Pigmented uveal tumours in a transgenic mouse model

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Abstract

Aims/background—The authors have developed transgenic mouse strains at the Arizona Cancer Center using a tyrosinase promoter to target expression of the mutated T24 Ha-ras gene in melanin producing cells. Histopathology and electron microscopy (EM) were performed to characterise the intraocular tumours observed phenotypically.

Methods—Transgenic TPras mice (n=8) and normal, age matched control mice (n=6) were sacrificed at 3 weeks, 6 weeks, 7 weeks, 4 months, 5 months, 9 months, and 11 months. Six were processed in formalin for light microscopic examination and eight in a glutaraldehyde/formalin solution for electron microscopic examination.

Results—Six of the TPras mice were found to have bilateral pigmented melanocytic/RPE proliferations of the uveal tract. The cytological characteristics of the tumours included low nuclear to cytoplasmic ratios (N:C ratios), bland nuclei, and abundant intracytoplasmic melanin. By EM two populations of cells were identified, including spindle-shaped cells with round to oval melanin granules and cuboidal cells with apically located, cigar-shaped, melanin granules, and basement membrane formation. A 3 week and an 11 month old TPras mouse had a higher grade, bilateral, melanocytic proliferation of the uveal tract which, although not metastatic, was morphologically melanoma. Cytological features included increased N:C ratios, nuclear pleomorphism, and prominent nucleoli. The uveal tract was normal in both eyes in all of the control animals.

Conclusion—Pigmented intraocular tumours developed in transgenic strains of mice that express a mutated Ha-ras gene in melanin producing cells. The morphology was most consistent with a melanoma in two of the mice and a benign melanocytic/RPE proliferation in the remaining mice.

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Choroidal melanoma is a primary intraocular cancer and is the most common ocular cancer among adults. Ocular cancers account for approximately 1% of all new cancers cases in the United States each year.[1] Other less common intraocular cancers in adults include melanoma of the iris and ciliary body. Although rare, this condition is of special concern within the field of ophthalmology because of its life threatening potential.

Primary ocular melanoma can arise from the choroid, ciliary body, iris, conjunctiva, or less commonly from the orbital structures, lacrimal system, cornea, and retina. It is the most common primary malignancy of the eye in adults.[2] There are several animal models of intraocular melanoma which have proved useful in studying tumour development and therapeutic modalities.[3] In addition, several research groups have been successful in developing experimental mouse[4–6] or transgenic mouse lines[7–11] for the study of intraocular pigmented tumours and melanoma. The latter authors used a tyrosinase promoter to regulate expression of the SV40 early region transforming sequences in melanocytes. The mice they generate develop pigmented ocular tumours at a young age. We have developed transgenic mouse strains at the Arizona Cancer Center using a tyrosinase promoter to target

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Table 1   *Summary of the pathological findings in the TPras mice and the control mice*

Number	Age	Type	Ocular pathology	Neuropathology	EM
			Pathological findings in mice eyes		
1	3 weeks	ras	iris/ciliary body moderately pigmented melanoma	normal	no
2	6 weeks	ras	melanocytic/RPE hamartoma	benign melanocytic proliferation	yes
3	7 weeks	ras	melanocytic/RPE hamartoma	no	yes
4	5 months	ras	melanocytic/RPE hamartoma	normal	yes
5	4 months	ras	melanocytic/RPE hamartoma	no	yes
6	9 months	ras	melanocytic/RPE hamartoma	benign melanocytic proliferation	no
7	11 months	ras	melanocytic/RPE hamartoma	normal	no
8	11 months	ras	iris/ciliary body moderately pigmented melanoma	no	yes
9	3 weeks	normal	normal	normal	no
10	5 weeks	normal	RPE proliferation	normal	yes
11	3 months	normal	normal	normal	yes
12	9 months	normal	normal	normal	no
13	9 months	normal	normal	no	yes
14	11 months	normal	normal	no	no

expression of the mutated T24 Ha-ras gene in melanin producing cells.[10] Two independent founder mice were found to have a distinct phenotype with muted agouti coat colour, deeply pigmented skin with multiple naevi and "twirling behaviour". Histopathology of the tissue revealed hyperpigmentation and/or melanocytic hyperplasia in the skin, eyes, inner ear, and meningeal membranes in the brain. Histopathology and electron microscopy (EM) were performed to characterise the intraocular tumours observed phenotypically. In this paper, we report the histopathological characterisation of the ocular tumours.

Methods

TPRAS TRANSGENIC MICE

An expression vector using mouse tyrosinase promoter sequences to drive expression of the mutated human Ha-ras (T24) gene was used to generate the TPras transgenic mice. The pTPras construct contains a 2.5 kilobase (kb) tyrosinase promoter fragment isolated from the plasmid GL3.[12] The promoter fragment was

Figure 1 (A) Light micrograph of melanocytic hamartoma in the uveal tract of TPras transgenic mice. The melanocytic proliferation is seen to fill the entire uveal tract. Cells are deeply pigmented and morphological features are not readily discernible (haematoxylin and eosin, original magnification ×10). (B) High power light micrograph of the bleached specimen demonstrates the cytological characteristics of the melanocytic/RPE hamartoma with oval nuclei with bland nuclear characteristics (haematoxylin and eosin, original magnification ×10). Inset (C): Bleached specimen demonstrates a proliferation of plump polyhedral to spindle-shaped cells diffusely filling the uveal tract.

ligated into pR8-T24, a promoterless construct which contains the genomic sequences of the mutant T24 c-Ha-ras gene[13] in the pIC-20R vector. A 6.5 kb fragment isolated from the pTPras plasmid was used for microinjection to generate the transgenic mice. The exact procedure used to generate the transgenic mice has been completely described by Powell and colleagues.[10]

The mice used for this study are on C57BL/6XSJL X C3He/N background. Expression of the T24 Ha-ras mRNA is detected in tissues containing melanocytes such as skin, eyes, and meningeal membrane. The mice exhibit a distinct phenotype consisting of a muted agouti coat colour, pigmented skin, and twirling behaviour.[10] Mice were randomly chosen to represent a range of ages and sacrificed for pathological evaluation. All animals were treated in accordance with the ARVO resolution on animals used in research.
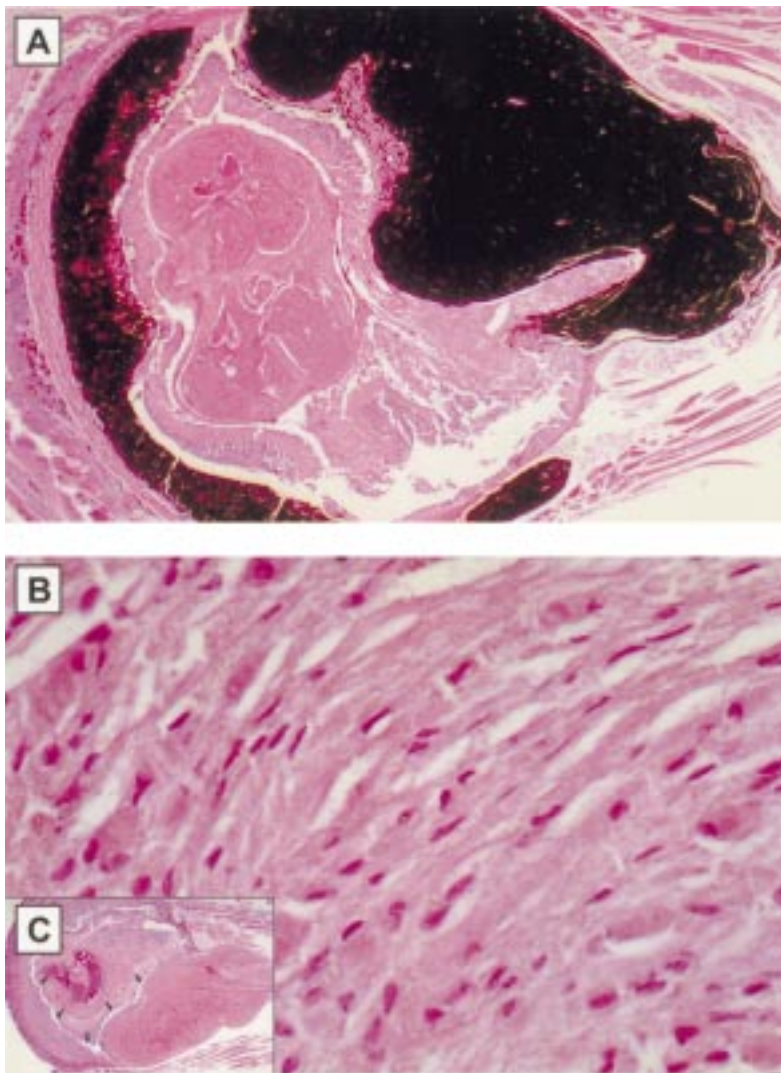
LIGHT AND ELECTRON MICROSCOPIC STUDIES

Whole mice heads were fixed in 10% buffered formalin. Specimens were then dehydrated in a series of alcohols and embedded in paraffin. Serial sections 4–6 μm thick were subsequently stained with haematoxylin and eosin for routine light microscopic evaluation. Selected samples were also fixed in 2.5% glutaraldehyde/0.1 molar cacodylate buffer solution, postfixed in osmium tetroxide, and subsequently embedded in LX 112 resin (Ladd Res Ind Inc, USA) for transmission electron microscopy. For transmission electron microscopy, semithin sections (1–2 μm) were stained with toluidine blue and sodium borate (1%) and examined by light microscopy. Ultrathin sections (60–70 nm) were stained with aqueous uranyl acetate followed by lead citrate and examined using a Jeol 100CX electron microscope.
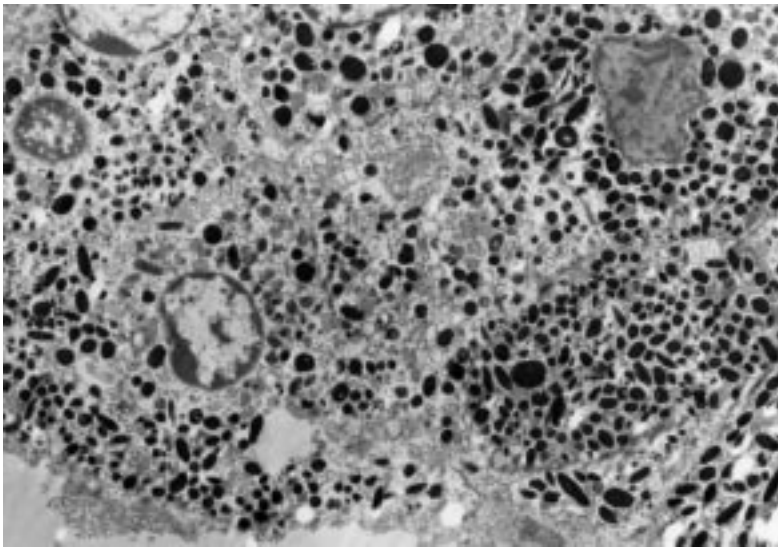
Results

Bilateral intraocular tumours arose in the TPras mice. The tumours were identified histopathologically and ultrastructurally as melanoma and mixed melanocytic/retinal pigment epithelial (RPE) hamartomas. In addition, a benign melanocytic proliferation of the choroid plexus was identified in two of the TPras mice. In this report, we examine the ocular tissues from TPras transgenic mice at several different ages and compare them with age matched negative littermates. Table 1 summarises the histopathological and ultrastructural findings in the TPras mice (n=8) and the control mice (n=6). The ocular tissue examined from one of the normal control littermates contained an RPE proliferation.

Six of the TPras mice were found to have pigmented hamartomatous proliferations of the uveal tract. These were composed primarily of proliferations of heavily pigmented, spindle-shaped melanocytes with low nuclear to cytoplasmic ratios, bland nuclei, and abundant intracytoplasmic melanin pigment (Fig 1). Occasionally, more atypical cytological characteristics were observed in these pigmented hamartomatous proliferations. These

Figure 2 An electron micrograph of a melanocytic hamartoma in the uveal tract of TPras transgenic mice. Both spindle cells and cuboidal cells with oval nuclei containing marginated heterochromatin are identified. Intracytoplasmic membranous vesicles, premelanosomes, phagosomes, and cigar-shaped and oval melanin granules are identified. The melanosomes measure between 0.4 and 1.50 µm. Atypical nuclear features are identified in one cell (lower left) which contains a prominent nucleolus (original magnification ×5510).

tumours massively filled the entire uveal tract. Four of the six tumours were examined by electron microscopy in addition to histopathological examination. The majority of the cells identified had ultrastructural characteristics which included spindle-shaped cells with oval to round nuclei and numerous intracytoplasmic oval melanosomes and premelanosomes (Fig 2). This cell type did not display intercellular junctions or basement membrane formation. Less frequently, cells which had cuboidal shape with rounded nuclei, apical to basal polarity, and intracytoplasmic cigar-shaped melanin granules were identified. These cells displayed intercellular junctions and basement membrane formation.

The remaining two TPras mice, a 3 week and an 11 month mouse had a higher grade melanocytic proliferation which was morphologically melanoma. The tumours were present in the iris, ciliary body, and the anterior chamber and were characterised histopathologically as a proliferation of moderately pigmented uveal melanocytes (Fig 3). Cytological features included increased nuclear to cytoplasmic ratios, nuclear pleomorphism, and prominent nucleoli. Ultrastructurally, the cells were spindle shaped with oval to round nuclei, increased nuclear to cytoplasmic ratios, marginated chromatin, irregular nuclear envelope, and prominent nucleoli. Intracytoplasmic melanin granules were demonstrated. The round to oval melanosomes measured 0.53 µm by 0.87 µm (range 0.41–0.75 µm and 0.75–1.08 µm) (Fig 3). The uveal melanocytic tumour in the 11 month TPras mouse also contained melanophages and areas of retinal pigment epithelial proliferation. None of the mice were shown to have metastatic tumours.

Two of the six TPras mice were also noted to have benign melanocytic proliferations in the brain. These were characterised histopathologically as small proliferations of moderately
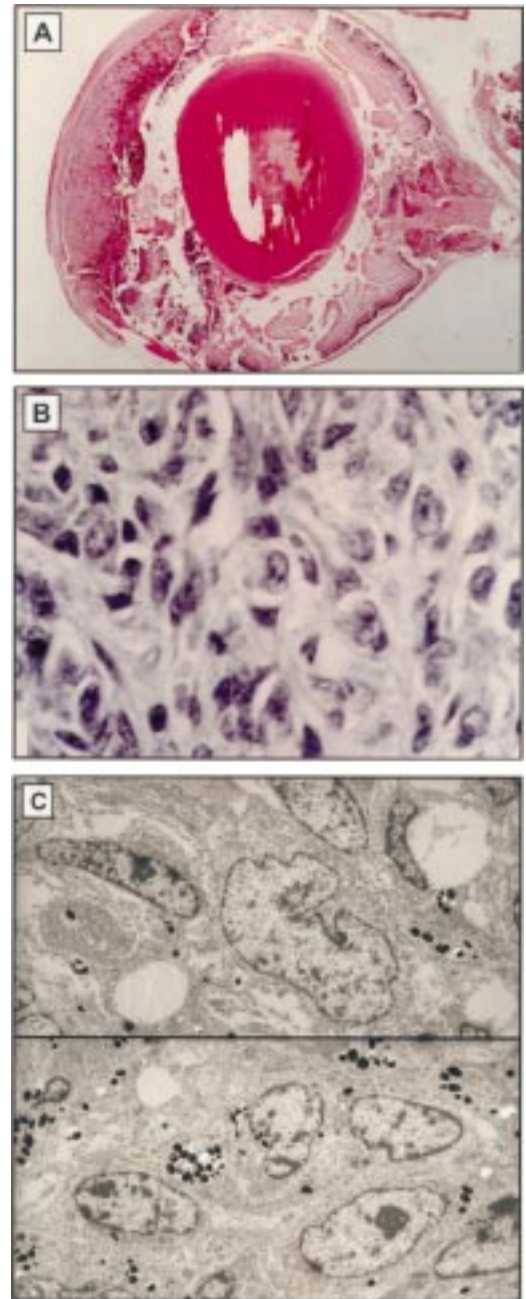
Figure 3 (A) Light micrograph of melanoma in the uveal tract of TPras transgenic mice. The melanocytic proliferation is seen to fill the entire uveal tract. Cells are moderately pigmented and morphological features are not readily discernible (haematoxylin and eosin, original magnification ×10). (B) High power light micrograph of the bleached specimen demonstrates a proliferation of pleomorphic, spindle-shaped cells. The cytological characteristics of the malignant melanoma in the 3 week TPras mouse are demonstrated. (C) Ultrastructurally, the cells are spindle shaped with oval to round nuclei, increased nuclear to cytoplasmic ratios, marginated chromatin, irregular nuclear envelope, and prominent nucleoli. Intracytoplasmic melanin granules are demonstrated (haematoxylin and eosin, original magnification ×160).

to heavily pigmented melanocytes in the choroid plexus. These melanocytes were entirely bland and morphologically characterised as plump polyhedral cells similar to naevus cells. Ultrastructural examination was not done because of the benign morphology on histopathological examination.

The uveal tract was normal in all six of the control animals (negative littermates).
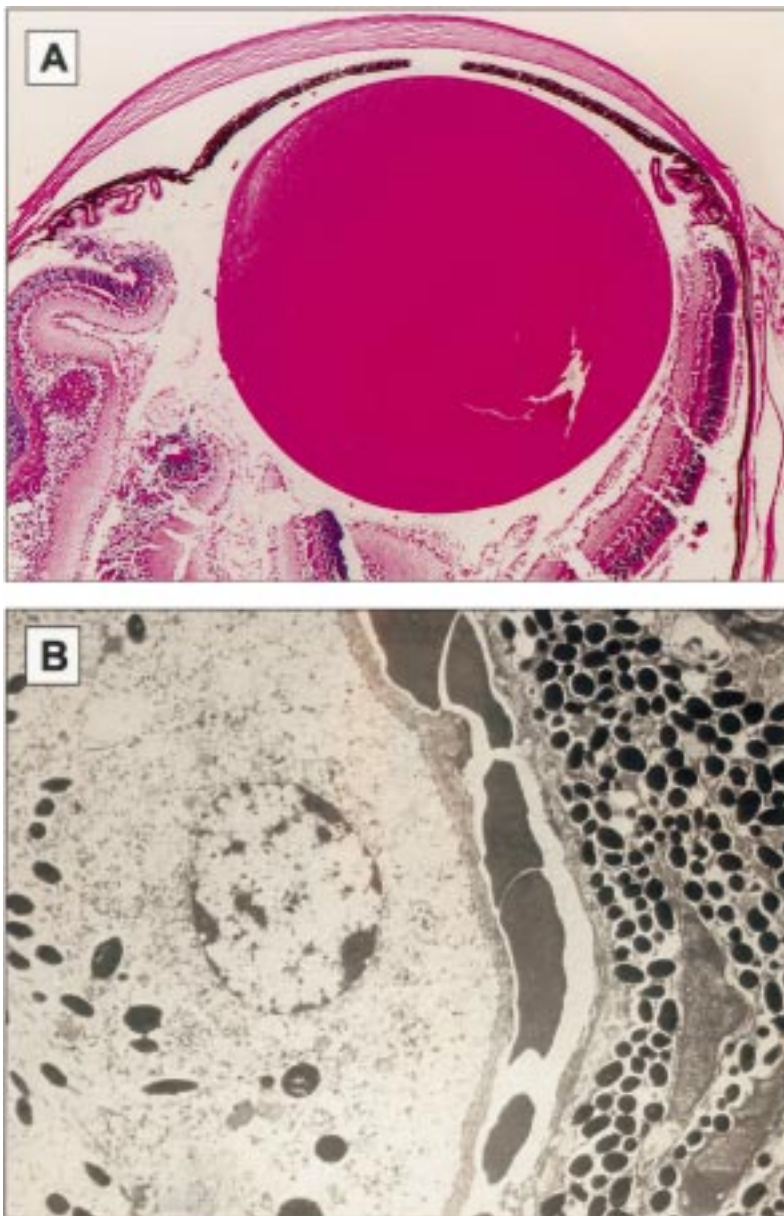
Figure 4   (A) Photomicrograph of the anterior uveal tract in one of the control (negative littermate) mice. The iris ciliary body and choroid are pigmented and normal in structure. There is no evidence of infiltration hyperplasia or hypertrophy of the uveal tract (original magnification ×44). (B) Electron micrograph demonstrating the retinal/choroidal junction in one of the control mice. Choroidal melanocytes are spindle-shaped cells with oval nuclei containing a bland chromatin pattern. Intracytoplasmic round and oval melanin granules are identified. The melanosomes measure 0.50 μm by 0.75 μm. The RPE is composed of cuboidal cells with oval nuclei containing marginated heterochromatin. There is apical to basal polarity and basement membrane formation. Intracytoplasmic rough endoplasmic phagosomes and membranous vesicles are identified. Intracytoplasmic cigar-shaped and oval melanin granules are identified and measure 0.44 μm by 1.58 μm (original magnification ×9180).

Microscopically, the uveal tract was composed of arteries, veins, and melanocytes amid loose stromal connective tissue (Fig 4). Ultrastructural examination was completed on the retinal/choroidal junction. Choroidal melanocytes were spindle-shaped cells with oval nuclei containing a bland chromatin pattern. Intracytoplasmic cigar-shaped and oval melanin granules were identified. The melanosomes measured 0.40 μm by 1.50 μm (range 0.27–0.44 μm by 1.09–2.03 μm). The RPE was composed of cuboidal cells with oval nuclei containing marginated heterochromatin. There was apical to basal polarity and basement membrane formation.

Intracytoplasmic rough endoplasmic phagosomes and membranous vesicles were identified.

An RPE proliferation, confirmed by electron microscopy, was discovered in one control mouse (5 weeks). This was characterised, histopathologically, as a melanotic proliferation of RPE cells between the retina and choroid. The cells were noted to form tubuloacinar configurations. Ultrastructural characteristics included apical to basal polarity, abundant basement membrane proliferation, and intercellular junctional complexes (Fig 5).

Discussion

In order to better understand the pathobiology of uveal malignant melanoma, numerous investigators have developed models of ocular melanoma. These models have been reviewed extensively and the potential limitations and advantages of each model have been discussed by Albert *et al*.[14] These have included hetero-transplantation models,[15–18] models induced by a DNA oncogenic virus (papova, C-type, retrovirus), and chemically induced models.[19–22] In addition, models with transplantation of melanoma cells into the anterior chamber and the posterior compartment of the eye have been established.[5 6] In the model of Grossniklaus,[6] mice were inoculated in the anterior chamber or posterior compartment with B16F10 melanoma cells. Melanoma was demonstrated in both compartments and these melanomas metastasised to the lungs. Newer models of ocular melanoma have included transgenic mouse models. These models are based on using the promoter region of the tyrosinase gene to target expression of oncogenes to pigment producing cells of different origins. Most of the models have been successful in producing intraocular pigmented tumours including RPE tumours, carcinomas, and ocular melanosis.[7–11] These models are summarised in Table 2.

Powell *et al*, in an attempt to develop an animal with cutaneous melanoma, reported hyperpigmentation and melanocytic hyperplasia in transgenic mice expressing the human T24 Ha-ras gene regulated by a mouse tyrosinase promoter.[10] The mutated Ha-ras was used to generate the transgenic mice based on observations of the occurrence of ras mutations in human cutaneous melanoma.[23] Phenotypically, these transgenic mice displayed a muted agouti coat, highly pigmented skin, and a twirling movement. Although no cutaneous melanoma developed, primarily benign melanocytic lesions were observed in the skin and tissues of the inner ear and uveal tract.[10] In our study, we determined that the cellular composition of the spontaneously arising pigmented ocular tumours that developed in the TPras transgenic mice expressing a mutated Ha-ras gene represented a proliferation of melanocytes intermixed with RPE cells. The cytological characteristics of the tumour cells included a spindle/dendritic shape with abundant intracytoplasmic melanosomes and premelanosomes. These cells had round to oval melanin granules. A second population of cells were cuboidal with cigar-shaped
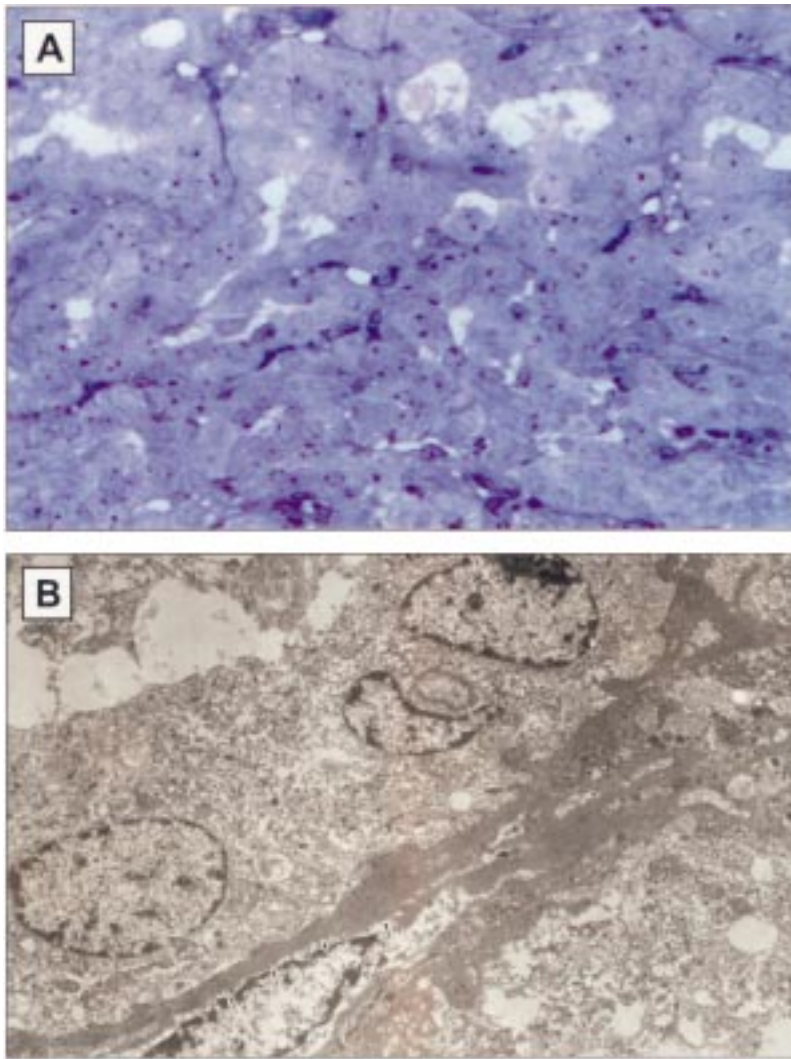
Figure 5   (A) Light micrograph demonstrating a retinal pigment epithelial adenomatous proliferation found in the uveal tract of a normal control litter mate. (B) Histopathologically, this was characterised as a melanotic proliferation of RPE between the retina and the choroid. The cells were noted to form tubuloacinar configurations (haematoxylin and eosin, original magnification ×160).

human eye. In this respect, several types of pigment containing cells are generally recognised within the eye, including iris pigment epithelium, pigmented ciliary epithelium, retinal pigment epithelium, and uveal melanocytes. Pigmented epithelial cells and melanocytes of the eye have different morphological characteristics, are known to arise embryologically from different tissue origins, and behave in biologically distinct ways.

Uveal melanocytes are generally recognised as dendritic cells with a spindle shape and an elongated, oval nucleus containing peripherally marginated chromatin. Numerous oval melanosomes are present in the cytoplasm and many of the melanosomes contain a striated substructure.[24 25] Intracytoplasmic melanin granules in uveal melanocytes measure 0.6–0.8 μm in diameter.[26] Melanocytes residing in skin, iris, and choroid are neural crest derivatives. Uveal melanocytes do not begin to produce pigment until late in embryological development and continue to produce pigment after birth.[24] Intraocular pigmented epithelial cells are morphologically epithelial rather than dendritic. The RPE cells contain larger ellipsoidal to needle-shaped pigment granules.[24 27] Intracytoplasmic melanin granules in the RPE are cigar shaped and measure 1 μm in diameter and 2–3 μm in length.[27] RPE cells arise from the outer layer of the optic cup. Melanin granules are found in the RPE at about the 10th week of gestation.[27] Between the eighth and 14th week of gestation, granules of all stages can be found including premelanosomes and melanosomes.[28] The RPE density also increases from birth to age 2 years.[29] In the mouse, the first pigment in the retinal region appears around day 11–11.5 of gestation.[30 31]

Uveal malignant melanoma is a cellular proliferation of cytologically malignant melanocytes. The cytological characteristics of uveal malignant melanoma were originally described by Callendar,[32] who recognised two main cell types, spindle and epithelioid. Spindle-type cells of uveal malignant melanoma are fusiform and usually arranged in tightly cohesive bundles. These spindle-type cells have been divided into subtypes A and B. Spindle A cells have a small, slender nucleus with a fine, delicate, chromatin structure, and ill defined or absent nucleoli. Often a nuclear fold passing along the length of the nucleus giving the appearance of a nuclear streak. Mitotic figures are rarely seen. Spindle B cells have a larger, plumper nucleus containing a coarse chromatin network and a small round, sharply defined, eosinophilic nucleolus. Mitotic figures are often observed. Callendar's

melanin granules characteristic of RPE cells. Some of the cells had intercellular junctions and basement membrane formation characteristic of pigment epithelial cells such as iris pigment epithelium, RPE, and ciliary pigment epithelium. These cells also displayed apical to basal polarity and abundant basement membrane formation typical of epithelial cells. Thus, we have concluded that the majority of the cellular proliferations in the uveal tract of the transgenic mice represented a combined melanocytic/RPE proliferation.

The identification of cell type in the uveal tumours of the TPras mice was based on morphological and ultrastructural characteristics of the intraocular pigmented cells in the

Table 2   Transgenic model of ocular tumours

| | | | | Predominant cell type | |
Investigator	Model name	Target gene	RPE	Melanocytes	Metastases
Bradl, Klein-Szanto, 1991	Tyr-SV40E	SV40	RPE proliferations	Not reported	Numerous organs
Anand, 1994	SV40	SV40	RPE proliferations	Not reported	Liver
Syed, 1995,1996	Tyr-Tag A, Tyr-Tag B	SV40	RPE, spindle, and epithelioid cells	Not reported	Muscle and spleen in Tag A mice
Powell, 1995	Tpras	T24 Ha-ras	RPE	Melanocytes and melanoma	Lung and lymph nodes (induced skin)

epithelioid cell is a larger, polygonal cell with considerable variation in both size and shape. These cells have abundant glassy cytoplasm. The nuclei of these cells are typically larger and rounder and the nuclear envelope is more angular with irregular indentations and outpouchings. The chromatin is very coarse and marginated. A very large eosinophilic nucleolus is present in the centre of the nucleus. Mitotic figures are abundant. Gamel *et al* [33] [34] further defined the nuclear characteristics of the various malignant melanoma cells. The modified Callendar classification now recognises spindle cell naevus, spindle cell melanoma, mixed cell melanoma, and epithelioid cell melanoma. [35]

In our study, we found that the majority of the uveal tumours were composed of a benign mixed melanocytic/RPE cell population in all but two of the TPras mice. However, focal areas of cytologically atypical cells were identified in many tumours. The remaining two TPras mice (the 3 week and 11 month old) had a higher grade melanocytic proliferation which was morphologically identical to melanoma. The uveal tumour was characterised histopathologically as a proliferation of moderately pigmented uveal melanocytes with malignant cytological features including increased nuclear to cytoplasmic ratios, nuclear pleomorphism, coarse chromatin network with margination, and prominent nucleoli. Pigment production in these cells was variable. Although the pigmented uveal tumour in two of the TPras transgenic mice most closely resembled human uveal malignant melanoma, true malignancy cannot be confirmed without known evidence of metastasis. We did not demonstrate metastases of the ocular tumours; however, we have found metastases to the lung and lymph node, from DMBA induced cutaneous melanoma in these same transgenic mice (Powell, personal communication). Additionally, injection of cell lines from these cutaneous tumours into SCID mice results in melanocytic tumours that invade adjacent muscle. [36] In ongoing studies, we will inject these melanocytes into the anterior chamber and posterior compartment of semisyngeneic mice and assess for metastases according to the model of Grossniklaus *et al*. [5] [6]

Clinically, in humans, there exists a spectrum of pigmented intraocular tumours, including those derived from melanocytes and those derived from pigment epithelial cells. In order to best characterise the melanocytic proliferations in the TPras transgenic mice, we have reviewed the histopathological and cytological characteristics of the spectrum of pigmented intraocular tumours known to occur clinically in humans. [37–65] Included in those presumably arising from the neural crest melanocytes are malignant melanomas, melanosis oculi, melanocytoma, diffuse choroidal spindle cell naevus, diffuse melanocytoma, and diffuse melanocytic uveal tumours associated with systemic malignant neoplasms. [37–52] [58] [65] The benign tumours all are hamartomatous proliferations of uveal melanocytes and malignant melanoma is believed to occur because of the malignant transformation of uveal melanocytes. Tumours of the RPE are mainly a result of hypertrophy and hyperplasia which most often occur as degenerative or reactive processes. [27] Primary hypertrophy of the RPE can also occur in humans and has been categorised into congenital or acquired and is manifested by several patterns, including solitary hypertrophy of the RPE, congenital hypertrophy of the RPE, acquired hypertrophy of the RPE. Hyperplasia of the RPE has also been subcategorised into localised hyperplasia of the RPE and diffuse hyperplasia of the RPE. [27] Finally, benign tumours of the RPE that are known to occur in humans include adenoma of RPE, [27] [62–64] combined hamartoma of the retina and RPE, [59–64] congenital hypertrophy of the RPE or grouped pigmentation. [62–64] Rarely, primary neoplasia of the RPE, defined by specific clinical and histopathological criteria is known to occur. [64]

It is difficult to ascertain with certainty what the human correlate of the uveal tumours in the transgenic mice is; however, the presence of cells morphologically identifiable as melanocytes, RPE cells, and melanoma cells as well as a spectrum of cytological atypia is intriguing. Two human clinical entities seem to be the most similar. Haas and coworkers described the histopathological and ultrastructural features of a diffuse choroidal melanocytic hamartoma in a 10 year old boy. [38] This tumour was composed of polygonal hyperpigmented cells and spindle-shaped cells. The cells contained well melanised melanosomes. Diffuse malignant melanoma represents 4.5% of all intraocular malignant melanomas, is more heavily pigmented, causes more RPE disturbance, grows at a faster rate, and the clinicopathological features have been reviewed by Font *et al*. [65] Both of these human entities resemble histopathologically the tumours found in the TPras transgenic mice.

This transgenic mouse model of ocular melanocytic proliferations is distinct from other transgenic models, which have been primarily proliferations of retinal pigment epithelial cells rather than mixed RPE/melanocytic tumours. In addition, our transgenic model is the only model that consistently produces a spectrum of benign melanocytic/RPE hamartoma, morphologically atypical melanocytic/RPE proliferations, and cytologically malignant melanoma. It is difficult to ascertain the reason for the alterations in the primary pigmented cell that is proliferating in these various transgenic models. We suspect that the expression of the SV40 gene and the Ha-ras gene during embryologic development occurs at different times, as does the production of pigment and proliferation of uveal melanocytes and RPE cells. Perhaps, expression of the different genes at different embryological stages allows for the selective or partially selective proliferation of melanocytes over RPE cells.

In future studies, we intend to use this model to study the process of malignant transformation of uveal melanocytes to melanoma. We have already established cell culture lines of both the benign melanocytes and/or RPE cells from the melanocytic/RPE hamartomas and we intend to establish cell cultures of the melanoma cells from the malignant melanomas. As the biology of ocular melanoma and molecular mechanisms involved in malignant transformation are poorly understood, we hope to use this model along with microarray technology[66] to study the molecular alterations occurring during the process of malignant transformation. Should metastases develop after injection of tumour cells into the anterior and posterior compartment, we hope to analyse the molecular alterations in metastatic tumours and compare them with primary tumours. We believe that this unique transgenic model of spontaneously arising pigmented intraocular tumours may be valuable in elucidating the pathobiological mechanisms involved in the process of malignant transformation.

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