probes.

Normalization and Statistical Analysis

Normalization and statistical management of data have been led using R resources and Bioconductor statistical packages (<http://www.bioconductor.org/>) and ArrayAssist software from Stratagene for ANOVA.

Normalization of data was achieved using pre-processing algorithm GC-RMA that led to intra and inter-chip quantile normalizations followed by log2 transformation of intensity values. This algorithm (Wu and Irizarry, 2004) is based on the RMA method and differs only in the background correction step. Its aim was to reduce the bias caused by not subtracting MM (mismatch) in the RMA algorithm. The GCRMA algorithm uses a rather technical procedure to reduce this bias and is based on the fact that the non-specific affinity of a probe is related to its original sequence. The algorithm computes a background value to be subtracted from each probe using its raw sequence. This requires access to the base sequences. The RMA method is in contrast to MAS5 a PM (perfect match) method (Irizarry et al., 2003). The method uses Quantile normalization which assumes that the distribution of gene abundance is nearly the same in all samples. In this method the signal values are sorted. The highest signal of each array is replaced by the average of all highest signals. The second highest signal is also replaced by the average of all highest signals and so forth. The downside of this method is that low signals may be lost. Statistically, this method seems to obtain very sharp normalizations.

Genes were defined as differentially expressed for t_n vs t_0 time points using on the one hand the t-test statistical and on the other hand one way ANOVA with Benjamini Hochberg multiple testing correction of the p-value to control the False Discovery Rate (FDR). The FDR control is necessary since tens of thousands of probes on a microarray are tested for significance at the same time and create automatically false positives by chance alone. The number of false positives that occur depends on a combination of the number of probes on the array and the P-value cutoff chosen.

Multiple hypotheses testing was controlled by applying Benjamini Hochberg FDR correction (FDR<1%) (Hochberg and Benjamini, 1990). Probe sets were defined as differentially expressed for t_n vs t_0 time points if the fold-change (FC) was higher than 2 and p-value < 0.05 using unpaired t-test. Furthermore, probe sets also found significant after ANOVA were used

for K-means clustering (n=6). K-means is an unsupervised learning algorithm (McQueen, 1967) to classify or to group objects (expression values) based on attributes/features into K number of groups. The grouping is done by minimizing the sum of squares of distances between data and the corresponding cluster centroid.

Reannotation of porcine Affymetrix Probesets

The porcine Affymetrix microarray is poorly annotated with less than 10% of immobilized probes describing a gene, making it hard to interpret any expression data. Tsai et al. (2006) described a strategy to improve its annotation:

- the Affymetrix probeset target sequence was retrieved from its website and if necessary extended if possible with the TIGR assembly (Lee et al., 2005).
- the extended sequences were compared by BLAST against the Ensembl human cDNA sequence library.
- If BLAST bit score was <50, extended sequences were further compared by BLAST against the Ensemble human genomic sequence library. These results were summarized into a final annotation.

Using this strategy, 19.675 of 24.123 transcripts (82%) on the Affymetrix porcine microarray could be identified, representing 11.256 unique human genes

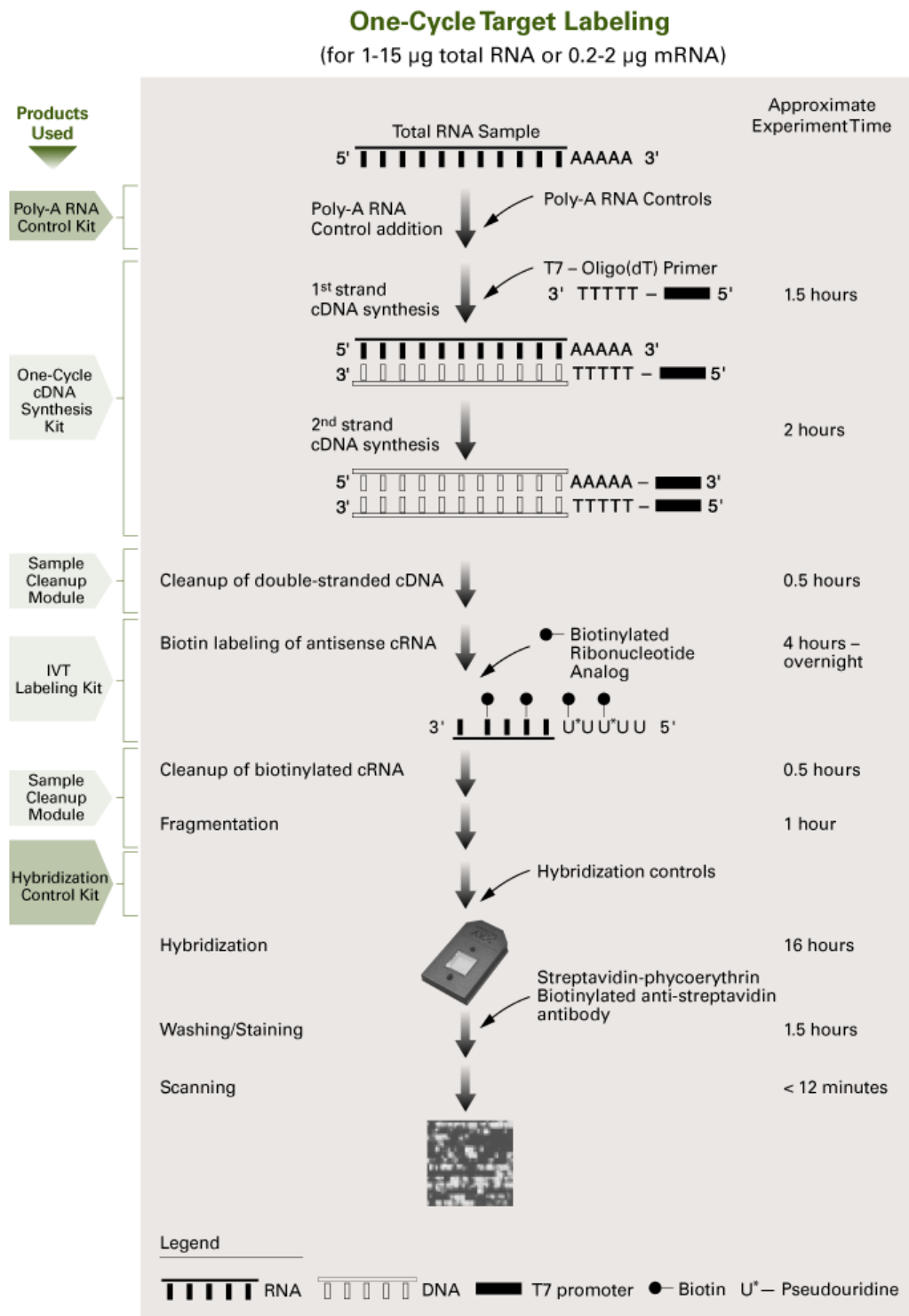


Fig. 4: One cycle Target protocol (adapted from GeneChip® One-Cycle Target Labeling Kit, User Manual, Affymetrix).

Total RNA is first reverse transcribed using a T7-Oligo(dT) Promotor primer in the first strand cDNA synthesis reaction. Following RNA H-mediated second-strand cDNA synthesis, the double stranded cDNA is purified and serves as a template in the subsequent *in vitro* transcription (IVT) reaction. The IVT reaction is carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets are then cleaned up, fragmented, and hybridized to GeneChip expression arrays

Paper II: Gene Expression Signature for Spontaneous Cancer Regression in Melanoma Pigs

Florian Rambow^{1,2}, Guillaume Piton^{1,2}, Stephan Bouet^{1,2}, Jean-Jaques. Leplat^{1,2}, Sylvain Baulande³, Angelique Marrau³, Mark Stam^{1,2}, Vratislav Horak⁴, Silvia Vincent-Naulleau^{1,2}

¹ INRA, UMR 314, Laboratoire de Radiobiologie et d'Etude du Génome, F-78350 Jouy-en-Josas, France

²CEA, DSV, iRCM, SREIT, Laboratoire de Radiobiologie et d'Etude du Génome, F-78350 Jouy-en-Josas, France

³ PartnerChip, Genopole, F-91000 Evry, France

⁴Institute of Animal Physiology and Genetics, 27721 Libechov, Czech Republic

Running title: Gene expression profiling in porcine melanoma regression

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Corresponding author: *F. Rambow*

Telephone: +33-1-34-65-28-05

Fax: +33-1-34-65-29-64

E-mail: florian.rambow@jouy.inra.fr

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Abstract

Incomplete spontaneous regression of melanoma is common. However, complete melanoma regression is still a very rare phenomenon. As melanoma is the most immunogenic human malignancy the mechanisms leading to regression, based on accumulative evidence, are the host immune response. 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 (MeLiM) are highly suitable for this study since 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 anti-melanoma therapy.

Introduction

Complete regression of cancer is the ideal outcome of any anti-tumor therapy. At present no such treatment exists for advanced melanoma since melanoma cells exhibit an extraordinary resistance to chemotherapy, radiotherapy and even immunotherapy [1]. Hence, its resistivity to treatment and aggressiveness make it the most fatal of all skin cancers, with mortality of patients with metastasis reaching >95% within 5 years [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) [3]. Complete regression of metastatic melanoma is an extremely rare occurrence with only 38 well documented cases [4]. However, the regression could be more common than reported since it is prone to escape detection [5]. Nevertheless, partial regression is observed more frequently with 7% to 61% in thin melanoma [6]. Clinically, partial regression is mainly characterized by a heterogeneous pigmentation of the tumor site. While on a histopathological level, the process starts with a dense infiltrate of lymphocytes, and ends with fibrosis and/or melanosis within a thickened papillary dermis [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 MeLiM pigs have been described as a suitable animal model to study melanoma and its regression since the tumors occur and vanish naturally and melanocytes are localized at the basal layer of the epidermis. Also, large litters allow studies of homogenous genetic background. Spontaneous complete tumor regression occurs in 96% of MeLiMs and is characterized by tumor-flattening, -drying, depigmentation, and infiltration of firstly melanophages and then lymphocytes [9]. The biggest difference between human and pig is the early onset of regression in MeLiM minipigs that occurs during childhood and its extreme efficiency. The elucidation of regression mechanisms is of valuable interest in order to find a more specific therapy to treat the disease. Therefore, we aimed to study the molecular changes leading to melanoma regression in MeLiM pigs using Porcine Genome Arrays (GeneChip®, Affymetrix). 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.

Material and Methods

Biological Samples

Time dependent gene expression profiling of spontaneously regressing melanomas was performed at five different time points such as t_0 = day+8 after birth (d+8), t_1 = d+28, t_2 = d+49, t_3 = d+70, and t_4 = d+91. 6 MeLiM pigs of the same litter were chosen that were homozygous for predisposition QTLs 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 anaesthesia.

At t_0 and t_1 $n = 6$ tumors, at t_2 and t_3 $n = 5$, 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 3 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 histology. Hematoxylin stained paraffin embedded sections were evaluated histologically according to human classification and for different criteria such as 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 n° 05-030).

RNA isolation

Total RNA was extracted from several 16 μ m tumor tissue cryosections using the PicoPure™ RNA extraction kit (Arcturus, CA). The RNA quality was carefully assessed by capillary electrophoresis using the 2100 BioAnalyzer (Agilent). The quantity as well as the 260/230nm ratio, 260/280nm and 405/415nm (detection of melanin contamination) were determined by spectrometry using Nanodrop (Thermo Fisher Scientific).

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. Expression data and raw expression data (CEL files) were generated using GCOS software (Affymetrix). Quality control was assessed based on 3'/5' ratios of GAPDH and β -actin control probesets.

Normalization and Statistical Analysis

Normalization and statistical analysis of microarray-data have been realized using R resources, Bioconductor statistical packages (<http://www.bioconductor.org/>), and the ArrayAssist software (Stratagene) for the analysis of variance (ANOVA) and k-means analysis. Raw intensity values were subjected to a pre-processing step using the GCRMA 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 t-test and one-way ANOVA analysis. The time post-birth was considered as central parameter for one-way ANOVA analysis. Multiple hypotheses testing was controlled by applying Benjamini Hochberg FDR correction. P-values of the ANOVA analysis were adjusted using the Benjamini Hochberg algorithm (FDR or adjusted p-value <0.01). For the t-tests, p-value adjustments were performed individually for each comparison. Probe sets were defined as differentially expressed for t_n vs. t_0 time points if the fold-change (FC) was bigger than 2 and p-value lower than 0.05 after unpaired t-test. Furthermore, probesets also found significant after ANOVA were used for k-means clustering (k=6). We used k = 6 clusters since most of the time the number of clusters is close to the number of time points. Also, we grouped our data by k = 9 clusters. Subsequent functional analysis however showed an “overclustering” of the data, as many genes of the same biological function were arranged in different k-means clusters, a. 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 Probesets

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 and others [12, 13]. Using this strategy, 19,675 of 24,123 transcripts (82%) 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 $e\text{-value} < e^{-10}$.

Functional pathway analysis by Ingenuity

1411 K-means clustered genes ($e\text{-value} < e^{-10}$) were analyzed by Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). Swiss Prot identifiers were imported into the Ingenuity Pathway Analysis Software. 1396 of these genes were mapped to the Ingenuity database. Cluster 1 with 406 IPA mapped genes (IMGs) contained 235 network eligible genes (NEGs), cluster 2 (280 IMGs) 184 NEGs, cluster 3 (243 IMGs) 178 NEGs, cluster 4 (242 IMGs) 172 NEGs, cluster 5 (128 IMGs) 105 NEGs, and cluster 6 (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 6 gene clusters and the different timepoints (t_1 - t_4) was performed. IPA mapped the clustered genes for each time point relative to t_0 as follows: t_1 = 53 genes (42 network eligible), t_2 = 234 genes (183), t_3 = 1049 genes (719), and t_4 = 1256 genes (833). After single IPA core analysis for each time point, IPA comparison was performed to analyze changes in biological states over time. Functional pathway analysis over time identified highly significant biological classes that changed during the progression and regression of melanoma. The readout of the comparison analysis were histograms representing the significance ($-\log p\text{value}$) of the functional association that is dependent on the number of genes in a class as well as biological relevance. The threshold line that appears in the bar chart represents a $p\text{-value}$ of 0.05. In addition, network analysis was performed for each time point to identify gene interaction.

qRT-PCR Validation

To validate differentially expressed genes identified by microarray analysis, we performed quantitative real time PCR as previously described [14]. We tested 18 genes in total (CCNB1, CDC2, BUB1B, BIRC5, KIF11, CKAP2, SCIN, KLRK1, CCL5, IL15, TVB1, SLC37A2, ATP6V0D2, IGHG1, TRGV9, MITF-M, PRF1, MLANA). 18 genes were chosen since we wanted to validate genes of the major gene signatures of interest (cell cycle and immune response). We chose 6 genes representative for the cell cycle signature (CCNB1, CDC2,

BUB1B, BIRC5, KIF11, and CKAP2) and 9 genes for the different facets of the immune response (SCIN, KLRK1, CCL5, IL15, TVB1, SLC37A2, ATP6V0D2, IGHG1, and TRGV9). 3 additional genes were selected that were of interest but not responsive or present on the microarray. PRF1 was represented on the porcine genechip with 2 probesets, but both were unresponsive. MITF-M and MLANA were not present on the porcine microarray

Gene List comparison

To confront our data with human melanoma expression profiles, we compared our MeLiM data against 3 human melanoma expression studies conducted with melanoma tissue and primary melanoma cell lines [15-17]. The aim was to identify overlapping genes as an indicator of biological relationship. In order to evaluate an eventual gene-overlap with a certain significance, we applied the hypergeometric distribution test (HDT) [15]. Briefly, the test calculates the probability of obtaining by chance a number k of annotated genes for a given term among a dataset of size n , knowing that the reference dataset contains m such annotated genes out of N genes. In our case, m was the number of genes in the target gene list, k = the number of genes in our gene list, n = the number of overlapping genes between m and k and N = the number of all possible genes. Since our porcine microarray did not represent a ~33K human pan genomic genechip, we corrected m (number of genes in reference data set) by subtracting genes which were not immobilized.

Since the HDT calculates the probability of having exactly n overlaps, we repeated the calculation up to 4 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.

TIL isolation and Flow cytometry

Single cell suspension of melanoma tissue was obtained by collagenase B (Roche) treatment. Double staining was performed with mouse anti-CD4 at 1:500 (IgG2a, clone PT90A, VMRD) and mouse anti-CD8 α antibody at 1:500 (IgG2b, clone PT81B, VMRD). Primary antibody staining was revealed by RPE labelled anti mouse IgG2a at 1:500 (Southern Biotech) and FITC labelled anti mouse IgG2b at 1:300 (Southern Biotech). Flow cytometry analysis was realized using a FACSCalibur (BD).

Immunohistochemistry

Cryosections were fixed in 95% ethanol for 10min. Non-specific binding sites were blocked by incubation with goat serum. Melanoma sections were incubated overnight with mouse anti CD3 (IgG1, clone PPT3, Santa Cruz at 1:50), mouse anti CD8 α (IgG2b, clone PT81B, VMRD at 1:400), and mouse anti SWC3a (IgG2b, clone 74-22-15a, BD Pharmingen at 1:200). CD3, CD8 and SWC3a protein stainings were revealed respectively by: Alexafluor® 555 labelled, goat anti mouse IgG1 at 1:500 (Invitrogen) and FITC labelled goat anti mouse IgG2b at 1:500 (Southern Biotech). Negative controls were assessed by replacing the primary antibodies with the non immune goat serum at the same concentration. Staining patterns were assessed independently by two different investigators.

Results

Sample characteristics

Tumors at t_0 and t_1 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 t_2 (d+49) with the occurrence of TILs (3 of 5 tumors), fibrosis, and melanophages. This observation pursued while tumors at t_3 and t_4 showed clear signs of regression such as 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. Whilst the highest RNA integration number (RIN) at t_0 was 9.2, the maximal RIN at t_4 was 7.8 (Table1) due to regression related phenomena like necrosis.

Table 1: Tumor characteristics of biological samples used for microarray analysis.

RIN=RNA integration number, UC=unclassified, NM=nodular melanoma, TIL=tumor infiltrating lymphocyte, n = number of excised tumors = number of used microarrays, N = number of different animals from which tumors were excised.

<i>Timepoint</i>	<i>n</i>	<i>N</i>	<i>RIN</i>	Clinical aspect			Histological aspect					
				<i>Tumor type / Clark level</i>	<i>Characteristics</i>	<i>Metastasis</i>	<i>% Tumor cells</i>	<i>TILs</i>	<i>Hair follicles</i>	<i>Vascularization</i>	<i>Regression</i>	<i>Fibrosis</i>
t 0 = day 8	6	6	7.6-9.2	NM / IV-V	Ulcerated nodule – plateau shaped tumor	0 of 5	60-97	0 of 6	++	++	0 of 6	0 of 6
t 1 = day 28	6	6	7.6-8.9	NM / IV-V	Bleeding ulcerated exophytic tumor	5 of 6	85-98	0 of 6	+	+++	0 of 6	0 of 6
t 2 = day 49	5	5	7.4-8.6	NM / IV-V	Dry plateau shaped – dry exophytic tumor	5 of 5	70-95	3 of 5	+	++	Doubtful 5 of 5	4 of 5
t 3 = day 70	5	4	7.3-8.5	UC	Grey plateau shaped, dry exophytic, ulcerated plateau shaped tumor	5 of 5	60-80	5 of 5	+	++	5 of 5	5 of 5
t 4 = day 91	3	3	7.1-7.8	UC	Grey plateau shaped tumor	3 of 3	30-60	3 of 3	++	++	3 of 3	3 of 3

Microarray analysis and Clustering

Time dependent gene expression profiling was carried out at 5 time points with 5 animals of the same family. At each time point 5 different tumors, one from each animal, were excised. After normalization and statistical analysis 1761 probe sets representing 1411 unique human genes ($e\text{-value} < e^{-10}$) were regulated over time and grouped into 6 clusters according to similar expression behaviour. Cluster 1 contained the most genes (409) whereas cluster 2, 3, and 4 were almost equal in numbers (248, 245 and 244). Cluster 5 and 6 harboured fewer genes such as 131 and 98, respectively (Supplement 1). The expression profiles of cluster 2, 3, and 5 indicated a general upregulation of genes over time, with cluster 5 showing the strongest upregulation at t_3 (Figure 1A). Cluster 1, 4, and 6 profiles showed a downregulation over time with cluster 6 marking a dramatic decrease in gene expression from t_3 on. Only minor differences were detected between t_0 and t_1 among the 6 clusters. However, at t_2 more prominent changes in gene expression profiles were observed (129 genes significantly up- and 107 downregulated) reflecting observed histological changes. Functional classes were assigned to gene clusters by Ingenuity Pathway Analysis (IPA) (Figure 1B). Genes of cluster 6 were mainly associated with *Hair and Skin Development and Function* ($p = 1.36 \times 10^{-14}$), whereas cluster 5 contained mainly *Immune Response* ($p = 3.28 \times 10^{-16}$) and *Cell Death* ($p = 1.7 \times 10^{-9}$) related genes. Cluster 4 genes were highly associated with *Cell Cycle* ($p = 9.39 \times 10^{-16}$) and *DNA Replication, Recombination, and Repair* ($p = 1.44 \times 10^{-11}$) functions. Genes of cluster 3 were mainly linked to *Cellular Movement* ($p = 1.61 \times 10^{-10}$) and also *Immune Response* ($p = 1.62 \times 10^{-7}$). Cluster 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.

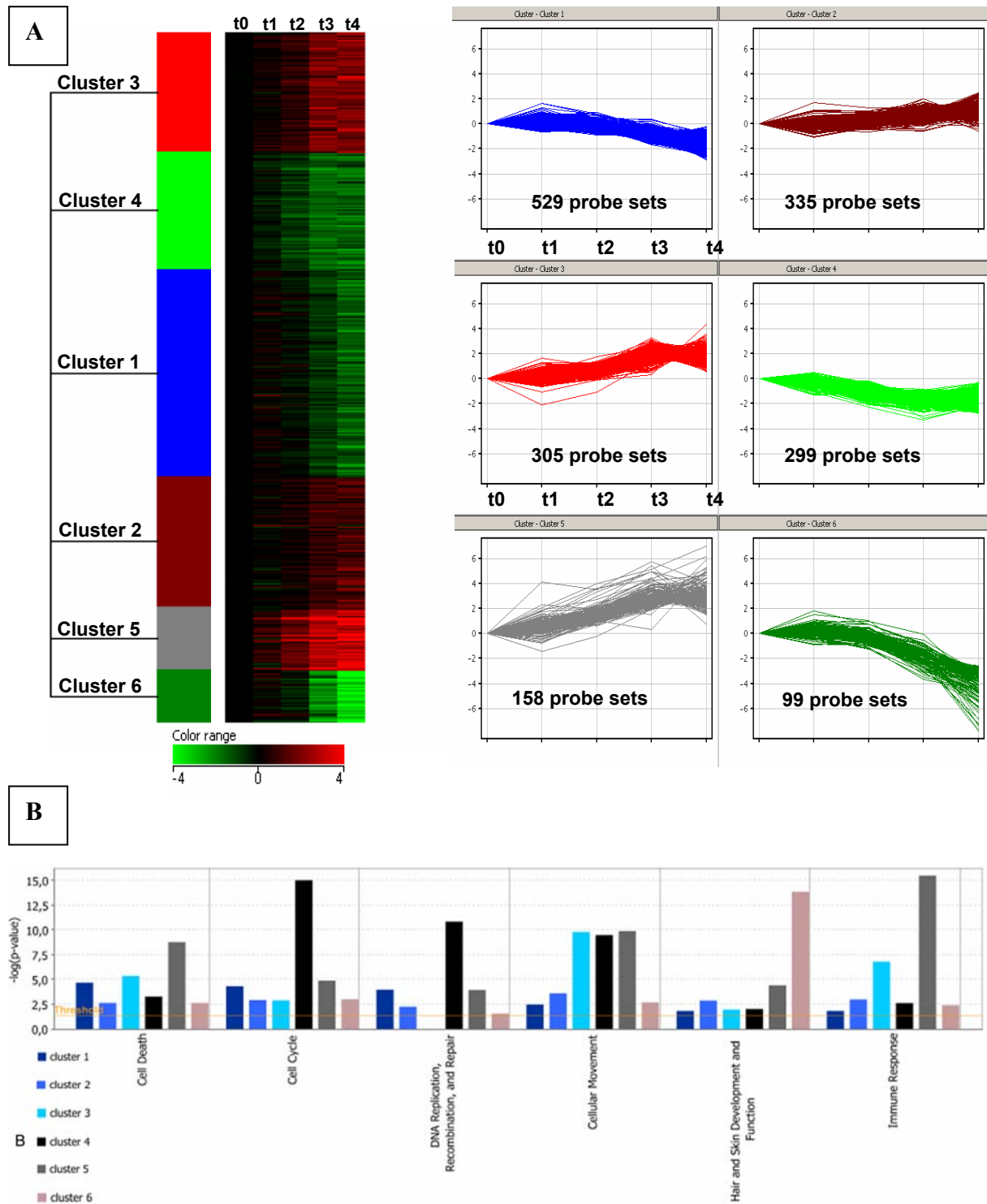


Figure 1: Figure 1: K-means clustered genes regulated during regression and their biological interpretation.

A) Gene expression profiles of K means clustered genes over time.

Y-axis represents the \log_2 Fold Change (FC), whereas the X-axis represents the 5 consecutive timepoints 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 biological relevance.

Time dependent analysis by IPA

To focus on expressional changes at each timepoint, functional interpretation and biological interaction of the clustered genes was achieved by Ingenuity Pathway analysis (IPA). As demonstrated in Figure 2A highly significant classes such as *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 per each time point, as well as their range of Fold Changes (FC) as a measure for their “weight” of regulation. Taken these criteria into account, t_1 (d+28) showed only minor transcriptomic changes relative to t_0 (d+8). However 18 genes were associated with *Cell Death* of which 12 were upregulated. Also at t_1 , 8 of 9 genes linked to *Immune Response* were upregulated (~ 10 fold) and 4 of 10 genes linked to *Cell Cycle* functions were downregulated (2.5 fold). Interestingly, at t_2 (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 ~7 fold compared to t_0 . *DNA Replication*, *Recombination and Repair*, a related class, showed the same trend with 86% of the genes down-regulated at t_2 . The cell cycle genes affected were mainly cyclin dependent kinases (CDC2, CDC6) and cyclins (CCNA2, CCNB1, CCNB3) as shown in the figure 2B, as well as 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 t_2 (Supplement 5). Furthermore, 48 of 72 genes involved in cell death were upregulated at t_2 . 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 t_2 . This immune response seemed to be established at t_3 with ~82% of the immune genes being strongly up-regulated (up to ~100 fold). There were no less than 6 canonical pathway classes demonstrating immune response associated signalling (Supplement 5). These canonical classes indicated a complex immune invasion since signalling pathways of chemokines, leukocyte extravasation and different immune cells such as T cells, NK cells, 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 t_3 . The phenomenon of depigmentation was clearly reflected on the expression level since 50% of pigmentation genes classified under *Hair and Skin Development and Function* were strongly downregulated.

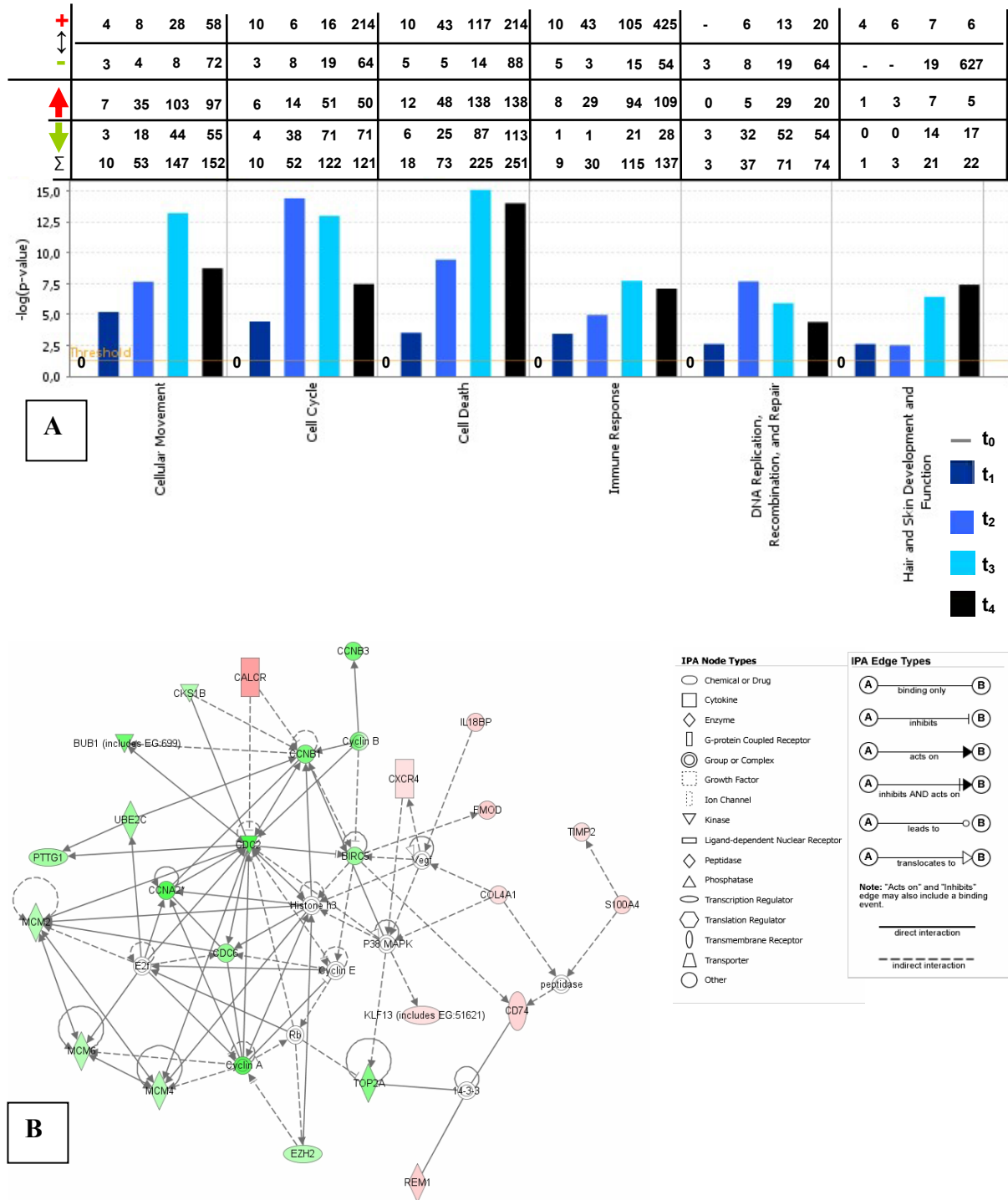


Figure 2: Functional analysis of clustered genes regulated over time (IPA).

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 biological relevance. Furthermore the number of genes up and downregulated as well as their fold change of regulation is noted.

B) Most significant Gene Network detected at t_2 by Ingenuity Pathway Analysis (IPA).

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 3 indicates that there is a 1/1000 (score = $-\log_{10}(pvalue)$) 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 downregulation, red gene symbols refer to upregulation. The colour intensity is correlated with fold change. Uncoloured 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.

Comparison to human melanoma expression studies

We compared our gene lists to those of different human melanoma gene expression profiling studies in order to identify overlapping genes [15-17]. All groups studied the progression of the disease either using melanoma tissue [17] or primary melanoma cell lines [15, 18]. The probability of finding common genes between two independent gene lists by random was calculated using the hypergeometric distribution test [15].

Compared to the 174 known genes associated with metastatic dissemination of cutaneous melanoma [17], we found 4 overlapping genes at t_1 , 27 at t_2 ($p < 2.86 \times 10^{-29}$), 36 at t_3 ($p < 9.69 \times 10^{-21}$), and 33 at t_4 ($p < 1.50 \times 10^{-15}$) (Supplement 3). 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 [16] showed again common genes associated with cell cycle progression, DNA replication and repair and apoptotic resistance such as HELLS, BIRC5, GINS1, NUSAP1, MELK and NCAP2 (Supplement 3). Again we found inverse regulation of those genes since they were down-regulated from t_2 on during regression. Ryu and others [18] identified also a signature for down-regulated genes in aggressive melanoma compared to primary human melanocytes harbouring genes of cell adhesion and differentiation-associated genes. We found a common decrease of mainly differentiation-associated genes such as KIT, OCA2, TYR, and CITED1 from t_3 onwards. Hoek and others [15] 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 (TGFB signature) seemed to be associated with a weak proliferative and high metastatic potential. At t_3 we identified 19 common genes ($p < 9.91 \times 10^{-6}$). 15 of 19 genes were associated with the neural crest signature while 10 of 15 being down- and 5 upregulated (Supplement 3). The last 4 common genes associated with the TGFB signature, were upregulated in our model. At t_4 , 20 overlapping genes ($p < 3.34 \times 10^{-5}$) were detected of which 16 were neural crest and 4 TGFB signature related. 11 of the neural crest genes were down-regulated, mostly melanocytic markers such as (TYR, CITED1, SILV, GPR143). 3 of 4 TGFB signature-related genes were upregulated but with low fold changes.

Immune Response

Focusing on immune response-related genes revealed distinct signatures that were regulated throughout spontaneous melanoma regression. A monocyte/macrophage-like signature was detected that reached maximal up-regulation on day 70 (t_3). This signature contained

macrophage-specific genes such as the recently described sugar transporter SLC37A2 [19], B7 family related phagocytic receptor VSIG4, a negative T cell regulator [20], as well as genes highly expressed in osteoclast-like cells such as tartrate resistant phosphatase (ACP5), transcription factor PU.1 (SPI1), calcitonin receptor (CALCR) [21], the lysosomal ATPase V0 subunit D2 (ATP6V0D2) [22], and integrin beta 3 (ITGB3). Moreover genes involved in differentiation and multinucleation of osteoclasts (CALCR, ATP6V0D2) were the most upregulated at t_3 . Markers for the monocytic lineage included sialic acid binding Ig-like lectin 5 (SIGLEC5) [23], and C-type lectin domain family 5 member (CLEC5A) [24] that were about 25 fold upregulated at t_3 (Figure 3A). Classical activation markers such as CD83 and CD86 remained stably expressed between t_3 and t_4 with minor fold changes. VSIG4 and CD74 reached maximal expression at t_4 as well as reactive oxygen species (ROS) production related genes such as CYBB and CYP2C9. We confirmed by immunohistochemical analysis an increase of tumor infiltrating monocytes during regression, characterized by SWC3a (SIRPA) expression (Fig. 4B).

Furthermore, we detected genes related to a T/NK response and different T cell receptors chains like alpha, beta and gamma (TCA, TVB1, and TRGV9). Genes of the T/NK signature were slightly upregulated at t_3 and highly upregulated at t_4 (d+91) (Figure 3B).

We confirmed by flow cytometry and immunohistochemical analysis an extensive infiltration of T cells at t_4 (Fig. 4B and 5) whereas ~43% were CD8 and ~22% CD4 positive.

Interestingly, the humoral response signature, containing different immunoglobulins, was most highly upregulated in the regression process. Immunoglobulin heavy chain (IGHM), kappa variable (IGKV1-5), and the Ig lambda constant (IGLC1) were increased more than 70 fold at t_4 (Figure 3C). The cytokine gene signature showed as expected late upregulation of T/NK cell related cytokines and receptors such as CCL5, IL2RG, IL15, and CCR5 (Figure 3D).

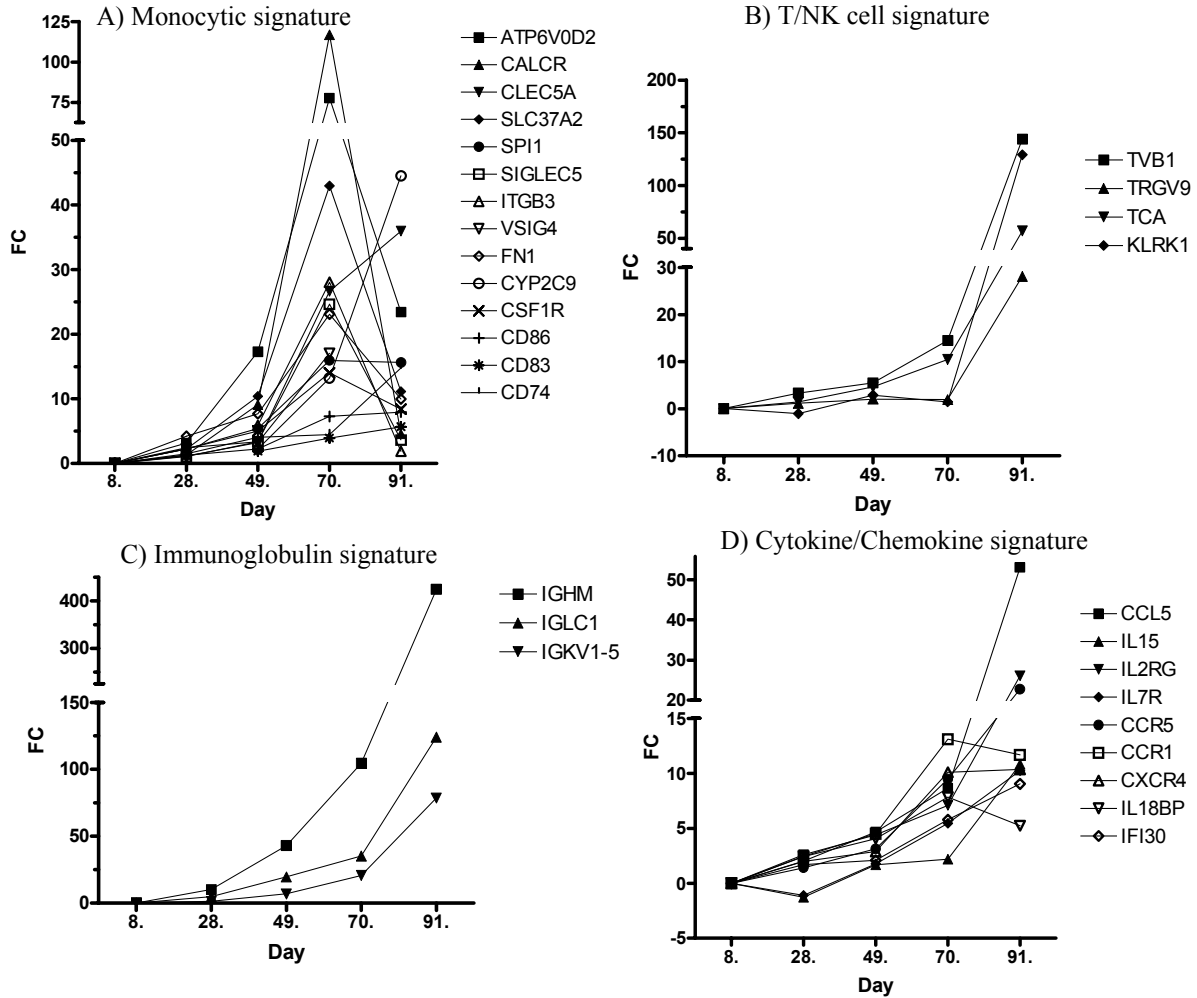


Figure 3: Regulation of immune response related genes during melanoma progression and regression. Fold changes relative to t_0

Merge of clinical, histological and transcriptomic data

To summarize the transition from melanoma progression to regression on a clinical, histological and transcriptomic level, most characteristic tumor samples and their corresponding top 15 up- and down-regulated genes (Supplement 4) per time point were demonstrated (Figure 4). Clinically, tumors of t_1 and t_0 were mostly exophytic and highly vascularized. Histological analysis revealed highly pigmented homogenous melanoma cells. T_2 showed first infiltrations of highly pigment macrophage-like cells and could be therefore considered as transitional stage between progression and regression. Fibrosis and drying were also detected. Most upregulated genes at t_2 were associated with a monocyte/macrophage like signature (ATP6V0D, SLC37A2, CALCR, FN1, ACP5, SPI1, ITGB3), whereas most down-regulated genes were implicated in the cell cycle (KIF11, CDC2, E2F7, CCNA2, CDKAP2, KIF4A, BUB1, CLSPN, CCNB3, MELK). On a clinical level at t_3 , tumors showed a drying surface as well as hypopigmentation, which were confirmed by histology. Here again, the top

15 upregulated genes were predominantly of monocyte/macrophage origin with additional genes such as CLEC5A and SIGLEC5 and the top 15 down-regulated genes were mostly involved in the cycle (CDK2, CDC2, KIF11) and also in melanogenesis (KIT). At t₄ a major loss of tumor volume was observed, along with depigmentation and lymphocyte infiltration. Gene expression showed an increase in T/NK cell (TVB1, TRAC, KLRK1, CCL5) and immunoglobulin associated genes (IGHM, IGKV1-5, IGLC1). The apoptosis related gene UBD was strongly upregulated. A loss of melanosomal gene expression was observed (SLC24A5, TYR, SLC45A2, CITED1, SILV, OCA2, GPR143), explaining the depigmentation effect.

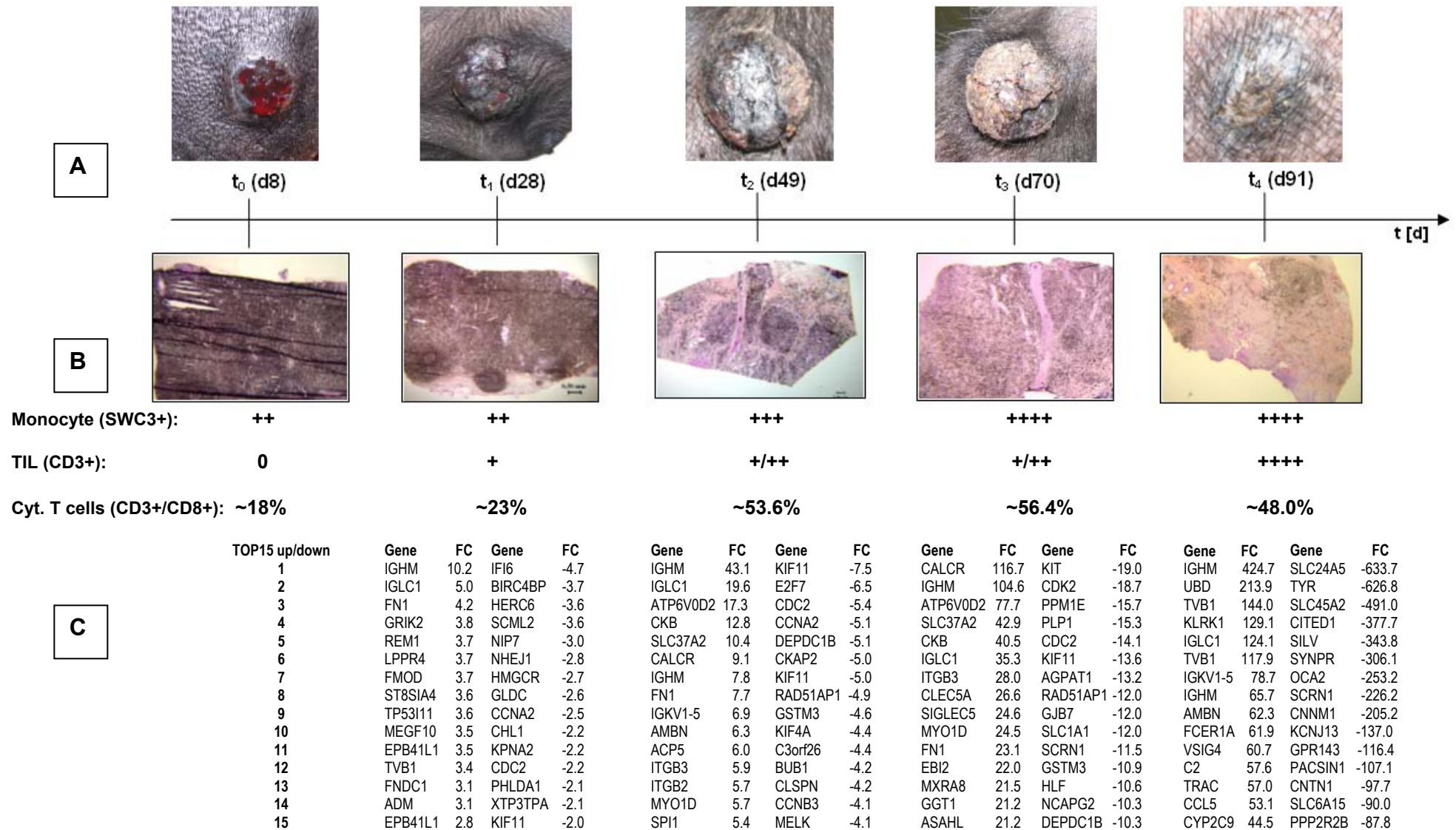


Figure 4: Clinical, histological, immunohistological and gene expression analysis of spontaneous melanoma development and regression.

A) Kinetics of clinical and histological progression and regression of MeLiM melanomas.

B) Immunohistological evaluation of tumors used for microarray analysis, characterizing monocytes by SWC3, T cells by CD3 and cytotoxic T cells by CD3 and CD8.

C) Gene expression analysis. T_0 was used as transcriptomic reference. Top 15 upregulated and top 15 downregulated genes per timepoint compared to t_0 are demonstrated.

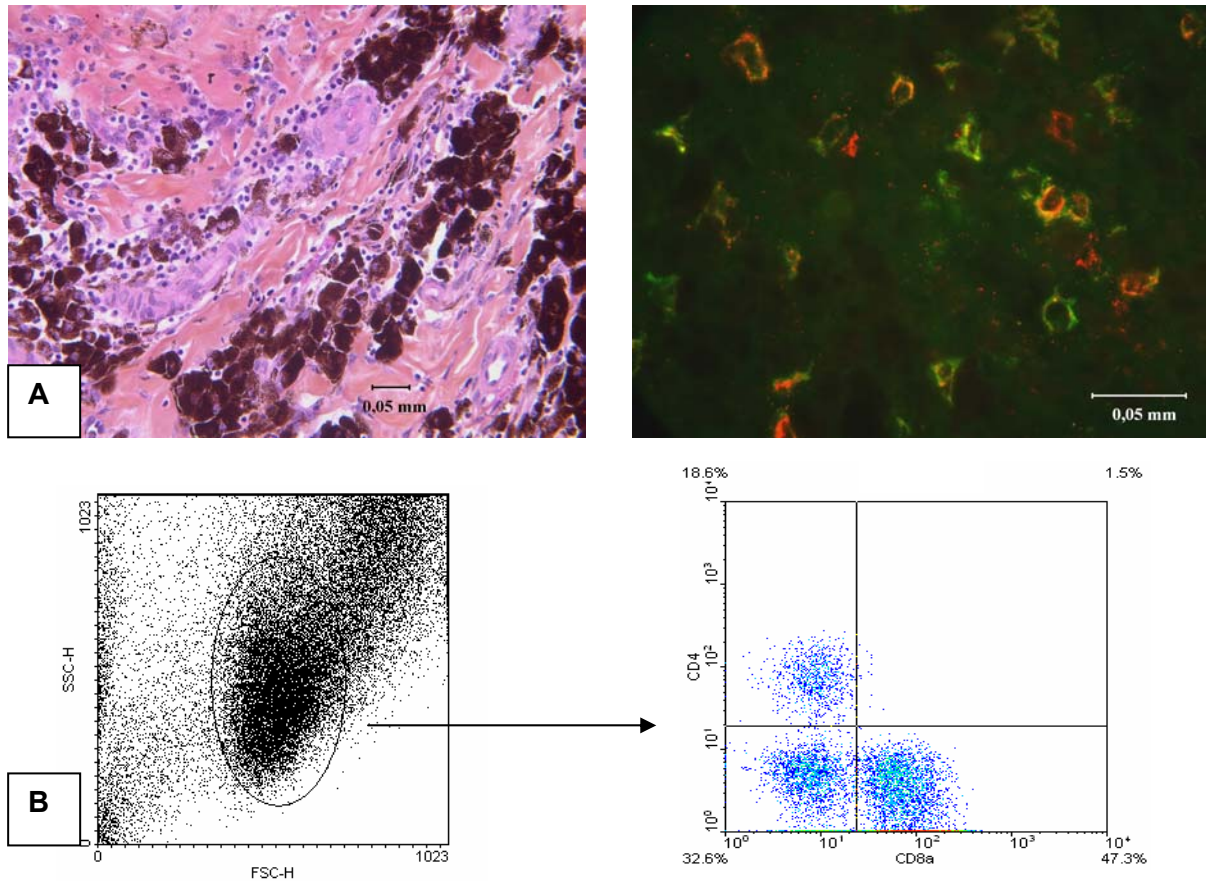


Fig. 5: Immunohistochemical and FACS analysis of Tumor infiltrating lymphocytes at t_4 .

A) Histological and Immunohistological analysis of melanoma sections at t_4 show extensive lymphocyte infiltrate. Lymphocytes were CD3 (Alexafluor@555) and/or CD8 (FITC) positive.

B) As analysed by flow cytometry, isolated tumor infiltrating lymphocytes at t_4 were mainly expressing CD8 (~42.8%, $n = 8$, SDV = 8.4%). CD4+ (22.2%, $n = 8$, SDV = 3.1%) TILs were also present.

Microarray Validation

To validate the microarray results, 9 immune response-related genes that were up-regulated over time and 6 cell cycle-related genes that showed a decrease over time were selected for quantitative RT-PCR (qRT-PCR) analysis (Supplement 2). Additionally, 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, KLRK1, PRF1) characteristic for immune effector cells such as cytotoxic T-cells and NK-cells, their signalling molecules (IL15, 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 cytotoxic T-cell (CTL) pressure [25], was also upregulated over time. MLANA was mostly downregulated (~27 fold) at t_4 whereas T cell response genes were expressed at highest. MITF-M showed already a decrease in expression (~4 fold) at t_3 , while

at t_4 it was downregulated up to 80 fold. Furthermore, we validated 6 genes associated with an early retardation of the cell cycle such as CCNB1, CDC2, BUB1B, BIRC5, KIF11, and CKAP2. All genes were at least 2 fold down-regulated from t_2 onwards, except BIRC5.

Table 2: Microarray validation by qRT-PCR analysis.

All expression values are relative to t0 (d+8). Same tumors used for microarray and qRT-PCR validation. For qRT-PCR analysis tumors for each time were pooled. FC=Fold change

Affy Probeset	Gene Symbol	Gene Title	Timepoint	FC (microarray)	FC (qRT-PCR)	Primers
Ssc.14243.1.S1_at	CCNB1	Cyclin B1	t1 t2 t3 t4	-1.55 -3.86 -6.82 -4.26	-1.39 -2.87 -3.06 -5.00	F: 5'-AGGAGCTTTTTGCCTATCCCTG-3' R: 5'-GCCAAGTGTGCATAACCACAA-3'
Ssc.873.1.S1_at	CDC2	Cell division cycle 2, G1 to S and G2 to M	t1 t2 t3 t4	-2.16 -5.36 -14.10 -4.99	-1.37 -2.82 -3.04 -2.73	F: 5'-TGTGCTTATGCAAGACTCCAGG-3' R: 5'- CCCTTGCAGGATTTGGTACAAA-3'
Ssc.7190.1.S1_at	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta	t1 t2 t3 t4	-1.16 -3.10 -5.03 -3.27	-1.49 -3.52 -3.76 -4.76	F: 5'-GGTCCCTCTGGAACTTAGCCA-3' R: 5'-GCTGCAAGTTCCTCAGAACAG-3'
Ssc.432.1.S1_at	BIRC5	Baculoviral IAP repeat-containing 5	t1 t2 t3 t4	-1.39 -2.95 -5.31 -3.63	1.06 -1.47 -1.48 -1.87	F: 5'-CCGGTTGTGCTTTCCTTTCTGT-3' R: 5'-CCTTGGCAATTTTGTCTTGGC-3'
Ssc.30748.1.S1_at	KIF11	Kinesin family member 11	t1 t2 t3 t4	-2.03 -7.52 -13.64 -4.82	1.10 -2.22 -1.96 -2.60	F: 5'-TGGAAAAGAAACAGCCTGAGCT-3' R: 5'-TCTTCAGCAGTGTGCACCAGA-3'
Ssc.26899.1.A1_at	CKAP2	Cytoskeleton associated protein 2	t1 t2 t3 t4	-1.95 -5.03 -5.90 -5.70	1.18 -2.18 -2.71 -3.86	F: 5'-TGCCTTGCACGTATTGAACA-3' R: 5'-CGCATTTCTTCAATAGGCTGAG-3'

Affy Probeset	Gene Symbol	Gene Titel	Timepoint	FC (microarray)	FC (qRT-PCR)	Primers
Ssc.6765.1.S1_at	SCIN	Scinderin (adseverin)	t1 t2 t3 t4	-1.90 3.76 16.01 32.44	-1.15 3.50 14.81 11.89	F: 5'-TGCCAATGAAGTTGAGAGAGCA-3' R: 5'-GGCTCATGGCCTTGTTTTACA-3'
Ssc.15871.1.S1_a_at	KLRK1	Killer cell lectin-like receptor subfamily K member 1	t1 t2 t3 t4	-1.01 2.88 1.49 129.10	-1.67 24.01 16.25 193.89	F: 5'-TCTCAAAATTCCAGTCTTCTGAAGATATA-3' R: 5'-AGGATCTGTTTGTTGGAATTTGTACTA-3'
Ssc.22030.1.S1_at	CCL5	Chemokine (C-C motif) ligand 5	t1 t2 t3 t4	2.01 4.67 8.66 53.06	4.70 6.79 9.90 16.82	F: 5'-TGGCAGCAGTCGTCTTTATCA-3' R: 5'-TGGCACACACCTGGCGGTTCTTTC-3'
Ssc.8833.1.S1_at	IL15	Interleukin 15	t1 t2 t3 t4	-1.28 1.67 2.19 10.83	1.18 2.74 9.29 8.76	F: 5'-GATGCCACATTGTATACTGAAAGTGA-3' R: 5'-GCGTAACTCCAGGAGAAAGCA-3'
Ssc.11075.3.S1_a_at	TVB1	T-cell receptor beta chain V region YT35	t1 t2 t3 t4	3.37 5.52 14.50 144.00	1.99 8.98 10.13 29.25	F: 5'-GCTGCCAAGTCCAGTTCTATGG-3' R: 5'-GATGGGCTTGGTCCAGTTGT-3'
Ssc.5887.1.A1_at	SLC37A2	Solute carrier family 37 (glycerol-3-phosphate transporter), member 2	t1 t2 t3 t4	2.24 10.41 42.94 11.14	2.94 5.65 32.64 6.86	F: 5'-TTGCCAAGCTGGTCAGTTACAC-3' F: 5'-GCCTATGATGCCACCAACATC-3'

Affy Probeset	Gene Symbol	Gene Titel	Timepoint	FC (microarray)	FC (qRT-PCR)	Primers
Ssc.22164.1.S1_at	ATP6V0D2	ATPase, H ⁺ transporting, lysosomal 38kDa, V0 subunit d2	t1 t2 t3 t4	3.15 17.30 77.68 23.41	2.34 8.50 38.87 12.78	F: 5'-AGGTACAGATGAATGTGCTGGC-3' R: 5'-TGCATTTCAGCTATCCACACGA-3'
Ssc.13778.1.S1_at	IGHG1	IGHG1 (IGHM)	t1 t2 t3 t4	10.23 43.05 104.61 424.72	19.08 104.33 192.24 428.81	F: 5'-GGACCATCTCCAAGGCTATAGG-3' R: 5'-TCCGTTGCTCTTCCACTCAA-3'
Ssc.180.1.S1_at	TRGV9	T cell receptor gamma variable 9	t1 t2 t3 t4	1.17 2.03 1.97 28.09	2.11 3.39 7.49 20.92	F: 5'-CCTGGAAGAGGTCCTGACAGAT-3' R: 5'-CCCAGAAAGGAAGTGCTGCTA-3'
Not on genechip	MITF-M	Microphthalmia-associated transcription factor-Melanocyte specific isoform	t1 t2 t3 t4	X	1.73 1.15 -4.38 -81.02	F: 5'-GCGTGGTTATGCTGGAAATGC-3' R: 5'-TGCTTTACCTGCTGCCTTTGG-3'
Not on genechip	MLANA	Melan A (MART1)	t1 t2 t3 t4	X	1.01 -1.23 -3.51 -27.35	F: 5'-ACAACTGTGGACCTGTGGTTCC-3' R: 5'-CAGAAGACCTGCTGGCTCTCAT-3'
Ssc.19693.1.A1_at Ssc.19693.1.S1_at Unresponsive probe sets	PRF1	Perforin 1 (pore forming protein)	t1 t2 t3 t4	X	-2.32 2.84 3.98 6.11	F: 5'-TTCGCGGCCCCAGAAGAC-3' R: 5'-CTGTAGAAGCGACACTCCACTGA-3'

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. 1411 genes were significantly regulated during melanoma regression. Functional analysis by Ingenuity Pathway Analysis (IPA) revealed highly significant functional gene classes such as Cell Death, Cell Cycle, Cellular Movement, Immune Response, Hair Skin development and Function, 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 upregulated at t_0 were connected to a highly proliferative gene signature [14]. Expression profiles at t_1 were not very different from t_0 indicating an ongoing growth phase. Nevertheless, immunoglobulin related genes (IGHM, IGLC1) were upregulated at t_1 as well as fibronectin (FN1) which is involved in tumor invasion.

Interestingly, we observed an early downregulation 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 downregulated genes at t_2 were connected with cell cycle and DNA repair mechanisms. The most significant gene network, detected by IPA analysis contained was mainly composed of genes (86%) involved in cell cycle mechanisms. Cyclins such as CCNB1, CCNA2 and cyclin-dependent kinases such as CDC2, CDC6 as well as spindle and chromosome structure-related proteins such as kinesins (KIF11, KIF2C), NEK2, MELK, TOP2A, BUB1 and BIRC5 playing roles in chromosome segregation and cell cycle checkpoint control, showed 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. BUB1B codes for a kinase involved in spindle checkpoint function, and BIRC5 acts as an inhibitor of apoptosis lately shown to be also recognized as an antigen by CD8+ T-cells in multiple myeloma patients [26]. We confirmed the downregulation of 6 cell cycle genes (KIF11, CCNB1, CDC2, BUB1B, BIRC5, CKAP2) by qRT-PCR. This is the first time that Kinesin family member 11 (KIF11), expression is described in melanoma. KIF11 encodes for a motor protein that is involved in spindle formation and has become a promising

target for antimitotic cancer therapy [27]. The majority of cell cycle genes that we found downregulated were involved in mitosis (~70%).

Most of these genes were recently reported to be related to aggressiveness of human melanoma but were regulated in the opposite way suggesting that the reversal of a metastatic signature could lead to tumor regression [15, 17, 18]. 3 genes (KPNA2, CDC2 and KIF11) which were associated with higher metastatic potential of human melanoma tissue showed already a trend of downregulation at t1 while no signs of regression were detected [17]. Even gene signatures of melanoma cell lines showed common but inversely expressed genes (NUSAP1, BIRC5, GINS1, NCAP2, MELK, and HELLS) that were cell cycle related [18]. At t3 when regression signs were already established, we identified 10 genes downregulated (PLP1, EDNRB, CITED1, TFAP2A, RAB38, TYR, MITF, GPR143, GPM6B, GPRC5B), whereas in humans the upregulation of these 10 genes was associated with a highly proliferative and weakly metastatic potential in primary melanoma cell lines [15].

The use of melanoma tissue in expression studies is often stated as a drawback since normal or reactive tissue constituent (e.g., vessels, inflammatory cells, normal epithelium) are interpreted as "contaminants" [28]. In our case, the interest was to obtain a global understanding of regression mechanisms related to melanoma cells and their surrounding, while the tumor micro-environment has recently been found to influence the progression of cancer by inducing phenotypic changes in cancer cells [29] and is therefore also very likely to be involved in regression [30].

To address questions of expression specificity, we laser microdissected tumor regions of homogenous melanoma cells at early timepoints such as t₀, t₁ and t₂ and confirmed the early downregulation 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 a induction of senescence is not very likely to be involved in the regression process. Terminal differentiation of melanoma cells into pigment laden macrophages (PLM) was also suggested as an early event in spontaneously regressing melanoma using the Sinclair swine, another spontaneous regressing melanoma model [31]. Indeed, differentiation occurs in human malignant melanoma via diverse pathways as

reviewed by Banerjee and Eyden [32]. However, genes frequently involved in human melanoma differentiation such as NCAM and SYP were not regulated. Induction of melanoma regression could either be initiated by the tumor cells itself or a consequence of extrinsic mechanisms like an anti-tumor immune response or beneficial interaction of both. Recent studies demonstrated that spontaneous regression is mediated by infiltration of leucocytes mostly of innate immunity [33, 34]. Spontaneous melanoma regression in MeLiM pigs 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 [35]. In our model, we detected a strong upregulation of monocyte/macrophage related genes from t_2 to t_3 that corresponded with the appearance of histiocyte-like cell infiltrates on a histological level. Tumor infiltrating macrophages were shown to play a dual role in cancerogenesis since they can enhance and inhibit tumor growth depending on their activation and secretion of effector molecules [36]. Molecules, associated with an inhibitory effect, such as IL10, IL13, IL4, and TGFB1 were present on our microarray but did not show significant regulation. Characteristics of these cells are hyperpigmentation, aneuploidy, increased size and irregular borders. Phenotypically similar cells in melanoma have been associated with osteoclast like giant cells [37], melanophages [38] and macrophage-melanoma fusion hybrids [39]. 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 [40], whereas high expression of ACP5 in mouse macrophages was shown to enhance cytotoxicity by increased ROS and superoxide production [41]. In the spontaneous regression/complete resistance (SR/CR) mouse model [42], macrophages were demonstrated to induce tumor apoptosis by cell contact dependent secretion of ROS and serine proteases. Even though antibodies against tumors were produced in SR/CR mice, they were not required for killing, suggesting that antibody dependent cellular cytotoxicity (ADCC) is not an effector mechanism in this model. Regression in MeLiM pigs is accompanied by an early upregulation 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 t_3 and t_4 even though at t_1 IGHM was already 10 fold upregulated compared to t_0 . 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 t_1 and healthy skin (data not shown). Furthermore a stronger upregulation of IgM levels would be expected if it is the initiating mechanism as normal pigs show already stable IgM serum levels throughout their first 3 weeks of life and are therefore fully armed to fight the tumor at t_1 [43].

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 MHCII expressing cells [44]. We detected a significant upregulation of different TCR chains at a late stage of regression (t₄), as well as T cell associated cytokines (CCL5 and IL15), 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⁺ than CD4⁺ T cells, suggesting a predominant cytotoxic T cell response in our case. γ/δ T cells as well as CD4⁺CD8⁺ T cells and CD16⁺CD8⁺ NK cells did not seem to play in regression since they were almost not detectable in tumor infiltrates compared to PBMCs (data not shown).

MeLiM pigs show signs of autoimmune reactions such as localised or systemic vitiligo-like depigmentation. This phenomenon could be explained by cross-antigenicity 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 [45]. Autoantibodies isolated from vitiligo patients were shown to trigger apoptosis in melanocyte cultures [46]. Furthermore antimelanoma antibodies were isolated from Sinclair swine, recognizing antigens expressed on the surface of normal melanocytes [47].

CD8⁺ T cells isolated from peripheral blood of vitiligo patients were also shown to frequently recognize the melanosomal protein MLANA and kill HLA-matched melanoma cells [48], playing therefore a role in the depigmentation process. We confirmed the downregulation of MLANA expression during spontaneous regression by qRT-PCR analysis. The expression of additional genes involved in melanogenesis, such as SILV, SLC24A5, OCA2, CITED1 and TYR was dramatically downregulated 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 reactive oxygen species (ROS), including OH[•], O₂^{•-} and H₂O₂ [49], whilst accumulation of melanin intermediates, produced in the absence of tyrosinase, TYRP1, and DCT, is cytotoxic [50]. 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 since more melanoma antigens would be presented on the surface that could be recognized by immune cells [51]. A

main immune escape mechanism such as the downregulation 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 MHCII molecule CD74, as well as costimulatory molecule CD86, seems to lead to the activation of CD8⁺ cytotoxic effector T cells. The major downregulation of melanoma antigens at t_4 is coherently accompanied by the upregulation 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 (TP53111, BIM) and extrinsic (TNFSF12, GZMB) death signals lacking a distinct classification. A possible explanation could be that our chosen timepoints for microarray hybridization missed the molecular detection of the ongoing apoptotic process since programmed cell death is a rather rapid process. We orientated our choice by taking into account major histological changes such as first signs of regression. Other apoptotic genes such as CLU, ELMO1, PLEKHF1, THY1, were detected along with a strong increase of ubiquitin D expression at t_4 . Clusterin was maximal upregulated at t_3 (~12 fold) suggesting a role in early regression, as we have shown and confirmed earlier by SSH and qRT-PCR [14]. CLU codes for a glycoprotein whose nuclear isoform seems pro- and its secretory form antiapoptotic [52]. 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 [12]. Future investigations include in vitro experiments to answer questions of cytotoxic key mechanisms as well as adoptive transfer of melanoma cells in mice (beige mice) with functionally defective monocytes, especially phagocytes and NK cells [53, 54].

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, harbouring potential targets for either chemotherapy (downregulation of a mitotic cell cycle gene signature) or immunotherapy (upregulation of a complex immune response signature).

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Supplementary Data

See Appendix.

Supplement 1: Gene lists of cluster 1-6

Corrected p value = Benjamini Hochberg (FDR=0.01) corrected p value (ANOVA)

Supplement 2: Downregulation of cell cycle related genes is related to melanoma cells during early regression

Supplement 3: Gene list comparison with human melanoma expression data.

This file contains 3 gene list comparisons against those of Winnepenninckx et al, Ryu et al, and Hoek et al. Common genes were noted as well as their regulation if it was mentioned and their corresponded regulation and fold change in melanoma regression.

Supplement 4: Top 15 up and downregulated genes per time point.

Supplement 5: IPA canonical pathway analysis

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Phenotypic characterization of tumor immune infiltrate

As previously described, the process of spontaneous tumor regression in melanoma pigs is accompanied by a biphasic cellular response. The two phases consist of an initial innate cellular response characterized by an infiltration of highly pigmented histiocyte like cells, followed by an adaptive T cell mediated response. Since we detected these cellular responses on a transcriptomic level by the identification of different immune signatures (Rambow et al., 2008b), we aimed to further characterise this phenomenon by the help of immunohistochemistry and flow cytometry. Both methods were briefly described elsewhere (Rambow et al., 2008b).

1. Characterization of highly pigmented cells

Histopathological analysis suggested a role for these highly pigmented cells since their presence correlates with the phenomenon of regression.

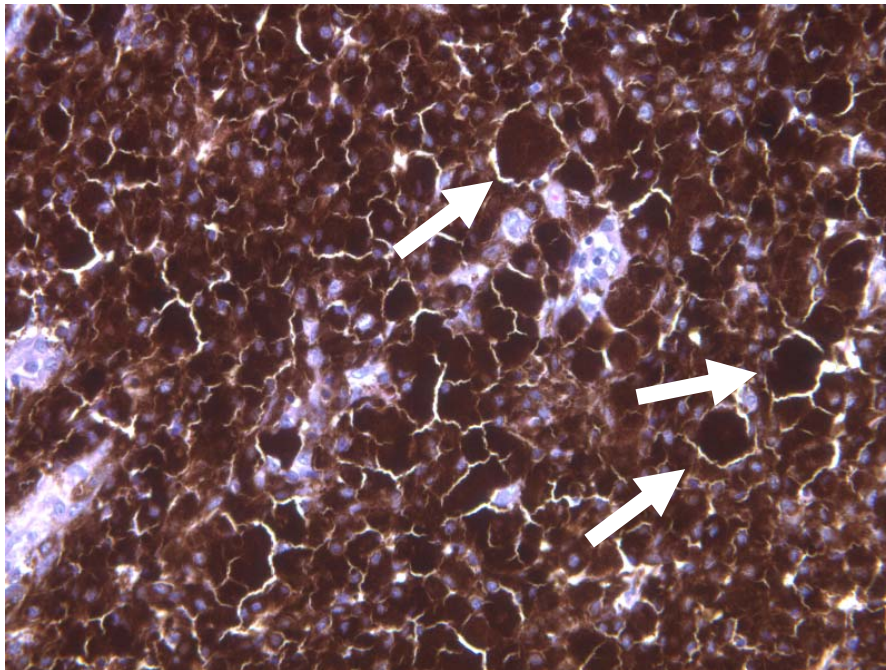
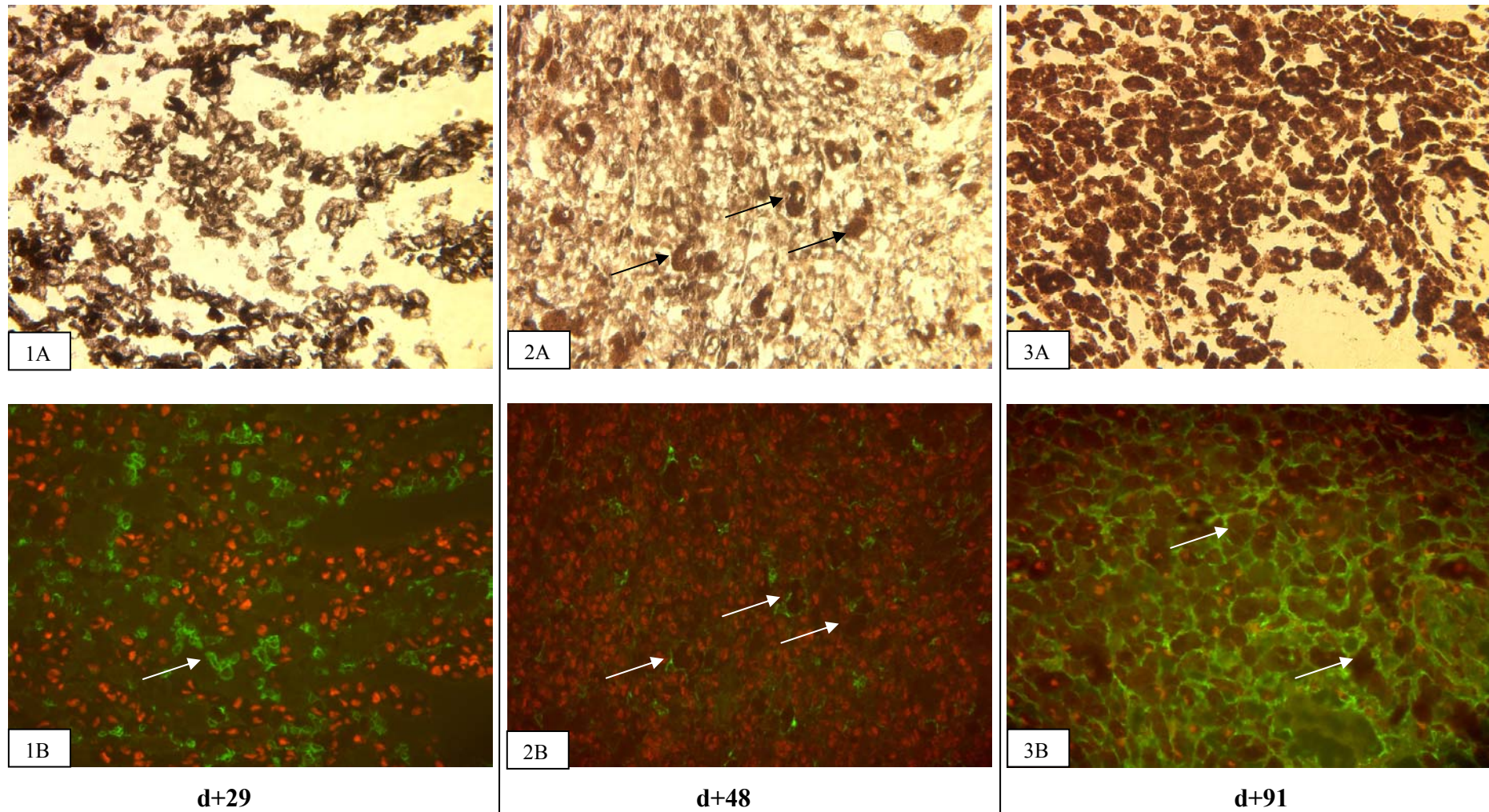


Fig. 8: Highly pigmented histiocyte-like cells

The interest was to characterize further these highly pigmented cells because their phenotype and biological role are speculative. Suggested roles and origins are quite diverse and range from melanophage like cells, differentiated melanoma cells, fusion hybrids between melanoma cells and macrophages and neoplastic cells of animal-melanoma origin. Histologically these cells show different characteristics such as large cells with irregular borders, hyperpigmentation, and de- or multinucleation.

In a first study we wanted to characterize these cells on a protein level using antibodies directed against SWC3 and MITF to get an idea of the either melanocytic or monocytic origin of these cells. The Swine Workshop Cluster 3 (SWC3) antigen is well accepted marker for porcine cells of the myeloid lineage such as macrophages, peripheral blood monocytes, dendritic cells and granulocytes. Cloning and molecular characterization of the protein recognized by the anti-SWC3 antibody lead to the identification of the SWC3 protein that belongs to the signal-regulatory protein (SIRP) α family (Alvarez et al., 2007). In humans, SIRP proteins are a family of transmembrane glycoproteins, involved in signal transduction, which are expressed at high levels in neurons and myeloid cells, including macrophages, monocytes, granulocytes and dendritic cells. Important to know, that SIRP α has cytoplasmic domains containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Tyrosinase phosphorylation of these motifs leads to a negative regulation of signal transduction cascades. MITF is a well established marker for cells of the melanocytic lineage. Important to know, MITF has different isoforms that could also be expressed in non-melanocytic cell types such as macrophages and mast cells (MITF-E,-J) for example (Murakami M 2007). Anyhow the melanocyte specific isoform MITF-M can be mimicked by a MITF expression restricted to the nucleus.

In a first study we were able to describe 3 different stages of MITF and SWC3 protein expression at the tumor site. Comparing panels 1A and 1B of Fig. 9, which demonstrate a typical melanoma tissue at d+28, SWC3 expression is mainly restricted to non-pigmented cells, most probably macrophages or dendritic cells, whereas MITF expression is found mainly in pigmented cells. Comparing panels 2A and 2B, that demonstrate a typical melanoma tissue at d+48, show huge highly pigmented cells which seem to be SWC3+ and MITF+ whereas others seem to be double negative for SWC3 and MITF staining. Comparing panels 3A and 3B, that demonstrate a typical melanoma tissue at d+91 (late regression), MITF expression seems to be weaker even though cells are still highly pigmented. SWC3 expression seems now to be ubiquitously expressed. Again highly pigmented cells can be found without MITF staining. As discussed later, additional markers would be needed to unravel the origin and role of these cells.



A) normal light

B) SWC3 staining revealed by FITC (green), MITF staining revealed by Alexafluor® 555 (red)

Fig. 9: MITF and SWC3 staining during regression

2. Phenotypic characterization of Tumor Infiltrating Lymphocytes (TIL)

Infiltration of the tumor site by lymphocytes is a common phenomenon in human melanoma. Also in pigs infiltration by TILs can be observed. This infiltration is the second arm of the biphasic cellular response against melanoma described in Sinclair, MMS Trolls and MeLiM pigs. We were interested to characterise the different subsets of tumor infiltrating lymphocytes by flow cytometry based phenotyping. The method was described elsewhere (Rambow et al., 2008a; Rambow et al., 2008b). We used $n = 9$ tumors for TIL isolation from different animals during late stage of regression. Pigs ranged from d+97 to d+126 in age. We investigated the expression of the following markers in TILs (isolated from melanomas) and PBMCs (isolated from blood) for each animal:

anti γ/δ	BD Pharmingen clone MAC320 cat# 551534 host:rat, 0,1mg
CD3	VMRD clone: 8EG host: mouse, 1,0mg/ml
CD16	Serotec MCA1971 host: mouse, 0,2mg
CD8 α	VMRD clone: PT81B host:mouse conc.:1mg/ml
CD4	VMRD clone: PT90A host:mouse conc.:1mg/ml

With the following markers we were able to distinguish between different cell types such as double positive T lymphocyte (CD4+CD8+), γ/δ T cells (γ/δ + CD3-), cytotoxic T cells (CD8+CD3+CD4-), T helper cells (CD4+CD3+CD8-) and Natural Killer (NK) cells (CD8+CD16+).

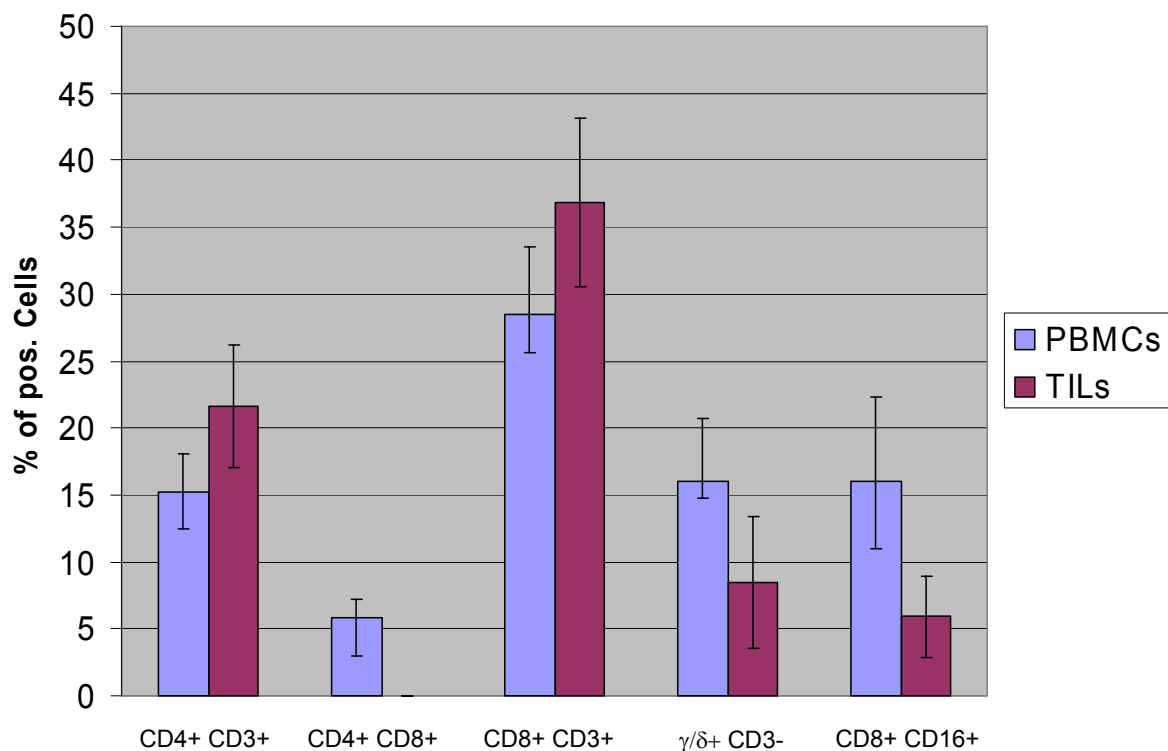


Fig. 10: Immune cell populations in PBMC and TIL isolates.

Comparing the different immune cell populations in the peripheral blood and melanomas during a late stage of regression revealed the following:

- T helper cells (CD4+CD3+) were slightly upregulated in melanomas (~15%) compared to peripheral blood (~22%).
- Double positive T cells were present in the blood with ~5% whereas no double positive T cells could be detected in melanomas.
- Cytotoxic T cells (CD8+CD3+) were found be the most abundant cell type in melanoma TILs (~37%). In the peripheral blood 29% of lymphocytes were shown to be cytotoxic T cells.
- γ/δ T cells were more abundant in peripheral blood (16%) than in melanoma tissue (9%).
- NK cells (CD8+CD16+) were also more abundant in peripheral blood (16%) than in melanoma tissue (~6%).

In the following Fig. 11, typical FACS density plots for immunophenotyped cells isolated from A) PBMC and B) TIL populations are demonstrated.

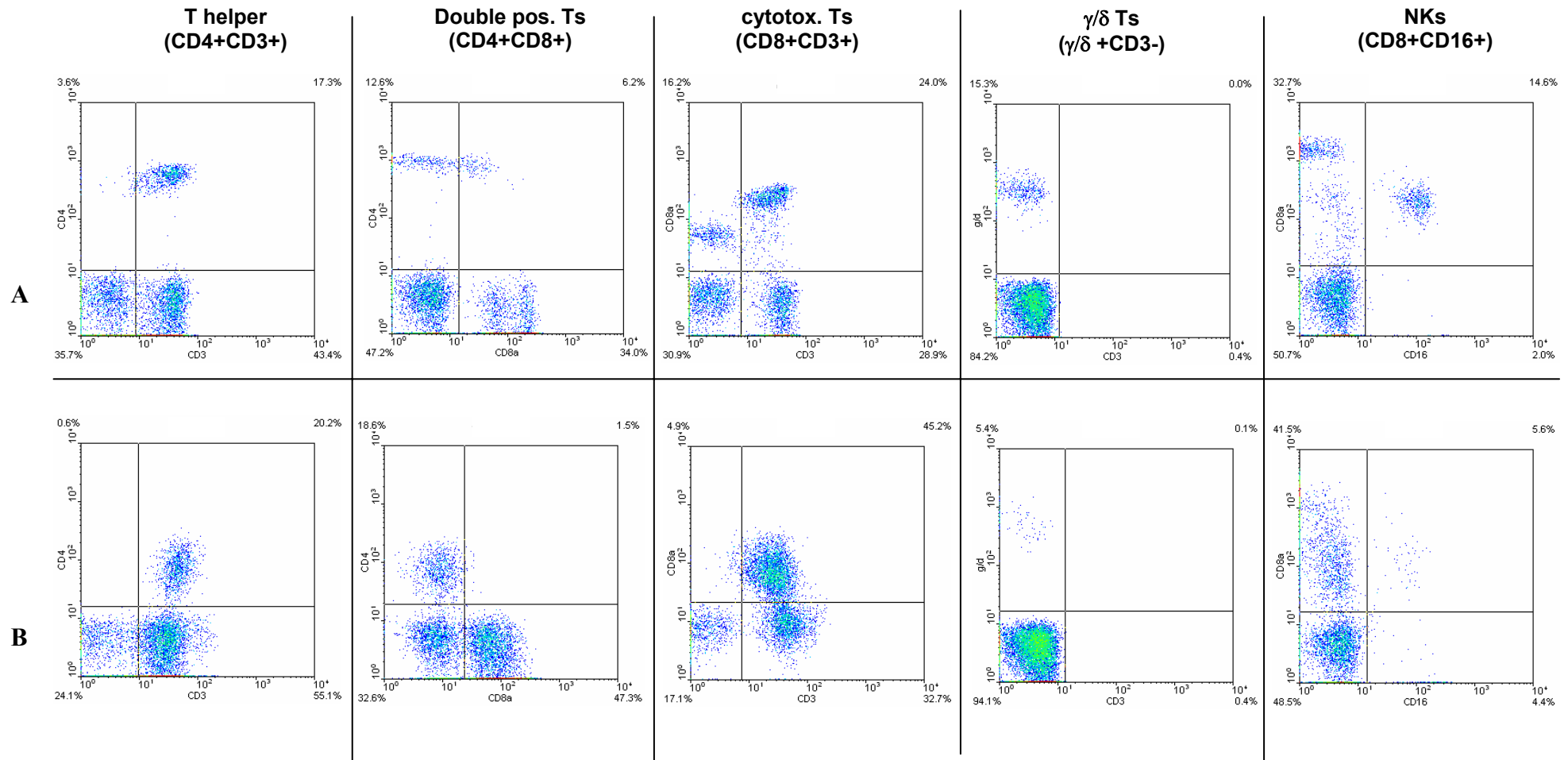


Fig. 11: Flow cytometry analysis of A) PBMCs and B) tumor infiltrating lymphocyte

CHAPTER 4

General Discussion

The objective of this PhD thesis was to investigate molecular and cellular mechanisms involved in spontaneous cancer regression using a porcine melanoma model.

Melanoblastoma bearing Libechov Minipigs (MeLiM) were used to study melanoma regression since they develop melanoma naturally *in utero* or shortly after birth. Most importantly, melanomas and even metastasis in MeLiM minipigs regress completely without any external influence.

To study the transition from growing to regressing melanoma on a molecular level, we applied gene expression profiling techniques such as Suppression Subtractive Hybridization (SSH) and microarrays. Gene expression profiling has become an invaluable tool in functional cancer genomics since identified gene expression profiles have the capacity to surrogate specific cancer phenotypes. A gene expression profile, or signature, is a simple representation of a biological state, in the form of a pattern that is unique to a specific circumstance. The underlying concept is that any biological condition, whether a developmental state, a cellular response to extracellular ligands or a pathological state, is reflected by changes in gene expression (Nevins and Potti, 2007).

Gene expression profiling is often used to screen for active genes that could shed light on mechanisms leading to a certain phenotype. These studies result normally in hundreds of target genes that require functional interpretation and external confirmation. However gene expression profiling has been shown to be a powerful tool in human melanoma that helped for example to understand the transition from highly proliferative to highly invasive melanoma cells (Hoek et al., 2008).

Besides transcriptional analysis of spontaneously regressing tumors, we investigated cellular responses by characterizing tumor infiltrating immune cells such as antigen presenting cells, lymphocytes, and highly pigmented histiocyte-like cells using flow cytometry and immunohistochemistry.

Differentially expressed genes between regressive and progressive melanoma

The aim of our first study was to obtain a global overview of transcriptional changes involved in the beginning of spontaneous regression. Therefore we compared RNA populations of whole tumor tissue isolated from growing and early-stage regressing melanoma by SSH. To

further differentiate between intrinsic (melanoma cells) and extrinsic (stroma, microenvironment) transcriptomic changes, we also compared subtractive libraries of cell cultures isolated from growing and regressing melanoma. We were able to identify a molecular profile for growing melanoma at d+8 that showed similar genes to progressive human melanoma (Rambow et al., 2008a). Genes were mainly involved in melanocyte differentiation such as *MLANA*, *SILV*, *TYR*, *TYRP1*, and the regulator of melanocyte transcription *MITF*. These molecules amongst other pigmentation related genes were downregulated in regressive melanoma tissue at d+52 suggesting a loss of melanoma cells as confirmed by a localized depigmentation effect. In some cases depigmentation spreads throughout the animal and results in a vitiligo-like phenotype (Vincent-Naulleau et al., 2004). Since vitiligo has been linked to an autoimmune T-cell and/or antibody-mediate response against melanocytic antigens, it stands to reason that the immune response could play a role in regression-associated depigmentation since melanoma cells and melanocytes share surface antigens (Ram and Shoenfeld, 2007). Vitiligo has been observed in malignant melanoma patients, although the incidence and significance vary widely (Schallreuter et al., 1991). However, it was shown recently that the development of vitiligo in metastatic melanoma patients treated with a certain immunotherapy correlated with a better therapeutic outcome (Boasberg et al., 2006), suggesting a beneficial role of vitiligo in melanoma patients.

Other genes identified by SSH analysis that were overexpressed at the beginning of regression were mainly involved in functional classes such as the immune system, cell cycle arrest, and tumor suppression. 3 genes confirmed to be significantly upregulated at early regression included *CLU*, *CD9* and *RARRES1*. Enhanced *CD9* and *RARRES1* expression were confirmed on the protein level supporting a functional role during early regression. This is the first time that *RARRES1* is reported in melanoma. Retinoic acid receptor responder 1 (*RARRES1*) is a retinoid regulated gene. Its expression is frequently down-regulated through DNA hypermethylation in several types of malignant tissues. As a putative tumor suppressor, *RARRES1* progressively declines in malignant tissues with a concomitant decrease in tumor differentiation and increase in disease progression (Wu et al., 2006). However, the exact endogenous function of *RARRES1* remains unclear. In our case *RARRES1* is naturally upregulated during spontaneous melanoma regression and suggests therefore a role for differentiation. Also, expression of *RARRES1* was shown to be induced in well-differentiated colon cancer cells after treatment with vitamin D (Wood et al., 2004).

The major advantage of the SSH technique is to identify new genes. This feature has not been fully explored yet on our data as the entire porcine genome has not been sequenced so far.

Interestingly, we observed various small size RNAs that aligned in introns and exons of genes suggesting the presence of microRNAs (miRNAs). MiRNAs are endogenous ~22nt noncoding small RNAs which regulate gene expression in a sequence specific manner (Zamore and Haley, 2005). Increasing evidence shows that expression of miRNAs is deregulated in human cancer (Calin et al., 2004; McManus, 2003). Specific over- or underexpression has been shown to correlate with particular tumor types such as ovarian cancer (Yang et al., 2008) and colorectal cancer (Cummins et al., 2006). MiRNAs overexpression could result in downregulation of tumor suppressor genes whereas their underexpression could lead to oncogene activation. Actually, we identified the microRNA21 (MIRN21) that was overexpressed in early regressing melanoma. MIRN21 was shown to regulate the expression of the tumor suppressor PTEN in hepatocellular cancer (Meng et al., 2007). Another example is let-7 which is downregulated in lung cancer (Takamizawa et al., 2004) and was shown to suppress RAS (Johnson et al., 2005). Just recently, common miRNAs that shared gene copy number changes for epithelial cancers such as breast, ovarian and melanoma were identified supporting the notion that copy number alterations of miRNAs and their regulatory genes are highly prevalent in cancer (Zhang et al., 2006).

It is worthwhile to mention that this gene expression study was conducted at only one specific time point during regression without biological replicates. Furthermore melanomas can be quite heterogeneous in terms of regression speed and therewith associated morphological and transcriptional changes. This heterogeneity of melanoma can be observed even on different lesions on the same animal and needs therefore precise clinical and histological evaluation as we performed.

Time dependent gene expression profiling during melanoma progression and regression

Tumor regression is a dynamic process that lasts about 3 months in our case. This process has been already characterized on a clinical and histological level (Vincent-Naulleau et al., 2004). Clinical signs associated with regression are drying and greying of the tumor site, and loss of tumor volume. Histological signs comprise infiltration of highly pigmented melanophage-like cells, occurrence of TiLs, and fibrosis.

The objective of the second study was to monitor gene expression changes over time to identify gene signatures that could explain the phenotype. We performed time dependent gene expression profiling on a family of MeLiM pigs using porcine microarrays. We collected

melanoma tissues every 3 weeks over 3 months starting at day 8 after birth. Microarray analysis revealed significant gene signatures that changed over time allowing us to speculate on mechanisms leading to spontaneous regression.

Firstly, we identified a downregulation of cell cycle related genes, 7 weeks after birth while on a clinical and histological level only a few first signs of regression were detectable. The cell cycle signature contained mostly genes involved in mitosis suggesting a mitotic arrest of melanoma cells at the very beginning of regression.

Secondly, 10 weeks after birth, we identified an upregulation of monocyte/macrophage related genes. This increase of genes of the innate immune system was also observed on the histological level since tumors were infiltrated by highly pigmented macrophage like cells (week 3 -13).

Thirdly, 13 weeks after birth, we observed an increase of T/NK related genes as well as genes of the immunoglobulin family. Furthermore melanocyte differentiation genes (pigmentation related genes) were strongly downregulated that correlated with loss of melanoma cells and depigmentation of the tumor site.

Comparison of obtained transcriptomic results

Even though the two techniques used to identify differentially expressed genes have major differences such as open vs. closed method, number of replicates, sensitivity for low abundance genes, and statistical confidence (Cao et al., 2004), we searched for common genes. We compared therefore SSH results of progressive melanoma tissue against regressive melanoma tissue to the corresponding timepoints such as d+8 and d+49 of the microarray study. Common genes upregulated in progressive melanoma that were identified by the two independent experiments included NUSAP1, CHL1, MAD2L1, TPX2, and HSD17B12. Interestingly, 3 of these 5 genes upregulated during melanoma progression are involved in the formation of spindle apparatus during mitosis such as NUSAP1 (Ribbeck et al., 2006), MAD2L1 (Michel et al., 2004), and TPX2 (Gruss et al., 2002) suggesting a higher mitotic rate. CHL1 is a member of the L1 gene family of neural cell adhesion molecules and was shown to play a role in cancer progression by enhancing cell migration and tumor growth in ovarian carcinoma (Gast et al., 2005). The enzyme 17-beta hydroxysteroid dehydrogenase-12 (HSD17B12) is a new enzyme involved in the production of estradiol (Luu-The et al., 2006). Expression of estrogen receptors that bind the biological active form of estrogen, 17-beta-

estradiol has been shown to be well established markers in breast cancer and was also proposed as a marker for melanoma progression (Tanemura et al., 2007). Common genes upregulated in regressive melanoma tissue included CLU, ATP6V0D2, ITGB2, and SH3BGRL3. The genes CLU, ATP6V0D2 and ITGB2 play a role in apoptosis and immune response as discussed in Rambow et al. (2008b). SH3 domain binding glutamic acid-rich protein like 3 (SH3BGRL3) is the new member of thioredoxin (TRX) super family, whose posttranslational modified form was identified as tumor necrosis factor alpha (TNF-alpha) inhibitory protein that may function as a regulator in all-trans retinoic acid-induced pathway (Xu et al., 2005). Although different animals of different genetic background were used for both studies and differences in applied techniques, common genes were identified that play a role in cell cycle, apoptosis and immune response. These functional groups are of major interest when speculating on regression mechanisms as supported by the complex findings of the microarray study.

Hypothesis of regression mechanism in MeLiM

Spontaneous melanoma development and subsequent regression in MeLiM pigs are continuous and complex phenomena with well defined changes on the clinical, histological, and molecular level. These changes should not be regarded as single events but rather as interrelated phenomena influencing each other. One may speculate on the key that leads to spontaneous melanoma regression, and create a plausible hypothesis:

Familial melanoma develops spontaneously without UV exposure in MeLiM swine. Melanoma development has been shown to be multigenic and inherited in an autosomal dominant way showing incomplete penetrance. However, almost all tumors that develop regress spontaneously, along with potential metastasis. This might suggest that regression is able to spread systemically in the animal. The regression process could be either genetically manifested or a consequence of genetically induced development.

Recently, Cui et al. have described a strain of mice (spontaneous regression-cancer resistant, SR/CR) that are resistant to many types of transplantable tumors by mounting an innate immune response against the tumors (Hicks et al., 2006; Cui, 2003). This resistance trait is inherited in a dominant manner and appears to be controlled by one chromosomal locus whereas this locus is not identified yet. Anyhow, this anti tumor immunity can be transferred to other mice, suggesting that the mutated gene encodes for a ligand uniquely expressed by a subset of leukocytes in the SR/CR mice that leads to regression (Smyth and Kershaw, 2003).

In the case of the MeLiM swine, the “regression-phenotype” has not been genetically assessed.

In contrast, the consequence-theory (Fig. 12) could be supported by different biological aspects. Speculating on genetic alterations leading to melanoma development in MeLiM includes MC1R and MITF amongst others (Du et al., 2007). These genes play an important role in the process of skin pigmentation (Levy et al., 2006; Sulem et al., 2007). Hence potential genetic alterations in pigmentation related and/or melanocyte differentiation related genes could be responsible for the hyperpigmented phenotype of MeLiM melanoma. In concordance, MITF was shown to regulate transcriptionally melanoma antigens such as MLANA and gp100 (Du et al., 2003). Furthermore the transfection of UIISO-MEL-6 cells with MITF increased the expression of melanocyte differentiation markers like TYR and TRP1 (Lekmine et al., 2007). An upregulation of melanocyte differentiation markers induced by potential gain of function mutations would lead possibly to an increase of melanoma antigens (TYR, MLANA, gp100) expressed on the tumor cell surface and therefore to a higher immunogenicity of the tumor. To additionally activate the immune system by increasing the immunogenicity, has been the rationale for melanoma vaccination therapy.

Besides the augmentation of melanoma antigens that could be recognized by extrinsic mechanisms like the immune system, mutations of melanogenesis related genes could also affect intrinsic mechanisms, namely the cell cycle.

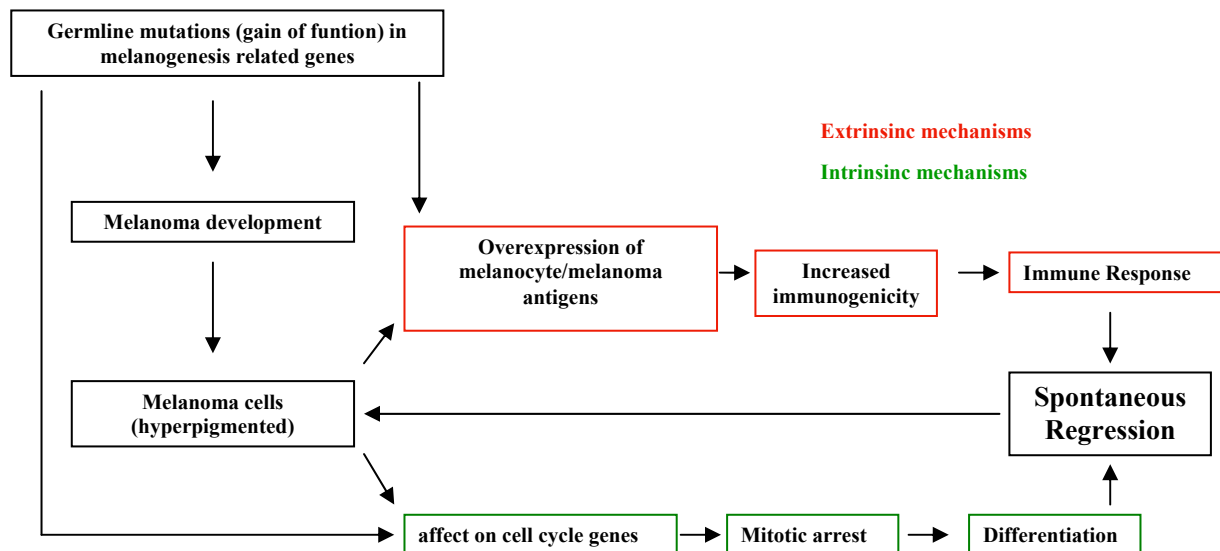


Fig. 12: “Regression as consequence” theory in MeLiM. Spontaneous regression of melanoma in MeLiM might be linked to initial germline mutations in melanogenesis regulating genes. Potential gain of function mutations lead to melanoma development. Melanoma cells are highly pigmented, and present more antigens on the surface. Increased immunogenicity leads to a more efficient immune response fighting the tumor and leading to regression. In parallel, gain of function mutations lead to a downregulation of cell cycle genes resulting in a mitotic arrest of melanoma cells (intrinsic mechanisms). Differentiated melanoma cells are less aggressive and more susceptible to extrinsic mechanisms (immune response) and end up in spontaneous regression.

Overexpression of MITF *in vitro* has been demonstrated to induce negative regulators of cyclin dependent kinases such as p21 and p27, leading to a less aggressive phenotype in transfected UIISO-MEL-6 cell lines *in vivo* (Lekmine and Salti, 2007). Other studies have shown that MITF effects are mediated via modulation of CDK2 (Du et al., 2003), p21, and p16INK4A (Carreira et al., 2005; Loercher et al., 2005). There is evidence that MITF links cell differentiation with cell cycle arrest in melanocytes by activating the cell cycle inhibitor p16INK4A which in turns induces retinoblastoma protein hypophosphorylation, thereby triggering cell cycle arrest (Loercher et al., 2005). Cell cycle exit, required for proper differentiation, could therefore be associated with MITF function. Subcutaneous injection of MITF-M overexpressing UIISO-MEL-6 cells into Balb/c mice showed inhibition of tumor growth compared to injection of wild type UIISO-MEL-6 cells suggesting an influence on the tumor phenotype, possibly by inducing differentiation (Lekmine and Salti, 2007). Furthermore p16INK4A itself is a well established tumor suppressor gene in melanoma, that normally induces senescence in naevi, and forms therefore a barrier against melanoma development (Gray-Schopfer et al., 2007).

Since we detected a downregulation of mitosis-related genes at the very beginning of the regression phase, it could be hypothesized that at week 7 after birth less melanoma cells undergo mitosis compared to week 1, suggesting a less aggressive phenotype. This finding was supported by a meta-analysis comparing our regression-profiles with human melanoma expression profiles. Interestingly, expression signatures associated with human melanoma progression and poor clinical outcome showed significant gene overlap but conversely regulated in swine expression profiling. If this cell cycle arrest is linked to differentiation in our case requires further investigation. However, differentiation as mediator for melanoma regression has been already suggested in the Sinclair swine since progressive melanoma cells transplanted into severe combined immunodeficient (SCID) regressed, leaving highly pigmented cells of porcine origin behind (Greene et al., 1997). Yet, gene expression analysis for potential differentiation markers has not been assessed in this study. Even though progressive tumors were chosen (d+9,10) by Greene et al., immunohistochemical analysis of MeLiM melanoma at d+8 revealed already SWC3+ monocytes, thus a take over of porcine immune cells into the SCID mouse could not be completely excluded.

We were also interested in the role of these big highly pigmented histiocyte-like cells in our model since they occur while regression. Characteristics of these cells are hyperpigmentation, aneuploidy, increased size and irregular borders. Phenotypically similar cells in melanoma have been associated with osteoclast like giant cells (Al-Brahim and Salama, 2005),

melanophages (Handerson et al., 2007), macrophage-melanoma fusion hybrids (Chakraborty and Pawelek, 2007), and differentiated melanoma cells (Greene et al., 1997). At a first time, we tried to get an insight into the origin of these cells by immunohistological staining for SWC3 and MITF. With SWC3, a porcine myeloid marker, and MITF (nuclear expression) as melanocytic marker, we characterized these highly pigmented cells during regression. At the very early beginning of regression (d+28) MITF expression was mainly restricted to pigmented melanoma cells, whereas SWC3 marked mainly non pigmented macrophages. At d+48, the differential staining was not clear cut any more since big highly pigmented cells were positive for SWC3 with either a MITF positive (weak) nucleus or without any nuclear MITF staining at all. At a late stage of regression (d+91), MITF staining was generally weak with SWC3 being almost ubiquitously expressed. Not knowing if one and the same cell is undergoing these different stages of SWC3 and MITF staining cannot lead to the exclusion of the above mentioned hypotheses. Hypothetically, differentiation of MITF expressing melanoma cells could lead to a transient expression of MITF and SWC3 whereas a late stage of differentiation could result in a weak or no staining for MITF and a strong SWC3 signal. On the contrary, SWC3 or SIRP α , a glycoprotein with immunoregulatory functions upon ligation with CD47, is mainly expressed on macrophages and dendritic cells (Takizawa and Manz, 2007) and has not yet been described as melanoma cell differentiation marker. In the case of simultaneous expression of MITF and SIRP α , the theory of fusion hybrids between macrophages and melanoma cells would be more likely (Pawelek et al., 2006) as markers of both lineages (monocytic and melanocytic) would be present. Anyway, melanophages (macrophages that engulfed a melanoma cell) would be more prone to express only the myeloid marker instead of both. As we were able to detect different combinations of MITF and SIRP α expression, it would be fair enough to conclude that at the regressing melanoma site melanophages, fusion hybrids and differentiated melanoma cells might be present with a similar morphological phenotype resembling a big highly pigmented cell. Looking at these two markers only makes it difficult to draw any conclusion about the origin or role of these cells and therefore needs further investigation.

How does the immune system fit into the regression hypothesis? Is the immune system solely responsible for the regression process?

Firstly, the concept of immunoediting that is the immune systems capacity to control and shape cancer, is well accepted nowadays (Dunn et al., 2002; Dunn et al., 2004). Immunoediting is the result of 3 processes that function either independently or in sequence (Dunn et al., 2006): 1) Cancer immunosurveillance, in which immunity functions as a tumor

suppressor in naïve hosts; 2) Equilibrium that is the hold-in-check of immunity against expansion of transformed cells that escaped; 3) Escape that is the growth of tumor cells due to immunosilencing.

Extensive experimental support exists for the elimination and escape processes since immunodeficient mice develop more carcinogen-induced and spontaneous cancers than wild-type mice, and tumor cells from immunodeficient mice are more immunogenic than those from immunocompetent mice (Koebel et al., 2007). There seems to be also a consensus in humans since the incidence of melanoma is increased in chronically immunosuppressed kidney-transplant patients (LeMire L. et al., 2006). In various tumor types, including breast (Marrogi et al., 1997), prostate (Vesalainen et al., 1994), renal cell (Nakano et al., 2001), esophageal (Schumacher et al., 2001), colorectal (Ohtani, 2007) and ovarian cancer (Tomsova et al., 2008) the overall presence of tumor infiltrating lymphocytes has been correlated with better prognosis. However, inflammatory cell infiltration of tumors does not lead automatically to better outcome due to tumor heterogeneity and diversity of the inflammatory cell phenotypes (Talmadge et al., 2007). Tumor infiltration by macrophages is generally associated with neoangiogenesis and negative outcome, whereas dendritic cell infiltration is typically linked to a positive outcome in association with their ability of antigen presentation. In general, infiltration by CD8⁺ T cells predicts a positive outcome while CD4⁺ T cells do not (Talmadge et al., 2007). Not only the infiltration-phenotype but also the timing when lymphatic infiltration occurs, is an important factor (Prehn, 2007).

Arguably, the evidence for a clinically valuable anticancer immune response is stronger in melanoma than any other human malignancy (Slingluff et al., 2006). Functional T cells restricted to melanoma antigens can be readily recovered from patients with melanoma, establishing the tumor's immunogenicity (Romero et al., 2006). Tumor infiltrating lymphocytes directed against specific melanoma antigens (MLANA, gp100) but not circulating melanoma specific T cells have been linked to better survival in resected advanced-stage melanoma patients (Haanen et al., 2006). Unfortunately, immunoediting ends in escape and progression of the disease for the majority of melanoma patients.

In the MeLiM swine, it seems that melanoma cells do not succeed in escaping the immune surveillance. Genes that play a role in classical immune escape mechanisms like the downregulation of MHC I molecules and immunosuppressive cytokines production (IL10) were not found to be significantly regulated by microarray analysis. Expression of MHC class I molecules such as HLA-A and HLA-B were shown to be lost in malignant melanoma tissue and cell lines as reviewed by Rebmann (Rebmann et al., 2007). But in our case, HLA-A- and

HLA-B-like expression levels seem to be stable during regression since their corresponding probe sets did not show significant regulation. A preliminary study, conducted in our lab, yielded to quantify the expression of swine leukocyte antigen class I genes (SLA1-3) in regressive melanoma tissue. It showed an upregulation of classical SLA-I genes in regressive melanoma tissue, indicating that MHC class I downregulation-escape seems unlikely.

However, we detected an upregulation of T cell response suppressive genes such as scinderin (SCIN) and the B7 family-related protein V-set and Ig domain-containing 4 (VSIG4) during regression (Rambow et al., 2008b). SCIN has been recently described to play a role in tumor resistance to T cell lysis by altering the actin cytoskeleton and influencing the formation of the lytic synapse (Abouzahr et al., 2006). VSIG4, a strong negative regulator of T cell proliferation and IL2 production, is expressed on antigen presenting cells, to render T cell unresponsive (Vogt et al., 2006). Even though there is evidence for escape mechanisms regression occurs. If MeLiM melanomas lack further classical immune escape mechanisms needs further detailed investigation.

Or could it be that the MeLiM immune system has outstanding anticancer immune characteristics and that these may be the main driver for regression?

Important to know, that spontaneous melanoma regression occurs also in other pigs. In the Munich Troll miniature swine, evidence showed a peripheral blood NK cell activity against allogeneic melanoma cells but NK cell activity of German Landrace sows against Troll melanoma cells was very similar (Buttner et al., 1991). Furthermore it was shown that Troll allogeneic melanoma cells were able to inhibit NK cell mediated cytotoxicity. This impairment of NK cell cytotoxicity by melanoma cells was also described in humans and linked to the low expression of NK surface molecules such as CD161 and NKG2D (Konjevic et al., 2007). In contrast, MeLiM regression was associated with a late but strong upregulation of NKG2D amongst other effector cell molecules (Rambow et al., 2008b).

Furthermore we detected a monocyte/macrophage signature at d+70, confirmed by SWC3+ immunohistological staining. This first cellular immune response was followed by an upregulated T/NK gene signature at d+91, as confirmed by CD3+ immunohistological staining. Flow cytometry analysis revealed higher percentage of CD8+ in TIL suspensions, and higher percentage of CD4+ in PBL suspensions. T cells characteristic for pig immunity such as CD4+/CD8+ T cells and γ/δ T cells (Takamatsu et al., 2006; Zuckermann and Husmann, 1996) were also investigated in TIL and PBL suspensions during late stages of regression (>d+97). Whereas about 16% of PBL suspensions were γ/δ positive, only ~8.5% of TIL suspensions showed γ/δ positive T cells (additional results chapter 3). Regarding

CD4⁺/CD8⁺ T cells, they were present in PBL suspensions (~6%) but absent in TIL suspensions. NK cells were also assessed, with ~16% CD8⁺/CD16⁺ NK cells in PBL suspensions and ~6% in TIL suspensions (additional results chapter 3). A potential humoral response has not been investigated yet in MeLiM pigs. These results were in concordance with the characterization of TILs in the Sinclair swine. They revealed significantly higher percentages of CD8⁺ T cells in tumors than in PBMCs, whereas CD4⁺ T cells were more abundant in PBL suspensions than TIL suspensions (Morgan et al., 1996). Furthermore γ/δ T cells were also detected in PBL suspensions of the Sinclair swine (Grimm et al., 1993). Together, morphological evidence suggests, that the cellular immune system is actively and progressively associated with spontaneous regression of melanoma in a biphasic way in the Sinclair model, whereas the first phase consist of macrophage infiltration prior to 60 days of age followed by the second phase that is complete elimination of melanoma, associated with TIL infiltration (Greene et al., 1994b). Even an immunohistological characterization of inflammatory infiltrates in regressing melanoma of crossbred Iberian x Duroc pigs revealed a role for CD3⁺ T cells, whereas CD79⁺ B cell infiltrate was low (Perez et al., 2002).

In all, this data suggest a role of pig immunity in melanoma regression that is not restricted to the MeLiM model. If the pig immune system is generally efficient against cancer has to be verified by adoptive transfer experiments. But the question rises why does melanoma develop first hand if there is a highly efficient immune response to fight it?

This delay of regression has been associated with the piglet's acquisition to gain self-immune status. The piglet's immune system however seems to be already armed in terms of T and B cells before regression take place (~3 months of age) (Butler et al., 2006; Brown et al., 2006). A speculative answer would be that the delay is caused by a change in melanoma expression affecting intrinsic mechanisms that render melanoma cells less aggressive and more vulnerable for immunosurveillance.

Gene expression profiling of melanoma patients that underwent immunotherapy has been already performed but a persistent immune response was not achieved. About 30 genes associated with a predictive clinical response were identified while half of them were related to T-cell regulation (Wang et al., 2002). Another comprehensive analysis of signatures associated with immune sensitivity was performed by serially following the response of basal cell carcinoma (BCC) to local applications of the TLR7 agonist Imiquimod (synthetic small nucleotides) (Panelli et al., 2007). The result of this analysis demonstrated that the eradication

of BCC is a complex multi factorial phenomenon. 539 genes displayed additional immune functions involving the activation of innate and adaptive immune effector mechanisms centered around the infiltration of CTL and NK cells. Although this local treatment does not at the moment represent a potential cure for a systemic cancer, it provides, in principle, a valuable model to study the mechanism of immune rejection.

For the first time, we provide transcriptional profiles for intrinsic (cell cycle) and extrinsic mechanisms (immune response) that lead to complete melanoma regression even of advanced metastatic disease.

Expression profiles and other regressing malignancies

Taking a step back from melanoma regression could offer an insight in other malignancies that regress spontaneously, and comparing those expression profiles could permit the identification of common mechanisms.

Neuroblastoma is a malignant tumor of the peripheral nervous system found almost exclusively in young children and arises from the embryonic neural crest (Reynolds, 2000). Neuroblastomas show remarkable biological heterogeneity, resulting in favourable or unfavourable outcomes. Unfavorable tumors often have several genetic aberrations (MYCN, PHOX2B) and grow aggressively, while favourable tumors regress or mature (Hiyama E, 2004). Spontaneous regression of neuroblastomas is a rare phenomenon but it is frequently observed in stage 4S tumors that are childhood tumors (age ≤ 12 months) with metastases limited to liver, skin and/or bone marrow (Reynolds, 2000; Miale and Kirpekar, 1994). Maturation from primitive neuroblast-like malignant cells to well-differentiated and benign tumors has been documented during regression (Haas et al., 1988) but most spontaneously regressing neuroblastomas do not leave behind well-differentiated tissue, instead they simply disappear (Reynolds, 2002). Both cellular (Bernstein et al., 1976) and humoral (Ollert et al., 1996) immunologic attack of neuroblastoma cells have been postulated as mechanisms of spontaneous regression but demonstration that immunologic effectors are causative in regressing tumors has been elusive. Moreover, the lack of regression in tumors with aggressive biologic features would argue against an immunological mechanism and suggests a mechanism dependent on tumor cell biology such as lack of telomerase activity (Hiyama et al., 1999). Thus spontaneous regression of neuroblastoma may occur via more than one mechanism.

Common features with the MeLiM model are striking such as an early onset of the disease, genetic complexity, spontaneous regression and common neural crest ancestors as cancer cell progenitors. To unravel molecular mechanisms underlying the process of spontaneous regression, gene expression profiling by microarrays was realized between stage 4 and 4S neuroblastoma. These studies failed to reliably discover discriminating gene expression patterns (Berwanger et al., 2002; Schramm et al., 2005) maybe due to the small size (~4K) of used cDNA microarray, or due to the small numbers of 4 and 4S stage tumors. Recently, a serial analysis of gene expression (SAGE) revealed about 500 differentially expressed genes between 4 and 4S tumors ($n = 76$) of which 41 were assessed by qRT-PCR. Finally, 18 of 41 genes that were differentially expressed, were able to discriminate the two subgroups *in silico* (Fischer et al., 2006). Genes identified led to the hypothesis that spontaneous regression in stage 4S neuroblastomas might be due a delayed activation of developmentally regulated apoptosis. We did not identify a significant overlap with the 41 genes identified by Fischer and colleagues suggesting different mechanisms involved in spontaneous melanoma regression in MeLiM pigs. The process of differentiation might be different depending on the cell type but finally renders cells mortal. Differentiation of neuroblastoma cells might be characterized by different markers than differentiation of melanoma cells. “Death by differentiation” has been already described as a rationale for anti melanoma therapy by using the melanoma differentiation associated gene 7 (MDA7), or IL24 (Fisher et al., 2007). MDA7 has been successfully shown to selectively induce apoptosis in multiple human cancers including melanoma as reviewed by Fisher (2005). A phase I clinical trial was conducted to test MDA7 by a replication incompetent adenovirus in patients with advanced solid cancers including melanoma with promising results (Lebedeva et al., 2005). The MDA genes (MDA5 or IFIH1, MDA1 or IL19, MDA6 or p21, MDA7 or IL24, MDA9 or SDCBP) were initially identified by treatment of human melanoma cells with a combination of IFN- β and mezerein (MEZ) which resulted in a loss of tumorigenic potential, irreversible growth arrest, antigenic modulation, enhanced melanin synthesis, profound changes in gene expression and terminal cell differentiation (Huang et al., 1999a; Huang et al., 1999b). Interestingly, we detected MDA5 and MDA9 by SSH analysis, but it needs to be verified if these MDA genes play a distinct role in MeLiM melanoma regression. However, an induction of differentiation by most likely a genetic mutation could be the case. Strange enough that tumors firstly develop meaning the potential induction of terminal differentiation might not be on a melanocyte but on a melanoma cell level. Again, potential mutations probably act synergistically by first inducing melanoma growth and then regression by terminal differentiation. Potential

candidate genes bearing these mutations could be linked to the pigmentation pathway such as TYR, MITF, CCND1, and MC1R since the phenotype of MeLiM melanoma shows severe hyperpigmentation from the start on.

General findings suggest that spontaneous regression in MeLiM pigs is a dynamic process that includes defined molecular changes. The changes indicate a role for the immune system as an active but not necessarily the unique player during the regression process but also intrinsic mechanism affecting the cell cycle, maybe by induction of differentiation, seem to be involved in this process. We found that the porcine melanoma transcriptome is similar to the human, qualifying the MeLiM swine as suitable model to study melanoma development and regression. Genes that were associated with spontaneous regression as well as the establishment of a complex immune response that was able to overcome tumor escape should be further investigated to optimize anti melanoma therapy.

Perspectives

We characterized spontaneous melanoma regression using the MeLiM model on a molecular and cellular level. By studying the transcriptome of melanoma tissue during tumor development and regression, we demonstrated clearly that the tumor phenotype at each stage could be explained by its gene expression signature. Besides the detection of a complex immune response, we deciphered a very early event during regression that hasn't been described yet, namely the cell cycle retardation. Further studies should focus on the question: what is the initiator and what is an epiphenomenon of regression.

Short- term perspectives

With our transcriptomic studies we identified a gene signature which suggests an early mitotic arrest for melanoma cells. This intrinsic mechanism could lead to a downregulation of the proliferative capacity of melanoma cells and render melanoma cells more susceptible for immunosurveillance. Different mechanisms such as differentiation or induction of senescence have been hypothesized that could result in a mitotic arrest and need further analysis. Therefore we should focus on genes that play a role in differentiation and/or senescence and check for differential expression following the kinetics of melanoma development and regression in MeLiM using qRT-PCR. Following genes could be of interest that were either not immobilized on the porcine DNAchip (Affymetrix) or where unresponsive:

- TERT (telomerase reverse transcriptase), telomerase repairs/elongates the telomeres and is crucial in cellular proliferation, usually overexpressed in cancer cells.
- p16INK4A, loss of p16INKA is associated with the overcome of senescence in melanocytes.
- p21 (MDA6), cyclin-dependent kinase inhibitor p21, is differentially expressed during growth, differentiation and progression in human melanoma cells

The most widely used biomarker for senescent and aging cells is senescence-associated beta-galactosidase (SA-beta-gal) and could be assessed on a protein level. In the context of terminal differentiation, it would be worthwhile investigating the nuclei structure of regressing melanoma cells via chromosome specific probes using confocal microscopy. Terminal differentiation

(denucleation) is the process in which cells lose their nuclei but remain functional before undergoing apoptosis.

Further attention should be paid on the characterization of the big highly pigmented histiocyte-like cells that often miss nuclei or show an abnormal structure of the same. More discriminative immunohistological markers should be identified to shed light on their origin and present function during regression. Our obtained expression profile (monocytic signature) could be helpful in finding new markers such as ACP5 or SLC37A2 amongst others.

To rule out the immune response as an initiator in spontaneous melanoma regression, one can think of porcine immunosuppression therapy. MeLiM pigs should undergo systemic immunosuppressive therapy (i.e. cyclosporine A or tacrolimus) 3 weeks after birth. Both immunosuppressive agents are calcineurin inhibitors and act on IL2 levels by inhibiting its production and thus leading to a decrease in the proliferation of naïve and activated T-lymphocytes. Another option to exclude a initial role of the immune response in melanoma regression would be the adoptive transfer into immunodeficient mice. The NOD-SCID mice for example have impaired T and B cell lymphocyte function as well as lacking NK functions and the ability to stimulate complement activity. If transferred melanoma cells would still regress in these mice, would then implicate that the immune system mediated by T, B, and NK cells might not play a role. The same is true for adoptive transfer of melanoma cells into mice bearing the “beige” mutation. The characteristic of these beige mice is an impairment of lysozomal granules. This phenotype is most pronounced within granule containing cells such as melanocytes and phagocytes. An impaired innate immune system could shed light on the importance of a monocyte/macrophage response as an inducer of regression.

Long-term perspectives

To fully explore the transcriptomic data obtained by SSH and microarray analysis, results should be reanalyzed once the whole genome sequence data of the pig will be available:

- Reannotation of microarray and functional interpretation of results (~350 significantly regulated probe sets could not be explored due to bad sequence homology).
- Re-BLAST of SSH data with focus on potential miRNAs and unknown genes.

Regression seems to be a consequence of genetically manifested tumor development. But it is impossible to identify chromosomal regions linked to regression by following segregation since we don't observe tumor development without regression (the 4% of animals that do not regress, die). An indirect way could include the identification of potential oncogenes or tumor suppressor genes leading to melanoma development and then following their gene expression during regression. Therefore microarray analysis should be performed comparing growing melanoma cells (laser microdissected) and normal melanocytes (laser microdissected) from the same animal. This could lead to the potential identification of tumor suppressor and oncogenes. Furthermore, the integration of obtained functional (expression study) and positional results (QTL study) would help to narrow down the number of candidate genes within the QTL regions.

MITF-M (melanocyte specific isoform of MITF), as discussed previously, could be such an oncogene that leads to melanoma development and an overproduction (gain of function mutation) of MITF-M could drive melanoma cells into differentiation, and also to a higher immunosusceptibility by overproduction of immunogenic antigens. To functionally target MITF-M, primary melanoma cell isolates could be manipulated on the MITF-M expression level before xenotransplanting them into immunodeficient mice. The manipulation of MITF-M on the expression level should be realized by dose dependent gene silencing using for example lentivirus-mediated RNA interference (RNAi) as MITF-M is known to be tightly regulated *in vivo*. Total gene knockout of MITF would not be a solution because it has been shown to result in embryonal lethality in mice, apart from the fact that gene knockout is not yet feasible in pigs. Conditional gene knockout of MITF-M in melanocytes has not been realized in mice either to our knowledge. However, total silencing of MITF-M would not be the aim in our case, since we are interested in the effects of different levels of MITF-M expression.

Another starting point for further studies could be the investigation of embryonal melanoma development using embryonal stem cells as the majority of the tumors develop *in utero*. Epigenetic processes such as imprinting, gene inactivation, paramutation and histone modification could also be involved in embryonal melanoma development. Knowing at what time (developmental stage) melanoma development occurs, could give an idea about which genetic pathways might be implicated and could narrow down the list of candidate genes carrying a mutation. Fair enough to mention that melanoma development occurs also spontaneously *ex utero*. Theoretically, the genetic information of a melanoma developing *in utero* and a melanoma

of the same animal developing *ex utero* should be the same, so that environmental factors would be responsible for after birth tumor development. Interestingly, the major environmental factor of human melanoma development, UV light, can be excluded since our pigs are held inside and are not exposed to UV irradiation. Other yet unknown environmental factors might play a role.

All these different facets of melanoma development and regression reflect well the integrative character of this PhD combining many biological disciplines. This was exactly the objective of the framework project RIVAGE (Regulation and Variability of Animal Genome Expression) in which this PhD was implemented. RIVAGE, an Early Stage Training Marie Curie project that included 13 PhD fellows at INRA Jouy en Josas, aimed to merge animal genetics and physiology approaches for identifying the general mechanisms governing genome expression.

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APPENDIX

Supplementary material for Rambow et al. 2008b:

Supplement 1: Gene lists of cluster 1-6

K means cluster 1 regulated porcine probes and their human homologs (e value: $e < e^{-10}$)			
Affy Probeset	Human Gene Symbol	e-value	ANOVA FDR corrected P-value
Ssc.5998.1.S1_at	AARSD1	0	3.04E-04
Ssc.6276.1.S1_at	ABHD6	0	5.99E-03
Ssc.18475.2.S1_at	ACOT1	0	9.59E-04
Ssc.18475.3.A1_at	ACOT4	0	2.30E-03
Ssc.16577.1.A1_at	ACOX2	1.00E-122	5.82E-03
Ssc.9954.1.S1_at	ACTL6A	0	9.21E-04
Ssc.1114.1.A1_at	ADAM10	2.00E-163	6.40E-04
Ssc.10911.1.A1_at	ADRBK2	0	3.79E-03
Ssc.10911.2.S1_at	ADRBK2	0	7.00E-03
Ssc.24153.1.S1_at	ADSSL1	1.00E-135	1.94E-03
Ssc.21889.1.S1_at	AGK	8.60E-32	5.04E-03
Ssc.9766.2.S1_at	AK2	0	2.75E-04
Ssc.2505.2.S1_at	AKAP11	1.10E-65	5.92E-04
Ssc.20904.1.A1_at	AKT3	1.80E-62	1.48E-03
Ssc.17843.1.A1_at	ANKRD32	0	6.58E-03
Ssc.29819.1.A1_at	ANKS1A	2.10E-70	1.51E-03
Ssc.20184.1.S1_at	ARHGEF12	9.00E-172	1.79E-03
Ssc.16896.1.A1_at	ARL6IP1	1.10E-69	3.71E-04
Ssc.2861.1.A1_at	ARMET	8.60E-58	1.28E-03
Ssc.7735.2.S1_at	ARPC1A	0	1.19E-03
Ssc.17027.1.S1_at	ATAD3A	0.00E+00	9.93E-04
Ssc.6541.1.S1_at	ATP2C1	3.00E-93	2.82E-03
Ssc.27177.2.S1_at	ATP8A1	0	7.53E-04
Ssc.7686.1.S1_at	ATRNL	3.60E-69	7.62E-03
Ssc.12724.1.S1_at	ATXN10	0	2.50E-04
Ssc.2503.1.S1_at	B3GNT1	0	1.42E-03
Ssc.10143.1.A1_at	BACE2	0	2.42E-04
Ssc.18206.1.S1_at	BACE2	0	6.82E-04
Ssc.15771.1.S1_at	BEST1	0	4.05E-03
Ssc.17537.1.S1_at	BHLHB3	0	9.89E-03
Ssc.27880.1.S1_at	BID	5.50E-54	3.35E-04
Ssc.13321.1.A1_at	BMI1	0	5.99E-03
Ssc.13929.1.S1_at	BRMS1L	6.30E-37	2.89E-03
Ssc.8577.1.A1_at	BTF3L4	3.00E-133	8.30E-03
Ssc.15034.1.S1_at	C10orf97	9.80E-62	2.20E-03
Ssc.24631.1.S1_at	C12orf64	4.10E-35	1.27E-04
Ssc.5411.1.S1_at	C14orf106	4.00E-25	1.60E-03
Ssc.12067.1.S1_at	C14orf122	0	9.16E-03
Ssc.1210.1.A1_at	C14orf130	0	6.27E-04
Ssc.12832.1.A1_at	C14orf179	2.00E-109	2.23E-03
Ssc.7554.2.S1_at	C16orf80	0	1.41E-03

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Ssc.19165.1.S1_at	C17orf80	2.40E-37	8.53E-04
Ssc.10076.1.S1_at	C19orf2	2.50E-11	1.15E-03
Ssc.1271.1.S1_at	C1orf121	3.00E-43	2.44E-03
Ssc.17410.1.A1_at	C20orf19	6.90E-48	2.62E-03
Ssc.1956.1.S1_a_at	C4orf27	0	9.59E-03
Ssc.17516.1.S1_at	C6orf168	0	3.55E-03
Ssc.4808.1.S1_at	C7orf41	0	1.60E-03
Ssc.20876.1.S1_at	C8orf32	0	1.01E-03
Ssc.18058.1.S1_at	C9orf151	6.10E-69	1.18E-03
Ssc.4599.1.A1_at	CACNA1C	5.00E-123	7.66E-04
Ssc.20151.1.S1_at	CADM1	4.00E-151	8.41E-03
Ssc.16719.1.A1_at	CALM2	0	9.85E-03
Ssc.18204.2.S1_at	CAMTA1	2.20E-47	3.80E-03
Ssc.6407.1.A1_at	CASD1	4.50E-55	3.02E-03
Ssc.6789.1.A1_at	CCDC106	0.00E+00	9.82E-03
Ssc.6218.1.S1_at	CCDC23	7.00E-79	4.89E-03
Ssc.25151.1.S1_at	CCDC90A	0	6.80E-03
Ssc.15749.1.S1_at	CCND2	0	6.70E-04
Ssc.18187.1.A1_at	CCNE1	7.00E-145	5.56E-03
Ssc.937.1.S1_at	CDC123	0	1.08E-04
Ssc.25221.1.A1_at	CDC14C	4.80E-16	1.23E-03
Ssc.17268.1.A1_at	CDC42EP5	9.00E-82	7.58E-03
Ssc.26024.1.S1_at	CDC7	4.00E-16	6.06E-03
Ssc.28012.1.A1_a_at	CDK5RAP2	2.40E-92	2.72E-03
Ssc.21082.1.S1_at	CDR2L	6.90E-33	4.63E-03
Ssc.8312.1.A1_at	CDYL	7.00E-130	8.54E-03
Ssc.22613.1.S1_at	CENPP	6.00E-157	9.08E-03
Ssc.15109.1.S1_at	CEP78	2.50E-16	1.65E-03
Ssc.274.1.S1_at	CLCN2	0	7.63E-03
Ssc.20491.1.A1_at	CLCN3	3.00E-117	7.86E-03
Ssc.20491.2.S1_at	CLCN3	3.00E-117	1.21E-03
Ssc.29052.1.S1_at	CLCN3	3.00E-117	3.60E-03
Ssc.8254.1.A1_at	CMPK	0	1.48E-03
Ssc.19203.1.A1_at	CMTM4	5.20E-55	7.24E-03
Ssc.19203.2.S1_at	CMTM4	5.20E-55	7.38E-03
Ssc.4848.1.S1_at	CNN3	0	1.60E-03
Ssc.31040.1.A1_at	CNOT6	1.50E-37	1.74E-04
Ssc.8408.1.A1_at	CNTD1	3.00E-130	6.04E-04
Ssc.24250.1.S1_at	CRABP2	6.00E-172	2.73E-03
Ssc.18546.1.S1_at	CRIM1	0	3.03E-03
Ssc.5792.1.S1_at	CTNNA1	0	2.53E-03
Ssc.1648.1.S1_at	CTNND1	0	6.71E-03
Ssc.18285.1.S1_at	CUEDC2	0	4.48E-04
Ssc.2472.1.S1_at	CYB5D2	2.00E-167	3.39E-03
Ssc.12713.1.A1_at	CYCS	1.20E-77	7.56E-04
Ssc.19575.1.S1_at	DACT1	9.00E-114	1.27E-04
Ssc.5337.1.S1_at	DDEF2	2.40E-23	2.51E-03
Ssc.25005.1.A1_at	DDHD1	2.00E-174	8.13E-03

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Ssc.4713.1.S1_at	DHPS	8.70E-24	9.41E-04
Ssc.6423.2.S1_at	DIP2C	1.70E-67	1.37E-03
Ssc.23822.1.S1_at	DKC1	5.60E-31	1.76E-03
Ssc.1632.1.S1_at	DKKL1	3.00E-167	7.69E-04
Ssc.7413.1.A1_at	DNMT3B	5.40E-42	2.61E-04
Ssc.9887.1.A1_at	DOCK7	0	2.48E-03
Ssc.5893.1.S1_at	DOCK7	2.00E-173	1.06E-03
Ssc.28912.1.S1_at	DOLK	0	1.65E-03
Ssc.10915.1.S1_at	DPY19L1	4.00E-174	5.87E-03
Ssc.29110.1.S1_at	DSCR2	0	4.13E-04
Ssc.21895.1.S1_at	DST	2.00E-127	2.00E-03
Ssc.22735.1.S1_at	DTNB	4.10E-73	4.22E-03
Ssc.16966.1.S1_at	DTX4	1.00E-58	2.12E-03
Ssc.25180.1.S1_at	DUSP22	3.40E-16	1.36E-03
Ssc.6394.1.S1_at	DZIP1	1.50E-28	1.19E-03
Ssc.21755.1.A1_at	EDEM3	4.00E-158	5.82E-04
Ssc.11308.1.A1_at	EDEM3	1.00E-88	3.08E-03
Ssc.25089.1.S1_at	EDG4	0	3.96E-03
Ssc.27504.1.S1_at	EFNB3	3.00E-163	5.64E-03
Ssc.11298.1.S1_at	EGFL8	0	2.78E-03
Ssc.25048.1.A1_at	EGLX	0	6.83E-03
Ssc.5226.1.S1_at	EHBP1	0	7.00E-03
Ssc.5226.2.A1_at	EHBP1	0	4.31E-04
Ssc.18474.1.S1_at	EIF2B3	0	8.16E-03
Ssc.30822.1.A1_at	EIF4EBP2	2.90E-52	6.05E-03
Ssc.10168.1.A1_at	ENC1	0	2.19E-03
Ssc.7839.1.A1_at	EPB41L3	2.40E-65	4.78E-03
Ssc.31024.1.A1_at	EXOC6	1.60E-40	5.10E-03
Ssc.6754.1.A1_at	FAM10A7	6.60E-23	9.23E-03
Ssc.6977.1.A1_at	FAM116A	7.00E-141	7.13E-04
Ssc.26240.1.S1_at	FAM129A	0	9.16E-03
Ssc.5524.1.S1_at	FAM129A	5.10E-25	7.57E-03
Ssc.26113.2.S1_at	FAM134B	0	8.17E-03
Ssc.1600.1.A1_a_at	FAM80B	3.80E-19	9.26E-03
Ssc.24981.1.S1_at	FAM91A1	1.10E-72	7.19E-03
Ssc.22028.1.S1_at	FANCL	4.60E-79	3.11E-03
Ssc.8389.1.A1_at	FBXL2	1.00E-40	4.81E-03
Ssc.21701.1.S1_at	FEN1	0	2.10E-03
Ssc.16381.1.S1_at	FGD1	0	2.03E-03
Ssc.30853.1.S1_at	FGF2	2.40E-15	3.78E-03
Ssc.28107.1.S1_at	FKSG44	2.00E-24	7.26E-04
Ssc.3998.1.S1_at	FLJ11184	5.80E-59	6.74E-03
Ssc.1696.1.A1_at	FMNL2	6.00E-166	3.84E-03
Ssc.5679.1.S1_at	FNTB	4.70E-84	1.42E-03
Ssc.24297.2.S1_a_at	FOXK2	2.20E-30	7.17E-03
Ssc.19592.3.S1_a_at	FREQ	3.20E-20	2.93E-03
Ssc.9439.1.A1_a_at	FUBP1	4.00E-150	3.09E-03
Ssc.7998.1.S1_at	FXR1	0	7.92E-03

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Ssc.30491.1.A1_at	GCSH	5.00E-101	5.60E-03
Ssc.24202.1.S1_at	GEMIN4	3.00E-173	5.73E-03
Ssc.19144.1.S1_at	GINS2	3.00E-115	2.47E-03
Ssc.11338.1.S1_a_at	GLRX5	2.00E-174	8.68E-04
Ssc.6873.1.S1_at	GNAI3	0	3.65E-03
Ssc.2760.1.A1_at	GNG12	6.60E-94	3.77E-04
Ssc.19552.1.S1_at	GNG7	1.70E-20	5.30E-03
Ssc.1308.1.S1_at	GPC1	0	6.01E-04
Ssc.14334.1.S1_at	GPM6B	0	4.68E-03
Ssc.8297.1.S1_at	GPM6B	4.00E-144	6.45E-03
Ssc.8297.2.A1_at	GPM6B	4.00E-144	7.36E-04
Ssc.17560.1.S1_at	GPR177	0	1.99E-04
Ssc.13930.1.S1_at	GPRASP1	3.10E-17	6.00E-03
Ssc.5105.2.S1_a_at	GPRC5B	9.60E-68	1.12E-04
Ssc.27289.1.S1_at	GRIK2	0	4.63E-03
Ssc.24942.1.S1_at	GRK4	9.70E-76	9.38E-03
Ssc.5008.1.A1_at	GSTA4	0	1.04E-03
Ssc.15702.1.S1_at	GSTK1	9.00E-165	5.88E-03
Ssc.15549.1.A1_at	GSTT1	0	4.10E-03
Ssc.7058.1.A1_at	GTF2A2	4.00E-166	5.88E-04
Ssc.6171.1.A1_at	HDHD2	0	2.77E-03
Ssc.19830.1.S1_at	HDHD3	5.00E-154	9.99E-03
Ssc.2926.1.S1_at	HMOX2	0	2.91E-03
Ssc.6887.1.S1_at	HNRPDL	2.00E-27	1.38E-03
Ssc.10538.1.A1_at	HNRPM	0	7.17E-03
Ssc.8819.1.A1_at	HPS3	0	1.51E-03
Ssc.26363.1.S1_at	HS2ST1	0	9.38E-03
Ssc.21858.1.A1_at	HSDL1	7.30E-18	3.17E-03
Ssc.3313.1.S1_at	HSPA4	2.00E-57	6.16E-03
Ssc.1344.1.A1_at	IGFL3	1.50E-13	7.27E-03
Ssc.5664.1.S1_at	IHPK2	4.00E-130	1.31E-03
Ssc.23179.1.A1_at	INPP5A	2.90E-57	1.99E-04
Ssc.5699.1.S1_at	IQCK	1.50E-17	4.02E-03
Ssc.10835.1.A1_at	ISG20L2	3.20E-56	2.84E-04
Ssc.15723.1.S1_at	ISYNA1	0	9.35E-03
Ssc.25199.1.S1_at	ITGAM	3.00E-16	3.37E-04
Ssc.30856.1.A1_s_at	JMJD1A	0	5.61E-03
Ssc.13524.1.A1_at	KCNC2	4.00E-132	8.64E-04
Ssc.11663.1.A1_at	KCNRG	0	1.36E-03
Ssc.1398.1.S1_at	KCTD15	9.40E-29	6.12E-04
Ssc.20481.1.A1_at	KIAA0020	0	6.23E-03
Ssc.21880.1.S1_at	KIAA1166	1.80E-11	5.56E-03
Ssc.24733.1.A1_at	KIAA1549	3.80E-81	5.08E-03
Ssc.6205.1.S1_at	KIFC3	1.70E-54	2.62E-03
Ssc.24918.1.S1_at	KLHDC5	1.00E-148	2.53E-03
Ssc.11668.1.A1_at	KPNA2	4.00E-150	5.06E-03
Ssc.2263.1.S1_at	LARGE	1.40E-36	2.06E-03
Ssc.14025.1.A1_at	LEF1	9.30E-74	2.88E-03

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Ssc.20677.1.S1_at	LEPROTL1	2.60E-94	2.24E-03
Ssc.13028.1.S1_at	LGI4	0	9.59E-03
Ssc.2282.1.A1_at	LIMA1	2.30E-48	2.19E-04
Ssc.12709.1.A1_at	LMNB2	1.60E-21	3.83E-03
Ssc.29810.1.A1_at	LOC126147	4.90E-92	3.37E-03
Ssc.25782.1.S1_at	LOC130355	1.60E-13	9.13E-04
Ssc.21832.1.S1_at	LOC134145	1.90E-94	1.65E-03
Ssc.6448.1.S1_at	LOC391356	3.00E-112	2.62E-03
Ssc.8009.1.A1_at	LRP1B	2.20E-77	2.14E-04
Ssc.19658.2.A1_at	LYRM4	6.80E-66	4.95E-03
Ssc.11376.1.A1_a_at	MAGED2	0	1.09E-04
Ssc.24271.1.S1_at	MAP1LC3A	0	7.11E-03
Ssc.19103.1.A1_at	MED8	2.50E-11	6.62E-03
Ssc.29707.1.A1_at	MEGF10	2.80E-35	5.04E-04
Ssc.11067.1.S1_a_at	MEGF9	7.00E-104	9.57E-03
Ssc.4306.1.A1_at	MESDC1	3.00E-133	5.27E-03
Ssc.24089.1.S1_a_at	MINA	5.00E-147	9.33E-04
Ssc.2010.1.S1_at	MRPL43	5.00E-157	1.31E-04
Ssc.5102.1.S1_at	MRPS27	0	2.41E-03
Ssc.11012.1.A1_at	MSRB2	7.00E-132	2.20E-03
Ssc.6185.2.S1_at	MSTO1	0	1.17E-03
Ssc.12327.2.S1_a_at	MSTP101	2.10E-30	8.99E-03
Ssc.24710.1.S1_at	MSTP101	0	7.89E-03
Ssc.11746.1.A1_at	MSTP150	4.00E-38	3.10E-03
Ssc.2719.1.A1_at	MTA3	0	1.97E-03
Ssc.22204.2.A1_at	MTMR12	2.00E-158	9.89E-03
Ssc.1957.1.A1_at	MYO5A	3.00E-104	2.07E-04
Ssc.12335.2.S1_a_at	N6AMT2	2.50E-36	3.40E-03
Ssc.25115.1.A1_at	NASP	0	5.73E-03
Ssc.9260.1.A1_at	NEK1	2.00E-102	1.17E-03
Ssc.14125.1.A1_at	NEK3	7.00E-113	2.69E-03
Ssc.6623.1.S1_at	NIPSNAP3A	0	6.79E-03
Ssc.10500.1.A1_at	NMNAT2	5.40E-46	8.54E-03
Ssc.11357.1.A1_at	NMRAL1	4.90E-88	5.60E-03
Ssc.16861.1.A1_at	no entry	3.40E-15	6.79E-03
Ssc.26172.1.S1_at	NO55	7.00E-165	6.60E-03
Ssc.25220.1.S1_at	NOL10	0	2.51E-03
Ssc.12312.1.A1_at	NOLC1	2.90E-25	4.19E-04
Ssc.17872.1.A1_at	NR2F1	2.00E-108	8.40E-03
Ssc.22051.2.S1_at	NSF	0	8.94E-03
Ssc.17330.2.A1_at	NSMCE4A	0	5.26E-04
Ssc.855.1.S1_at	NUDT1	2.00E-124	4.67E-04
Ssc.1388.1.S1_at	NUDT2	2.00E-168	3.07E-03
Ssc.21840.1.S1_at	NUDT5	4.00E-108	4.43E-04
Ssc.30818.1.S1_at	NUFIP1	0	7.16E-03
Ssc.4556.1.S1_at	NUP107	0	9.60E-03
Ssc.2790.1.S1_at	NXN	1.80E-80	9.69E-03
Ssc.4812.2.S1_a_at	NY-SAR-48	3.00E-119	8.42E-03

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Ssc.25198.1.A1_at	OAT	0	4.38E-03
Ssc.22715.1.S1_at	OCIAD2	6.20E-83	2.00E-03
Ssc.20404.1.S1_at	PARP1	0	3.93E-03
Ssc.1705.1.S1_at	PCBD1	5.00E-147	4.06E-03
Ssc.10928.1.A1_at	PCTK2	1.00E-154	1.63E-03
Ssc.10928.2.A1_at	PCTK2	1.00E-154	3.59E-03
Ssc.12925.1.A1_at	PDIA6	0	7.62E-04
Ssc.8274.1.A1_at	PFN2	0	1.49E-03
Ssc.1006.1.S1_at	PGM1	5.00E-135	3.78E-03
Ssc.9819.1.S1_at	PGRMC1	0	1.24E-03
Ssc.7523.1.A1_at	PHB	1.80E-26	1.23E-03
Ssc.29076.1.A1_at	PHKA1	6.50E-86	3.40E-03
Ssc.11748.1.S1_at	PHLDB1	0	8.83E-03
Ssc.29410.1.A1_at	PLCB4	1.00E-105	6.05E-03
Ssc.29372.1.A1_at	PLCXD3	2.70E-48	7.80E-03
Ssc.25216.1.S1_at	PLCZ1	3.30E-14	2.36E-03
Ssc.26028.1.S1_at	PNMA1	0	1.85E-03
Ssc.22290.1.A1_at	PNMA1	1.70E-11	6.44E-04
Ssc.5300.1.S1_at	POMGNT1	0	1.32E-03
Ssc.7446.1.S1_a_at	PPID	0	2.75E-03
Ssc.13719.1.A1_at	PPM1B	0	1.91E-03
Ssc.2406.1.S1_at	PPM1B	0	1.89E-03
Ssc.4482.1.A1_at	PPP2R5C	1.00E-109	1.90E-03
Ssc.4482.2.S1_at	PPP2R5C	1.00E-109	2.79E-03
Ssc.4363.1.S1_at	PPP5C	0	5.65E-03
Ssc.13637.1.A1_at	PRKCA	6.10E-26	2.74E-04
Ssc.6371.1.A1_at	PRNP	0	1.51E-03
Ssc.838.1.S1_at	PSCD3	1.00E-26	1.40E-04
Ssc.24175.1.A1_at	PSEN2	0	1.82E-03
Ssc.1539.1.S1_at	PSIP1	0	1.83E-03
Ssc.3608.1.S1_at	PTDSS2	2.40E-20	4.70E-03
Ssc.16187.1.S1_at	PTGDS	1.10E-70	3.51E-03
Ssc.9101.1.A1_at	PTPN13	0	6.72E-04
Ssc.29458.1.A1_at	PTPRG	1.90E-39	1.94E-03
Ssc.21264.1.A1_at	PTPRM	5.00E-119	2.46E-03
Ssc.5069.1.S1_at	PTPRS	3.50E-41	5.85E-04
Ssc.25561.1.A1_at	PWP1	0	7.07E-03
Ssc.4857.2.S1_a_at	PXMP2	5.90E-76	7.00E-03
Ssc.7755.1.A1_at	Q9P151	5.90E-54	5.54E-03
Ssc.17250.1.S1_at	QDPR	0	7.47E-03
Ssc.3249.1.S1_at	QSOX1	7.40E-17	5.92E-04
Ssc.11796.1.S1_at	RAB34	0	6.50E-03
Ssc.24392.1.S1_at	RAB5B	6.00E-173	5.14E-03
Ssc.24392.2.A1_at	RAB5B	6.00E-173	4.16E-03
Ssc.30706.1.A1_at	RABEPK	0	6.70E-05
Ssc.2801.1.S1_at	RALGPS2	3.80E-72	2.31E-03
Ssc.9137.1.A1_at	RANBP9	0	1.17E-03
Ssc.23139.1.S1_at	RAP2B	2.00E-119	6.03E-03

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Ssc.8143.1.A1_at	RAPGEF2	0	2.28E-04
Ssc.14236.1.A1_at	RARS2	8.00E-170	9.03E-03
Ssc.22216.1.A1_at	RBBP8	0	4.21E-03
Ssc.4765.1.A1_at	RBM22	5.00E-170	3.14E-04
Ssc.8220.1.A2_at	RBM39	0	1.36E-03
Ssc.10394.1.A1_at	RBM8A	3.00E-106	1.24E-03
Ssc.19177.1.S1_at	RCC2	1.50E-87	1.28E-03
Ssc.4977.1.S1_at	RFC2	0	1.36E-03
Ssc.7099.2.A1_a_at	RFK	0	1.81E-03
Ssc.19333.3.S1_at	RFP	0	1.18E-03
Ssc.14997.1.A1_at	RFP	4.90E-25	4.84E-04
Ssc.4202.1.S1_at	RFXANK	0	8.56E-04
Ssc.27978.1.A1_at	RMND5A	1.50E-99	6.73E-03
Ssc.11114.1.A1_at	RPA1	2.00E-135	1.49E-03
Ssc.2132.1.S1_a_at	RPS6KA5	5.20E-78	3.51E-03
Ssc.10623.2.S1_at	RTN3	6.70E-79	8.11E-03
Ssc.19420.1.S1_at	RUVBL1	0	4.09E-03
Ssc.31023.1.A1_at	RWDD3	8.00E-101	2.75E-04
Ssc.2855.1.S1_at	RWDD4A	4.00E-65	1.09E-04
Ssc.3952.1.S1_at	SAE2	0	1.72E-03
Ssc.2544.1.S1_at	SALL2	2.70E-63	1.72E-03
Ssc.18447.1.S1_at	SCAMP5	1.70E-36	9.47E-03
Ssc.27564.1.S1_at	SEC22C	1.10E-58	5.72E-03
Ssc.10124.1.A1_at	SEC61A2	8.00E-172	2.02E-03
Ssc.22824.1.S1_at	SEPHS1	0	1.95E-03
Ssc.7576.3.S1_at	SFRS1	0	4.22E-03
Ssc.7281.2.A1_at	SFRS10	0	3.35E-04
Ssc.19345.2.S1_at	SFRS14	8.00E-165	8.75E-03
Ssc.9241.1.A1_at	SFRS6	6.00E-21	6.24E-03
Ssc.5220.1.S1_at	SFRS9	0	1.95E-03
Ssc.3772.1.A1_at	SGCE	0	3.50E-03
Ssc.8905.1.A1_at	SHC4	3.00E-178	1.16E-03
Ssc.18682.1.S1_at	SHROOM2	2.00E-19	6.00E-04
Ssc.18635.1.S1_at	SIVA1	8.60E-84	7.64E-04
Ssc.4979.1.S1_at	SLC1A6	9.00E-173	9.12E-03
Ssc.17012.1.A1_at	SLC24A5	1.40E-26	2.20E-03
Ssc.27419.1.S1_at	SLC25A13	1.80E-88	1.76E-03
Ssc.2879.1.S1_at	SLC25A25	6.30E-58	2.62E-03
Ssc.8379.1.A1_at	SLC35A1	2.50E-59	2.37E-03
Ssc.12664.2.S1_at	SLCO3A1	0	5.54E-03
Ssc.12664.1.S1_a_at	SLCO3A1	8.00E-166	3.50E-03
Ssc.12664.1.S1_at	SLCO3A1	8.00E-166	2.81E-03
Ssc.12091.1.A1_at	SMARCA1	5.00E-142	2.03E-03
Ssc.1449.1.S1_at	SMC1A	6.50E-53	2.05E-03
Ssc.3875.2.A1_at	SNURF	0	2.49E-03
Ssc.22685.1.S1_at	SOCS6	0	2.42E-03
Ssc.12492.2.S1_at	SORT1	0	2.85E-04
Ssc.22700.1.S1_at	SOX5	2.10E-78	2.63E-03

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Ssc.3786.1.S1_at	SPIRE1	1.00E-158	1.32E-03
Ssc.16046.1.S1_s_at	SPTBN1	2.80E-57	1.49E-03
Ssc.28763.1.A1_at	SR35	8.00E-135	1.27E-04
Ssc.1450.1.S1_at	SRM	0	9.66E-03
Ssc.17833.2.S1_at	SS18L1	1.10E-93	2.73E-03
Ssc.26167.1.S1_at	ST3GAL3	1.30E-33	5.99E-03
Ssc.4387.1.A1_at	ST3GAL4	0	7.83E-03
Ssc.2464.1.S1_at	STC1	3.00E-173	6.81E-03
Ssc.18296.2.S1_a_at	SULF2	0	7.46E-03
Ssc.18296.1.A1_a_at	SULF2	9.90E-30	6.62E-03
Ssc.10395.1.A1_at	TAF9	0	8.54E-03
Ssc.9498.1.S1_at	TAX1BP3	2.00E-175	1.76E-03
Ssc.7929.1.A1_at	TBC1D15	0	6.67E-03
Ssc.4949.2.S1_a_at	TBP	0	2.05E-03
Ssc.7564.1.A1_at	TCP11L1	5.00E-30	3.13E-03
Ssc.2557.1.S1_at	TEAD2	0	1.41E-03
Ssc.11043.1.A1_at	TEX2	4.40E-56	5.26E-04
Ssc.11043.2.A1_at	TEX2	4.40E-56	6.20E-03
Ssc.30327.1.A1_at	TEX9	2.10E-36	3.12E-03
Ssc.21208.1.S1_at	TFAP2A	0	2.12E-03
Ssc.28628.1.S1_at	TIAL1	3.00E-110	2.92E-03
Ssc.1814.1.S1_at	TIMM17B	0	2.26E-03
Ssc.25535.1.S1_at	TM6SF1	1.00E-34	7.57E-03
Ssc.9518.1.A1_at	TMCC1	2.00E-103	2.02E-03
Ssc.18123.1.A1_at	TMCC1	6.90E-93	2.50E-04
Ssc.16964.2.S1_a_at	TMCO3	8.00E-83	5.88E-04
Ssc.7797.1.S1_at	TMED8	1.10E-46	2.20E-03
Ssc.26936.1.S1_at	TMEM169	8.60E-11	5.04E-03
Ssc.24847.1.S1_at	TMEM170	3.10E-44	7.52E-03
Ssc.11219.1.S1_at	TMEM176B	4.40E-12	6.25E-04
Ssc.27416.1.S1_at	TMEM186	6.70E-61	8.41E-03
Ssc.24995.1.S1_at	TMEM66	0	2.85E-04
Ssc.9010.1.A1_at	TMEM9	0	1.88E-04
Ssc.1559.1.S1_at	TMEM93	0	5.01E-04
Ssc.24702.1.S1_at	TMTC4	8.90E-20	7.75E-04
Ssc.21601.1.S1_at	TOE1	0	7.19E-03
Ssc.14186.1.A1_at	TOMM20	4.90E-18	1.19E-03
Ssc.29366.1.A1_at	TOP1	8.30E-51	4.94E-03
Ssc.7694.1.A1_at	TOPBP1	9.00E-103	2.75E-04
Ssc.10918.1.A1_at	TPRKB	0	7.47E-03
Ssc.15492.1.S1_at	TRIB1	2.10E-65	2.39E-04
Ssc.5504.1.A1_at	TRIM37	6.90E-76	3.96E-04
Ssc.24451.1.S1_at	TRPC1	2.00E-136	1.36E-03
Ssc.8445.1.A1_at	TRPC1	2.00E-136	1.17E-03
Ssc.21124.1.S1_at	TSG118.1	8.10E-74	7.05E-04
Ssc.10825.1.A1_at	TSGA14	1.30E-23	3.77E-03
Ssc.16583.1.S1_at	TSPAN6	0	1.36E-03
Ssc.30361.1.A1_at	TTC8	4.10E-16	1.37E-03

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Ssc.13586.1.A1_at	TULP4	1.20E-57	2.36E-03
Ssc.11673.1.S1_at	UBE2E3	0	6.28E-03
Ssc.24422.1.S1_at	UBE2S	0	4.91E-03
Ssc.6296.1.S1_at	UBL7	0	9.23E-03
Ssc.4072.1.A1_at	UBXD1	0	2.84E-04
Ssc.27550.1.S1_at	UGCG	0	1.60E-03
Ssc.4912.1.A1_at	ULK2	1.10E-65	2.53E-03
Ssc.18729.1.A1_s_at	ULK2	2.60E-16	1.27E-03
Ssc.20772.1.S1_at	USP39	0	8.88E-03
Ssc.12984.1.A1_s_at	VPS36	4.90E-28	1.17E-03
Ssc.4177.1.A1_at	WASF3	2.80E-72	2.34E-03
Ssc.24000.1.S1_at	WASL	0	9.95E-03
Ssc.9339.2.S1_a_at	WDFY1	0	8.73E-03
Ssc.24857.1.S1_at	WDFY1	2.00E-107	2.11E-03
Ssc.16873.1.S1_at	WDR44	0	6.67E-03
Ssc.11083.1.S1_at	WNK4	8.00E-121	6.57E-03
Ssc.12888.1.S1_at	WRB	6.00E-164	3.43E-03
Ssc.8761.1.A1_at	WSB2	2.00E-114	9.80E-03
Ssc.26094.2.S1_at	YTHDF2	0	2.75E-03
Ssc.3684.1.S1_at	ZC3H7B	4.30E-88	2.99E-03
Ssc.27899.1.S1_at	ZC3H7B	3.60E-25	5.19E-03
Ssc.5808.1.S1_at	ZCCHC3	4.00E-179	2.65E-04
Ssc.30576.1.A1_at	ZFP1	1.30E-12	5.08E-03
Ssc.22060.1.A1_at	ZKSCAN1	1.80E-43	1.44E-03
Ssc.22060.2.S1_at	ZKSCAN1	1.80E-43	6.11E-03
Ssc.19284.1.A1_at	ZNF300	0	5.22E-03
Ssc.30849.1.A1_at	ZNF318	3.00E-124	1.36E-03
Ssc.28523.1.S1_at	ZNF496	3.60E-11	9.96E-04
Ssc.18877.1.A1_at	ZNF507	0	4.36E-03
Ssc.2035.1.S1_at	ZNF530	4.80E-16	1.36E-03
Ssc.10619.1.A1_at	ZNF677	8.50E-11	6.27E-03
Ssc.23846.1.S1_at	ZNF7	0	8.12E-03

K means cluster 2 regulated porcine probes and their human homologs (e value: $e < e^{-10}$)			
ID	Gene Symbol	e-value	ANOVA FDR corrected P-value
Ssc.3625.1.A1_at	AAK1	6.80E-12	6.12E-04
Ssc.19385.1.A1_at	ADAMTS7	0	7.41E-03
Ssc.27438.1.S1_at	AGA	0	3.30E-04
Ssc.2487.1.S1_at	AIFM2	0	2.03E-03
Ssc.28768.1.S1_at	AIP	0	3.59E-03
Ssc.18593.1.S1_at	AKAP13	2.20E-45	1.17E-03
Ssc.16750.1.A1_at	ALCAM	0	8.83E-03
Ssc.8917.1.A1_at	ALDOA	0	3.44E-03
Ssc.1187.1.S1_at	ALDOC	0	4.06E-03
Ssc.6009.1.S1_a_at	AMY2B	0	8.16E-04
Ssc.26213.1.S1_at	ANKRD13A	0	5.92E-03

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Ssc.2652.1.A1_at	ANTXR2	6.00E-142	1.97E-04
Ssc.20255.1.S1_at	ARHGAP10	1.50E-67	1.55E-03
Ssc.4632.2.A1_at	ARHGEF3	6.30E-36	4.56E-03
Ssc.21450.1.S1_at	ARHGEF6	4.90E-18	1.18E-03
Ssc.24155.1.S1_at	ARID4A	0	4.97E-04
Ssc.24407.1.S1_at	ARID4A	0	5.88E-04
Ssc.5509.2.S1_at	ARL4A	0	6.20E-03
Ssc.8501.2.A1_at	ARL6IP5	0	2.19E-03
Ssc.1732.1.S1_at	ARL6IP5	5.40E-19	9.80E-04
Ssc.9454.1.S1_at	ARRDC2	5.00E-18	6.65E-04
Ssc.1027.1.S1_at	ASAH1	3.80E-53	4.19E-03
Ssc.8012.1.A1_at	ATP8B1	2.60E-28	1.28E-03
Ssc.12348.2.S1_at	B2M	9.20E-15	7.20E-03
Ssc.14898.2.S1_at	B4GALNT1	0	3.59E-03
Ssc.16963.1.S1_at	B4GALT5	2.00E-127	7.19E-03
Ssc.25700.1.S1_at	BANK1	3.30E-58	1.72E-03
Ssc.4578.2.S1_at	BCAR3	0	2.32E-05
Ssc.16850.1.S1_at	BIN1	0	1.36E-03
Ssc.1338.1.S1_at	BLVRB	7.00E-171	9.66E-03
Ssc.28502.1.S1_at	C11orf24	9.60E-24	2.54E-03
Ssc.2850.1.A1_at	C22orf25	0	1.93E-03
Ssc.9071.1.A1_at	C3orf41	4.80E-10	7.73E-03
Ssc.30665.1.S1_at	C4orf32	1.40E-27	6.26E-03
Ssc.19686.1.S1_at	CACNB4	0	6.39E-04
Ssc.8116.1.S1_at	CALCR	0	7.27E-03
Ssc.18360.1.S1_at	CAPG	0	1.33E-03
Ssc.235.1.S1_a_at	CAST	0	2.24E-03
Ssc.28706.1.S1_at	CBX7	3.70E-65	2.31E-03
Ssc.18879.1.A1_at	CCDC21	1.00E-117	1.36E-03
Ssc.271.1.A1_at	CD55	1.60E-24	9.68E-03
Ssc.18220.3.A1_at	CDC37L1	7.00E-130	6.51E-03
Ssc.15576.1.S1_at	CEBPG	4.00E-137	5.30E-03
Ssc.3565.1.A1_at	CHRD	2.40E-89	5.10E-03
Ssc.9718.1.A1_at	CITED2	0	3.19E-03
Ssc.30779.1.S1_at	CLDN10	0	3.50E-03
Ssc.19539.1.A1_at	CLN8	9.60E-39	3.63E-03
Ssc.25364.1.S1_at	CMYA5	1.00E-122	3.71E-03
Ssc.9385.1.S1_at	CNNM4	1.40E-35	1.83E-03
Ssc.11312.1.A1_at	COG3	8.00E-105	3.52E-03
Ssc.4345.1.S1_at	COL4A1	0	6.96E-03
Ssc.1078.1.A1_at	CREG1	4.50E-14	2.86E-03
Ssc.6155.1.S1_at	CTSC	0	9.80E-03
Ssc.28893.1.A1_at	CTSD	0	7.82E-03
Ssc.3671.3.A1_at	CTSW	4.40E-78	7.00E-03
Ssc.24520.1.A1_at	CWF19L2	4.50E-67	4.52E-03
Ssc.21.1.S1_s_at	DDX58	1.70E-51	7.62E-03
Ssc.15252.1.S1_at	DGKZ	0	7.88E-04
Ssc.10191.1.A1_at	DKFZp586F1418	0	2.20E-03

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Ssc.4962.1.S1_at	DLG3	1.10E-37	6.34E-03
Ssc.5831.1.S1_at	DNAI2	7.40E-11	1.09E-04
Ssc.13345.1.A1_at	DNAJB4	5.00E-153	9.23E-03
Ssc.18422.1.S1_a_at	DNASE1L1	0	7.83E-03
Ssc.18422.1.S1_at	DNASE1L1	0	5.32E-03
Ssc.6019.1.S1_at	DNASE2	6.00E-145	6.79E-03
Ssc.26862.1.A1_at	DOCK5	8.10E-17	5.60E-03
Ssc.22480.1.S1_at	DTX1	0	7.87E-03
Ssc.6191.1.S1_at	DTX3L	7.10E-25	5.67E-03
Ssc.12589.1.A1_at	DYNLT3	2.00E-152	7.35E-04
Ssc.21134.1.S1_at	DYNLT3	6.70E-41	7.15E-03
Ssc.9629.1.A1_at	DYRK2	0	3.19E-03
Ssc.23854.1.S1_at	EEA1	0	8.55E-04
Ssc.19722.1.S1_at	ELTD1	7.00E-115	9.41E-04
Ssc.19119.1.A1_at	EMX2	0	8.79E-03
Ssc.19431.1.S1_at	ENPP3	1.00E-106	4.54E-03
Ssc.5631.1.S1_at	EPB41L1	0	8.74E-03
Ssc.2070.1.S1_at	EPB41L1	4.30E-66	1.55E-03
Ssc.21456.1.S1_at	ERO1L	0	5.63E-03
Ssc.23486.1.S1_at	ETHE1	0	1.65E-03
Ssc.6168.1.S1_at	ETV7	3.00E-108	1.29E-03
Ssc.1516.1.A1_at	FARP1	1.00E-128	6.08E-03
Ssc.4479.1.S1_at	FBLN2	0	7.09E-03
Ssc.18138.1.A1_at	FBXL20	0	5.90E-03
Ssc.24944.1.S1_a_at	FCGR1A	2.00E-122	3.80E-03
Ssc.29301.1.A1_at	FLJ11286	9.00E-82	2.53E-03
Ssc.10636.1.A1_at	FLJ21062	0	2.98E-03
Ssc.13128.1.A1_at	FLJ22680	1.70E-34	5.33E-03
Ssc.13644.1.A1_at	FLJ25371	2.60E-33	6.01E-03
Ssc.4943.1.A1_at	FLJ31025	6.90E-95	5.09E-03
Ssc.2828.1.A1_at	FLJ90651	0	7.92E-03
Ssc.1801.1.A1_at	FNBP1	0	4.69E-03
Ssc.1801.2.S1_a_at	FNBP1	0	2.14E-03
Ssc.1801.2.S1_at	FNBP1	0	3.43E-03
Ssc.4103.1.A1_at	FNDC3B	0	2.42E-04
Ssc.14544.1.S1_at	FOLR2	5.00E-102	6.29E-03
Ssc.2012.1.A1_at	FRMD3	2.00E-110	5.30E-03
Ssc.5744.2.S1_at	FUCA1	0	5.88E-04
Ssc.1093.3.S1_at	FZD4	0	7.06E-03
Ssc.4584.1.S1_at	GFOD2	0	2.91E-03
Ssc.15306.1.S1_at	GGH	3.00E-159	8.57E-03
Ssc.20974.1.A2_at	GNS	4.60E-48	1.73E-03
Ssc.14167.1.A1_at	GOLGA4	0	7.53E-04
Ssc.26127.1.S1_at	GPR107	0	6.93E-03
Ssc.19334.1.S1_at	GRB2	0	1.80E-03
Ssc.25475.1.S1_at	GRIA3	3.70E-70	8.73E-03
Ssc.9056.1.A1_at	GRIP1	1.60E-57	5.27E-03
Ssc.30917.1.A1_at	GSK3B	3.80E-29	2.56E-03

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Ssc.4939.1.A1_at	GZMB	1.10E-27	2.87E-03
Ssc.24494.1.A1_at	HDC	7.00E-137	1.08E-04
Ssc.30724.1.S1_at	HERC6	2.00E-110	9.64E-03
Ssc.13496.1.A1_at	HISPPD2A	1.20E-40	5.72E-04
Ssc.29031.1.A1_at	HIST1H2BE	3.00E-151	7.61E-03
Ssc.27038.1.A1_at	HORMAD1	1.50E-26	6.82E-04
Ssc.15266.1.S1_at	HPGD	0	2.63E-04
Ssc.24670.1.S1_at	HPS5	0	4.83E-03
Ssc.14293.1.A1_at	HSPA12A	2.50E-69	7.43E-03
Ssc.29504.1.A1_at	HYDIN	3.50E-32	7.80E-03
Ssc.11694.1.S1_at	ID2	0	4.79E-03
Ssc.14914.1.A1_at	ID2	4.00E-174	6.79E-03
Ssc.22620.1.S1_at	IFIT2	4.00E-149	5.73E-04
Ssc.422.1.S1_at	IGF2R	1.90E-33	4.16E-03
Ssc.8833.1.S1_at	IL15	3.00E-104	1.53E-04
Ssc.25858.1.S1_at	IL17D	3.50E-10	5.96E-03
Ssc.16489.1.S1_at	IL7R	8.80E-19	2.10E-03
Ssc.19537.1.S1_at	IRF2	0	5.30E-03
Ssc.16218.1.S1_at	ITGA2	0	1.36E-03
Ssc.2786.1.S1_at	ITGA7	1.00E-67	6.77E-03
Ssc.16418.1.A1_at	ITGBL1	0	3.80E-03
Ssc.7460.1.A1_at	IVNS1ABP	0	6.34E-03
Ssc.25973.1.A1_at	JDP2	3.60E-95	5.04E-03
Ssc.5485.1.S1_at	KCNN3	0	2.35E-03
Ssc.4963.1.A1_at	KCTD10	0	6.67E-03
Ssc.20300.1.S1_at	KIAA0323	1.00E-16	3.10E-04
Ssc.9953.1.A1_at	KIAA1598	5.10E-73	1.72E-03
Ssc.9953.2.S1_at	KIAA1598	5.10E-73	2.91E-03
Ssc.24781.1.A1_at	KIAA1712	3.00E-159	7.70E-03
Ssc.7275.1.S1_at	KYNU	3.00E-102	4.31E-04
Ssc.8098.1.A1_at	LAMP2	4.00E-166	9.48E-03
Ssc.4789.1.A1_at	LASP1	6.60E-46	1.96E-03
Ssc.836.1.S1_at	LDHD	0	4.16E-03
Ssc.5228.1.S1_at	LGALS8	3.00E-132	5.31E-03
Ssc.5548.1.A1_at	LHFPL2	0	3.37E-04
Ssc.2598.1.S1_at	LOXL2	2.00E-144	5.77E-03
Ssc.2466.1.S1_at	LRP10	0	1.74E-04
Ssc.863.2.S1_at	LY6E	1.60E-33	1.09E-04
Ssc.2563.1.S1_at	M6PR	0	4.11E-03
Ssc.19360.1.S1_at	MAN1C1	0	2.84E-03
Ssc.21192.3.S1_at	MAP4K4	0	1.42E-03
Ssc.2476.1.S1_at	MARCH2	0	5.66E-03
Ssc.13763.2.A1_at	MAT2B	0	2.36E-03
Ssc.1081.1.A1_at	MBNL1	0	3.30E-03
Ssc.1081.3.A1_at	MBNL1	0	1.70E-03
Ssc.29716.1.A1_at	MCTP1	3.60E-13	8.56E-04
Ssc.9096.1.S1_at	MDFIC	0	1.08E-04
Ssc.18528.2.A1_at	MFSD1	4.00E-114	6.34E-03

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Ssc.20367.1.S1_at	MGC129938	2.00E-105	3.58E-03
Ssc.25129.1.A1_at	MID1IP1	4.90E-69	1.89E-03
Ssc.9382.1.A1_at	MKL1	6.60E-17	3.59E-03
Ssc.3218.1.S1_at	MKNK1	6.20E-33	5.54E-03
Ssc.5385.1.S1_at	MOBKL2C	4.30E-18	6.77E-03
Ssc.30253.1.A1_at	MORC3	1.10E-56	2.62E-03
Ssc.22360.1.S1_at	MRPL1	0	5.22E-03
Ssc.15768.1.S1_at	MS4A2	1.60E-17	2.63E-04
Ssc.14392.1.A1_at	MSMB	1.60E-13	7.06E-03
Ssc.2260.1.A1_at	MTHFR	1.00E-148	5.04E-03
Ssc.28510.1.S1_at	MYLE	6.00E-154	8.85E-03
Ssc.21565.1.S1_at	NAGPA	0	8.38E-03
Ssc.1755.2.A1_at	NANS	1.40E-69	5.53E-03
Ssc.9495.1.S1_at	NCF2	0	1.31E-03
Ssc.6822.1.A1_at	NCOA7	1.60E-79	2.10E-03
Ssc.15046.1.A1_at	NCOA7	1.70E-64	1.60E-03
Ssc.4936.1.S1_at	NECAP2	0	1.46E-03
Ssc.13296.1.A1_at	NEFH	3.00E-121	7.19E-03
Ssc.18497.1.S1_at	NEIL1	0	4.60E-04
Ssc.27184.1.S1_at	NISCH	4.10E-22	2.38E-04
Ssc.12918.1.S1_at	NMI	6.60E-89	8.35E-03
Ssc.9919.1.A1_at	NMI	2.50E-16	3.07E-04
Ssc.21065.1.S1_a_at	NMNAT1	1.70E-88	1.18E-03
Ssc.1533.1.S1_at	NPC1	0	1.83E-03
Ssc.21971.2.S1_at	NPDC1	4.00E-74	2.77E-03
Ssc.22369.1.A1_at	NR3C1	0	6.19E-04
Ssc.15952.1.S1_at	NR3C1	6.10E-53	2.39E-03
Ssc.24899.1.S1_at	NRP2	6.00E-145	5.92E-03
Ssc.13776.1.S1_at	NT5C2	0	5.27E-03
Ssc.25733.1.S1_at	OGFOD1	9.00E-139	4.97E-03
Ssc.18377.2.S1_at	P2RX4	0	5.10E-03
Ssc.13060.1.S1_a_at	PAPD5	0	9.54E-03
Ssc.13060.3.A1_a_at	PAPD5	0	3.88E-03
Ssc.22491.1.A1_at	PAPD5	6.00E-160	1.38E-04
Ssc.2599.1.S1_at	PAQR8	0	5.60E-03
Ssc.13068.1.A1_at	PARP12	4.40E-93	8.99E-03
Ssc.25815.1.A1_at	PARP3	4.30E-60	1.97E-03
Ssc.8798.1.A1_at	PBX1	3.70E-29	1.63E-03
Ssc.2814.1.S1_at	PBXIP1	2.00E-158	8.60E-03
Ssc.17347.1.S1_at	PC	0	4.48E-03
Ssc.17574.1.S1_at	PDE2A	8.80E-59	8.11E-03
Ssc.11395.1.S1_at	PI4K2A	0	8.60E-03
Ssc.3856.1.S1_at	PI4K2A	1.10E-26	3.84E-03
Ssc.18551.1.S1_at	PKIG	1.10E-91	5.73E-03
Ssc.20678.1.S1_at	PKN1	0	6.37E-03
Ssc.307.1.S1_at	PPP1R12A	2.00E-158	1.62E-03
Ssc.16528.1.S1_at	PQLC2	0	7.13E-03
Ssc.16722.1.S1_at	PQLC3	4.00E-103	1.17E-03

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Ssc.8658.1.A1_at	PRKACB	4.30E-88	3.12E-03
Ssc.9639.1.A1_at	PRRX1	8.80E-12	1.90E-04
Ssc.26669.1.A1_at	PSD4	7.60E-15	2.55E-03
Ssc.21993.1.S1_at	RAB10	0	5.31E-04
Ssc.1560.3.S1_a_at	RALA	4.30E-97	1.92E-03
Ssc.8269.1.A1_at	RAPH1	1.40E-37	1.34E-03
Ssc.10534.3.A1_a_at	RASA2	0	6.70E-03
Ssc.27241.2.S1_at	RASAL2	2.00E-116	8.29E-03
Ssc.29086.2.S1_a_at	RBPJ	0	4.30E-03
Ssc.29086.3.S1_a_at	RBPJ	0	3.83E-03
Ssc.5960.1.A1_at	RBPJ	5.00E-105	4.70E-03
Ssc.21769.1.S1_at	RERG	0	6.70E-03
Ssc.20812.2.S1_at	RFTN1	4.50E-50	9.05E-03
Ssc.16039.1.A1_at	RGS1	1.20E-36	3.76E-03
Ssc.8706.1.S1_at	RGS3	0	3.03E-03
Ssc.5987.1.A1_at	RHPN2	3.30E-52	4.02E-03
Ssc.20781.1.S1_at	RNF123	9.40E-22	2.31E-03
Ssc.3959.1.S1_at	RNF130	0	1.09E-04
Ssc.6221.1.S1_at	RNF149	0	2.74E-03
Ssc.3742.1.S1_at	RNF166	0	9.46E-03
Ssc.15672.1.S1_at	RRAS	0	9.29E-03
Ssc.3899.1.S1_at	RRBP1	9.30E-85	2.27E-03
Ssc.24376.1.S1_at	RUFY1	0	1.82E-03
Ssc.5185.1.A1_at	RUSC1	4.00E-57	5.25E-04
Ssc.29090.1.A1_at	SASH1	1.00E-132	3.60E-03
Ssc.21074.1.A1_at	SAT1	0	7.13E-03
Ssc.25537.1.S1_at	SCGN	0	2.79E-03
Ssc.10793.1.A1_at	SDC2	0	9.96E-03
Ssc.21519.1.S1_s_at	SDC2	0	5.82E-03
Ssc.8939.1.S2_at	SDHB	0	6.77E-03
Ssc.9611.1.A1_at	SESN1	4.00E-100	2.48E-03
Ssc.2009.1.S1_at	SESN2	7.70E-29	4.88E-03
Ssc.18798.1.A1_at	SLC10A3	0	2.53E-03
Ssc.6273.1.A1_at	SLC25A38	0	1.01E-03
Ssc.24721.1.A1_at	SLC27A6	0	8.85E-03
Ssc.19836.2.S1_at	SLC31A2	4.10E-47	5.72E-04
Ssc.24978.2.S1_at	SLC37A2	0	4.46E-04
Ssc.26810.1.A1_at	SLC38A6	1.00E-21	1.51E-03
Ssc.6034.1.S1_at	SLC45A3	8.60E-61	6.83E-03
Ssc.24342.2.A1_at	SLIT2	0	3.66E-03
Ssc.27954.1.S1_at	SNX11	7.40E-41	4.36E-03
Ssc.7207.1.S1_at	SP100	6.80E-89	5.36E-03
Ssc.7207.3.A1_at	SP100	6.80E-89	6.70E-03
Ssc.12376.1.A1_at	SP110	3.00E-61	9.00E-03
Ssc.11163.1.S1_at	SP140	6.00E-107	1.55E-03
Ssc.23781.1.S1_at	ST6GALNAC4	0	4.01E-04
Ssc.9288.1.A1_at	STAMBPL1	0	1.60E-03
Ssc.4484.1.S1_at	STARD3	6.00E-120	5.63E-04

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Ssc.19568.1.S1_at	STAT6	0	1.23E-03
Ssc.24586.1.S1_at	SYNJ1	6.00E-180	1.30E-03
Ssc.1050.1.S1_at	TALDO1	0	2.52E-03
Ssc.16975.1.A1_at	TANC2	0	7.71E-03
Ssc.1166.1.S1_at	TBC1D20	0	3.04E-04
Ssc.24538.1.S1_at	TCF4	6.10E-51	3.65E-04
Ssc.31176.1.A1_at	TCN2	6.80E-32	6.49E-03
Ssc.26103.1.S1_at	TFEB	2.40E-50	2.38E-03
Ssc.23477.1.S1_at	TFIP11	0	9.70E-03
Ssc.76.3.S1_a_at	TGFB1	0	7.13E-03
Ssc.92.1.S1_at	TGFB2	0	9.21E-03
Ssc.12131.1.A1_at	TGFB2	1.80E-58	7.28E-03
Ssc.23305.1.S1_at	TMEM144	1.10E-10	6.72E-03
Ssc.10752.1.A1_at	TMEM65	3.10E-41	3.93E-03
Ssc.2131.1.S1_at	TMTC1	1.10E-28	2.94E-03
Ssc.24234.1.A1_at	TNFAIP8	0	7.17E-03
Ssc.1758.1.S1_at	TNIP1	0	4.18E-03
Ssc.12569.1.A1_at	TPD52	0	4.35E-03
Ssc.12569.2.A1_at	TPD52	0	5.85E-03
Ssc.5030.1.A1_at	TRIM22	1.40E-13	8.85E-03
Ssc.26642.1.S1_at	TRIM24	0	3.08E-03
Ssc.9774.1.A1_at	TRPS1	3.50E-32	8.97E-03
Ssc.2515.1.S1_at	TSC2	0	3.39E-03
Ssc.2682.1.S1_at	TSC22D3	0	7.00E-03
Ssc.22072.2.S1_at	TSPAN2	1.80E-67	2.53E-03
Ssc.11504.1.A1_at	UNC13D	2.10E-63	4.16E-03
Ssc.30447.1.A1_at	USP15	0	7.67E-03
Ssc.24224.1.A1_at	USP25	0	2.80E-03
Ssc.10160.1.A1_at	WIPF1	1.00E-154	1.01E-03
Ssc.12384.1.A1_at	XK	1.80E-34	6.70E-05
Ssc.3392.1.A1_at	ZFP36L2	5.40E-56	4.47E-03
Ssc.2259.1.A1_at	ZMIZ1	0	2.32E-03
Ssc.6433.1.S1_at	ZNFX1	0	9.60E-03

K means cluster 3 regulated porcine probes and their human homologs (e value: e<e-10)

ID	Gene Symbol	e-value	ANOVA FDR corrected P-value
Ssc.7818.1.A1_at	ABCB10	2.30E-31	7.11E-03
Ssc.6425.3.A1_at	ABCC5	4.00E-155	6.77E-03
Ssc.22033.1.S1_at	ACP2	0	7.94E-04
Ssc.19980.1.S1_at	ADAM28	1.00E-42	1.01E-03
Ssc.13385.2.S1_a_at	ALCAM	0	2.00E-03
Ssc.6009.1.S1_at	AMY2B	0	2.91E-03
Ssc.29929.1.S1_at	ANGPTL2	2.50E-64	2.53E-03
Ssc.17592.1.S1_at	ANKH	4.00E-178	5.31E-04
Ssc.8854.1.A1_at	ARHGAP27	2.20E-15	6.76E-03
Ssc.1230.1.A1_at	ARHGDIB	4.00E-167	2.94E-03
Ssc.8984.1.S1_at	ARHGEF2	4.00E-168	7.53E-04

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Ssc.18230.1.S1_at	ARHGEF6	1.70E-61	6.95E-04
Ssc.23770.1.S1_at	ARSB	0	1.36E-03
Ssc.14898.1.A1_at	B4GALNT1	0	8.16E-04
Ssc.5848.1.S1_at	B4GALT5	0	8.04E-03
Ssc.4578.1.A1_at	BCAR3	0	3.28E-04
Ssc.12561.1.A1_at	BCAT1	1.20E-67	3.50E-03
Ssc.27431.1.A1_at	BCAT1	3.70E-12	5.41E-03
Ssc.27319.1.S1_at	BCL2L11	8.80E-39	2.40E-03
Ssc.15518.1.A1_at	BCL6	7.00E-122	6.95E-03
Ssc.12872.1.A1_at	C13orf18	1.00E-14	2.39E-03
Ssc.19637.1.S1_at	C16orf77	4.00E-167	1.60E-03
Ssc.7476.1.A1_at	C1orf54	1.20E-75	4.17E-03
Ssc.27811.1.S1_at	C1QTNF6	1.10E-10	6.76E-03
Ssc.15439.1.A1_at	C21orf100	2.40E-10	3.82E-03
Ssc.5611.1.S1_at	C22orf16	2.40E-59	1.81E-03
Ssc.1285.1.S1_at	C3orf64	8.80E-12	1.51E-03
Ssc.20917.1.S1_at	C5orf13	2.90E-19	3.62E-03
Ssc.6365.1.A1_at	C6orf98	2.30E-94	2.85E-04
Ssc.5108.1.S1_at	C9	1.60E-85	2.64E-03
Ssc.24254.1.S1_at	CASC4	0	9.35E-03
Ssc.235.3.S1_a_at	CAST	8.50E-87	1.91E-03
Ssc.18955.2.A1_at	CD300A	2.80E-19	7.95E-04
Ssc.12095.1.S1_at	CD83	6.40E-81	1.08E-04
Ssc.3205.1.S1_at	CD99	6.40E-12	1.91E-03
Ssc.18466.1.A1_at	CDH11	1.00E-60	3.64E-03
Ssc.75.1.S1_at	CDH5	0	8.54E-03
Ssc.15991.1.S1_at	CEBPB	3.10E-23	9.23E-04
Ssc.11307.1.A1_at	CEECAM1	6.50E-20	3.84E-03
Ssc.2295.1.S1_at	CENTG3	2.00E-147	3.18E-03
Ssc.1644.1.S1_a_at	CLCN7	2.80E-66	3.29E-04
Ssc.1644.1.S1_at	CLCN7	2.80E-66	1.27E-04
Ssc.3107.1.S1_at	CLEC14A	2.00E-11	7.27E-03
Ssc.1909.1.S1_at	CLIP3	2.00E-175	3.82E-03
Ssc.18938.1.A1_at	CPZ	0	3.66E-03
Ssc.15316.1.S1_at	CRIP1	1.70E-74	1.50E-03
Ssc.9029.1.S1_at	CST3	1.10E-33	2.22E-04
Ssc.17323.1.S1_at	CSTB	4.00E-42	9.23E-04
Ssc.3593.1.S1_at	CTSH	0	2.42E-04
Ssc.17203.1.A1_at	CTSS	0	8.35E-03
Ssc.17203.2.S1_at	CTSS	0	9.10E-03
Ssc.2187.1.S1_at	CUTL2	3.00E-152	3.64E-04
Ssc.6145.1.A1_at	CYBRD1	8.30E-80	2.03E-04
SscAffx.21.1.S1_at	CYP7A1	3.00E-172	8.80E-04
Ssc.9118.1.S1_at	DAB2	0	7.51E-03
Ssc.4511.1.S1_at	DHRS3	0	4.92E-03
Ssc.16494.1.A1_at	DOK3	2.70E-21	1.09E-04
Ssc.18504.1.S1_at	DSCR1L1	0	4.97E-04
Ssc.18577.2.A1_at	DYRK3	0	2.37E-03

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Ssc.25839.1.S1_at	ECE2	4.20E-15	5.64E-03
Ssc.1935.1.S1_at	EDEM1	3.00E-132	1.65E-03
Ssc.6068.1.A1_at	EDG1	7.40E-78	6.56E-03
Ssc.29063.1.A1_at	EEA1	0	1.72E-03
Ssc.4267.1.A1_at	EFEMP2	0	2.89E-03
Ssc.21173.1.S1_at	EFHD2	2.50E-51	1.21E-03
Ssc.5648.1.A1_at	ELMO1	3.90E-33	3.05E-04
Ssc.12128.2.S1_at	EMB	0	1.09E-04
Ssc.2078.1.S1_at	EMILIN1	0	8.01E-03
Ssc.4913.1.A1_at	ENPP1	7.00E-94	4.07E-04
Ssc.9771.1.A1_at	ERG	5.40E-99	9.58E-04
Ssc.1557.1.S1_at	ESAM	1.00E-179	8.48E-03
Ssc.18895.1.A1_at	FAM40B	2.70E-35	1.09E-04
Ssc.8340.1.A1_at	FAM49A	3.00E-53	8.95E-03
Ssc.7641.1.S1_at	FAM69A	0	9.96E-03
Ssc.19232.1.A1_at	FAT4	6.10E-35	5.66E-03
Ssc.19640.1.A1_at	FCER1A	3.60E-28	4.54E-04
Ssc.2368.1.S1_at	FLI1	1.50E-54	9.62E-03
Ssc.3935.1.S1_at	FLJ20489	0.00E+00	1.95E-03
Ssc.22055.1.A1_at	FNDC3B	0	4.91E-04
Ssc.5547.1.A1_at	FOSL2	4.00E-138	9.07E-03
Ssc.12018.1.A1_at	FOXP1	4.00E-138	9.99E-03
Ssc.20113.1.S1_at	FRMD4B	1.40E-77	1.74E-03
Ssc.5744.1.A1_at	FUCA1	0	2.42E-04
Ssc.1093.2.A1_at	FZD4	0	1.65E-03
Ssc.25230.1.S1_at	FZD4	3.40E-29	3.77E-04
Ssc.20913.1.S1_at	GADD45A	0	8.12E-04
Ssc.25172.1.S1_at	GAS6	1.70E-89	8.95E-03
Ssc.15306.3.S1_at	GGH	3.00E-159	1.91E-03
Ssc.116.1.S1_at	GGT1	0	2.50E-04
Ssc.23363.1.S1_at	GLA	0	3.49E-04
Ssc.7423.1.S1_at	GLA	0	1.99E-04
Ssc.27256.1.S1_at	GM2A	1.80E-19	2.08E-03
Ssc.31053.1.A1_at	GOLGB1	0	3.58E-03
Ssc.14246.1.S1_at	GSN	0	7.14E-03
Ssc.8549.1.A1_at	GUCY1A3	1.10E-11	9.22E-03
Ssc.2329.1.A1_at	GYS1	3.20E-19	4.84E-04
Ssc.4587.1.S1_at	HAPLN4	4.30E-35	8.54E-03
Ssc.8451.1.A1_at	HECTD2	6.00E-106	1.60E-03
Ssc.11025.1.S1_at	HLA-DMB	7.10E-66	6.39E-03
Ssc.26893.1.A1_at	HMCN1	2.10E-54	3.62E-03
Ssc.29012.1.S1_at	HNMT	0	7.75E-03
Ssc.114.1.S1_at	HSPA6	0	6.08E-04
Ssc.809.1.S1_at	IFI30	2.40E-63	8.15E-04
Ssc.20101.1.S1_at	IFI6	4.00E-36	7.28E-03
Ssc.30752.3.S1_at	IFIT1	2.00E-111	1.72E-03
Ssc.8909.1.A1_at	INHBB	8.00E-113	7.61E-03
Ssc.27879.1.S1_at	IQSEC1	1.60E-10	1.65E-03

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SscAffx.1.1.S1_at	ISG20	1.80E-84	3.61E-03
Ssc.16418.2.S1_at	ITGBL1	0	8.46E-03
Ssc.4141.1.A1_at	KCTD12	6.00E-31	6.87E-03
Ssc.4141.2.S1_at	KCTD12	6.00E-31	7.19E-03
Ssc.28834.1.A1_at	KIAA1328	2.70E-69	1.08E-04
Ssc.9719.1.S1_at	KIAA1598	0	2.24E-03
Ssc.19311.1.A1_at	KLF13	4.50E-64	5.04E-03
Ssc.23774.1.S1_at	LCP1	0	2.37E-03
Ssc.23774.3.S1_at	LCP1	0	8.83E-03
Ssc.29951.1.S1_at	LCP1	2.90E-10	1.63E-03
Ssc.19531.1.A1_at	LFNG	0	8.63E-03
Ssc.3630.1.S1_at	LIMS2	0	7.67E-03
Ssc.29636.1.A1_at	MAMDC2	1.60E-37	4.61E-03
Ssc.14371.1.S1_at	MAP1A	0	7.13E-03
Ssc.3988.1.S1_at	MAP1A	1.00E-160	3.30E-03
Ssc.6093.1.S1_at	MAP1B	8.50E-43	9.78E-03
Ssc.22705.1.A1_at	MBNL1	3.10E-14	9.60E-03
Ssc.15378.1.A1_at	MCTP1	4.30E-16	3.60E-04
Ssc.9096.2.A1_at	MDFIC	0	2.61E-04
Ssc.24864.1.A1_at	MEIS1	0	8.54E-03
Ssc.24938.1.S1_at	METRNL	2.30E-63	2.00E-03
Ssc.19861.1.S1_at	MFNG	1.10E-21	8.73E-03
Ssc.21925.1.S1_at	MFRP	4.00E-170	7.77E-03
Ssc.18528.1.S1_at	MFSD1	4.00E-114	7.87E-03
Ssc.23899.1.A1_at	MKX	2.00E-110	5.90E-03
Ssc.734.1.S1_at	MMP14	0	3.46E-03
Ssc.19356.1.S1_at	MPP1	0	7.99E-04
Ssc.17756.1.S1_at	MRAS	5.90E-29	2.43E-03
Ssc.7893.1.A1_at	MTSS1	0	5.26E-03
Ssc.7893.2.A1_at	MTSS1	0	6.00E-03
Ssc.1017.1.S1_at	MXRA5	6.00E-112	6.31E-03
Ssc.21780.1.A1_at	MYO1E	0	3.54E-03
Ssc.10346.1.A1_at	NCKAP1L	4.90E-97	5.92E-04
Ssc.12918.2.A1_at	NMI	6.60E-89	4.32E-03
Ssc.6055.1.A1_at	SLC46A3	4.70E-89	3.32E-03
Ssc.4287.1.S1_at	LOC339483	5.80E-52	5.99E-03
Ssc.973.1.A1_at	MSTP119	2.20E-47	1.43E-03
Ssc.20294.1.S1_at	FLJ39622	7.00E-20	2.29E-03
Ssc.3541.1.A1_at	NOSTRIN	6.00E-112	6.70E-05
Ssc.19035.1.S1_at	GALNACT1	9.60E-29	6.39E-03
Ssc.18091.1.A1_at	IPO11	1.20E-91	3.80E-03
Ssc.6172.1.S1_at	DKFZp686C1662	2.20E-51	5.97E-03
Ssc.24441.2.S1_a_at	NPAS2	6.00E-109	5.13E-03
Ssc.248.1.S1_at	NPL	0	5.87E-03
Ssc.16354.1.S1_at	NR1H3	0	2.58E-03
Ssc.14361.1.A1_at	OLFM1	0	3.64E-04
Ssc.6491.1.S1_at	OLFML1	3.00E-162	4.47E-03
Ssc.10917.1.A1_at	OXR1	1.20E-31	1.01E-03

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Ssc.21537.1.A1_at	PARVB	0	1.09E-04
Ssc.4128.1.A1_at	PDE4DIP	2.50E-61	1.46E-04
Ssc.9595.1.S1_at	PDGFRB	2.50E-24	2.00E-03
Ssc.6050.1.A1_at	PECAM1	5.10E-12	4.28E-03
Ssc.27786.1.S1_at	PFKFB3	4.20E-46	8.41E-03
Ssc.23978.1.S1_at	PHACTR3	8.00E-159	4.17E-03
Ssc.27557.1.S1_at	PID1	1.20E-27	3.43E-03
Ssc.11109.1.S1_at	PIK3CG	0	4.04E-03
Ssc.1257.1.S1_a_at	PIK3IP1	0	1.96E-03
Ssc.10754.1.A1_at	PIK3R1	1.10E-84	7.42E-05
Ssc.18551.1.S2_at	PKIG	2.90E-91	1.36E-03
Ssc.7881.1.A1_at	PLCXD2	1.00E-14	9.75E-03
Ssc.15267.1.S1_at	PLEKHF1	5.70E-27	3.54E-04
Ssc.26067.1.S1_at	PODXL	1.80E-38	1.29E-03
Ssc.18011.1.S1_at	POPDC3	1.00E-142	1.27E-03
Ssc.14475.3.S1_a_at	PPARG	0	6.65E-04
Ssc.18135.1.S1_at	PRELP	7.40E-22	5.86E-03
Ssc.2011.1.A1_at	PRKCH	2.30E-20	6.73E-04
Ssc.1813.1.S1_at	PRODH	0	2.92E-03
Ssc.23920.1.A1_at	PRRX1	7.30E-21	3.60E-03
Ssc.3547.1.S1_at	PRSS11	0	5.26E-03
Ssc.2140.1.S1_at	PTK2B	8.80E-57	3.32E-03
Ssc.12975.1.S1_at	PTN	0	1.32E-03
Ssc.23248.1.S1_at	PTPRC	0	1.65E-03
Ssc.23494.1.S1_a_at	PTPRC	1.10E-35	6.36E-03
Ssc.5147.1.S1_at	LDLRAD2	1.10E-34	9.60E-03
Ssc.9726.2.S1_at	DRAM	1.00E-20	1.16E-03
Ssc.3250.1.S1_at	FLJ11151	0	1.24E-03
Ssc.2551.1.A1_at	RAB31	1.20E-28	2.74E-04
Ssc.17304.3.S1_at	RAC2	0	4.13E-04
Ssc.11258.1.A1_at	RASSF2	1.70E-63	8.40E-03
Ssc.11787.1.S1_at	RASSF2	1.30E-21	4.36E-03
Ssc.5894.1.S1_at	RASSF4	5.30E-25	5.88E-04
Ssc.27902.1.S1_a_at	RBKS	1.00E-144	4.92E-04
Ssc.24393.1.S1_at	RCN3	0	2.54E-03
Ssc.25990.1.S1_at	RUNX1T1	1.50E-85	9.33E-03
Ssc.979.1.S1_at	S100A1	3.00E-148	8.40E-03
Ssc.18980.1.A1_at	S100A6	8.80E-45	6.77E-03
Ssc.23516.1.S1_at	SATB1	0	4.09E-03
Ssc.20832.1.S1_at	SCTR	3.80E-11	1.99E-04
Ssc.25217.1.S1_a_at	SELM	3.40E-86	8.38E-03
Ssc.18375.1.A1_at	SEMA4D	6.10E-55	1.31E-03
Ssc.5968.1.A1_at	SGPL1	6.90E-49	3.58E-03
Ssc.1403.1.S1_at	SH3BGRL3	9.00E-155	8.18E-04
Ssc.4029.1.S1_a_at	SH3GLB2	0	7.97E-03
Ssc.11185.1.A1_at	SH3KBP1	0	2.24E-03
Ssc.11185.2.S1_at	SH3KBP1	0	6.39E-03
Ssc.30088.1.A1_at	SH3KBP1	4.60E-90	1.35E-03

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Ssc.7692.1.S1_at	SH3PXD2B	3.30E-14	1.94E-03
Ssc.27790.1.A1_at	SIGLEC5	5.60E-34	1.30E-06
Ssc.24360.1.S1_a_at	SLC17A5	0	1.74E-04
Ssc.29232.1.A1_at	SLC1A2	4.50E-43	3.53E-06
Ssc.4739.1.S1_at	SLC24A6	9.00E-138	1.76E-03
Ssc.24978.1.S1_at	SLC37A2	0	4.13E-04
Ssc.23804.1.S1_at	SLC7A3	3.40E-21	2.26E-03
Ssc.7743.1.A1_at	SLIT2	1.20E-31	2.70E-03
Ssc.4705.2.A1_at	SMOC2	1.10E-18	9.55E-03
Ssc.10995.1.S1_at	SNN	3.30E-98	9.59E-04
Ssc.15360.1.A1_a_at	SOX18	9.00E-118	5.54E-03
Ssc.7207.2.A1_at	SP100	6.80E-89	4.36E-03
Ssc.27039.1.S1_at	SPECC1L	2.20E-67	3.24E-03
Ssc.2543.1.S1_at	SPOCK2	4.10E-56	5.87E-03
Ssc.3285.1.S1_at	ST14	0	6.77E-03
Ssc.11698.1.A1_at	ST8SIA4	1.20E-19	6.85E-03
Ssc.23834.1.S1_at	SUSD3	2.10E-73	5.87E-04
Ssc.18272.2.S1_at	TCIRG1	0	6.62E-04
Ssc.4466.1.S1_at	TEC	7.00E-141	6.70E-05
Ssc.4753.1.A1_at	TEK	6.70E-67	4.92E-03
Ssc.3753.1.S1_at	TFRC	7.00E-157	2.88E-03
Ssc.1082.1.S1_at	TIMP2	0	6.36E-04
Ssc.11257.1.S1_at	TIMP2	0	6.95E-04
Ssc.1077.1.A1_at	TMEM127	6.80E-34	1.09E-04
Ssc.24445.1.A1_at	TMEM47	0	5.20E-03
Ssc.8464.1.A1_at	TMEM47	0	8.83E-03
Ssc.17636.1.S1_at	TMEM58	0	2.31E-03
Ssc.26879.2.S1_at	TNFSF12	3.00E-159	3.37E-04
Ssc.5614.1.S1_at	TPS1	3.60E-59	7.54E-04
Ssc.180.1.S1_at	TRGV9	8.30E-61	3.08E-03
Ssc.21942.3.A1_a_at	TSKU	0	2.91E-03
Ssc.21942.1.S1_at	TSKU	2.80E-12	7.75E-04
Ssc.26272.2.S1_at	TSPAN9	1.00E-28	3.51E-03
Ssc.5936.1.A1_a_at	TSPO	5.00E-147	1.60E-03
Ssc.17121.1.S1_at	TWIST1	0	8.27E-03
Ssc.12415.1.A1_at	UNC93A	4.90E-46	5.36E-03
Ssc.12682.1.A1_at	VAV3	0	3.11E-03
Ssc.12790.1.A1_at	VEGFC	1.00E-97	2.24E-03
Ssc.16640.3.S1_at	VSIG4	0	5.32E-03
Ssc.18550.1.S1_at	WAS	3.00E-68	3.37E-03
Ssc.9299.1.S1_at	ZEB1	0	6.73E-03
Ssc.21986.1.S1_at	ZNF238	0	1.83E-03

K means cluster 4 regulated porcine probes and their human homologs (e value: e<e-10)

ID	Gene Symbol	e-value	ANOVA FDR corrected P-value
Ssc.14114.1.A1_at	ABCD3	5.00E-147	4.04E-03
Ssc.11816.1.A1_at	ABCD3	7.60E-26	1.53E-03

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Ssc.6276.2.S1_at	ABHD6	0	7.71E-03
Ssc.2176.1.A1_at	ACSL3	0	9.33E-04
Ssc.6654.1.A1_at	ACSL3	1.80E-10	8.17E-04
Ssc.6323.1.S1_at	ADFP	0	1.19E-03
Ssc.9766.3.A1_a_at	AK2	0	8.61E-04
Ssc.7621.1.A1_at	ANLN	0	7.72E-03
Ssc.23221.1.S1_at	APOF	1.90E-23	5.77E-03
Ssc.7546.1.A1_at	ARHGAP11A	1.40E-17	2.54E-03
Ssc.24195.1.A1_at	ARHGAP19	1.50E-20	1.09E-04
Ssc.29706.1.A1_at	ATAD2	6.60E-12	1.89E-03
Ssc.27454.1.S1_at	ATIC	0	3.35E-04
Ssc.21717.1.S1_a_at	AURKC	0	1.43E-03
Ssc.18164.1.A1_at	BCHE	0	8.60E-03
Ssc.4845.1.S1_at	BECN1	0	1.72E-03
Ssc.432.1.S1_at	BIRC5	1.00E-150	6.83E-03
Ssc.7195.1.A1_at	BUB1	5.00E-170	3.80E-03
Ssc.7190.1.S1_at	BUB1B	0	1.76E-03
Ssc.19626.1.S1_at	C10orf76	0	4.01E-03
Ssc.14286.1.A1_at	C10orf78	0	3.46E-03
Ssc.18205.1.S1_at	C12orf32	6.80E-27	2.10E-03
Ssc.5452.1.S1_at	C13orf21	7.60E-23	2.10E-03
Ssc.13499.1.A1_at	C13orf27	0	7.43E-03
Ssc.19268.1.S1_at	C14orf143	4.00E-126	7.48E-03
Ssc.4924.1.S1_at	C16orf35	4.00E-131	5.84E-04
Ssc.27187.1.S1_at	C18orf24	0	4.45E-03
Ssc.5983.1.A1_at	C1orf112	1.00E-154	7.37E-04
Ssc.1271.3.S1_at	C1orf121	3.00E-43	9.02E-03
Ssc.19192.1.S1_at	C20orf108	1.00E-120	1.13E-03
Ssc.17410.2.S1_at	C20orf19	6.90E-48	1.23E-03
Ssc.2953.1.A1_at	C21orf45	2.00E-147	2.49E-03
Ssc.28155.1.A1_at	C3orf26	6.60E-46	6.15E-03
Ssc.17900.1.S1_at	C6orf162	8.60E-88	5.87E-04
Ssc.19205.1.A1_at	C7orf24	7.00E-172	2.15E-03
Ssc.19205.2.S1_at	C7orf24	7.00E-172	1.82E-03
Ssc.19940.1.S1_at	CCDC5	0	1.93E-03
Ssc.23877.1.S1_at	CCNA2	0	2.47E-03
Ssc.23877.2.A1_at	CCNA2	0	2.89E-03
Ssc.14243.1.S1_at	CCNB1	0	2.24E-03
Ssc.26568.1.A1_s_at	CCNB3	2.10E-22	2.10E-03
Ssc.873.1.S1_at	CDC2	0	2.36E-03
Ssc.5721.1.S1_at	CDC45L	0	4.58E-03
Ssc.5721.3.S1_a_at	CDC45L	0	8.78E-03
Ssc.11879.1.A1_at	CDC6	1.50E-98	5.72E-04
Ssc.29855.1.A1_at	CDCA7	2.60E-14	1.65E-03
Ssc.21251.1.A1_a_at	CENPT	8.00E-164	5.31E-04
Ssc.9993.1.A1_at	CEP57	4.20E-96	5.70E-03
Ssc.8308.1.A1_at	CHL1	4.00E-144	8.67E-03
Ssc.26899.1.A1_at	CKAP2	2.00E-148	1.27E-03

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Ssc.4403.1.A1_at	CKS1B	3.00E-151	5.83E-04
Ssc.8334.1.A1_at	CLIP4	3.80E-58	8.65E-03
Ssc.19346.1.S1_at	CLSPN	1.00E-25	2.12E-03
Ssc.22370.1.S1_at	DCC1	7.00E-143	9.35E-03
Ssc.7594.1.A1_at	DEPDC1B	5.00E-151	2.14E-03
Ssc.7139.1.S1_at	DHFRP1	0	4.37E-03
Ssc.21920.1.A1_at	DHRS13	0	3.09E-04
Ssc.17458.1.S1_at	DMXL2	3.00E-145	3.88E-03
Ssc.17243.1.S1_at	DNAJA4	0	6.95E-03
Ssc.24459.1.S1_at	DTL	5.40E-30	6.65E-04
Ssc.19928.1.S1_a_at	DUT	8.00E-167	7.31E-03
Ssc.21718.1.S1_at	E2F7	8.50E-81	2.58E-03
Ssc.29094.1.A1_at	ECT2	2.10E-22	6.65E-04
Ssc.12970.1.S1_at	EIF1AX	6.50E-21	9.51E-04
Ssc.26786.1.S1_at	EIF4EBP2	2.40E-10	5.86E-03
Ssc.30857.1.S1_at	ENC1	0	5.76E-03
Ssc.24356.1.S1_at	EPB41L4B	0	4.89E-03
Ssc.20009.1.S1_at	EPS8	0	2.14E-03
Ssc.24916.1.S1_at	ESPL1	0	2.98E-03
Ssc.5073.1.A1_at	EZH2	0	3.80E-03
Ssc.8901.1.A1_at	FAM57A	3.00E-126	2.89E-03
Ssc.24094.1.S1_at	FAM64A	2.00E-114	1.03E-03
Ssc.21217.1.A1_at	FAM72A	1.00E-53	7.00E-03
Ssc.1600.3.S1_at	FAM80B	0	1.96E-04
Ssc.11984.1.A1_at	FANCD2	0	1.02E-03
Ssc.17991.1.A1_at	FLJ40869	4.20E-61	8.51E-03
Ssc.9856.1.A1_at	FLJ42117	5.00E-35	3.32E-04
Ssc.2570.1.A1_at	G6PD	1.50E-10	8.16E-03
Ssc.16391.1.A1_at	GAS2	5.00E-146	2.48E-03
Ssc.5401.1.S1_at	GINS1	2.00E-172	2.69E-03
Ssc.13716.1.A1_at	GJB7	3.20E-12	1.38E-03
Ssc.6707.1.A1_at	GLDC	8.70E-28	9.14E-03
Ssc.24336.1.S1_at	GMPS	0	8.40E-03
Ssc.8208.1.A1_at	GPAM	2.10E-71	1.65E-03
Ssc.5680.1.S1_at	GRHPR	0	8.35E-03
Ssc.12273.1.A1_at	GSTM3	0	4.84E-04
Ssc.4108.1.S1_at	H2AFV	0	7.61E-03
Ssc.7221.1.S1_at	H2AFV	0	5.31E-04
Ssc.8163.1.A1_at	HDDC2	1.10E-95	7.40E-03
Ssc.6171.3.A1_at	HDHD2	0	7.41E-03
Ssc.7910.1.A1_at	HELLS	0	1.36E-03
Ssc.7910.2.A1_a_at	HELLS	0	1.03E-03
Ssc.10664.1.S1_at	HLF	0	5.84E-04
Ssc.226.1.S1_at	HMGB2	0	8.15E-03
Ssc.16088.1.S1_at	HMGCR	0	5.54E-03
Ssc.26770.1.S1_at	HMMR	0	8.11E-03
Ssc.18308.1.A2_at	HNRPA3	0	2.46E-03
Ssc.1301.1.S1_at	HSD17B10	0	6.71E-04

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Ssc.7654.1.A1_at	HSPA2	0	2.02E-03
Ssc.17070.1.A1_s_at	INPP5F	1.50E-11	5.73E-04
Ssc.8360.1.A1_at	INPP5F	1.50E-11	1.65E-03
Ssc.15819.1.S1_at	KCNB2	0	5.73E-04
Ssc.25336.1.S1_at	KIAA1430	1.90E-21	3.06E-03
Ssc.11164.2.S1_a_at	KIAA1794	0	8.75E-03
Ssc.30748.1.S1_at	KIF11	1.00E-166	2.77E-03
Ssc.7361.1.A1_at	KIF11	4.20E-12	9.76E-03
Ssc.7361.2.S1_at	KIF11	4.20E-12	4.56E-03
Ssc.3227.1.A1_a_at	KIF20A	1.60E-94	2.45E-03
Ssc.3227.1.A1_at	KIF20A	1.60E-94	8.98E-03
Ssc.2154.3.S1_a_at	KIF2C	0	1.91E-03
Ssc.1667.1.S1_at	KIF4A	3.10E-93	3.66E-03
Ssc.15824.1.S1_at	KPNA2	0	4.77E-03
Ssc.10493.1.A1_at	LANCL1	5.30E-19	5.30E-03
Ssc.21730.1.S1_at	LMNB1	4.00E-139	3.59E-03
Ssc.20518.1.A1_at	LOC81691	0	3.43E-03
Ssc.9551.1.S1_at	LOC87769	5.90E-43	2.74E-03
Ssc.9551.3.S1_a_at	LOC87769	5.90E-43	8.38E-03
Ssc.829.1.A1_at	LPIN2	3.60E-75	1.88E-03
Ssc.6526.1.S1_a_at	LRBA	4.00E-115	7.32E-03
Ssc.6526.3.A1_a_at	LRBA	2.30E-62	7.02E-03
Ssc.5129.1.S1_at	MAD2L1	0	5.87E-04
Ssc.22495.1.S1_at	MBOAT2	0	3.77E-04
Ssc.1408.2.S1_at	MCM2	0	2.15E-03
Ssc.4612.1.S1_at	MCM4	0	6.90E-03
Ssc.7202.1.A1_at	MCM6	1.50E-48	2.73E-03
Ssc.17030.1.S1_at	MCM7	0	7.40E-03
Ssc.22206.1.S1_at	MELK	0	1.76E-03
Ssc.8554.2.S1_at	MRPL18	2.00E-135	1.19E-03
Ssc.21205.1.S1_at	MRPL19	0	7.84E-03
Ssc.2010.2.S1_a_at	MRPL43	5.00E-157	3.77E-04
Ssc.25270.1.S1_at	MSH2	0	2.74E-03
Ssc.24063.1.S1_at	MSH6	0	1.09E-04
Ssc.7153.1.A1_at	NASP	0	6.71E-04
Ssc.19159.1.S1_at	NCAPG2	1.00E-123	1.56E-03
Ssc.7367.1.S1_at	NDC80	0	5.96E-03
Ssc.13876.1.S1_at	NEK2	0	1.65E-03
Ssc.2045.1.S1_at	NHEJ1	0	1.83E-03
Ssc.2045.2.S1_at	NHEJ1	0	5.07E-03
Ssc.26386.1.A1_at	NIP7	0	4.87E-03
Ssc.26386.2.S1_a_at	NIP7	0	9.29E-03
Ssc.24009.1.S1_at	NT5C	1.00E-169	1.93E-03
Ssc.5026.2.S1_at	NUDT3	0	8.27E-03
Ssc.25023.1.S1_at	NUF2	0	5.14E-03
Ssc.21965.1.S1_at	NUP35	3.00E-118	2.72E-04
Ssc.21605.1.S1_a_at	NUSAP1	2.60E-71	1.33E-03
Ssc.21605.2.S1_at	NUSAP1	2.60E-71	5.25E-04

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Ssc.16990.1.S1_at	ORC6L	0	7.27E-03
Ssc.11423.2.A1_at	ORMDL1	0	3.67E-03
Ssc.7004.1.A1_at	ORMDL1	1.20E-52	1.31E-03
Ssc.12493.1.A1_at	PAFAH1B2	0	4.37E-03
Ssc.11553.1.A1_at	PAICS	0	8.15E-03
Ssc.9387.1.A1_at	PCNA	0	5.86E-03
Ssc.9387.2.S1_at	PCNA	3.40E-83	5.99E-03
Ssc.21684.1.S1_at	PHF16	9.00E-100	3.77E-03
Ssc.9311.1.A1_at	PHLDA1	2.30E-36	5.41E-04
Ssc.25792.1.S1_at	PIGP	5.00E-140	5.04E-03
Ssc.29168.1.A1_at	PLA2G4A	2.70E-42	6.44E-03
Ssc.25215.1.A1_at	PLK4	0	2.00E-03
Ssc.7919.1.A1_at	PMS1	0	8.26E-03
Ssc.5371.3.S1_a_at	POLE2	6.50E-88	8.57E-03
Ssc.26263.1.S1_at	POLQ	3.90E-33	1.60E-03
Ssc.9937.1.S1_at	POLR3K	4.00E-124	9.80E-03
Ssc.26720.1.S1_at	PPA2	1.00E-116	2.42E-03
Ssc.8726.1.A1_at	PPAT	5.00E-27	6.70E-05
Ssc.13216.1.S1_at	PPEF1	6.10E-57	7.68E-03
Ssc.2754.1.S1_at	PRC1	2.50E-76	3.53E-03
Ssc.25975.1.S1_at	PRMT3	1.40E-89	5.44E-03
Ssc.2868.1.S1_at	PSIP1	2.80E-80	1.44E-03
Ssc.27561.1.S1_at	PSRC1	0	6.55E-04
Ssc.3276.1.S1_at	PTPLAD1	6.20E-22	7.53E-04
Ssc.9174.2.A1_a_at	PTPMT1	4.60E-29	1.01E-03
Ssc.11630.1.S1_at	PTTG2	0	4.10E-04
Ssc.27206.1.A1_at	RAB6B	1.00E-133	8.32E-03
Ssc.2927.1.S1_at	RAB6B	4.80E-19	6.65E-03
Ssc.30706.2.S1_at	RABEPK	0	6.59E-04
Ssc.30706.3.S1_a_at	RABEPK	0	3.35E-03
Ssc.19675.1.S1_at	RACGAP1	2.00E-176	7.11E-03
Ssc.13793.1.A1_at	RAD51AP1	1.00E-100	2.36E-03
Ssc.26573.1.S1_at	RALGPS2	9.30E-47	5.64E-03
Ssc.2521.1.S1_at	RAP1GAP	8.20E-29	6.65E-03
Ssc.4676.1.A1_at	RAVER2	4.20E-27	6.41E-03
Ssc.8818.1.A1_at	RFC3	0	9.75E-04
Ssc.27374.1.A1_at	RFC5	6.20E-11	5.41E-04
Ssc.7099.1.S1_at	RFK	2.50E-12	9.23E-04
Ssc.21431.3.A1_s_at	RNF113A	0	9.89E-03
Ssc.23207.1.S1_at	RNF2	0	2.95E-03
Ssc.25344.1.S1_at	RNF2	1.10E-39	1.35E-03
Ssc.757.1.S1_at	SCAMP1	0	3.54E-04
Ssc.11791.1.S1_at	SCARB1	0	2.98E-03
Ssc.13665.1.A1_at	SCML2	1.00E-136	9.39E-03
Ssc.4384.1.S1_at	SEPT10	1.60E-31	4.82E-04
Ssc.5676.1.S1_at	SERBP1	1.10E-81	7.53E-04
Ssc.6071.1.S2_at	SET	0	7.74E-04
Ssc.26307.1.S1_at	SFRS10	0	1.96E-03

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Ssc.3798.1.A1_at	SFXN4	7.10E-49	2.52E-03
Ssc.22020.1.S1_at	SLC19A2	1.70E-80	4.94E-03
Ssc.1139.1.A1_at	SLC4A4	0	7.23E-03
Ssc.25667.1.S1_at	SMC3	0	1.80E-03
Ssc.7850.1.A1_at	SMC4	1.00E-135	8.61E-04
Ssc.28608.1.S1_at	SPAG5	0	3.36E-03
Ssc.9896.1.A1_at	ST8SIA1	0	3.55E-03
Ssc.7701.1.A1_at	STMN1	0	3.77E-04
Ssc.5863.1.S1_at	STX6	1.90E-10	9.80E-03
Ssc.12099.1.A1_at	STX7	1.10E-31	7.00E-04
Ssc.4132.1.A1_at	SUB1	0	5.27E-03
Ssc.24424.1.A1_at	SUV39H2	0	8.29E-03
Ssc.12013.1.A1_at	SYTL4	3.10E-17	1.27E-04
Ssc.27842.1.S1_at	TEX10	0	5.30E-03
Ssc.27593.1.S1_at	TGFB3	0	3.43E-03
Ssc.11220.1.S1_at	TGIF2	5.10E-28	1.89E-03
Ssc.18469.1.S1_at	THRAP6	0	5.60E-03
Ssc.1675.1.S1_at	TIMM13	8.00E-125	1.27E-04
Ssc.8300.1.A1_at	TIP1	4.70E-12	5.88E-04
Ssc.22187.1.A1_at	TMCC3	1.20E-27	3.64E-04
Ssc.8314.1.A1_at	TMEM17	2.00E-104	6.08E-03
Ssc.12855.1.S1_at	TMEM27	0	1.79E-03
Ssc.16363.1.S1_at	TMOD3	0	4.92E-03
Ssc.31207.1.S1_at	TMPO	0	3.06E-03
Ssc.5645.1.A1_at	TMPO	1.10E-53	1.27E-03
Ssc.11139.1.S1_at	TOMM20	0	9.23E-03
Ssc.24240.1.A1_a_at	TOMM34	1.20E-11	1.36E-03
Ssc.14506.1.S1_at	TOP2A	0	3.24E-03
Ssc.12104.1.S1_at	TPX2	1.70E-97	5.87E-03
Ssc.7277.1.A1_at	TRAK2	4.80E-32	1.48E-03
Ssc.21185.1.S1_at	TRAPPC6A	2.00E-137	4.48E-03
Ssc.26266.1.S1_at	TYBN	1.60E-75	4.31E-03
Ssc.4696.1.S1_at	UACA	0	1.36E-03
Ssc.27540.2.S1_at	UBE2C	0	3.62E-03
Ssc.13668.1.A1_a_at	UCHL3	0	3.58E-03
Ssc.27995.1.A1_at	UHRF1	3.50E-12	5.04E-03
Ssc.6338.2.S1_at	USP1	0	6.24E-03
Ssc.8564.1.A1_at	VLDLR	0	1.19E-03
Ssc.24353.1.S1_at	VRK1	0	9.84E-03
Ssc.24353.2.S1_at	VRK1	0	3.97E-03
Ssc.4177.2.S1_at	WASF3	2.80E-72	2.52E-03
Ssc.23999.1.S1_a_at	WDHD1	8.00E-128	7.29E-04
Ssc.16830.1.S1_at	XRCC1	4.90E-63	4.31E-04
Ssc.1313.1.A1_at	XTP3TPA	9.00E-124	2.89E-03
Ssc.7502.1.A1_at	YES1	4.60E-91	6.29E-03
Ssc.6801.1.S1_at	YES1	3.40E-66	1.01E-03
Ssc.5013.1.A1_at	ZADH1	0	1.23E-03
Ssc.26450.1.A1_at	ZDHHC23	3.40E-58	1.80E-04

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Ssc.13616.1.S1_at	ZFP106	3.90E-75	3.13E-03
Ssc.10709.1.A1_at	ZNF271	6.50E-68	2.65E-03

K means cluster 5 regulated porcine probes and their human homologs (e value: $e < e^{-10}$)

ID	Gene Symbol	e-value	ANOVA FDR corrected P-value
Ssc.575.1.S1_at	ACP5	0	3.91E-04
Ssc.314.1.S1_at	ADM	5.00E-170	6.54E-03
Ssc.140.1.S1_at	AMBN	7.00E-173	4.29E-04
Ssc.820.1.S1_at	ANPEP	2.00E-174	1.75E-03
Ssc.19344.1.A1_at	ARHGAP25	0	1.36E-03
Ssc.15398.1.A1_at	ARHGAP30	1.00E-165	1.14E-03
Ssc.3445.3.A1_at	ARL7	0	3.47E-03
Ssc.24984.1.S1_at	ASAH1	8.10E-17	1.06E-03
Ssc.22164.1.S1_at	ATP6V0D2	1.70E-88	2.75E-04
Ssc.4283.1.S1_at	BIN2	1.40E-21	6.55E-04
Ssc.8594.1.A1_at	BLNK	1.20E-38	1.83E-03
Ssc.15419.1.S1_at	BTK	1.00E-110	1.51E-03
Ssc.23963.1.S1_at	C13orf15	9.00E-180	2.58E-03
Ssc.9553.1.A1_s_at	C13orf15	9.00E-180	2.89E-03
Ssc.19364.1.S1_at	C2	0	6.12E-04
Ssc.14275.1.A1_at	CALCR	2.20E-71	1.09E-04
Ssc.2897.1.S1_at	CBLB	0	1.09E-04
Ssc.22030.1.S1_at	CCL5	1.60E-79	7.00E-03
Ssc.18359.1.S1_at	CCR1	0	1.60E-03
Ssc.26328.1.S1_at	CCR5	0	1.49E-03
Ssc.27201.1.S1_a_at	CCRL2	2.20E-36	2.45E-03
Ssc.15296.3.S1_at	CD53	0	5.04E-03
Ssc.6222.1.S1_a_at	CD74	3.00E-129	9.14E-03
Ssc.16160.1.S1_at	CD86	1.60E-70	1.55E-03
Ssc.31189.1.S1_at	CHPT1	3.00E-159	1.49E-03
Ssc.19688.1.S1_at	CHRNA1	6.80E-50	9.09E-03
Ssc.9914.1.A1_at	CKB	0	4.07E-04
Ssc.5.1.S1_a_at	CLECSF5	5.60E-91	4.31E-04
Ssc.17297.1.A1_at	CLIC2	1.10E-82	2.07E-03
Ssc.11992.1.A1_at	CLU	3.00E-144	9.63E-04
Ssc.9362.1.A1_at	COL1A2	3.40E-16	2.61E-04
Ssc.13176.1.S1_at	CORO1A	0	8.97E-03
Ssc.4779.1.A1_at	CPM	4.00E-167	7.56E-04
Ssc.10931.1.S1_at	CRYAB	0	2.52E-03
Ssc.6369.1.A1_at	CSF1	3.30E-20	6.04E-04
Ssc.5826.1.A1_at	CSF1R	2.70E-69	3.68E-04
Ssc.17203.3.S1_at	CTSS	0	8.15E-03
Ssc.16769.1.S1_at	CTSZ	0	1.94E-03
Ssc.7176.1.A1_at	CXCR4	0	3.72E-04
Ssc.151.1.S1_at	CYBB	0	5.70E-03
Ssc.8261.1.A1_at	CYP2C9	1.60E-18	4.29E-04
Ssc.5950.1.S1_at	DNM1	2.80E-46	2.89E-03

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Ssc.3621.1.S1_at	DPYSL3	1.10E-65	7.27E-03
Ssc.9726.1.A1_at	DRAM	1.00E-20	1.36E-03
Ssc.26709.1.S1_at	EBI2	0	1.09E-04
Ssc.12128.1.A1_at	EMB	0	2.78E-05
Ssc.508.1.S1_at	FCER1G	7.00E-118	2.57E-03
Ssc.3692.1.A1_at	FER1L3	0	1.95E-03
Ssc.8063.1.A1_at	FLJ20273	3.30E-32	5.70E-03
Ssc.30602.1.A1_at	FLJ46041	9.30E-30	2.24E-03
Ssc.11858.1.S1_at	FMOD	0	4.90E-03
Ssc.8843.1.A1_at	FN1	1.20E-59	5.60E-03
Ssc.6656.1.A1_at	FNDC1	0	2.00E-03
Ssc.17159.1.S1_at	GIMAP	2.80E-27	8.75E-03
Ssc.20464.1.S1_at	GLIPR1	1.00E-126	3.83E-03
Ssc.709.1.S1_at	GLRX	2.40E-74	1.19E-03
Ssc.15370.1.S1_at	GMFG	0	7.80E-03
Ssc.26337.1.S1_at	ICAM2	1.20E-12	5.29E-04
Ssc.30758.1.S1_s_at	IFI30	2.40E-63	1.62E-03
Ssc.6801.1.S1_at	IGHM	3.40E-66	5.82E-03
Ssc.13778.1.S1_at	IGHM	2.00E-61	2.88E-03
Ssc.19946.1.S1_at	IGLC1	2.20E-53	5.04E-03
Ssc.16985.1.S1_at	IL18BP	3.60E-38	3.62E-03
Ssc.15739.1.S1_at	IL2RG	0	8.98E-03
Ssc.14561.1.S1_at	ITGB2	0	3.30E-04
Ssc.44.1.S1_at	ITGB3	0	1.27E-03
Ssc.15892.1.S1_at	KCNAB2	0	1.72E-03
Ssc.8355.1.A1_at	KIAA1598	0	3.02E-03
Ssc.12761.1.A1_at	KIAA1671	0	1.83E-03
Ssc.16912.1.S1_at	KIAA1671	2.40E-97	4.85E-04
Ssc.16912.2.S1_at	KIAA1671	2.40E-97	1.13E-03
Ssc.15871.1.S1_a_at	KLRK1	1.40E-76	1.56E-03
Ssc.7275.2.A1_at	KYNU	3.00E-102	1.48E-03
Ssc.22037.1.S1_at	LAPTM5	1.00E-174	6.70E-05
Ssc.22037.2.S1_at	LAPTM5	1.00E-174	3.77E-04
Ssc.2624.1.S1_at	LOC196463	2.50E-11	1.80E-04
Ssc.2624.2.S1_at	LOC196463	2.50E-11	1.09E-04
Ssc.3219.1.S1_at	LRP1	0	3.75E-03
Ssc.6785.1.S1_at	LRP1	2.90E-74	4.45E-03
Ssc.10965.2.S1_at	MAFB	0.00E+00	5.92E-03
Ssc.26060.1.A1_at	MAFB	0.00E+00	1.36E-03
Ssc.19359.1.A1_at	MBP	7.20E-24	7.56E-04
Ssc.19359.2.S1_at	MBP	7.20E-24	1.36E-03
Ssc.30059.1.A1_at	MXRA8	2.30E-67	4.15E-03
Ssc.4520.1.S1_at	MYO1D	4.90E-36	2.34E-04
Ssc.21091.1.S1_at	MYO1F	0	3.82E-03
Ssc.21570.1.S1_at	NCKAP1L	0	3.40E-03
Ssc.26470.1.A1_at	NEBL	4.50E-24	1.20E-03
Ssc.30598.1.A1_at	NRP2	7.40E-88	2.14E-04
Ssc.6079.1.A1_at	NUAK1	6.90E-51	2.62E-03
Ssc.17449.1.S1_at	OLFM1	4.50E-34	1.60E-03

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Ssc.18343.1.A1_at	PDPN	2.00E-47	9.00E-03
Ssc.12817.1.S1_at	PLXNA2	0	3.14E-04
Ssc.4792.1.A1_at	PRKCB1	0	2.55E-03
Ssc.30761.1.A1_at	PSD4	0	5.40E-04
Ssc.18425.1.S1_at	PTPRCAP	1.90E-48	5.76E-03
Ssc.10487.2.A1_at	RARRES1	3.00E-147	1.65E-03
Ssc.11787.2.A1_at	RASSF2	1.30E-21	3.54E-03
Ssc.17853.1.A1_at	REM1	0	7.05E-04
Ssc.20812.1.S1_at	RFTN1	4.50E-50	7.53E-04
Ssc.5274.1.S1_at	RGS10	0	2.14E-04
Ssc.3139.1.A1_at	RGS2	0	9.67E-03
Ssc.30055.1.A1_at	RUNX1	1.40E-31	7.07E-03
Ssc.23014.1.S1_at	S100A4	7.00E-142	1.50E-03
Ssc.6765.1.S1_at	SCIN	0	4.03E-04
Ssc.957.1.S1_at	SERPING1	1.00E-124	5.60E-03
Ssc.19648.1.S1_at	SLC15A3	0	1.27E-04
Ssc.19836.1.S1_at	SLC31A2	4.10E-47	2.85E-04
Ssc.5887.1.A1_at	SLC37A2	0	2.72E-04
Ssc.1126.1.A1_at	SMPDL3A	0	7.29E-04
Ssc.11006.1.S1_at	SNX10	0	6.70E-05
Ssc.20177.1.S1_at	SPI1	0	3.60E-04
Ssc.7907.1.A1_at	ST6GAL1	5.40E-17	1.76E-03
Ssc.18272.1.A1_at	TCIRG1	0	1.27E-04
Ssc.16234.1.S1_at	TCN1	3.70E-18	3.24E-03
Ssc.11075.3.S1_a_at	TCRB	2.00E-126	9.82E-03
Ssc.11075.1.S1_a_at	TCRB	6.00E-120	8.57E-03
Ssc.20133.1.A1_at	THY1	2.00E-135	7.99E-04
Ssc.27360.1.A1_at	TIAM1	4.70E-55	7.67E-03
Ssc.1526.1.S1_at	TMEM119	3.60E-34	2.92E-03
Ssc.2971.1.S1_at	TNS1	1.70E-39	2.02E-03
Ssc.4258.1.S1_at	TP53I11	3.00E-33	8.49E-04
Ssc.428.5.S1_at	TRAC	1.60E-40	5.16E-03
Ssc.507.1.A1_at	TYROBP	7.10E-59	6.08E-04
Ssc.21582.1.S1_at	UBD	2.90E-75	5.59E-03
Ssc.26300.2.S1_at	UNC93B1	0	6.73E-04
Ssc.5910.1.A1_at	VAV1	0	2.41E-03
Ssc.5910.3.S1_at	VAV1	0	3.55E-03
Ssc.16640.1.A1_at	VSIG4	2.00E-54	2.30E-03
Ssc.5530.2.S1_at	ZNF205	4.00E-159	6.52E-03
Ssc.11208.1.S1_at		3.50E-62	2.26E-03

K means cluster 6 regulated porcine probes and their human homologs (e value: e<e-10)			
ID	Gene Symbol	e-value	ANOVA FDR corrected P-value
Ssc.5414.1.S1_at	ABLIM3	1.50E-21	2.63E-04
Ssc.3386.1.A1_at	AGPAT1	0.00E+00	2.50E-04
Ssc.3386.2.S1_at	AGPAT1	0.00E+00	2.75E-04
Ssc.27177.1.S1_at	ATP8A1	0	5.47E-04
Ssc.29413.1.A1_at	B3GALT2	6.80E-43	1.91E-04

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Ssc.837.1.A1_at	BPI	9.10E-34	7.43E-03
Ssc.5171.1.S1_at	C2orf32	0	4.84E-04
Ssc.8325.1.A1_at	C6orf65	3.60E-18	2.67E-04
Ssc.24298.1.S1_at	C9orf30	0	1.38E-03
Ssc.7538.1.S1_at	CDH1	7.00E-133	1.18E-03
Ssc.16532.1.S1_at	CDK2	0	2.13E-05
Ssc.16532.2.S1_at	CDK2	0	5.31E-04
Ssc.27616.1.S1_at	CDK2	2.90E-43	6.70E-05
Ssc.27019.1.S1_at	CHN2	1.90E-60	1.34E-04
Ssc.3326.1.S1_at	CITED1	0	3.30E-04
Ssc.8035.1.S1_at	CNNM1	1.90E-48	6.70E-05
Ssc.23997.1.S1_at	CNTN1	0	7.79E-04
Ssc.6343.1.S1_at	CNTN1	7.50E-28	1.06E-03
Ssc.4863.1.S1_at	CTNNAL1	0	6.04E-04
Ssc.16839.1.S1_at	DDAH1	5.00E-28	8.12E-04
Ssc.4436.1.S1_at	EDNRB	3.00E-102	2.32E-03
Ssc.3295.1.S1_at	EDNRB	4.60E-15	2.13E-05
Ssc.3295.2.A1_at	EDNRB	4.60E-15	4.31E-04
Ssc.22031.2.A1_at	ETV5	1.20E-58	2.39E-03
Ssc.27266.1.S1_at	GPR143	5.90E-23	1.08E-04
Ssc.3450.1.S1_at	HOXD8	3.60E-35	7.11E-03
Ssc.22043.1.S1_at	ID4	0	1.48E-03
Ssc.18284.1.A1_at	KCNJ13	3.00E-65	1.38E-04
Ssc.7231.1.A1_at	KIF21A	3.80E-96	2.20E-03
Ssc.16096.2.S1_a_at	KIT	0	6.40E-04
Ssc.27543.1.S1_at	LPIN1	3.50E-26	4.55E-04
Ssc.7297.1.S1_at	MAOB	0	4.31E-04
Ssc.9275.1.A1_at	MET	5.30E-66	6.72E-04
Ssc.28450.1.S1_at	MFSD2	0	6.01E-04
Ssc.27601.1.S1_at	MITF	0	2.75E-04
Ssc.30810.1.S1_at	MITF	1.00E-120	1.38E-04
Ssc.13047.1.A1_at	MLPH	2.50E-25	2.31E-04
Ssc.8169.1.A1_at	MPPED2	0	2.06E-03
Ssc.30671.1.A1_at	MPZL1	2.00E-162	1.01E-04
Ssc.21255.1.S1_at	MPZL1	6.40E-75	1.09E-04
Ssc.30996.1.S1_at	MPZL1	6.40E-75	1.11E-04
Ssc.6843.1.A1_at	MYO5A	6.60E-36	9.32E-05
Ssc.13921.1.S1_at	NBEA	0	2.74E-04
Ssc.5372.1.S1_at	NFRKB	6.30E-37	8.68E-04
Ssc.6035.1.S1_at	NIPA1	3.70E-44	4.25E-04
Ssc.18127.1.A1_at	NIPA1	4.50E-42	1.39E-04
Ssc.761.1.S1_at	NR4A3	0	1.12E-04
Ssc.761.2.S1_a_at	NR4A3	0	9.23E-04
Ssc.29672.1.A1_at	NRXN1	7.00E-15	2.50E-04
Ssc.15775.1.S1_at	OCA2	0	6.70E-05
Ssc.10845.1.A1_at	PACSIN1	2.70E-68	9.80E-04
Ssc.27600.1.S1_at	PAX3	6.00E-154	9.83E-04
Ssc.13476.1.A1_at	PEG10	4.40E-72	6.70E-05

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Ssc.6951.1.A1_at	PKNOX2	5.70E-35	2.50E-04
Ssc.8177.1.A1_at	PLP1	6.00E-179	4.31E-04
Ssc.8177.1.A2_at	PLP1	6.00E-179	2.28E-04
Ssc.18519.1.S1_at	PPM1E	1.20E-46	5.79E-04
Ssc.103.1.S1_at	PPP2R2B	0	2.22E-04
Ssc.9170.1.A1_at	PRKD1	5.60E-33	5.18E-04
Ssc.24503.1.S1_at	Q96EQ5	3.50E-67	6.20E-03
Ssc.21186.1.S1_a_at	RAB15	4.60E-47	2.39E-04
Ssc.21186.3.S1_at	RAB15	4.60E-47	2.00E-03
Ssc.6940.1.A1_at	RAB38	6.00E-110	2.63E-04
Ssc.6940.1.A1_s_at	RAB38	6.00E-110	2.42E-04
Ssc.22795.1.A1_at	RHOBTB3	4.30E-17	2.50E-04
Ssc.30053.1.A1_at	RNF180	2.90E-29	3.67E-03
Ssc.9198.1.S1_at	SAH3	1.30E-84	2.43E-03
Ssc.4965.1.A1_at	SCRN1	2.90E-38	1.08E-04
Ssc.6589.1.S1_a_at	SDC3	6.40E-61	3.19E-03
Ssc.21689.1.S1_at	SHROOM2	2.00E-19	1.36E-03
Ssc.29472.1.S1_at	SILV	0	1.84E-04
Ssc.3909.1.A1_at	SLC16A9	2.00E-56	4.97E-04
Ssc.7570.2.S1_at	SLC1A1	3.50E-42	2.71E-04
Ssc.12727.1.A1_at	SLC24A4	1.70E-81	2.72E-04
Ssc.12727.2.A1_at	SLC24A4	1.70E-81	5.26E-04
Ssc.25520.1.S1_at	SLC24A5	2.00E-151	2.34E-04
Ssc.25468.1.S1_at	SLC45A2	3.00E-169	3.28E-05
Ssc.6211.1.A1_at	SLC6A15	1.00E-120	1.62E-04
Ssc.24711.1.S1_at	SMYD4	2.60E-59	1.09E-04
Ssc.13295.1.A1_at	SNX25	0	2.19E-04
Ssc.1534.1.A1_at	SORBS1	9.90E-52	3.68E-04
Ssc.21796.1.S1_at	SORL1	4.70E-22	3.32E-04
Ssc.12492.1.A1_at	SORT1	0	4.84E-04
Ssc.12492.3.S1_at	SORT1	0	1.95E-04
Ssc.11403.1.S1_at	SPTBN1	0	3.82E-03
Ssc.8369.1.A1_at	SRG1	2.00E-140	2.75E-04
Ssc.21594.1.S1_at	STXBP6	1.60E-58	5.88E-03
Ssc.2157.1.A1_at	SYNPR	2.80E-62	1.22E-04
Ssc.6974.1.A1_at	SYTL2	0	2.63E-05
Ssc.27277.1.S1_at	TES	0	2.50E-04
Ssc.7552.1.A1_at	TES	1.40E-17	3.77E-04
Ssc.17350.1.S1_at	TFAP2B	0	6.55E-04
Ssc.19052.1.S1_s_at	TMEM9	0	2.13E-05
Ssc.26383.1.A1_a_at	TMTC2	9.00E-145	2.43E-03
Ssc.21963.1.S1_at	TRAF4	0	1.08E-04
Ssc.22638.1.S1_at	TYR	6.00E-141	1.09E-04
Ssc.30156.1.A1_at	ZDHC2	1.20E-19	2.72E-04
Ssc.19179.1.A1_at	NP_055654	1.10E-89	1.70E-03

Supplement 2: Downregulation of cell cycle related genes is related to melanoma cells during early regression

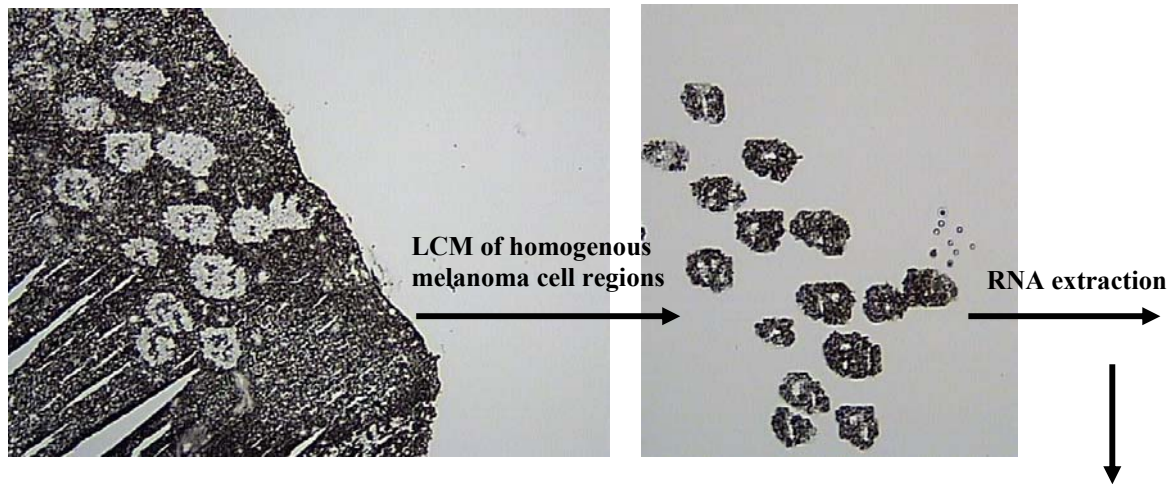
Microarray analysis and subsequent verification of altered gene expression of qRT-PCR were performed using whole tumor tissue sections. The whole tissue sections were rather homogenous in terms of melanoma cell distribution at early timepoints such as d+8 (t_0) and d+28 (t_1) but become more heterogenous with the onset of regression (from t_2 on) since different cells infiltrated the tumor site. To address questions regarding expression specificity of cell cycle related genes, we laser-microdissected homogenous regions of melanoma cells at t_0 , t_1 and t_2 and checked for CCNB1 and CDC2 expression.

Comment on Method:

The following tumor samples were used for laser microdissection (LCM):

- t_0 (d+8): 0497-L1 (52A2E4)
- t_1 (d+28): 0493-L7 (52A4B3)
- t_2 (d+49): 0495-L3 (52B2B5)

8 μ m cryosections were mounted on membrane glass slides (Leica, PEN-membrane 2.0 μ m) that were UV-activated for 30min before and then stored in 75% ethanol at -20°C. Cryosections were dehydrated in an ethanol gradient (75%, 95%, 95%, 100%, 100%) for 1min each. After, slides were tapped dry and incubated two times for 5min in xylene. Then slides were stored at room temperature in an evacuated dissector until use for LCM. Areas of homogeneously distributed melanoma cells (ranging from 19.6-46.0mm²) were selected and microdissected using the “cut and capture” method, based on the UV laser cutting option (Arcturus, Veritas™) with the following settings: cutting laser at medium laser power (10-13), spot size 1-2 μ m. Microdissected tissue was captured on CapSure® LCM high sensitivity caps (Arcturus). RNA extraction was performed by incubating the cap in 40 μ l of extraction buffer for 30min at 42°C. Then lysates from up to 10 caps were pooled and passed on a purification column. One column for each sample was used, following the PicoPure™ (Arcturus) extraction protocol. RNA was finally eluted in 20 μ l of elution buffer. RNA quantity and quality was assessed by Nanodrop and Bioanalyzer (Picochip) analysis.



Name	Address	Day	timepoint	Date LCM	selected area [mm ²]	Conc. RNA ng/ul agilent pico	RIN
0493-L7	52A4B3	28	t1	26.09.2007	19.37	52.78	7.4
0495-L3	52B2B5	49	t2	19.10.2007	33.91	49.95	7.8
0497-L1	52A2E4	8	t0	19.10.2007	46.01	65.63	7.8

11µl of total RNA were reversed transcribed into cDNA by using the Superscript™ III RT-enzyme (Invitrogen) following the manufacturer's first strand protocol. Relative quantification (ddCt method) of CCNB1 and CDC2 expression by qRT-PCR analysis was performed as described elsewhere (Rambow et al. 2008b). Briefly, about 1,3ng of cDNA served as template, whereas the total PCR reaction volume was 20µl using the SybrGreen protocol.

	Time	FC (qRT-PCR) LCM	FC (microarray) whole tissue	FC (qRT-PCR) whole tissue
CCNB1	t1/t0	-0.72	-1.55	-1.43
	t2/t0	-7.73	-3,86	-4.85
CDC2	t1/t0	-2.17	-2.16	-1.36
	t2/t0	-8.16	-5.36	-4.51

Fold changes of the different experiments were demonstrated. Comparing the fold changes (FC) of the different experiments for CCNB1 revealed a common downregulation no matter if whole tissue sections or laser-microdissected (LCM) melanoma cells were used. At t_2 it seemed that the downregulation of CCNB1 compared to t_0 was even more pronounced, as the gene was almost 8 times downregulated in laser microdissected melanoma cells. Whole tissue fold changes obtained by microarray and q-RT-PCR analysis suggested a less drastic downregulation of 4 to 5 times. The same was true for CDC2 expression. At t_2 CDC2

expression in LCM melanoma cells was about 8 times downregulated whereas in whole tissue tumor sections the downregulation reached changes around 5 fold. Knowing that LCM expression verification was performed with only one sample for each time point, this additional result suggests that the early downregulation of cell cycle related genes detected by microarray analysis and qRT-PCR analysis in tissue sections, might be mainly derived from melanoma cells.

Supplement 3: Gene list comparison with human melanoma expression data.

WINNEPENNICKX et al.		GENE LIST COMPARISON	147 of 254 Winnep. Reference genes are immobilized on porcine gene chip	
		Winnenpenninckx et al. 2007	Rambow et al.	
Number	Common gene	up/down in short survival	up/down at t1/t0	FC
1	KPNA2	up	down	-2.21
2	CDC2	up	down	-2.16
3	KIF11	up	down	-2.03
4	PTGDS	down	up	2.48
		Winnenpenninckx et al. 2007	Rambow et al.	
Number	Common gene	up/down in short survival	up/down at t2/t0	FC
1	KIF11	up	down	-7.51
2	CDC2	up	down	-5.36
3	MELK	up	down	-4.12
4	CCNB1	up	down	-3.86
5	NEK2	up	down	-3.56
6	TOP2A	up	down	-3.52
7	DCC1	up	down	-3.28
8	KIF2C	up	down	-3.26
9	CDC6	up	down	-3.22
10	BUB1	up	down	-3.09
11	KPNA2	up	down	-3.01
12	PRC1	up	down	-3.00
13	BIRC5	up	down	-2.94
14	ANLN	up	down	-2.90
15	SPAG5	up	down	-2.89
16	PTTG2	up	down	-2.72
17	ECT2	up	down	-2.61
18	RACGAP1	up	down	-2.51
19	CDC45L	up	down	-2.38
20	CKS1B	up	down	-2.24

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21	NASP	up	down	-2.23
22	MCM4	up	down	-2.22
23	MCM6	up	down	-2.19
24	UHRF1	up	down	-2.16
25	ATAD2	up	down	-2.15
26	PCNA	up	down	-2.13
27	CST3	down	up	2.04
		Winnenpenninckx et al. 2007	Rambow et al.	
Number	Common gene	up/down in short survival	up/down at t3/t0	FC
1	CDC2	up	down	-14.06
2	KIF11	up	down	-13.63
3	MELK	up	down	-8.95
4	CDC6	up	down	-7.99
5	BUB1	up	down	-7.36
6	NEK2	up	down	-7.17
7	CCNB1	up	down	-6.82
8	KIF2C	up	down	-6.54
9	CTNNAL1	up	down	-6.12
10	CDC45L	up	down	-6.07
11	KPNA2	up	down	-5.49
12	PTTG2	up	down	-5.43
13	BIRC5	up	down	-5.30
14	DCC1	up	down	-5.20
15	ATAD2	up	down	-4.91
16	TOP2A	up	down	-4.82
17	ECT2	up	down	-4.79
18	SPAG5	up	down	-4.75
19	PRC1	up	down	-4.43
20	PCNA	up	down	-4.24
21	UHRF1	up	down	-3.86
22	PAICS	up	down	-3.81
23	ANLN	up	down	-3.71
24	MCM4	up	down	-3.39

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25	NASP	up	down	-3.29
26	RACGAP1	up	down	-3.16
27	GMPS	up	down	-3.15
28	MSH6	up	down	-3.09
29	MCM6	up	down	-3.05
30	CKS1B	up	down	-2.89
31	H2AFV	up	down	-2.45
32	RFC5	up	down	-2.41
33	HSPA4	up	down	-2.21
34	PWP1	up	down	-2.10
35	EMX2	down	up	2.53
36	CST3	down	up	3.83
		Winnenpenninckx et al. 2007	Rambow et al.	
Number	Common gene	up/down in short survival	up/down at t4/t0	FC
1	CTNNAL1	up	down	-13.65
2	CDC6	up	down	-8.26
3	PAICS	up	down	-6.20
4	BUB1	up	down	-6.02
5	CDC45L	up	down	-5.05
6	CDC2	up	down	-5.01
7	KIF11	up	down	-4.82
8	NEK2	up	down	-4.58
9	MELK	up	down	-4.53
10	ATAD2	up	down	-4.40
11	CCNB1	up	down	-4.25
12	ANLN	up	down	-4.16
13	NASP	up	down	-4.12
14	DCC1	up	down	-3.89
15	KIF2C	up	down	-3.88
16	PRC1	up	down	-3.81
17	PTTG2	up	down	-3.71
18	BIRC5	up	down	-3.62

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19	TOP2A	up	down	-3.18
20	UHRF1	up	down	-3.18
21	MCM4	up	down	-2.97
22	GMPS	up	down	-2.97
23	MSH6	up	down	-2.89
24	KPNA2	up	down	-2.84
25	PCNA	up	down	-2.72
26	MCM6	up	down	-2.64
27	ECT2	up	down	-2.63
28	CNN3	up	down	-2.41
29	SPAG5	up	down	-2.41
30	RFC5	up	down	-2.22
31	RACGAP1	up	down	-2.20
32	EMX2	down	up	2.68
33	CST3	down	up	3.82

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RYU et al.	GENE LIST COMPARISON		53 of 98 Ryu et al reference genes immobilized on porcine gene chip			
	Ryu et al. 2007		Ryu et al. 2007		Rambow et al.	
Common gene	altered expression up/down in aggressive melanoma cells involved in cell cycle, prolif., repair	FC	down in aggressive melanoma cells compared to melanocytes	FC	up/down at t1/t0	FC
PTGDS			down	-9.0	up	2.5
PRKCB1			down	-5.6	up	2.0
	Ryu et al. 2007		Ryu et al. 2007		Rambow et al.	
Common gene	altered expression up/down in aggressive melanoma cells involved in cell cycle, prolif., repair	FC	down in aggressive melanoma cells compared to melanocytes	FC	up/down at t2/t0	FC
NUSAP1	up	4,1			down	-2.8
BIRC5	up	5,1			down	-2.9
GINS1	up	4,7			down	-3.1
NCAPG2	up	4,6			down	-4.0
MELK	up	5,9			down	-4.1
PRKCB1			down	-5.6	up	3.3
MYO1D			down	-17.7	up	5.7
	Ryu et al. 2007		Ryu et al. 2007		Rambow et al.	
Common gene	altered expression up/down in aggressive melanoma cells involved in cell cycle, prolif., repair	FC	down in aggressive melanoma cells compared to melanocytes	FC	up/down at t3/t0	FC
NCAPG2	up	4,6			down	-10.3
MELK	up	5,9			down	-8.9

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GINS1	up	4,7			down	-5.5
BIRC5	up	5,1			down	-5.3
NUSAP1	up	4,1			down	-4.8
HELLS	up	8,5			down	-4.3
KIT			down	-94.0	down	-19.0
MET			down	-7.2	down	-7.2
EDNRB			down	-7.6	down	-5.2
CITED1			down	-17.7	down	-4.8
KCNJ13			down	-11.7	down	-4.6
CDH1			down	-35.1	down	-4.0
TYR			down	-13.6	down	-3.5
OCA2			down	-11.4	down	-3.4
GSTT1			down	-22.1	down	-2.7
MYO5A			down	-6.7	down	-2.3
HNMT			down	-8.7	up	3.0
PRKCB1			down	-5.6	up	6.9
PHACTR3			down	-5.7	up	9.6
MBP			down	-19.7	up	16.4
MYO1D			down	-17.7	up	24.5
	Ryu et al. 2007		Ryu et al. 2007		Rambow et al.	
Common gene	altered expression up/down in aggressive melanoma cells involved in cell cycle, prolif., repair	FC	down in aggressive melanoma cells compared to melanocytes	FC	up/down at t4/t0	FC
HELLS	up	8,5			down	-3.2
BIRC5	up	5,1			down	-3.6
GINS1	up	4,7			down	-3.7
NUSAP1	up	4,1			down	-3.9
MELK	up	5,9			down	-4.5
NCAPG2	up	4,6			down	-7.0
OCA2			down	-11.4	down	-

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						253.2
KCNJ13			down	-11.7	down	-137.0
TYR			down	-13.6	down	-626.8
CITED1			down	-17.7	down	-377.7
MYO1D			down	-17.7	up	4.9
MBP			down	-19.7	up	15.0
GSTT1			down	-22.1	down	-4.3
CDH1c			down	-35.1	down	-6.8
SOCS6			down	-5.0	down	-2.6
PRKCB1			down	-5.6	up	11.6
PHACTR3			down	-5.7	up	7.6
PAX3			down	-6.2	down	-72.4
MYO5A			down	-6.7	down	-14.5
MET			down	-7.2	down	-15.0
EDNRB			down	-7.6	down	-22.5
HNMT			down	-8.7	up	6.4
PTGDS			down	-9.0	down	-6.7
KIT			down	-94.0	down	-36.4
HOEK et al.	GENE LIST COMPARISON					
	223 cohort specific genes	linked with greater metastatic potential	159 of 223 Hoek et al reference genes			
	neural crest signature	high proliferation and weak metastatic	are immobilized on porcine gene chip			
	TGFb signature	weak proliferation and high metastatic				
	hoek et al 2006	223 cohort specific genes	Rambow et al.			
Number	Common gene	neural crest / TGFb signature	up/down at t1/t0	FC		
1	NUAK1		up	2.2		
2	CHL1		down	-2.2		
3	CLCN3		up	2.6		

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	hoek et al 2006	223 cohort specific genes	Rambow et al.		
Number	Common gene	neural crest / TGFb signature	up/down at t2/t0	FC	
1	CLCN7	neural crest	up	2.5	
2	PDGFRB	neural crest	up	2.4	
3	ACP5		up	6.0	
4	MAP1B		up	2.7	
5	CSF1		up	2.5	
6	NUAK1		up	2.5	
7	CHL1		down	-2.1	
	hoek et al 2006	223 cohort specific genes	Rambow et al.		
Number	Common gene	neural crest / TGFb signature	up/down at t3/t0	FC	
1	PLP1	neural crest	down	-15.3	
2	EDNRB	neural crest	down	-6.5	
3	CITED1	neural crest	down	-4.8	
4	TFAP2A	neural crest	down	-4.3	
5	RAB38	neural crest	down	-3.5	
6	TYR	neural crest	down	-3.5	
7	MITF	neural crest	down	-3.4	
8	GPR143	neural crest	down	-3.4	
9	GPM6B	neural crest	down	-3.0	
10	GPRC5B	neural crest	down	-2.2	
11	RRAS	neural crest	up	2.8	
12	PODXL	neural crest	up	4.0	
13	VEGFC	neural crest	up	5.2	
14	CLCN7	neural crest	up	5.6	
15	S100A1	neural crest	up	6.5	
16	TCF4	TGFb	up	2.8	
17	LOXL2	TGFb	up	4.2	
18	PDGFRB	TGFb	up	5.0	

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19	THY1	TGFb	up	10.5		
20	MPPED2		down	-6.1		
21	CHL1		down	-5.7		
22	CDK2		down	-5.5		
23	SLC45A2		down	-4.9		
24	MLPH		down	-3.2		
25	MYO5A		down	-3.0		
26	CLCN3		down	-2.9		
27	ZFP106		down	-2.5		
28	NUDT3		down	-2.5		
29	ASAH1		up	2.2		
30	IVNS1ABP		up	2.9		
31	MAP1B		up	3.2		
32	SLIT2		up	3.4		
33	EFEMP2		up	4.2		
34	FRMD4B		up	6.3		
35	TIMP1		up	6.4		
36	NUAK1		up	6.5		
37	GM2A		up	6.7		
38	ACP5		up	11.0		
39	GAS6		up	11.8		
40	FOSL2		up	12.7		
41	CSF1		up	14.0		
	hoek et al 2006	223 cohort specific genes	Rambow et al.			
Number	Common gene	neural crest / TGFb signature	up/down at t4/t0	FC		
1	TYR	neural crest	down	-626.8		
2	CITED1	neural crest	down	-377.7		
3	SILV	neural crest	down	-343.8		
4	GPR143	neural crest	down	-116.4		
5	PLP1	neural crest	down	-54.1		
6	EDNRB	neural crest	down	-45.1		
7	MITF	neural crest	down	-13.5		

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8	TFAP2A	neural crest	down	-12.7		
9	RAB38	neural crest	down	-12.4		
10	GPRC5B	neural crest	down	-7.2		
11	GPM6B	neural crest	down	-2.2		
12	CLCN7	neural crest	up	2.5		
13	RRAS	neural crest	up	3.0		
14	S100A1	neural crest	up	3.7		
15	PODXL	neural crest	up	3.9		
16	VEGFC	neural crest	up	5.7		
17	FGF2	TGFb	down	-5.6		
18	TCF4	TGFb	up	2.0		
19	PDGFRB	TGFb	up	2.6		
20	THY1	TGFb	up	7.0		
21	SLC45A2		down	-491.0		
22	CDK2		down	-64.3		
23	MLPH		down	-26.1		
24	MPPED2		down	-25.4		
25	MYO5A		down	-14.5		
26	CLCN3		down	-5.4		
27	NUDT3		down	-4.7		
28	BACE2		down	-3.0		
29	PSEN2		down	-2.9		
30	CRIM1		down	-2.8		
31	RPS6KA5		down	-2.8		
32	ZFP106		down	-2.7		
33	CHL1		down	-2.1		
34	ASAH1		up	2.3		
35	IVNS1ABP		up	2.7		
36	MAP1B		up	3.6		
37	EFEMP2		up	3.7		
38	TRIM22		up	4.7		
39	NUAK1		up	4.8		
40	CSF1		up	5.2		
41	GM2A		up	6.0		

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42	ACP5		up	6.1		
43	HPGD		up	6.9		
44	SLIT2		up	10.4		
45	FRMD4B		up	11.1		

Supplement 4: Top 15 up and downregulated genes per time point.

Genes upregulated at t1 (d28)

Probe Set ID	Gene	Gene Symbol	Gene symbol IPA	Fold Change	Cluster
Ssc.13778.1.S1_at	immunoglobulin heavy constant mu	IGHM	IGHM	10.2	5
Ssc.19946.1.S1_at	immunoglobulin lambda constant 1	IGLC1	IGLC1	5.0	5
Ssc.8843.1.A1_at	fibronectin 1	FN1	FN1	4.2	5
Ssc.27289.1.S1_at	glutamate receptor. ionotropic. kainate 2	GRIK2	GRIK2	3.8	1
Ssc.17853.1.A1_at	RAS (RAD and GEM)-like GTP-binding 1	REM1	REM1	3.7	5
Ssc.19179.1.A1_at	plasticity related gene 1	NP_055654	LPPR4	3.7	6
Ssc.11858.1.S1_at	fibromodulin	FMOD	FMOD	3.7	5
Ssc.11698.1.A1_at	ST8 alpha-N-acetyl-neuraminide alpha-2.8-sialyltransferase 4	ST8SIA4	ST8SIA4	3.6	3
Ssc.4258.1.S1_at	tumor protein p53 inducible protein 11	TP53I11	TP53I11	3.6	5
Ssc.29707.1.A1_at	multiple EGF-like-domains 10	MEGF10	MEGF10	3.5	1
Ssc.2070.1.S1_at	erythrocyte membrane protein band 4.1-like 1	EPB41L1	EPB41L1	3.5	2
Ssc.11075.3.S1_a_at	T-cell receptor beta chain C region	TVB1	TRB@	3.4	5
Ssc.6656.1.A1_at	fibronectin type III domain containing 1	FNDC1	FNDC1	3.1	5
Ssc.314.1.S1_at	adrenomedullin	ADM	ADM	3.1	5
Ssc.5631.1.S1_at	erythrocyte membrane protein band 4.1-like 1	EPB41L1	EPB41L1	2.8	2

Genes downregulated at t1 (d28)

Probe Set ID	Gene	Gene Symbol	Gene symbol IPA	Fold Change	Cluster
Ssc.20101.1.S1_at	interferon. alpha-inducible protein 6	IFI6	IFI6	4.7	3
Ssc.6172.1.S1_at	XIAP associated factor-1	DKFZp686C1662	BIRC4BP	3.7	3
Ssc.30724.1.S1_at	hect domain and RLD 6	HERC6	HERC6	3.6	2
Ssc.13665.1.A1_at	sex comb on midleg-like 2 (Drosophila)	SCML2	SCML2	3.6	4
Ssc.26386.2.S1_a_at	nuclear import 7 homolog (S. cerevisiae)	NIP7	NIP7	3.0	4
Ssc.2045.2.S1_at	nonhomologous end-joining factor 1	NHEJ1	NHEJ1	2.8	4
Ssc.16088.1.S1_at	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	HMGCR	HMGCR	2.7	4
Ssc.6707.1.A1_at	glycine dehydrogenase (decarboxylating)	GLDC	GLDC	2.6	4
Ssc.23877.1.S1_at	cyclin A2	CCNA2	CCNA2	2.5	4
Ssc.8308.1.A1_at	cell adhesion molecule with homology to L1CAM (close homolog of L1)	CHL1	CHL1	2.2	4
Ssc.15824.1.S1_at	karyopherin alpha 2 (RAG cohort 1. importin alpha 1)	KPNA2	KPNA2	2.2	4
Ssc.873.1.S1_at	cell division cycle 2. G1 to S and G2 to M	CDC2	CDC2	2.2	4
Ssc.9311.1.A1_at	pleckstrin homology-like domain. family A. member 1	PHLDA1	PHLDA1	2.1	4
Ssc.1313.1.A1_at	XTP3-transactivated protein A	XTP3TPA	XTP3TPA	2.1	4
Ssc.30748.1.S1_at	kinesin family member 11	KIF11	KIF11	2.0	4

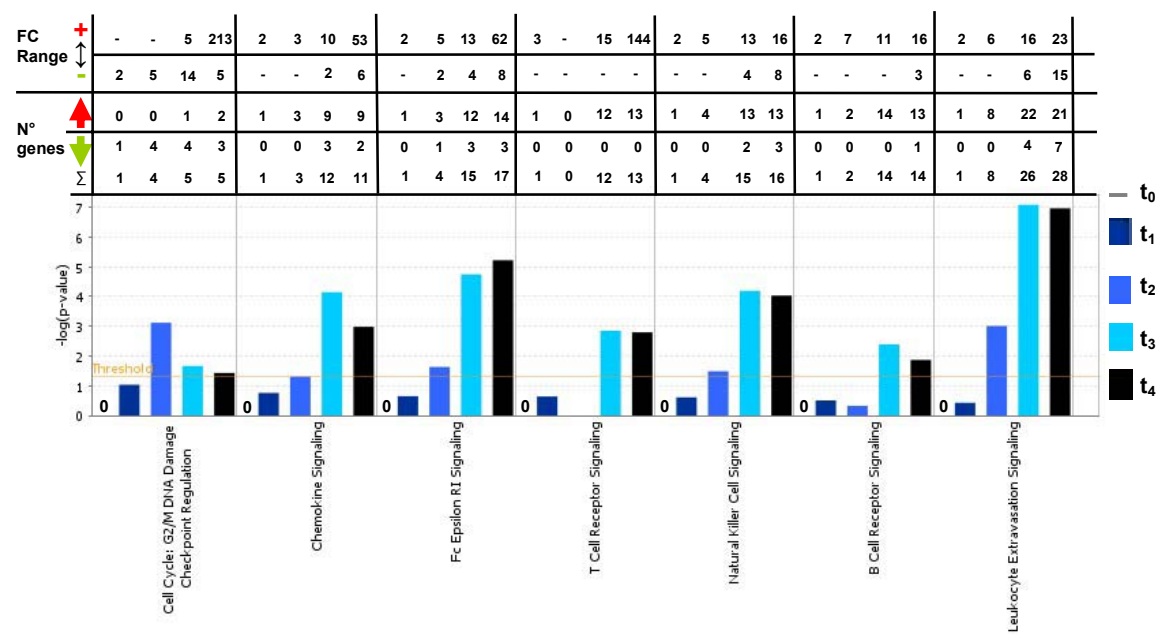
Genes upregulated at t2 (d49)					
Probe Set ID	Gene	Gene Symbol	Gene symbol IPA	Fold Change	Cluster
Ssc.13778.1.S1_at	immunoglobulin heavy constant mu	IGHM	IGHM	43.1	5
Ssc.19946.1.S1_at	immunoglobulin lambda constant 1	IGLC1	IGLC1	19.6	5
Ssc.22164.1.S1_at	ATPase. H+ transporting. lysosomal 38kDa. V0 subunit d2	ATP6V0D2	ATP6V0D2	17.3	5
Ssc.9914.1.A1_at	creatine kinase. Brain	CKB	CKB	12.8	5
Ssc.5887.1.A1_at	solute carrier family 37 (glycerol-3-phosphate transporter). member 2	SLC37A2	SLC37A2	10.4	5
Ssc.14275.1.A1_at	calcitonin receptor	CALCR	CALCR	9.1	5
Ssc.11070.1.S1_at	immunoglobulin heavy constant mu	IGHM	IGHM	7.8	5
Ssc.8843.1.A1_at	fibronectin 1	FN1	FN1	7.7	5
Ssc.11208.1.S1_at	Ig kappa chain V-I region	IGKV1-5		6.9	5
Ssc.140.1.S1_at	ameloblastin	AMBN	AMBN	6.3	5
Ssc.575.1.S1_at	acid phosphatase 5. tartrate resistant	ACP5	ACP5	6.0	5
Ssc.44.1.S1_at	integrin. beta 3 (platelet glycoprotein IIIa. antigen CD61)	ITGB3	ITGB3	5.9	5
Ssc.14561.1.S1_at	integrin. beta 2 (complement component 3 receptor 3 and 4 subunit)	ITGB2	ITGB2	5.7	5
Ssc.4520.1.S1_at	myosin ID	MYO1D	MYO1D	5.7	5
Ssc.20177.1.S1_at	spleen focus forming virus (SFFV) proviral integration oncogene spi1	SPI1	SPI1	5.4	5
Genes downregulated at t2 (d49)					
Probe Set ID	Gene	Gene Symbol	Gene symbol IPA	Fold Change	Cluster
Ssc.30748.1.S1_at	kinesin family member 11	KIF11	KIF11	7.5	4
Ssc.21718.1.S1_at	E2F transcription factor 7	E2F7	E2F7	6.5	4
Ssc.873.1.S1_at	cell division cycle 2. G1 to S and G2 to M	CDC2	CDC2	5.4	4
Ssc.23877.1.S1_at	cyclin A2	CCNA2	CCNA2	5.1	4
Ssc.7594.1.A1_at	DEP domain containing 1B	DEPDC1B	DEPDC1B	5.1	4
Ssc.26899.1.A1_at	cytoskeleton associated protein 2	CKAP2	CKAP2	5.0	4
Ssc.7361.2.S1_at	kinesin family member 11	KIF11	KIF11	5.0	4
Ssc.13793.1.A1_at	RAD51 associated protein 1	RAD51AP1	RAD51AP1	4.9	4
Ssc.12273.1.A1_at	glutathione S-transferase M3 (brain)	GSTM3	GSTM3	4.6	4
Ssc.1667.1.S1_at	kinesin family member 4A	KIF4A	KIF4A	4.4	4
Ssc.28155.1.A1_at	chromosome 3 open reading frame 26	C3orf26	C3orf26	4.4	4
Ssc.7195.1.A1_at	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	BUB1	BUB1	4.2	4
Ssc.19346.1.S1_at	claspin homolog (Xenopus laevis)	CLSPN	CLSPN	4.2	4
Ssc.26568.1.A1_s_at	cyclin B3	CCNB3	CCNB3	4.1	4
Ssc.22206.1.S1_at	maternal embryonic leucine zipper kinase	MELK	MELK	4.1	4

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Genes upregulated at t3 (d70)					
Probe Set ID	Gene	Gene Symbol	Gene symbol IPA	Fold Change	Cluster
Ssc.14275.1.A1_at	calcitonin receptor	CALCR	CALCR	116.7	5
Ssc.13778.1.S1_at	immunoglobulin heavy constant mu	IGHM	IGHM	104.6	5
Ssc.22164.1.S1_at	ATPase, H ⁺ transporting, lysosomal 38kDa, V0 subunit d2	ATP6V0D2	ATP6V0D2	77.7	5
Ssc.5887.1.A1_at	solute carrier family 37 (glycerol-3-phosphate transporter), member 2	SLC37A2	SLC37A2	42.9	5
Ssc.9914.1.A1_at	creatine kinase, brain	CKB	CKB	40.5	5
Ssc.19946.1.S1_at	immunoglobulin lambda constant 1	IGLC1	IGLC1	35.3	5
Ssc.44.1.S1_at	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	ITGB3	ITGB3	28.0	5
Ssc.5.1.S1_a_at	C-type lectin domain family 5, member A	CLECSF5	CLEC5A	26.6	5
Ssc.27790.1.A1_at	sialic acid binding Ig-like lectin 5	SIGLEC5	SIGLEC5	24.6	5
Ssc.4520.1.S1_at	myosin ID	MYO1D	MYO1D	24.5	5
Ssc.8843.1.A1_at	fibronectin 1	FN1	FN1	23.1	5
Ssc.26709.1.S1_at	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	EBI2	EBI2	22.0	5
Ssc.30059.1.A1_at	matrix-remodelling associated 8	MXRA8	MXRA8	21.5	5
Ssc.116.1.S1_at	gamma-glutamyltransferase 1	GGT1	GGT1	21.2	3
Ssc.24984.1.S1_at	N-acylsphingosine amidohydrolase (acid ceramidase)-like	ASAH1	ASAH1	21.2	5
Genes downregulated at t3 (d70)					
Probe Set ID	Gene	Gene Symbol	Gene symbol IPA	Fold Change	Cluster
Ssc.16096.2.S1_a_at	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	KIT	19.0	6
Ssc.16532.2.S1_at	cyclin-dependent kinase 2	CDK2	CDK2	18.7	6
Ssc.18519.1.S1_at	protein phosphatase 1E (PP2C domain containing)	PPM1E	PPM1E	15.7	6
Ssc.8177.1.A1_at	proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated)	PLP1	PLP1	15.3	6
Ssc.873.1.S1_at	cell division cycle 2, G1 to S and G2 to M	CDC2	CDC2	14.1	4
Ssc.30748.1.S1_at	kinesin family member 11	KIF11	KIF11	13.6	4
Ssc.3386.2.S1_at	1-acylglycerol-3-phosphate O-acyltransferase 1 (lysophosphatidic acid acyltransferase, alpha)	AGPAT1	AGPAT1	13.2	6
Ssc.13793.1.A1_at	RAD51 associated protein 1	RAD51AP1	RAD51AP1	12.0	4
Ssc.13716.1.A1_at	gap junction protein, beta 7	GJB7	GJB7	12.0	4
Ssc.7570.2.S1_at	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	SLC1A1	SLC1A1	12.0	6
Ssc.4965.1.A1_at	secernin 1	SCRN1	SCRN1	11.5	6
Ssc.12273.1.A1_at	glutathione S-transferase M3	GSTM3	GSTM3	10.9	4
Ssc.10664.1.S1_at	hepatic leukemia factor	HLF	HLF	10.6	4
Ssc.19159.1.S1_at	non-SMC condensin II complex, subunit G2	NCAPG2	NCAPG2	10.3	4
Ssc.7594.1.A1_at	DEP domain containing 1B	DEPDC1B	DEPDC1B	10.3	4

APPENDIX

Genes upregulated at t4 (d91)					
Probe Set ID	Gene	Gene Symbol	Gene symbol IPA	Fold Change	Cluster
Ssc.13778.1.S1_at	immunoglobulin heavy constant mu	IGHM	IGHM	424.7	5
Ssc.21582.1.S1_at	ubiquitin D	UBD	UBD	213.9	5
Ssc.11075.3.S1_a_at	T-cell receptor beta chain C region.	TVB1	TRB@	144.0	5
Ssc.15871.1.S1_a_at	NKG2-D type II integral membrane protein (NKG2-D activating NK receptor) (NK cell receptor D) (Killer cell lectin-like receptor subfamily K member 1).	KLRK1	KLRC4	129.1	5
Ssc.19946.1.S1_at	immunoglobulin lambda constant 1	IGLC1	IGLC1	124.1	5
Ssc.11075.1.S1_a_at	T-cell receptor beta chain C region.	TVB1	TRB@	117.9	5
Ssc.11208.1.S1_at	Ig kappa chain V-I region	IGKV1-5	-	78.7	5
Ssc.11070.1.S1_at	immunoglobulin heavy constant mu	IGHM	IGHM	65.7	5
Ssc.140.1.S1_at	ameloblastin	AMBN	AMBN	62.3	5
Ssc.19640.1.A1_at	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	FCER1A	FCER1A	61.9	3
Ssc.16640.1.A1_at	V-set and immunoglobulin domain containing 4	VSIG4	VSIG4	60.7	5
Ssc.19364.1.S1_at	complement component 2	C2	C2	57.6	5
Ssc.428.5.S1_at	T-cell receptor alpha chain C region	TRAC	TRA@	57.0	5
Ssc.22030.1.S1_at	chemokine (C-C motif) ligand 5	CCL5	CCL5	53.1	5
Ssc.8261.1.A1_at	cytochrome P450, family 2, subfamily C, polypeptide 9	CYP2C9	CYP2C9	44.5	5
Genes downregulated at t4 (d91)					
Probe Set ID	Gene	Gene Symbol	Gene symbol IPA	Fold Change	Cluster
Ssc.25520.1.S1_at	solute carrier family 24, member 5	SLC24A5	SLC24A5	633.7	6
Ssc.22638.1.S1_at	tyrosinase (oculocutaneous albinism IA)	TYR	TYR	626.8	6
Ssc.25468.1.S1_at	solute carrier family 45, member 2	SLC45A2	SLC45A2	491.0	6
Ssc.3326.1.S1_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1	CITED1	CITED1	377.7	6
Ssc.29472.1.S1_at	silver homolog (mouse)	SILV	SILV	343.8	6
Ssc.2157.1.A1_at	synaptoporin	SYNPR	SYNPR	306.1	6
Ssc.15775.1.S1_at	oculocutaneous albinism II (pink-eye dilution homolog, mouse)	OCA2	OCA2	253.2	6
Ssc.4965.1.A1_at	secernin 1	SCRN1	SCRN1	226.2	6
Ssc.8035.1.S1_at	cyclin M1	CNNM1	CNNM1	205.2	6
Ssc.18284.1.A1_at	potassium inwardly-rectifying channel, subfamily J, member 13	KCNJ13	KCNJ13	137.0	6
Ssc.27266.1.S1_at	G protein-coupled receptor 143	GPR143	GPR143	116.4	6
Ssc.10845.1.A1_at	protein kinase C and casein kinase substrate in neurons 1	PACSIN1	PACSIN1	107.1	6
Ssc.23997.1.S1_at	contactin 1	CNTN1	CNTN1	97.7	6
Ssc.6211.1.A1_at	solute carrier family 6, member 15	SLC6A15	SLC6A15	90.0	6
Ssc.103.1.S1_at	protein phosphatase 2 (formerly 2A), regulatory subunit B, beta isoform	PPP2R2B	PPP2R2B	87.8	6



Supplement 5: IPA canonical pathway analysis

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Training course

Conferences:

1. World congress on Melanoma, 06-10.09.05, Vancouver, Canada, Poster presentation:
USE OF SUBTRACTIVE cDNA LIBRARIES TO ANALYZE MOLECULAR EVENTS INVOLVED IN
REGRESSION OF CUTANEOUS MELANOMA IN THE MeLiM MODEL
2. Marie Curie Actions: 28-30.9.05, Pisa, Italy, Oral Presentation:
ANALYZING MOLECULAR EVENTS INVOLVED IN REGRESSION
OF CUTANEOUS MELANOMA USING THE SWINE MeLiM MODEL
3. Marie Curie Actions: 03-04.05.06 Paris, France, Oral Presentation:
ANALYZING MOLECULAR EVENTS INVOLVED IN REGRESSION OF CUTANEOUS MELANOMA
USING THE SWINE MeLiM MODEL
4. 13th meeting of the European Society for Pigment Cell Research (ESPCR), Barcelona,
Spain, 24-27.09.06, Poster presentation:
THE MeLiM SWINE AS A SUITABLE MODEL TO ANALYZE MOLECULAR EVENTS INVOLVED IN
REGRESSION OF CUTANEOUS MELANOMA
5. 14th meeting of the European Society for Pigment Cell Research (ESPCR),
Bari, Italy, 14-17.10.07, Oral Presentation:
TIME DEPENDENT GENE EXPRESSION PROFILING DURING SPONTANEOUS MELANOMA REGRESSION
in MeLiM PIGS
6. 4th International Melanoma Congress, New York, USA, 01-04.11.07,
Poster presentation:
TRANSCRIPTOMIC ANALYSIS of SPONTANEOUS MELANOMA REGRESSION in MeLiM PIGS

Theoretical trainings:

1. Theoretical Training TT1 (Eukaryotic gene expression), INRA, Jouy: 23-27.01.06
2. Theoretical Training TT4 (Immunogenetics), INRA Jouy: 0-24.03.06
3. Seminar “Ending a PhD and integrating the workplace”, Jouy: 17-18.10.07
4. Intensive French Course: 17-28.10.05, E.L.F.E, Paris
5. European Research School, “Post-genomics in Animal Science”, Josas: 25-28.03.08

Lab training:

Generation of *in vitro* porcine dendritic cells. Institute de Virology et d’Immunoprophylaxis (IVI), Mittelhäusern, Suisse, 13-22.09.05