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MASTER IN BIOMEDICINE

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Award date:
2014

Awarding institution:
University of Namur

Link to publication

UNIVERSITÉ DE NAMUR

Faculté de Médecine

STUDY OF THE ROLE OF ABCB5 IN MELANOMA-INITIATING CELLS: GENERATION OF TWO MOUSE MODELS THAT EXPRESS ABCB5-IRESeGFP AND SPONTANEOUSLY DEVELOP MELANOMA

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Etude du rôle d'ABCB5 dans les cellules initiatrices du melanoma: Génération de deux modèles murins exprimant ABCB5-IRESeGFP et développant spontanément des mélanomes

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Résumé

Le mélanome est l'un des trois cancers de la peau les plus répandus, les autres étant le cancer basocellulaire et le cancer squameux. Le mélanome cutané provient ainsi de mélanocytes néoplasiques. ABCB5, un membre de la sous-famille B des ABC transporteurs, a été décrit comme étant exprimé dans les mélanocytes.

Ce transporteur a été identifié comme un marqueur des cellules initiatrices du mélanome. Cependant, les informations à ce sujet sont controversées. Afin de clarifier cela, nous avons créé un modèle murin qui développe spontanément des mélanomes et qui exprime ABCB5 couplé à la GFP. Le but est de vérifier si le paradigme des cellules souches cancéreuses est applicable au cas du mélanome et de valider les données indiquant qu'ABCB5 est un marqueur des cellules souches cancéreuses.

En outre, une cartographie du profil d'expression d'ABCB5 est intéressante pour améliorer les connaissances actuelles concernant la localisation de ce transporteur. De plus, ces données nous permettront de spéculer sur son rôle dans les cellules saines ainsi que sur son implication potentielle dans certaines pathologies.

Nous avons travaillé sur les conditions de détection de la GFP dans le modèle murin ABCB5-IRESeGFP. En outre, certains marquages tels que le Fontana-Masson et le Crézyl violet ont été réalisés pour mettre en évidence la mélanine et les neurones de la substance noire. Bien que nous n'avons pas pu détecter la GFP dans le cerveau de la souris, les données suggèrent qu'ABCB5 est exprimé au niveau des entérocytes.

Mémoire de master en sciences biomédicales
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ABCB5 is, a member of the subfamily B of ABC transporter that was identified as a marker of melanoma initiating cells. However, these data remain highly controversial.

In order to clarify that, we generated a mouse model spontaneously developing melanoma and expressing ABCB5 coupled to the GFP. The long-term goal is to check if the paradigm of the Cancer Stem Cells is applicable in the case of melanoma and to validate whether ABCB5 is a marker of melanoma initiating cells. Mapping ABCB5 protein expression in the animal is important to improve the current knowledge on the localization of the transporter. More importantly, this information will allow us to speculate on its potential role in normal organisms as well as in pathological conditions.

We have worked on the conditions of GFP detection in ABCB5-IRESeGFP mice as well as of several staining including Fontana-Masson and Cresyl Violet to target melanin and neurons in substantia nigra, respectively. Although we could not detect GFP in mouse brain sections, our data suggest that ABCB5 is expressed at the level of enterocytes.

Study of the role of ABCB5 in melanoma-initiating cells: Generation of two mouse models that express ABCB5-IRESeGFP and spontaneously develop melanoma

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Abstract

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Keywords

ABCB5, mouse models, melanoma initiating cells

Introduction

Melanomas arise from melanocytes and are the most aggressive of the skin cancers, which also include basal cell carcinoma, squamous cell carcinoma, sarcomas and some rare type of carcinomas. Cutaneous melanomas represent the most common subtype of melanomas. Acral melanomas develop specifically on the palms, soles, and subungual surfaces[1],[2]. Mucosal melanoma arises in mucosal tissues in the respiratory, digestive, and genital tracts [3]. Lastly, ocular melanomas majority develop from the uvea, composed of the iris, ciliary body and choroid [4].

The worldwide increase in incidence of melanomas can be explained by increased exposure to UV radiations, but also by several other risk factors including the environment, the lifestyle and the host characteristics, see for review Markovic and colleagues [5], [6].

Melanoma sometimes begins with benign nevus, a group of clonal melanocytes blocked in senescence, probably due to the activation of oncogene-induced senescence (e.g. h-Ras, Braf) in these cells [7],[8].

With the acquisition of additional mutations, the senescence can be overcome and the nevus can become dysplastic and spread, while remaining in the epidermis. It's the radial growth phase (RGP). In the third state, the cells acquire a profile permitting them to invade the other cell layer, especially the dermis, it's the vertical growth phase (VGF). Thence, the expansion continues and cells acquire the capacity to metastasize [7]–[9].

The acquisition of the invasive characteristics is possible by modifications of cell adhesion properties. The passage from RGP to VGP is characterized by the loss of E-cadherin replaced by N-cadherin, and the expression of $\alpha V\beta 3$ integrin (allowing the expression of some pro-survival proteins, and the degradation of the collagen by the metalloproteinase 2 induction) [8]. A mutation in β-catenin (protein linked to cadherins) gene can also have a role in melanoma development, inducing expression of proteins involved in cell proliferation and survival [8].

The passage from normal melanocytes to malignant melanomas triggered by DNA damages [7], [9].
These potential DNA damages are multiple but some occurs more frequently. It's the case for a mutation found in BRAF gene. Indeed, this alteration is found in 66% of melanoma. BRAF belongs to the MAPK-family and is activated by RAS. BRAF has an impact on MEK, which activate ERK. This latter is involved in the differentiation and the cell growth.
The V600E mutation in BRAF gene is present in melanoma and gives an oncogene character to the protein. It is, so, constitutively activated.
Mutation of BRAF is found in benign and dysplastic nevi, so it could have a role in senescence after having allowed their proliferation.

Another genetic alterations are required for cancer to develop [7], [9]–[12][13].

Mutations in RAS (including N-RAS and H-RAS) are also detected, but only at a frequency of 10 to 15% [10], [11].

CDKN2A is a gene encoding two proteins, INK-4A (p16^{INK-4A}) and ARF (P19^{ARF} in mice and P14^{ARF} in human).
INK-4A is an inhibitor of cyclin-dependent kinase 4A. So retinoblastoma protein isn't phosphorylated and prevents the passage of the cell from G1 to S phase (cell is in senescence). Arf acts in blocking mdm2 and so increases the p53 expression. Loss of heterozygosity (LOH) or mutations in the CDKN2A gene can imply a deregulation of the natural pathway and lead to a tumoral phenotype. They are found in 50% of melanoma. Mutations in both INK-4a and ARF genes promote more efficiently the development of melanoma than mutations in only one gene. Moreover, mutations in Ink-4a are frequently found along with mutation in Braf [10], [11], [14].

In some melanoma, we can find a hyperactivity of RTK (Receptor tyrosine kinase). But this abnormality had been less studied [10].

Another melanomagenesis pathway iq driven by MITF (microphtalmia associated transcription factor). Physiologically this transcription factor is implicated in pigmentation and melanocyte survival. The overexpression of MITF accompanied of BRAF can transform cells in culture [8].

The growth and survival of melanoma cells can also be exacerbated by the activation of PI3/AKT pathway. Under the action of extracellular growth signals, the level of PIP3 increases, implying the phosphorylation and so, the activation of AKT. Active AKT inhibits the apoptosis and promotes the activation of cell cycle.
The level of PIP3 is regulated by the tumor suppressor PTEN. In some melanoma, there is a LOH for this gene [10], [12].

Mouse models were generated to further study this cancer in vivo. Some lights are shed on two of them, which are used in this master thesis. Those used are Tyr-Cre:Pten$^{(-/-)}$ Braf$^{(V600E)}$ and TiRP-10B;Ink4a/Arf$^{flox/flox}$.

In the first, the Cre recombinase is under the control of the Tyrosinase promoter that allows the induction of Braf$^{(V600E)}$ expression only in melanocytes. The enzyme is CreER^{T2}, which means it is inducible by the tamoxifen (4-HT) and so the enzyme isn't constitutively activated [12].

In the second, a first CreER allows the activation of Hras and P1A (gene coding for an antigen, indeed, Huibers and colleagues works on cancer immunotherapy, but not reliable in our case). A second CreER allows the deletion of Ink4a/ARF. This second enzyme is stuck by Hsp90 in the cytosol without 4-HT [11], [15].

Beside tumor genetic characteristics, cancer stem cells have been intensively studied. The cancer stem cell paradigm was first coined by Lapidot et al. (1994) who described these types of cells in acute myeloid leukaemia [16], [17].

Cancer stem cells can be defined as a distinct cell subpopulation within a tumor, able to recapitulate tumor heterogeneity after chemotherapy. According to this theory, the CSC could be a factor explaining relapse of patients after chemotherapy.

Cancer stem cells have the characteristics of stem cell, including the self-renewal, and the hierarchical division [18], [19]. The identification of some specific markers allows distinguishing the CSCs from bulk tumor cell population [17]–[20].

This identification is reliable because if possible, new therapeutic perspectives could be considered to target these drug-resistant cells.

In the case of melanoma, these cells are termed Melanoma initiating cells (MICs).

Their existence is controversial as exemplified by the case of ABCB5, a potential marker of MIC

The ATP-Binding Cassette transporter B5 (ABCB5) is little characterized, and is being studied in our laboratory. This transporter is predominantly expressed in pigmented cells (e.g. retinal pigment epithelial cells and melanocytes) [21], [22], [23], [24]. Recent studies suggest that the expression of ABCB5 protein is linked to development of multidrug resistance in melanoma, and was identified as a potential marker of melanoma-initiating cells [21][25], [26] [27],[28].

Schatton et al. , showed that ABCB5$^+$ cells are more tumorigenic than ABCB5$^-$ cells in non-obese diabetic severe combined immunodeficiency mice (NOD/SCID). [27], [29].

However, Quintana et al. have not observed differences in term of tumorigenicity between ABCB5$^+$ and ABCB5$^-$ cells in NSG mice (NOD/SCID IL2Rγ ^{null}). Interestingly, these authors tested several additional potential markers (32) without seeing any differences between positive or negative cells for their marker. These experiments showed that all cells would have the capacity to develop melanoma, without needed particular specificities. This conclusion implies that the cells in tumor wouldn't be hierarchically organised [30], [31]

Based on these two controversial studies, two important questions arise: "Does cancer stem cells exist in the case of melanoma?" and if yes, "is ABCB5 a marker of them?".

The aim of this study is double. First, we will map the ABCB5 protein expression profile using the ABCB5-IRESeGFP mouse strain. There is no antibody directed against the murine form of that transporter, reason why the eGFP cassette was inserted in the 3' UTR region of that gene. Mapping ABCB5 protein expression

in the animal is important to improve the current knowledge on the localization of the transporter. More importantly, the findings could highlight new leads and speculations on its potential role in normal organisms as well as in pathological conditions.

In the meantime, we will breed the ABCB5-IRESeGFP mice with mouse strains spontaneously developing melanoma. The aim of these models is to validate or not the paradigm of the cancer stem cells in melanoma and, to verify whether ABCB5 is a marker of these cells. If so, it could open new perspectives for the treatment of the disease.

Materials and Methods

Generation of transgenic mice

ABCB5-IRESeGFP strain was constructed by Gillet and colleagues using C57Black6 mice. The construction was performed by the insertion of an IRES-eGFP cassette in the 3'UTR region of the gene. This cassette contains also a neomycine gene floxed. It can be removed by crossing those mice with Cre-expressing strains.

Tyr-Cre:Pten$^{(-/-)}$**Braf**$^{(V600E)}$ were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and developed by Bosenberg *et al.* [12]. In this mouse, the exons 4 and 5 of Pten gene are floxed and removed by Cre enzyme (CreER).
Cre is under the control of the Tyrosinase promoter and inducible by 4-hydroxytamoxifen(4-HT). 4-HT is diluted in DMSO to a concentration of 50mg/ml and apply to skin on young mice (day 2, 3 and 4). The same protocol can be applied on shaved mice of 6 to 8 weeks old.
For a general induction, an intra-peritoneal injection (500µg Tamoxifen/20g mouse) during 3 consecutive days is made [12].

TiRP-10B;Ink4a/Arf $^{flox/flox}$ is a mouse strain generously shared by Huibers *et al.* (Ludwig Institute, Brussels, Belgium). It consists of a mouse tyrosinase promoter and CreER site floxed. Ras is also present followed by P1A gene. The activation of Ras is achieved by Cre.

The mice are also Ink4a/Arf $^{flox/flox}$ for the exon 2 and 3.

The Cre recombinase is active by adding 4-HT.
The administration of Tamoxifen is performed by subcutaneous (SC) injection of 4-HT with 2 weeks of interval, per the indication of Huibers and colleagues [11].

Genotyping of mouse

Tails are clipped and digest in lysis buffer (KCl 500mM, Tris HCl 10mM, Tween 1%) plus proteinase K overnight (o/n) at 55°C to obtain genomic DNA. Next, the microtube is mixed and centrifuge.
The PCR is performed using DreamTaq Green PCR mastermix 2X (Fisher Scientific, Aalst, Belgium), and appropriate primers (see Table 1).

For **ABCB5-IRESeGFP mice**, the program is: 94° for 3', next a denaturation step at 94° for 30'' followed by annealing step at 56°C for 30'', the elongation is performed at the temperature of 72°C for 50 seconds. The steps 2 to 4 are repeated 39 times followed by 10' at 72°.
PCR must be performed with two set of primers (i.e. PG_1 and PG_6): one set allowing detecting knockin (KI) mice and the other for detecting WT. The WT band is 401bp long, whereas KI is 350bp long.

For **Tyr-Cre:Pten**$^{(-/-)}$**Braf**$^{(V600E)}$ **strain**, one PCR must be performed Braf detection. The program used is 94°C 3', 94°C 30'', 60°C 30'', 72°C 45'', these

steps are repeated 34X followed by 5' at 72°C. Following PCR, the targeted allele is 307bp and the WT allele is 187bp long. The primers used are PG_2

For Pten, according to Jackson Laboratory guidelines, the program is as follows: 3' at 94°C, 30'' at 94°C, 1' at 60°C, 72°C for 1min steps 2-4 repeated 35X followed by 72°C for 2'.

The mutant appears at 328bp and the WT at 156bp. For this one, the migration must be performed on agar 2%. For TyrCre, the program is 3' at 94°C, 30'' at 94°C, 45'' at 58°C, 72°C for 45'' steps 2-4 repeated 34X followed by 72°C 5'.

For **TiRP-10B;Ink4a/Arf**$^{flox/flox}$ mouse strain, the primers used are P16 F and R. The program is as follows: 40'' at 94°C, 30'' at 60°C and 50'' at 72°C. These steps are repeated 30 times

A **sexing** PCR can also be performed. The program is as follows: step at 94° for 3', next a denaturation step at 94° for 30'' followed by annealing step at 56°C for 30'', the elongation is performed at the temperature of 72°C for 40 seconds. The steps 2 to 4 are repeated 29 times.

The PCR (as the others) is performed using a C1000 Touch Thermo Cycler (Bio-Rad laboratories, Nazareth Eke, Belgium).

Frozen sections

Freezing of the organs is performed in isopentane/liquid nitrogen. Each organ is embedded in OCT (Bright instrument company limited, Huntingdon, England). The sections are made with cryostat (Leica microsystem CM.1900, Groot Bijgaarden, Belgium).

The microscopic slides must be fixed in PFA (paraformaldehyde) and covered by cover-glasses by adding Mowiol (Sigma-Adlrich, Saint-Louis, USA).

Imaging is performed using Olympus microscope at 400 ISO with 3 exposition times (2''-1, 3''-1'')

Paraffin-embedded tissues

First, the tissues must be fixed with acetic acid (Formol 4% and glacial acetic glacial 1%) for at least 24h.

The organs must be placed in the VIP (Sakura-Tissue-Tek) for dehydration (twice in formol 4% at 35°C for 90', 4 times in methanol 100% for 45', 4 times in pure toluene at 35°C, 2 times 45' and 2 times 60', lastly, 4 times in paraffin 60' at 60°c).

The coating of the sample is made withShandon Histocentre 2 (GMI, Minnessota, USA). Once cooled, the paraffin block must be roughed out with a microtome – here, a Leica RM 2145 (GMI, Minnessota, USA). For soften the samples, they are placed in Molliflex (VWR International Ltd, Poole, England) for 3 hours at least. After this step the blocks are putted on ice, and can be cut in tape and stored in a histological folder.

The tape assembly is performed by the application of tap water on a slide. The fragment of organ coated in paraffin is put on the water, and the slide is put on a warm plate for some seconds.

The assembly is performed with DPX (anhydrous mounting liquid).

The conservation of the slides is possible in box at room temperature.

Removal of paraffin is performed by successive baths. First two times in toluene for 3', followed by 2 times 3' in absolute methanol, the slides have to go in methanol 70% for the same time and then submerged for 10' in water. This step, as HES coloration, is performed using Medite Tissue stainer (Burgloff, Germany).

HES staining

It's performed in the Medite Tissue stainer (Burgloff, Germany) where slides are immersed in several baths containing hemalun (7' followed by a washing step in water), followed by a bath in ethanol-HCl (at least 10'), erythrosine (6' followed by a

washing step in water), a bath in absolute ethanol (1') and absolute isopropanol (3X2 min), and safran (8'). The process ends with 3 baths in absolute isopropanol and toluene.

Fontana-Masson

The slides are washed with dH_2O (5') before passing in ammoniacal silver solution (60' in the dark). Three new washing steps are necessary before putting slides in cajal for 10'. Two washes are then performed before placing the slides in $Na_2S_2O_3$ (5g/100ml dH_2O) for 5 min.
A counter-staining can be performed with Toluidin blue.The assembly is performed using DPX.

Cresyl violet

This staining is performed to be highlighted Nissl block at the neuronal level. Cresyl violet 1% is prepared as follows: 1g cresyl, 100ml of dH_2O, 3ml of glacial acetic acid, 3ml of sodium acetate, pH 3,5). The slides are put in two baths of ethanol 100% (one for 2' and one overnight). Few seconds in dH_2O are necessary before the staining (1-2 hours). Lastly, two baths in ethanol 95% are performed. The storage of the slides is made in a mix of absolute ethanol and toluol (50% of each).If it's necessary the slides can be clarified in toluol.The assembly is done with DPX.

Hoechst staining

Slides are washed in PBS/glycin 0,1M PH7,2 for 5', then in PBS/BSA0,5%/triton0,1% for 1h. Hoechst can be applied diluted 100x in PBS/BSA. The slides must incubate 15' in wet chambers. The assembly is performed with mowiol.

Immunohistochemistry (peroxidase)

The aim of this experiment is to reveal GFP expression. The paraffin must be removed of the slide and demasking of the antigen can be performed in warm citrate buffer (100°C during 20'). Before to apply Antibodies (Abs), several washes in Glycine 0,1M/PBS pH7.2, in H_2O_2 3%, in PBS/BSA (0.1%) must be carried out.
The primary antibody (GFP antibody 600401215, 1/1000, Rockland, USA) can be put on the organ overnight. The slides can be washed in PBS/BSA and then incubated with the secondary Ab (Vectastain ABC Kit anti rabbit:1/100, Vector Laboratories, Burlingame, CA, USA) for 45'. Each incubation is performed in humid chambers. The streptavidine, peroxidase mix (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) is then added for 45'. The revelation is performed with DAB^+ (Dako, Heverlee, Belgium). Assembly is performed with DPX after dehydration in isopropanol and toluol baths.

Cell culture

A375 were maintained in DMEM (Lonza, Verviers, Belgique) supplemented with 5% FBS and 100 units of penicillin/streptomycin/mL (Lonza, Verviers, Belgium) at 37°C and 5% CO_2 humidified air.

Cell transfection

Cell count is performed using Vi-Cell counter (Beckman Coulter, Analys, Suarlée, Belgium) and cells are then plated to have 125.000 cells per well. The transfection with the GFP-expressing construct is performed in DMEM and Turbofect (Thermoscientific, Rockford, IL, USA).

Protein extraction

Lysis buffer (Tris pH7 50mM, EDTA 80mM, NaCl 150mM, triton x-100 4%) and protease inhibitors (Thermoscientific, Rockford, IL USA) were added to cell pellet.

For the organs, the lysis buffer is added proportionally to the weight of the concerned organ.

The organs stay 15' in buffer on wet ice. A cold centrifugation is performed and the supernatant is recovered.

Western-blot.

Protein quantification is performed using BCA kit as per manufacturer's recommendations (ThermoScientific, Rockford,IL, USA). The plates are read in SpectraMax i3 (Molecular Devices, Sunyvalle, CA, USA). The samples are warmed at 37°C for 15-20', placed in SDS-PAGE gels (running and stacking gel) and the migration is performed at 100V during 1h30.

The transfer is carried out for 1h30 at 100V. The PVDF membranes are placed in blocking solution for at least 1h. Next, they are incubated with the primary antibody (1/1000) overnight.

Following PBS-Tween washes, membranes were incubated with HRP-conjugated secondary antibody (Polyclonal Goat anti-Rabbit Immunoglobulins/ HRP, 1/10000, Dako, Glostrup, Denmark).

The substrate (SuperSignal West Pico Chemoluminescent Substrate, Thermo Scientific, Rockford, USA) of the enzyme is then added for the revelation.

Results

Generation of mouse strains

Gillet and colleagues developed reporter mice for ABCB5 who express the fluorescent marker eGFP. The construct was prepared in inserting a IRES-eGFP cassette in the 3'UTR region of ABCB5 gene. This cassette contains also a neomycine gene floxed (**Figure 1 A**).

We bred these mice with a mouse strain expressing Cre recombinase to remove the neomycine-encoding gene. The offspring were used to establish the cartography of ABCB5 transporter expression.

We also wanted to generate a mice strain that express ABCB5 along with eGFP and spontaneously develop melanoma. The aim of this strain generation is to assess the role of ABCB5 as a marker of melanoma-initiating cells. To do this, we worked with two strains that develop spontaneously melanoma (**Figure 1 B, C**).

To date, we generated the following mouse strains: ABCB5-IRESeGFP X Tyr-Cre:Pten$^{(-/-)}$Braf$^{(V600E)}$ mice and ABCB5-IRESeGFP X TiRP-10B; Ink4a/Arf$^{flox/flox}$. The tumor induction is different for the two strains. For the Tyr-Cre:Pten$^{(-/-)}$Braf$^{(V600E)}$, the administration of 4-HT is performed on the ear, flank and tail of young mice with a paint brush. In order to obtain tumor on back skin of adult mice, the same protocol is applied to shaved mice of 6 to 8 weeks old. For a general induction, an intra-peritoneal injection during 3 consecutive days is made [12]. For TiRP-10B; Ink4a/Arf$^{flox/flox}$ the induction consists of subcutaneous (SC) injection of 4-HT with 2 weeks of interval[11].

Time constraints did not allow us to further characterize these mice. In the upcoming weeks, they will be monitored for melanoma development.
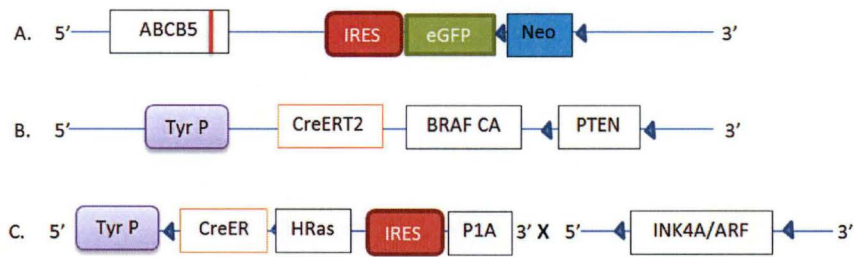
Figure 1: A. ABCB5-IRESeGFP mouse construct B. Tyr-Cre:Pten$^{(-/-)}$Braf$^{(V600E)}$ construct C. TiRP-10B;Ink4a/Arf$^{flox/flox}$. The red line represents the STOP codon.

Localization of ABCB5 in mouse tissues

Besides mouse strain generation and genotyping, the cartography of ABCB5 was performed using the ABCB5-IRESeGFP mice.

As a first step, frozen sections were performed from seven mice to identify the intrinsic fluorescence emitted by eGFP in different organs, and so to generate a preliminary list of eGFP-positive organs (data not showed). Some organs were clearly negative (e.g: muscles, bladder, spleen, etc…), whereas some appeared to be positive (e.g: eye, testis) However, the background noise was very important when compared with wild type mice, and the architecture of the organs was altered.

In a second step, we prepared sections from paraffin-embedded tissues (PET), especially from testis, eye, intestine, and the back skin of six mice.

We started with a basic HES staining and analyzed the slides in confocal microscopy.

Figure 2 shows HES staining of mouse testis. Panel A and B illustrates testis of ABCB5-IRESeGFP transgenic mouse, while panel C shows testis taken from wild-type (WT). No difference in term of fluorescence was observed between the ABCB5 transgenic mouse and the WT. Genotyping will be tested again to confirm the genetic background of these mice.
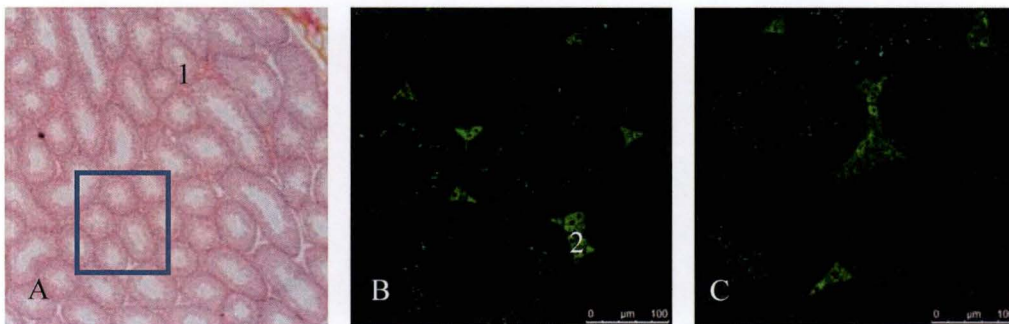

Figure 2: Mouse testis A. HES staining with the 4x zoom. The frame localizes the area extended in the next pictures B. ABCB5-IRESeGFP mouse testis C. WT mouse testis. Number 1 in panel A localizes the seminiferous tubules, number 2 in panel B indicates Leydig cells.

Concerning the eye (**Figure 3 A→C**) the most external part (3a) seemed to express specific fluorescence, but further observations allowed us to determine that this corresponds to an artefact, a gap

between the choroid and the retina, rather than being pigmentary epithelial tissue where ABCB5 should be found to be expressed
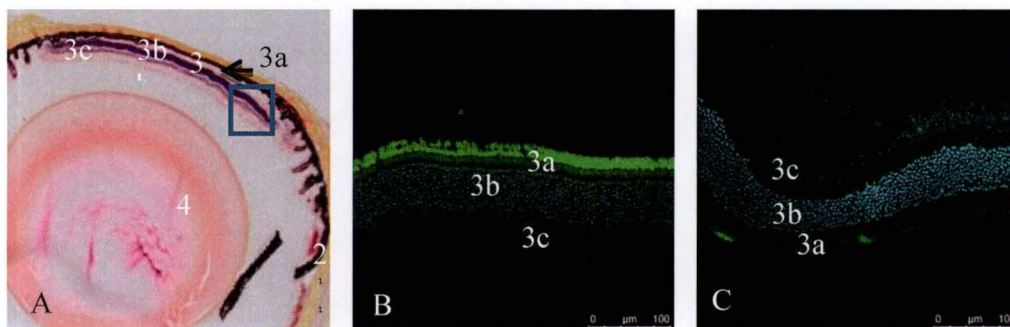
Figure 3: A. An eye, frontal view with HES staining imaged using 4x zoom B. ABCB5-IRESeGFP eye C. WT eye. Number 1 is the sclera, number 2 the choroids (black line), number 3 the retina (3a indicates retinal pigmentary epithelium, 3b photoreceptors nuclei, 3c, bipolar cell nuclei and 4, the lens.

The third organ tested is the intestine (**Figure 4 A→C**). The data suggest that ABCB5 is expressed in this tissue, especially in enterocytes. Additional slides will be further analyzed to confirm this finding.
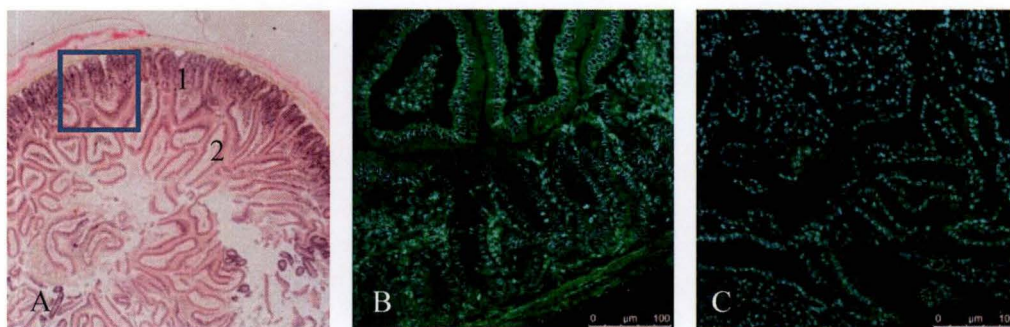

Figure 4: A. HES staining of mouse intestine. B. ABCB5-IRESeGFP mouse intestine. C. WT mouse intestine. Number1 indicates the Lieberkuhn crypts, number 2 shows the villous.

Determining that a tissue expresses GFP can be subjective. Therefore, we wanted to perform immunohistochemistry using the peroxidase revelation technique. **Figure 5** shows the results obtained with that technique on testis. However, the staining was not specific. Indeed, we see no significant difference between both mouse strains. This negative result may be explained by a lack of specificity of the anti-GFP antibody. This hypothesis will be further tested.

Afterwards, Fontana-Masson staining was performed to highlight the melanin and so the melanocytes. It is known in the literature that ABCB5 is expressed in this cell type.
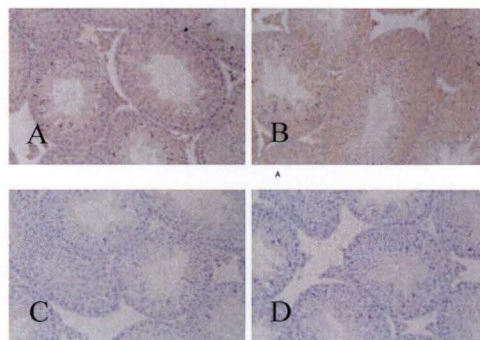

Figure 5: A. Testis mouse ABCB5-IRESeGFP labelled with primary and secondary antibodies (Abs). B. Testis mouse WT with the primary and secondary Abs. . C. Testis ABCB5-IRESeGFP with the secondary Ab (Ctl -), D. Testis WT with the secondary antibody.

Adult back skin and tail skin were stained with Fontana-Masson (**Figure 6**).The results suggest that melanin is secreted in the tail, but is not detected in the adult back skin. However this observation doesn't necessarily mean that melanocytes are absent, rather they do not produce melanin.
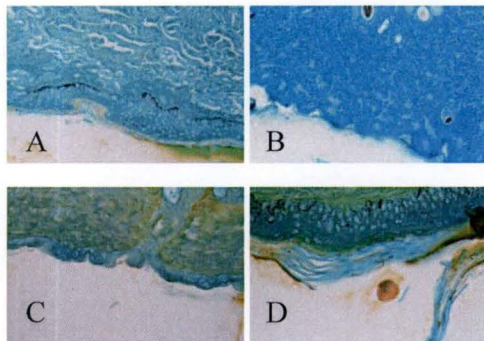

Figure 6: Fontana-Masson staining of skin. A. Positive control (Creole pork skin) that underwent the same treatment than the other slides. We can see the black line specific of the Fontana stain. B. It's the same lame but this one has not been in contact with the silver solution. C. Back skin of ABCB5-IRESeGFP mouse was in contact with the silver stain. No melanin is produced. D. Mice tail of ABCB5-IRESeGFP mice, We can observe black stain in the epidermis.

It is not clear whether ABCB5 is expressed in substantia nigra pars compacta (SNC). One can speculate that it is as this tissue is composed of pigmented dopaminergic neurons. The Crezil violet is a coloration that marks the endoplasmic reticulum accumulated at the neuron level: the Nissl bodies.The aim of doing this staining was to localize the substantia nigra (See **S1** in Appendix).
However, the observation at the confocal microscope showed no particular signal.

Validation of the anti-GFP Antibody by Western-Blot

Another purpose of the project was to monitor the expression of ABCB5-GFP in different tissues by western-blot analysis.

However, the efficiency of the anti-GFP antibody in western blot was not yet confirmed.
For this reason, we started validating this antibody on protein lysate of A375 cells transfected with a GFP-expressing construct.
A picture was made by fluorescent microscope to confirm the transfection efficiency (**Figure 7**). The primary antibody was recognized by a secondary peroxidase-conjugated antibody.
The results indicate that GFP was efficiently expressed in transfected A375 cells. The Western-Blot (WB) shows that the antibody recognizes the GFP in transfected cells (Figure 7 E), but that it is unspecific. This will be further validated in a second independent experiment.
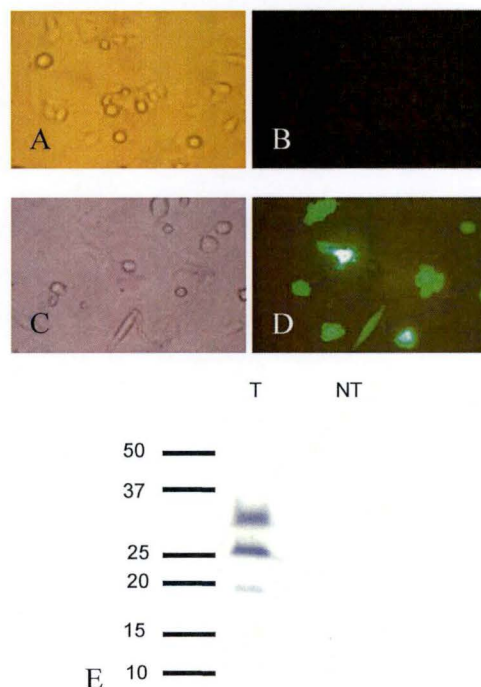

Figure 7: A375 non-transfected (NT) cells exposed successively at white light (A) and fluorescent light (B). A375 transfected cells (T) at white (C) and fluorescent (D) light. E. Western-blot performed with 15µg of proteins, and revealed following 1'' exposure.

Discussion

ABCB5 is a transporter that is little studied. Its physiological role has yet to be determined as well as its role in melanomagenesis. Most of the studies published in the literature have been focusing on the ABCB5 role in multidrug resistance in cancer. Furthermore, ABCB5 was proposed to be a marker of melanoma-initiating cells (MICs) [27]. This finding is particularly important as if it is validated, then one could develop therapeutic strategies to overcome multidrug resistance through targeting these MICs.

This project contributed to generate mouse strains that express eGFP along with ABCB5 and that spontaneously develop melanoma. Moreover, we also started optimizing the experiment conditions to stain mouse tissues in order to map ABCB5 transporter *in vivo*.

Mapping of ABCB5 in mouse tissue represent a challenge due to the complexity of fine-tuning the staining conditions and potential difficulties to find a specific anti-GFP antibody. With frozen sections, the most important issue was the tissue degradation. To conserve the tissue architecture, we can perfuse the animal with Paraformaldehyde (PFA) to fix the tissues, and so to guarantee their integrity before the freezing. This technique will allow us to detect the GFP in good conditions, and to maintain a reasonable architecture of the tissues. Preparing frozen sections is less cumbersome than paraffin-embedded tissues, which can possibly denature the GFP. However the opinions diverge on this point.

A problem that we experienced was the lack of specificity of the anti-GFP antibody used to perform immunohistochemistry. Western-blot revealed that the antibody detects multiple bands. This will be verified in a second experiment to confirm the data. We could also hypothesize to explain that the antibody is not compatible with the acetic formol. Thus, it could be interesting to re-test the antibody on fixed-tissue using another fixative and compare the two sets of results.

Another hypothesis that may explain the difficulties to detect fluorescence is that the GFP is most likely expressed at low level since this is a recurrent observation with ABCB5.
An indirect way to support this hypothesis is to perform a RT-qPCR. Total RNA was prepared during this master thesis. *In situ* hybridization could eventually be undertaken to detect either ABCB5 or eGFP at the RNA level, even though we have to bear in mind that RNA expression could not be correlated with protein expression. We could envision inducing ABCB5 expression in treating mice with a chemotherapeutic agent that was shown to be the substrate of ABCB5 (e.g. compounds from taxane or anthracycline classes) [26].

It is critical to fully exploit the ABCB5-IRESeGFP mice. The expression of ABCB5 should be studied during the embryo development. Melanocytes are derived from neural crest and migrate along the dorsolateral pathway to colonize the skin. So, 3 to 4 days old pups could be sacrificed to detect ABCB5 expression in melanocytes found in back skin. Colocalization could be done using using a melanocytes specific staining.

In the meantime, we generated two mouse strains by breeding and TiRP-10B;Ink4a/Arf $^{flox/flox}$ with ABCB5-IRESeGFP mice.
The aim of this project is to collect tumor cells and to perform FACS cell sorting according to GFP and so, the ABCB5 expression. These cells will be characterized for their expression of markers that are known to be expressed in cancer stem cells (NANOG, c-KIT, c-MYC for example) [20].

The GFP$^+$ cells will be injected at different dilution in nude mice to evaluate their capacity to reconstitute heterogeneous tumors. Moreover, their gene expression profile will be compared with those of embryonic stem cells, melanoblasts and melanocytes to reveal specific gene clusters for each cell types. This information would be the cornerstone to develop therapeutic strategies with limited side effects on healthy tissues.

It will also be interesting to compare both mouse models that develop melanoma through different pathways. If MIC presence is confirmed, do they have a different gene expression profile? Are they resistant to the same chemotherapeutic agents?

In summary, those few months have been used to optimize the conditions of GFP detection in ABCB5-IRESeGFP mice. Some advances have been made in that way. Indeed, our data suggest that ABCB5 is expressed in the intestinal villous. In addition, the laboratory has now two mouse strain ready to be used to further study the role of ABCB5 in melanoma-initiating cells.

Acknowledgements

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Appendix

Table 1: List of primers

Name	Sequences	Concentration
PG1_GFPlong_F	CTCACAGACTCTCTACAATAC	10µM
PG1_GFPlong_R	ACCCATCATGTGGATGCTGA	10µM
PG2BRAF_F	TGAGTATTTTTGTGGCAACTGC	10µM
PG2BRAF_R	CTCTGCTGGGAAAGCGGC	10µM
PG3TyrCRE_F	CAATGGTAGGCTCACTCTGGGAGATGATA	10µM
PG3TyrCRE_R	AACACACACTGGCAGGACTGGCTAGG	10µM
PG4TyrCRECTL_F	CAGGGTGTTATAAGCAATCCC	10µM
PG4TyrCRECTL_R	CCTGGAAAATGCTTCTGTCCG	10µM
P10_GFP_F	CTGACCCTGAAGTTCATCTG	10µM
P10_GFP_R	TAGTTGTACTCCAGCTTGTG	10µM
Pten_F	CAAGCACTCTGCGAACTGAG	10µM
Pten_R	AAGTTTTTGAAGGCAAGATGC	10µM
P16_F	CCTGACTATGGTAGTAAAGTGG	
P16_R	ACGTGTATGCCACCCTGACC	

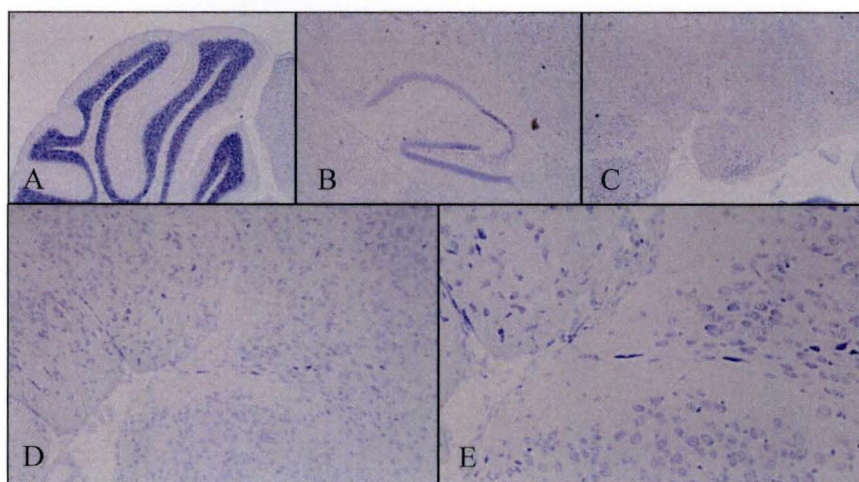### Supplementary Figure S1: Cresyl violet staining

Figure S1: A. The mouse cerebellum B. The hippocampus. These structures help to localize C. The Substantia nigra. D and E are zooms of that region, 10X and 20X zoom, respectively.