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Expression of porcine endogenous retroviruses (PERVs) in melanomas of Munich miniature swine (MMS) Troll

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Abstract

Porcine endogenous retroviruses (PERVs) are integrated in the genome of all pig breeds. Since some of them are able to infect human cells, they might represent a risk for xenotransplantation using pig cells or organs. However, the expression and biological role of PERVs in healthy pigs as well as in porcine tumours is largely unknown. Since we and others have recently shown overexpression of a human endogenous retrovirus, HERV-K, in human melanomas, we studied the expression of PERVs in melanomas of selectively bred Munich miniature swine (MMS) Troll. This breeding herd of MMS Troll is characterised by a high prevalence of melanomas, which histologically resemble various types of cutaneous melanomas in humans. Several genetic factors have been defined when studying inheritance of melanomas and melanocytic nevi in MMS Troll. Here we show that the polytropic PERV-A and PERV-B as well as the ecotropic PERV-C are present in the genome of all melanoma bearing MMS Troll investigated. Most interestingly, in the spleen, but not in other organs, recombinant PERV-A/C proviruses were found. PERV expression was found elevated in melanomas when compared to normal skin and viral proteins were expressed in melanomas and pulmonary metastasis-derived melanoma cell cultures. During passaging of these cells *in vitro* the expression of PERV mRNA and protein increased and virus particles were released as shown by RT activity in the supernatant and by electron microscopy. Genomic RNA of PERV-A, -B and -C were found in pelleted virus particles. Although PERV expression was elevated in melanomas and pulmonary metastasis-derived cell cultures, the function of the virus in tumour development is still unclear.

1. Introduction

Porcine endogenous retroviruses (PERVs) are an integrated part of the genome of all pig breeds (Ericsson et al., 2001, Patience et al., 2001) and were found to be released as infectious particles from normal pig cells (Martin et al., 1998a, Tacke et al., 2000, Tacke et al. 2003, McIntyre et al., 2003). Three subtypes of PERV, which differ in the *env* region encoding for the receptor binding site, are known (Le Tissier et al., 1997; Patience et al., 2001, Patience et al., 1997; Takeuchi et al., 1998). Two of them, PERV-A and PERV-B, are polytropic viruses and able to infect human cells *in vitro* (Martin et al., 2000; Patience et al., 1997; Specke et al., 2001; Takeuchi et al., 1998; Wilson et al., 1998, 2000). Therefore they may represent a risk for xenotransplantation when pig cells or organs will be used. PERV-C is an ecotropic virus, infecting only porcine cells. Recombinant PERVs with the long terminal repeat (LTR) of PERV-C and the receptor-binding domain (tropism) of PERV-A are able to adapt to human cells and this adaptation is associated with higher titres and an increase in the length of the LTR (Denner et al., 2003). PERV-A, -B and -C belong to the genera of the gammaretroviruses and are closely related to the murine and feline leukaemia viruses which are able to induce tumours and immunodeficiencies in the infected host (Denner, 1998). The tumour development by gammaretroviruses depends on high level replication, insertional mutagenesis, and alterations in the viral LTR (Athas et al., 1995). The expression of PERV and their biological role in normal pigs as well as in pig tumours is insufficiently analysed.

We (Buscher et al., 2005, 2006) and others (Muster et al., 2003) have recently shown that a human endogenous retrovirus, HERV-K, is highly expressed in human melanomas. Malignant melanomas commonly arise from pigment-producing cells in the epidermis and take an aggressive course of disease in man. In order to establish an animal model, a breeding herd of

MMS Troll with a high spontaneous rate of both congenital and postnatally occurring cutaneous melanocytic lesions, including invasive cutaneous melanomas with metastases, were established at the University of Munich and maintained as closed colony since 1986 (Wanke et al., 1998). Founder animals were derived from stock MMS Troll originally developed from Hanford and Columbian miniature swine at the Medical Service Munich (Sambraus, 1987). The overall prevalence of cutaneous melanoma in this breeding colony of MMS Troll is almost 50% (Wanke et al., 1998). 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 histologically resemble various types of cutaneous melanomas in humans but with a less aggressive course. MMS Troll represent a suitable animal model to study melanomagenesis, metastasis and melanoma regression, associated with vitiligo (Wanke et al., 1998) and provide the opportunity to study a possible participation of endogenous retroviruses in tumour development.

PERV particles have been found in the blood of leukaemic pigs, some of them treated by radiation (Frazier, 1985). PERVs were also isolated from pig lymphomas (Suzuka et al., 1985, 1986) and from transformed porcine lymphocytes treated with phytohemagglutinin and 5-azacytidine (Kaeffer et al., 1990), but it is still unclear whether they played a role in the induction or progression of these tumours.

In the present study, we analysed the genetic distribution of PERV-A, PERV-B and PERV-C in MMS Troll and their expression in melanomas in comparison to normal skin of the same individual. An enhanced expression of PERV in porcine melanomas was found by real-time PCR and virus particles were found released from melanoma pulmonary metastasis-derived cell cultures. This is consistent with findings showing high-level expression of HERV-K in human melanomas and further supports similarities between both entities.

2. Materials and methods

2.1. Animals

Munich miniature swine (MMS) of the strain Troll were kept under spf conditions at the Friedrich-Loeffler Institute (FLI), Tübingen, and as a closed colony at the Institute of Veterinary Pathology, University of Munich. Four melanoma-bearing MMS Troll ranging from four to eight weeks of age were included in the study. Animals were sacrificed by barbiturate overdose and complete necropsies were performed.

2.2. Cell culture

A melanoma cell culture was established from an aseptically taken pulmonary metastasis (animal #007, showing a cutaneous melanoma at the back (Fig. 1A) and was placed into a tissue culture flask. After 1 hour of attachment to the plastic surface RPMI 1640 culture medium supplemented with L-glutamine and 10% fetal bovine serum (PAA, Pasching, Austria) was added to cover the surrounding plastic surface of the flask. After one week the tissue pieces that showed cells spreading were removed and the culture medium in the flask was replaced. Thereafter the medium was changed every two days until the cells grew up to confluence. Initially all cells showed deep strong pigmentation which gradually faded during the first five passages of the cells until it was no more visible in light microscopy. The cells were split 1:2 every three to four days and were continuously grown until they reached 60 passages. Thereafter, two flasks of a low passage (10) and a high passage (54) were sent to the cell depository bank of the FLI in Riems/Greifswald, Germany (kindly maintained by Dr. R. Riebe). Maintenance of PERV-

producing porcine kidney PK-15 cells and human kidney 293 cells producing PERV/5^0 was described (Denner et al., 2003). The intestinal epithelial cell line IPEC-J2 was obtained from Dr. Schierack (Free University, Berlin, Germany) and cultured in DMEM/F12 (Schierack et al., 2006).

2.3. Histology, Immunohistochemistry

Tissue samples with melanomatous lesions were fixed in 10% neutral buffered formalin and embedded in paraffin or plastic. Sections were routinely stained with hematoxylin and eosin (H&E) with or without prior bleaching with 5% hydrogen peroxide in aqua dest. for 24 hours. Immunohistochemistry was performed on paraffin sections with or without prior bleaching, using the indirect immune-alkaline phosphatase method. Incubation steps with a monoclonal mouse anti-human HMB45 antibody (DAKO Diagnostica, Germany), diluted 1:250 in phosphate buffered saline (PBS, pH 7.4) and an alkaline phosphatase conjugated goat anti-mouse IgG (DAKO), diluted 1:50 in PBS (pH 7.4) took place in a humidity chamber at room temperature. Immunoreactivity was visualized using fuchsin as chromogen.

2.4. Isolation of RNA, DNA and protein

Melanoma biopsies and tissue samples were homogenized in liquid nitrogen and resuspended in TRI-Reagent (Sigma, Taufkirchen, Germany, 1 ml per 50-100 mg of tissue). After addition of 0.2 ml chloroform and centrifugation (12,000 x g, 15 min, 4°C) total RNA was purified from the aqueous phase using the RNeasy kit (Qiagen, Hilden, Germany), including an on-column DNase digestion. RNA was stored at -80°C until assayed. For isolation of total RNA

146 from cell cultures, 10^6 cells were lysed in 1 ml TRI-Reagent and processed as described above.
147 DNA and protein were isolated from the interphase or the organic phase of the TRI-
148 Reagent/chloroform mixture, respectively, according to the manufacturer's instructions and
149 stored at -20°C until use.
150
151 *2.5. PERV-specific PCR, RT-PCR and real time RT-PCR*
152
153 Provirus integration was analyzed by PCR, using primers for *env* of all three subtypes A,
154 B (Le Tissier et al., 1997) and C (Takeuchi et al., 1998) as well as primers for *pol* (Czauderna *et*
155 *al*., 2000) and *gag* (Paradis et al., 1999). For each reaction 100 ng DNA were used. The
156 temperature conditions were chosen as follows: 94°C, 15 min; 34 cycles (94°C, 30 sec; annealing
157 temperature, 30 sec; 72°C, 1 min); 72°C, 5 min. An one-step reverse transcription (RT) PCR
158 (Invitrogen, Karlsruhe, Germany) was performed using primers detecting viral full length RNA
159 (forward: 5' TGCTGTTTGCATCAAGACCGC, reverse: 5' ACAGACACTCAGAACA
160 GAGAC), spliced *env*-mRNA (forward: 5' TGCTGTTTGCATCAAGACCGC, reverse: 5'
161 ATGGAGGCGAAGCTTAAGGGGA) as well as primers specific for the *env, pol* and *gag* as
162 described above. For each reaction 100 ng total RNA and the following temperature conditions
163 were used: 50°C, 30 min; 94°C, 2 min; 30 cycles (94°C, 45 sec, annealing temperature, 45 sec,
164 72°C 1 min); 72°C, 7 min. Integrity of RNA was determined by amplifying the house keeping
165 gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, for: 5' CTGCCCCTTCTG
166 CTGATGC; rev: 5' TCC ACGATGCCGAAGTTGTC). False-positive results due to residual
167 DNA were excluded by parallel experiments omitting the reverse transcriptase. Quantitative real-
168 time one-step RT-PCR system (Invitrogen, Karlsruhe, Germany) was performed using the
169 MX4000 thermocycler (Stratagene, La Jolla, CA, USA), a FAM-labelled probe (5' FAM-

AGAAGGGACCTTGGCAGACTTTCT-BHQ1 (Sigma, Steinheim, Germany) and primers specific for PERV *gag* (for: TCCAGGGCTCATAATTTGTC, rev: TGATGGCCATCCAACATCGA) were applied. PERV expression was normalised to the expression of the house keeping genes GAPDH and cyclophilin. Therefore a HEX-labelled probe and primers specific for porcine GAPDH (for: 5' ACATGGCCTCCAAGGAGTAAGA, rev: GATCGAGTTGGGGCTGTGACT, 5' HEX-CCACCAACCCCAGCAAGAGCACGC-BHQ1 (Operon, Cologne, Germany, Duvigneau et al., 2005) as well as a Cy5-labelled probe and primers specific for porcine cyclophilin (for: 5' TGCTTTCACAGAATAATTCCAGGATTTA, rev: 5' GACTTGC CACCAGTGCCATTA, 5' Cy5- TGCCAGGGTGGTGACTTCACACGCC-BHQ2, Operon, Duvigneau et al., 2005) were used. For each reaction 50 ng total RNA and the following temperature conditions were used: 50°C, 15 min; 95°C, 2 min; 45 cycles (95°C, 15 sec; 54°C, 30 sec). Expression in each tissue was normalised to porcine GAPDH, porcine cyclophilin and total RNA, respectively and related to expression in the PERV-producing porcine kidney cell line PK-15. Data were analyzed using the ΔΔCT-method (Livak, 2001). The length of the viral LTR was analyzed by PCR using the following primers and temperature conditions: LTR-repeat-up (5' TCTTGGTGACAACATGTCTC) and LTR-repeat-down (5' AGTGTGGAGTCG GGACAGCT), 95°C, 10 min; 35 cycles (95°C, 45 sec; 60°C, 45 sec; 72°C, 1 min); 72°C, 7 min. PERVs released from a pulmonary metastasis-derived cell line at passage 95 were pelleted (rotor SW-28, 26.000 rpm, 2 h, 4°C, ultracentrifuge L8-07, Beckman Coulter, Krefeld, Germany); RNA was analysed using one-step reverse RT PCR and primers described above. The presence of PERV-A/C recombinants was investigated by PCR using the following primers: PERV-A *env* VRBF for (5' CCTACCAGTTATAATCAATTTAATTATGGC, Wood et al., 2004) and two different PERV-C *env* primers, TMR (5' CTCAAACCACCCTTGAGTAGTTTCC (Wood et al., 2004) and PERV-C rev (5' TATGTTAGAGGATGGTCCTGGTC (Martin et al., 2006). PCR

194 reactions were performed as described above and with the following temperature conditions:
195 95°C, 10 min; 40 cycles (95°C, 30 sec; 55°C, 30 sec; 72°C, 2 min); 72°C, 10 min.
196
197 *2.6. Western blot analysis*
198
199 Protein (50 µg) isolated from melanomas and pulmonary metastasis-derived cell cultures
200 as described above, recombinant PERV proteins p15E (Fiebig et al., 2003) and p27Gag (Irgang et
201 al., 2003) and pelleted virus (purified by ultracentrifugation from culture supernatant of PERV-
202 A/C-infected 293 cells) were subjected to SDS-PAGE in 10% gels using tricine buffered systems
203 (Tacke et al., 2000) and transferred to PVDF-membranes (Millipore, Bedford, MA, USA) by
204 semidry electroblotting. Unspecific binding was blocked using 0.05% Tween20 and 5% skimmed
205 milk in PBS for 1 hour at room temperature. Goat anti-PERV-p15E (Fiebig et al., 2003) and goat
206 anti-PERV-p27Gag (Irgang et al., 2003) sera were incubated for 12 h at 4°C or for 2 h at room
207 temperature at a 1:200 dilution, followed by a 2 h incubation at room temperature with a 1:2000
208 dilution of a peroxidase-conjugated secondary anti-goat serum (Dako, Hamburg, Germany).
209 Loading of equal protein amounts was verified using mouse antibodies against *ß*-actin (Sigma) at
210 a 1:5000 dilution, followed by 2 h incubation at room temperature with a 1:1000 dilution of a
211 peroxidase-conjugated secondary anti-mouse serum (Dako). Antibody binding was visualized
212 using an enhanced chemiluminescence-based system (Pierce/Perbio Science, Bonn, Germany).
213
214 *2.7. Immunofluorescence*
215
216 Cells were grown on coverslips placed into 6 well plates, fixed with 3.7% formaldehyde
217 for 30 minutes at room temperature, washed with PBS and permeabilised with 1% Triton X 100

in PBS. Cells were washed again and incubated with 5% skimmed milk in PBS for 20 minutes to block unspecific binding. Cells were stained with a goat anti-PERV-p15E serum or goat anti-PERV-p27Gag serum respectively, diluted 1:200 in PBS/2.5 % milk for 1 hour at 37°C. After washing the cells were incubated with the corresponding FITC-labelled secondary antibody at a dilution of 1:160 for 1 hour at 37°C in a humidified box. After the last wash, the cells were covered in ProLong Anti Fade reagent (Molecular Probes, Göttingen, Germany) and analysed using the CLSM 510 laser-scanning microscope (Zeiss, Göttingen, Germany) with appropriate filters.

2.8. Reverse transcriptase (RT) activity

Different passages of the pulmonary metastasis cell culture derived from MMS Troll #007 were seeded in a 24 well microtitre plate at a density of 2 x 10^5/well. At confluency, the supernatants were harvested, cells were removed by centrifugation and cell free supernatants were analysed using the C-type RT activity assay (Cavidi Tech, Uppsala, Sweden) according to the manufacturer's instructions.

2.9. Electron microscopy

For transmission electron microscopy cells of the pulmonary metastasis-derived cell culture were cultured until 95% confluence. After resuspension the cells were fixed in glutaraldehyde (2.5%, v/v) buffered with HEPES (0.05 M; pH 7.2) for 1 h at room temperature. For stabilizing the cells were agarose-block embedded by mixing equal volumes of cell suspension and low melting point agarose (3% in PBS). These agar blocks were than postfixed

with OsO_4 (1% in ddH_2O; Plano, Wetzlar, Germany) and block-stained with uranyl acetate (2% in ddH_2O; Merck, Darmstadt, Germany). After stepwise dehydration in graded alcohol the samples were embedded in Epon and polymerised at 60°C over night. Ultra thin sections were prepared with an ultramicrotome (Ultracut S, Leica, Germany) and placed on naked 400-mesh grids or on pioloform covered 100-mesh grids. The sections were stained with lead citrate and stabilised with ca. 1.5 nm carbon (carbon evaporation; BAE 250, Bal Tec, Liechtenstein). Transmission electron microscopy was performed with an EM 902 (Zeiss) and the images digitised using a slow scan CCD-camera (Pro-scan, Scheuring, Germany).

2.10. Virus transmission experiments

Uninfected 293 cells were incubated with the supernatant of the pulmonary metastasis-derived cell culture in its 87^{th} passage, (1.5 ml per $5x10^5$ cells) in the presence or absence of polybrene (6µg/ml). Every three days DNA was isolated and tested for PERV proviruses using PCR.

2.11. RT-PCR for melanoma markers, real-time RT-PCR for tyrosinase

One-step RT-PCRs were performed as described above using primers specific for the human genes of the melanoma markers MIA, MART and gp100 as described by Perez et al., 2000. Specific primers for the porcine tyrosinase (accession number: NM_001025212) were designed (ssTyr1for: 5'-GCTTTGGCAACTTCATGGGATTTA, ssTyr1rev: 5'-CGAAGTCTGGGC TGGTAGTATGTT) and used in an one-step RT-PCR (SuperScript III One-Step RT-PCR Platinum Taq Kit, Qiagen, Hilden, Germany) under the following conditions:

50°C, 30 min; 94°C, 2 min; followed by 30 cycles (94°C, 45 sec; 53.8°C, 45 sec, 72°C, 1 min, followed by 72°C, 7 minutes. Using the same primers, a Sybr Green real time PCR was established (Brilliant Sybr Green QPCR MasterMix, Stratagene, La Jolla, CA, USA). cDNA synthesis was performed using gene specific primers and the RevertAid First Strand cDNA synthesis kit according to the manufacturer's instructions (Fermentas, Leon-Rot, Germany). Primer efficiency was evaluated with a serial dilution of cDNA (0-30 ng) from normal skin under the following conditions: 95°C, 10 min, followed by 40 cycles (95°C, 30 sec; 53°C, 20 sec; 72°C, 40 sec), followed by incubation at 95°C, 1 min and 41 cycles (55°C↑, 30 sec.). In parallel, a Sybr Green real-time PCR was performed for porcine GAPDH using the same conditions and primers as described above. Expression of tyrosinase was normalised according to GAPDH and estimated according the ΔΔCT-method (Livak, 2001).

3. Results

3.1. Morphology, histological and immunohistochemical characterisation of the melanomas

The MMS Troll included in this study exhibited single or multiple black skin tumours, which were frequently ulcerated (Fig. 1A). Melanomatous lesions were found in regional lymph nodes and a variety of internal organs, including lungs, liver and spleen (Fig. 1B, C, Table 1). Histologically, the skin tumours were composed of heavily pigmented melanoma cells, invading the dermis and subcutaneous tissue as well as pigment laden macrophages (Fig. 2A). Regional lymph nodes were heavily infiltrated by melanoma cells, resulting in the destruction of tissue architecture (Fig. 2B). Using immunohistochemistry, melanoma cells of both cutaneous melanomas and metastases showed a strong reactivity with the monoclonal antibody HMB-45

(Fig. 2C, D).

3.2. Genetic distribution of PERV in MMS Troll

Since PERV-C is not present in all pig strains, the presence of the human-tropic PERV-A and PERV-B as well as of the ecotropic (replicating only in pig cells) PERV-C was studied. All three proviruses were found in the genome of pig #007 (Fig. 3A), as well as in the genome of all other MMS Troll (#371, #372, #472, #499) tested so far. Furthermore, the presence of two different PERV-A/C recombinant proviruses was observed in the spleen of MMS Troll #371 (Fig. 3B, C) and #372 but not in other organs, indicating that these viruses were exogenous and were *de novo* integrated into spleen cells. The proviruses differed in the length of the PERV-A sequence in the *env* gene of PERV-C. PERV/5^0 provirus in 293 cells was detected with one of the primer sets. In all investigated tissues of animals #007, #472 and #499 no PERV-A/C recombinants could be detected.

3.3. PERV expression in porcine melanomas

Melanomas and normal tissues of MMS Troll were studied for expression of PERV using RT-PCR. In all analysed cutaneous melanomas, lymph nodes with metastases, spleens with metastasis and normal skin and spleen samples from MMS Troll animals full length RNA, specific for *gag* and *pol* corresponding to all three PERVs, were detected. In addition, in all tissues spliced mRNA was observed (Fig. 4A). The presence of spliced *env* mRNA theoretically allows translation of Env protein. In order to measure the expression of PERV quantitatively, a one-step RT real-time PCR was established using *gag* specific primers, allowing to quantify the

314 expression of full length mRNA. The RT real-time PCR was characterised by a coefficient of
315 correlation of 0,980 and an almost optimal efficiency of amplification. The expression of PERV
316 was in most cases higher in cutaneous melanomas when compared to the expression in normal
317 skin (Fig. 4B). Regardless whether the PERV expression was normalised to the expression of the
318 house keeping genes GAPDH or cyclophilin or to total RNA, similar expression patterns were
319 found.
320
321 *3.4. PERV expression in cell cultures*
322
323 When tested for PERV expression in cultured pulmonary metastasis-derived melanoma
324 cells using the same PCR methods, expression of full length and spliced *env* mRNA was shown.
325 Interestingly, the amount of PERV-specific full length mRNA increased with the number of
326 passages of the melanoma cells in vitro (Fig. 4C). Whereas in passage 21 the expression of PERV
327 was less than 80% of the expression in PK-15 cells, at passage 87, the expression of PERV was
328 7-times higher than that in PK-15 cells. Regardless whether the PERV expression was normalised
329 to the expression of the house keeping genes GAPDH (Fig. 4C) or cyclophilin or to total RNA,
330 similar expression patterns were found. In contrast, when the intestinal epithelial cell line IPEC-
331 J2 (Schierack et al., 2006) was investigated, a low expression of PERV was found (20% of the
332 expression in PK-15 cells).
333 Since the length of the viral LTR and the virus titre was shown to increase during passaging of
334 PERV in human cells (Denner et al., 2003), the length of the LTR was studied in these melanoma
335 cells after each passage. No changes in the length of the LTR were observed (Fig. 4D). The LTR
336 corresponded to the LTR of PERV/3^0, containing only one 37bp repeat with one nuclear factor Y
337 (NF-Y) binding site (Denner et al., 2003).

338 *3.5. PERV protein expression in primary tumours and cell cultures*

339

340 In order to analyse whether the increased expression of PERV mRNA is also associated
341 with an increased expression of viral proteins, protein preparations from melanomas and
342 pulmonary metastasis-derived cell cultures were analysed using a Western blot assay and
343 antibodies specific for p15E and Gag. The detection limits for p15E and Gag in the Western blot
344 analysis were about 50 ng and 100 ng, respectively. Low amounts of viral p15E were found in
345 primary tumours, e.g., in a melanoma of animal #372, but not in the normal skin of the same
346 animal (Fig. 5A). When testing ten melanoma samples, four normal skin samples, three spleen
347 samples and one lymph node with metastasis by Western blot analysis, three of the melanoma
348 samples, but none of the normal tissues were positive (data not shown). In the pulmonary
349 metastasis-derived cell culture, p15E protein was detected and the highest expression was seen in
350 passage 88 (Fig. 5B), correlating with the expression of RNA (Fig. 4C). Expression of low
351 amounts of Gag precursor molecules was also found in primary tumours as well as in the cell
352 cultures (data not shown). The expression of p15E in the pulmonary metastasis-derived
353 melanoma cell culture was verified using immunofluorescence (Fig. 5C). When non-
354 permeabilised melanoma cells were analysed, expression of p15E was detected on the cell
355 surface and in the cytoplasma (Fig. 5C, a), indicating a cell surface localisation as well as the
356 ability of the antibodies to intrude into the cells. When cells were permeabilised, p15E was
357 detected predominantly in the cytoplasm (Fig. 5C, b, c). Consistent with the mRNA data (Fig.
358 4C), the expression was higher in late passage melanoma cells (Fig. 5C, d).

359

360

361

362 *3.6. Release of virus particles from melanoma cells*

363

364 To analyse, whether the pulmonary metastasis-derived melanoma cells released virus
365 particles, the RT activity was measured in the culture supernatants. Consistent with the increase
366 in the amount of viral full length RNA and protein, the released RT activity also increased during
367 passaging of the cells (Fig. 6A). In the supernatant of the 93rd passage of the melanoma cell
368 cultures, higher RT activity was detected than in the PK-15 cell culture supernatant. In order to
369 analyse, which type of PERV was released, the genomes were analysed in pelleted virus particles
370 from the supernatant of pulmonary metastasis-derived melanoma cells at passage 95 using RT
371 PCR. Genomes of all three PERV subtypes A, B and C were detected (Fig. 6B). Since no
372 recombinant PERV-A/C could be detected in spleen cells from animal #007, such viruses were
373 also not found in supernatant of the pulmonary metastasis-derived melanoma cells of this animal.
374 By electron microscopy PERV particles were found in a preparation of melanoma cells of
375 the 93rd passage (Fig. 6C). In order to analyse, whether these particles were infectious, uninfected
376 293 cells were incubated with the supernatant from cells of passage 87 showing a very high
377 PERV expression. After 42 days of incubation, no provirus was detected in 293 cells treated with
378 supernatant from passage 87; however, control cells treated in parallel with supernatant derived
379 from 293 cells producing PERV/5^{0} were infected.

380

381 *3.7. Melanoma markers in MMS Troll tissue*

382

383 Various markers were tested in order to verify that the tissue tested for PERV expression
384 contained tumour material. Since no markers for porcine melanomas were described so far, three
385 human melanoma markers, MIA (melanoma inhibitory activity), MART-1 (melanoma antigen

386 recognized by T-cells) and gp100 (recognized by cytotoxic T lymphocytes) (Perez et al., 2000)
387 were used for analysis. However, primers specific for human MIA, MART-1 and gp100 were not
388 found to be suitable for application in pigs. Since the sequences of porcine MIA, MART-1 and
389 gp100 were not available in the NCBI database, however the sequence of the porcine tyrosinase
390 was described (accession number: NM001025212), specific primers for this gene were designed
391 for the first time and used in a one-step RT-PCR. The size of the amplicon was 177bp.
392 Tyrosinase expression was analysed quantitatively, using Sybr Green real time PCR. The
393 expression of tyrosinase was always higher (up to 215 fold) in cutaneous melanomas than in
394 normal skin, lymph node and spleen (Fig. 7).
395
396 **4. DISCUSSION**
397
398 The results of this study provide first evidence for an elevated expression of PERVs in
399 melanomas of selectively bred MMS Troll characterised by a high prevalence of cutaneous
400 melanomas. In addition, a cell line derived from a pulmonary metastasis showed enhanced
401 expression as well as release of PERV. Melanomas in these animals and in other pig breeds
402 (Pérez et al., 2002) represent an excellent model system to study the genetic background, the
403 involvement of endogenous retroviruses and immunological reactions in the host.
404 This is also the first report showing recombinant PERV-A/C proviruses integrated in spleen cells,
405 but not in other cells of the same animal (Fig. 3B, C). Although numerous copies of PERVs were
406 found integrated in the genome of all pig strains (Ericsson et al., 2001; Patience et al., 2001), only
407 the human-tropic PERV-A and PERV-B were found in all strains, the ecotropic PERV-C was
408 absent in some (Niebert et al., 2005). Exogenous PERV-A/C recombinants not present in the
409 germ line were reported previously (Bartosch et al., 2004, Wood et al., 2004, Scobie et al., 2004,

Martin et al., 2006). Such PERV-A/C recombinants were associated with higher virus titers and genetic alterations in the genome (Wilson et al., 2000, Denner et al., 2003, Harrison et al., 2004). The presence of recombinants in the spleen, but not in the melanomas suggest activation, recombination and *de novo* integration mainly in lymphocytes as shown previously (Wilson et al.. 1998; Wood et al., 2004). This correlates with data showing an elevated expression of PERV in stimulated pig lymphocytes (Tacke et al., 2000, 2003) as well of murine endogenous retroviruses in immunologically stimulated mice (Hirsch et al., 1972, Denner et al., 1977).

The results showing elevated expression of full-length PERV mRNA in pig melanomas (Fig. 4A-C) are consistent with findings showing an enhanced expression of the human endogenous retrovirus HERV-K in human melanomas (Muster et al., 2003; Buscher et al., 2005, 2006). The transmembrane envelope (TM) protein of HERV-K was expressed in 50% of the tested primary tumours (Buscher et al., 2005).

An increased expression of PERV was also observed in pulmonary metastasis-derived cell cultures. The cell cultures were all amelanotic since they lost their capacity to produce melanin during the very first passages (Buettner et al., 1991). The elevated expression of PERV in this cell line resulted in release of virus particles as shown by RT activity (Fig. 6B) and electron microscopy (Fig. 6C). The presence of PERV-A, -B and -C viral genomes was shown in pelleted particles (Fig.6A). However, elevated PERV expression was not automatically associated with immortalisation of pig cells. The expression of PERV in late passages is much higher as in the well characterised cell line PK-15. In contrast, the epithelial cell line IPEC-J2, expressed only low amounts of PERV. The particles released from the melanoma cell line did not infect human 293 cells, suggesting, that they are defective or that the concentration was too low. In contrast, supernatant from 293 cells producing PERV/5^0 used as positive control, infected 293 cells.

433 Elevated expression of PERV and release of virus particles has also been shown for other pig
434 tumour cells, e.g. lymphoma cells either untreated (Suzuka et al., 1985, Suzuka et al., 1986) or
435 treated with BrdU or DMSO (Sandström et al., 1973, Moenning et al., 1974) or treated with PHA
436 and 5-azacytidine (Kaeffer et al., 1990) as well as leukaemia cells (Frazier, 1985). In addition,
437 PERV was also produced by oviduct cells (Bouilant et al., 1975) and by porcine primary aortic
438 endothelial cells (PAEC) (Martin et al., 1998a). Virus production was shown for PAEC from
439 German, Russian, French land-breed as well as from Yucatan micro and Göttinger mini pig
440 (Martin et al., 1998b). PERV-A, PERV-B, PERV-C as well as PERV-A/C were produced by
441 mitogen-stimulated PBMCs (Wilson, et al., 1998; Wilson et al., 2000; Tacke et al., 2000; Tacke
442 et al., 2003). The high expression of PERV in the spleen samples with and without metastases of
443 the animals with melanomas (Fig. 4B) may be based partially on the immunological stimulation
444 by the tumour.
445 Since analysis of a PERV-A/C recombinant derived from pig PBMCs (Wilson et al. 1998) and
446 passaged on human 293 cells showed, that an increase in virus titre was associated with an
447 increase in the number of transcription factor NF-Y binding sites in the LTR of the virus (Wilson
448 et al. 2000, Denner et al. 2003) the length of the LTR of the proviruses in the melanoma cell line
449 was studied (Fig. 4D). However, a multimerisation of the NF-Y binding sites was not observed,
450 suggesting that alterations in the expression pattern of transcription factors or demethylation was
451 reason for the enhanced expression. Methylation of CpG dinucleotides was shown to regulate
452 expression of the human endogenous retrovirus HERV-K in primary human testicular tumours
453 (Gotzinger et al., 2006).
454 The expression of the viral p15E protein in the MMS melanomas and pulmonary metastasis-
455 derived cell culture, which was shown by immunofluorescence and Western blot analysis (Fig. 5)
456 may be of great importance for tumour progression. Different retroviruses such as murine

leukaemia virus (MuLV) and immunodeficiency viruses (HIV-1 and HIV-2) were found to induce immunodeficiencies in the infected host (for review see Denner, 1987, 1998). The mechanism, how retroviruses induce immunodeficiencies, is still unclear, however there is accumulating evidence that the TM protein (p15E in the case of MuLV and PERV; gp41 in the case of HIV-1) may be involved (Oostendorp et al., 1993, Denner, 2000). Recently it was clearly shown that the expression of p15E of an endogenous mouse retrovirus is required for melanoma tumour growth *in vivo* (Mangeney et al., 2005). Similarly, the TM protein gp37 of HERV-K was found expressed on the surface of human melanoma cells (Buscher et al., 2005, 2006). The TM proteins of different retroviruses including HIV-1 have been shown to increase IL-10 production (Denner, 1998) and increased IL-10 levels were observed in melanoma patients (Dummer and Becker et al., 1995, our unpublished results).

Furthermore, PERV (and in the case of human melanomas HERV-K) may actively participate in malignant transformation. Since PERVs do not contain an oncogene, transformation may only be the result of insertional mutagenesis, either by disrupting a tumour suppressor gene or by activation of a cellular oncogene, which may contribute to the multistep process required for carcinogenesis. A murine retrovirus associated with melanomas in C57BL/6 mice for example also does not contain an oncogene, but it was found to be inserted into the c-maf proto-oncogene in transformed cells (Li et al., 1999). The situation is different in the case of HERV-K, which possess two potential oncogenes, *rec* and *Np9*. Rec encodes a 14kD protein localized in the nucleolus of HERV-K particle-producing GH teratocarcinoma cells (Lower et al., 1995). The Rec protein resembles the HIV Rev protein with respect to structural features, intracellular localization and function (Magin et al., 2000). When expressed in nude mice, Rec supports tumour growth (Boese et al., 2000). The 9kDa protein Np9 was found in biopsies of mamma

carcinomas and germ cell tumours and in transformed cell lines, but not in normal tissues (Armbruester et al., 2002).

For immunohistochemical analyses of human melanomas, a number of markers has been described (Guo et al., 1998; Perez et al., 2000; Reinke et al., 2005) among them HMB45, which recognizes the gp100 protein (Adema et al., 1994; Bakker et al., 1994). HMB45 was also successfully used for immunostaining of porcine melanomas (Fig. 2 C, D). In addition, a real-time PCR assay was developed and used to analyse porcine melanomas. Since the porcine sequences of MIA, MART-1 and gp100 were not known and primers specific for the human genes of these melanoma markers (Perez et al., 2000) did not work in the pig system (not shown), a Sybr green based real-time PCR for porcine tyrosinase was established. The importance of tyrosinase as marker for melanomas is not well known. Investigating a panel of 35 human melanomas and other tumour specimens, a sensitivity of 80%, a specificity of 100%, and an accuracy of 88%, (Guo et al., 1998) was found, whereas another study reported a sensitivity of 71%, a specificity of 40%, and an accuracy of 56% (Perez et al., 2000). At least our experience with tyrosinase in the pig model is positive, there was a higher expression of tyrosinase in melanomas and metastatic lymph nodes in comparison to normal skin, lymph node and spleen. The low expression of tyrosinase in spleens suggests that the investigated pieces of the organ did not contain many metastases and the elevated PERV expression associated with recombination and de novo integration of PERV-A/C recombinants is indeed due to immune activation.

To summarise, these data show that the melanoma of MMS Troll represent an excellent model for investigating melanomagenesis and the role of endogenous retroviruses in tumour development.

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691 **Legends**

692

693 Fig. 1. Cutaneous melanoma on the back of a MMS Troll (A), melanoma metastases in the liver
694 (B) and the lungs (C).

695

696 Fig. 2. Histology (A, B) and immunohistochemical analysis (C, D) of a cutaneous melanoma (A,
697 C) and a lymph node metastasis (B, D) of a MMS Troll. (A) Cutaneous melanoma; invasion of
698 subcutaneous adipose tissue, paraffin section, H&E after bleaching. (B) Melanoma metastasis in
699 the regional lymph node; tumour cells have largely replaced the lymphatic tissue; plastic section,
700 H&E. (C) Invasive cutaneous melanoma; melanoma cells are immunohistochemically stained
701 with the antibody HMB45 (red); scattered pigment-containing macrophages (brown), paraffin
702 section, indirect immune-alkaline phosphatase immunohistochemistry. (D) Melanoma metastasis
703 in a regional lymph node; melanoma cells are immunohistochemically stained with the antibody
704 HMB45, paraffin section, indirect immune-alkaline phosphatase immunohistochemistry.

705

706 Fig. 3. PERV provirus integration and PERV-A/C recombination. (A) Detection of PERV-A,
707 PERV-B and PERV-C proviruses in the genome of MMS animal #007 using PCR and specific
708 primers for *gag*, *pol*, *env*-A, *env*-B, *env*-C. (B, C) Analysis of the presence of two different
709 PERV-A/C proviruses in organs of animal #371 and in 293 cell infected with PERV/5^0 (293/5^0)
710 using primers VRBF and TMR (B) and VRBF and PERV envC rev (C).

711

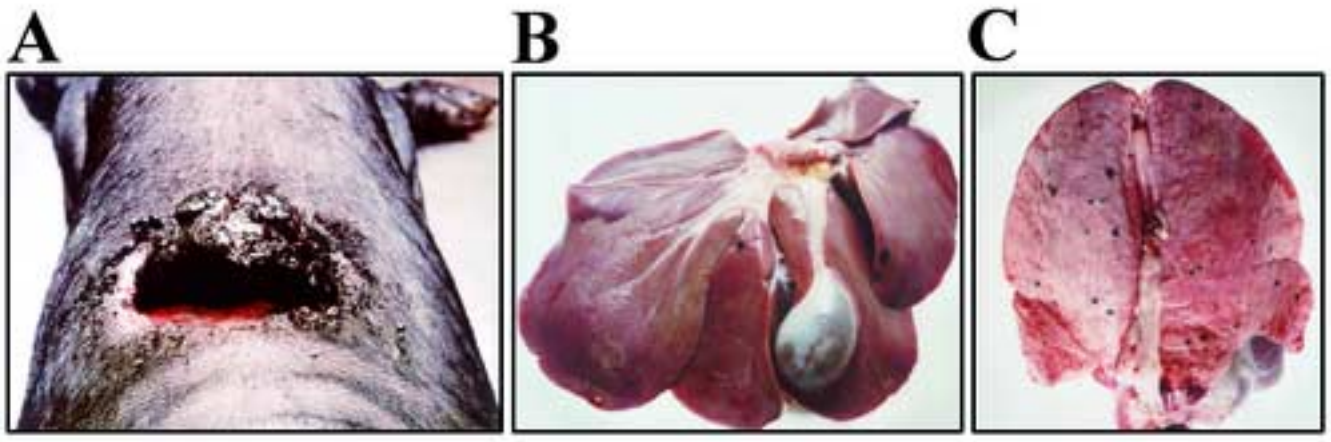
712 Fig. 4. PERV expression in melanomas and metastasis-derived cells (A) PERV expression in the
713 melanoma of the animal #372 using one-step RT-PCR, primers specifically detecting full-length
714 and spliced *env* mRNA were used. (B) PERV expression in melanomas and normal tissues of

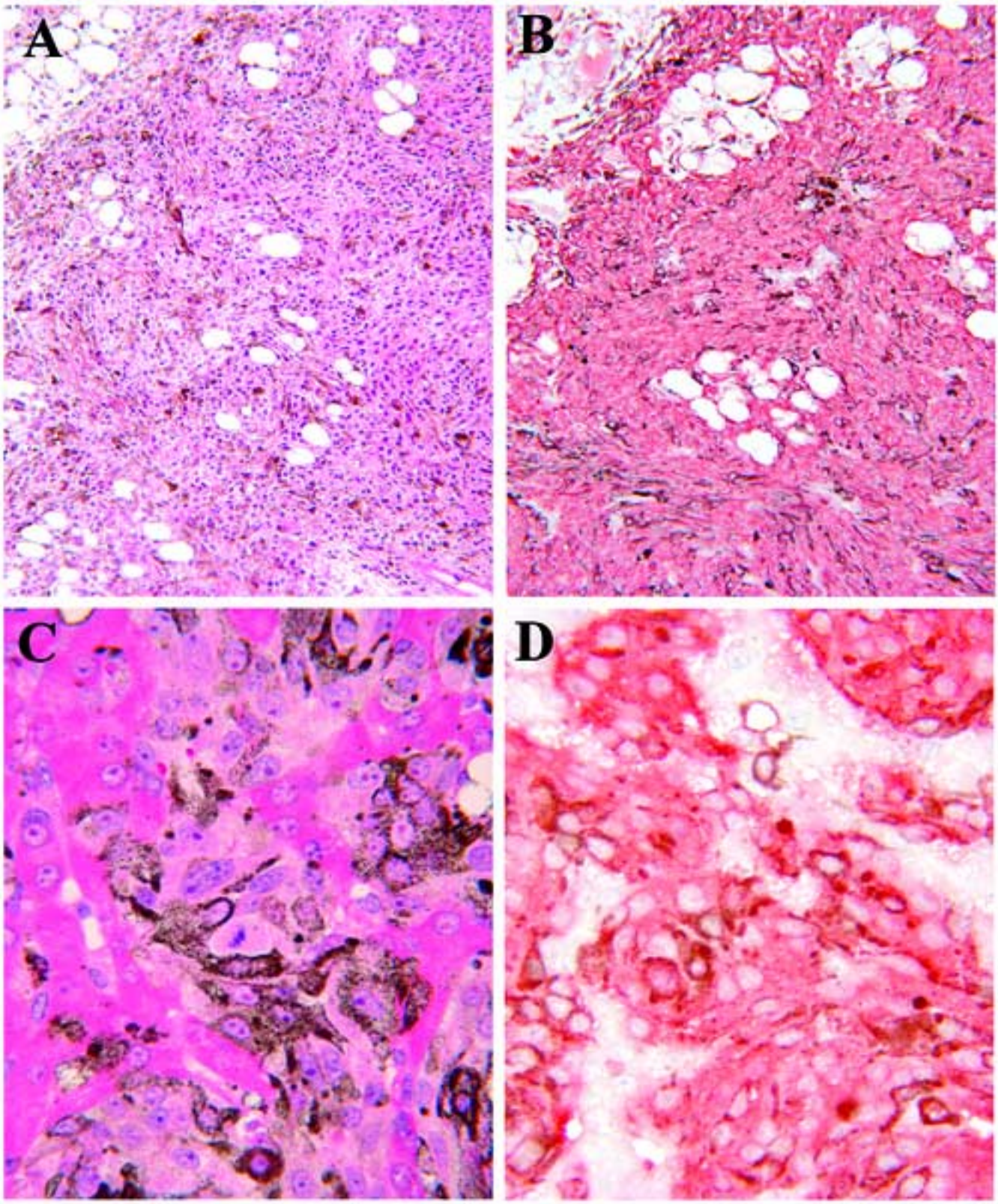
four different MMS Troll (#371, #372, #472, #499) in comparison to the expression in PK-15 cells (which was set 100%) using a quantitative one-step RT real-time PCR, data were normalised to the expression of GAPDH. In the case of animal 372 three different melanomas were analyzed. (C) Expression of full length mRNA in cells of different passages, measured by one-step RT real-time PCR normalised to GAPDH and in relation to the expression in PK-15 cells (which was set 100%). (D) Absence of alterations in the length of the LTR during passaging of metatstasis-derived cells. PCR was performed with DNA from cells in passage 21 and 87, plasmids with the LTR from PERV/3^0 and PERV/5^0 were used as controls.

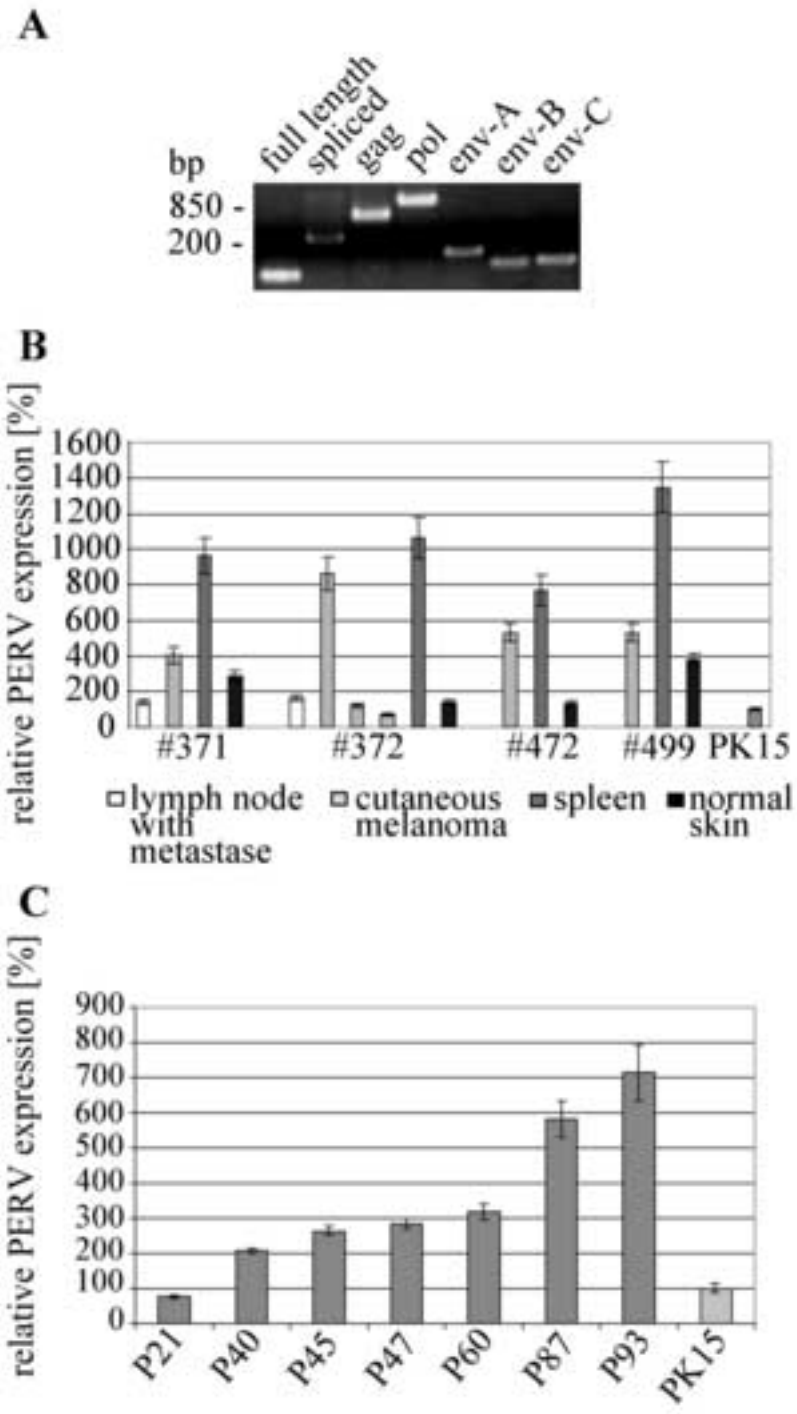
Fig. 5. (A) Expression of p15E protein in a primary melanoma and normal skin of animal #372 and (B) in cells of different passages (P) of metatstasis-derived cells as measured by Western blot analysis using a specific antiserum. Pelleted PERV (virus) and recombinant p15E (rp15E) were used as controls. β-Actin antibodies were used to verify loading of equal protein amounts. (C) Detection of p15E protein expression by immunofluorescence in a porcine pulmonary metastasis-derived cell culture at passage 87 using a specific antiserum for p15E. (a) non-permeabilised, (b) (c) permeabilised cells, different magnification and (d) permeabilised PK-15 cells.

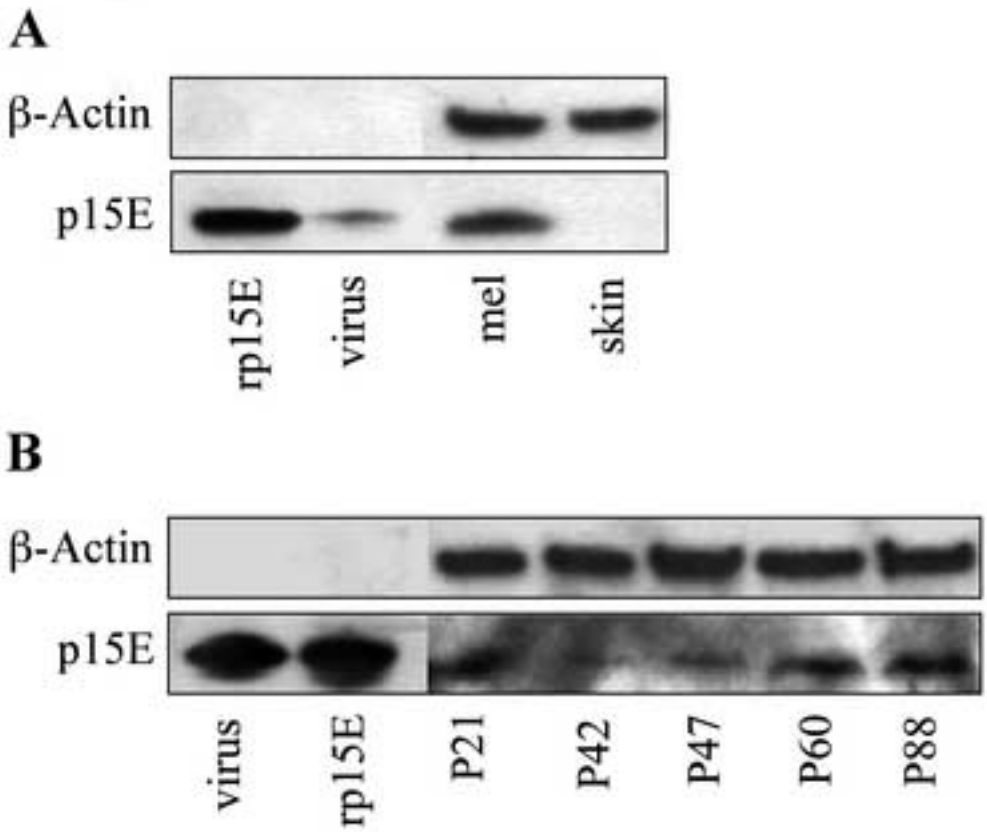
Fig. 6. Release of virus particles from melanoma cells. (A) RT activity in the supernatant of pulmonary metastasis-derived cells of different passages as measured by a C-type RT activity assay. (B) Detection of PERV-A, -B and -C in virus pellets from supernatants of these cells at passage 95. (C) Detection of PERV particles in two (a,b) pulmonary metastasis-derived cell culture preparations (passage 93) by transmission electron microscopy.

738 Fig. 7. Expression of porcine tyrosinase in melanomas, other tissues and normal skin of four
739 different MMS using a Sybr Green RT real time PCR. The value for normal skin was set 1.
740

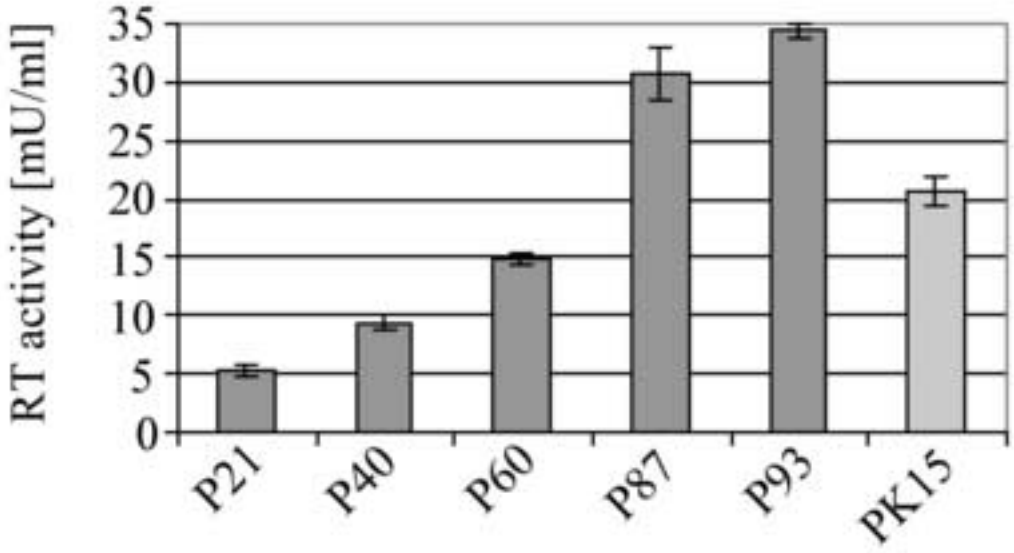








A



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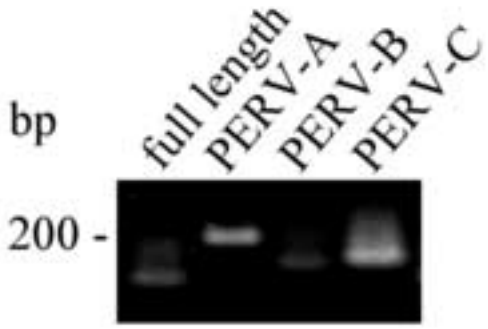

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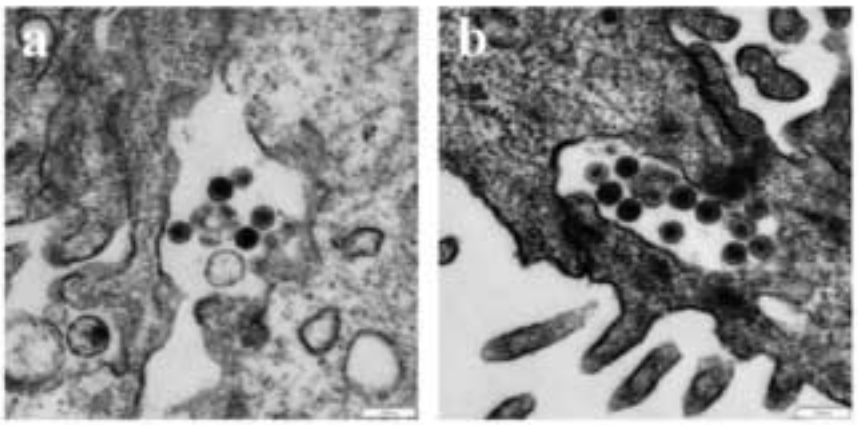

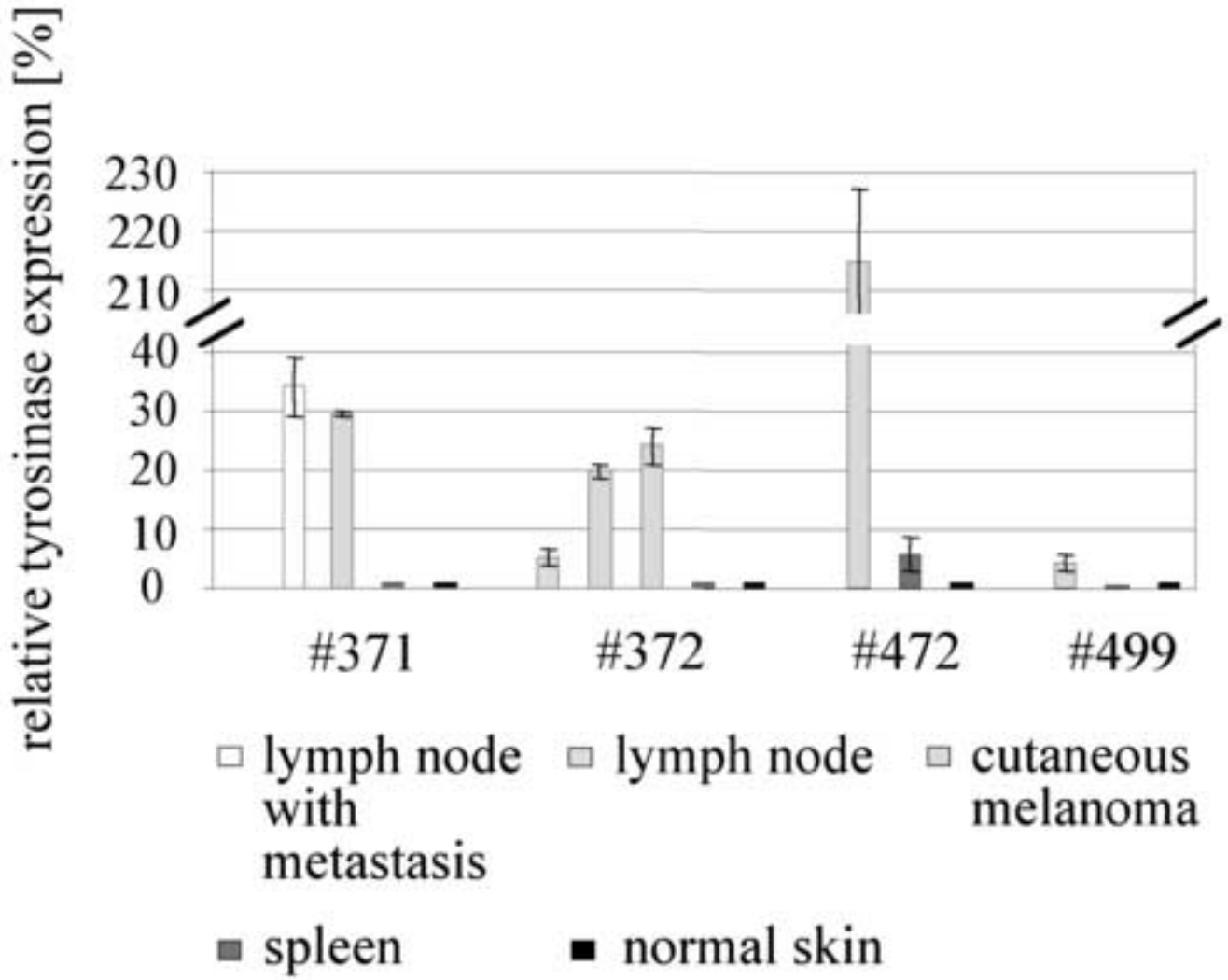

Table 1 Cutaneous melanocytic lesions and metastases in different organs

Animal	Skin	Regional lymph nodes	Spleen	Metastases in other organs
#371	1 melanoma multiple nevi	metastasis	metastases	lungs, heart, intestine, liver, kidney
#372	3 melanomas multiple nevi	metastasis	metastases	tongue, lungs, heart, stomach, intestine, liver, kidney
#472	5 melanomas multiple nevi	metastasis	metastases	lungs, stomach, intestine, liver and kidney
#499	1 melanoma multiple nevi	metastasis	none	none