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INVITED REVIEW

Cross-species models of human melanoma

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Abstract

Although transformation of melanocytes to melanoma is rare, the rapid growth, systemic spread, as well as the chemoresistance of melanoma present significant challenges for patient care. Here we review animal models of melanoma, including murine, canine, equine, and zebrafish models, and detail the immense contribution these models have made to our knowledge of human melanoma development, and to melanocyte biology. We also highlight the opportunities for cross-species comparative genomic studies of melanoma to identify the key molecular events that drive this complex disease.

Keywords: melanoma; cross-species analysis; genome analysis

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Introduction

Melanocytes produce melanin that protects skin from the effects of ultraviolet light, and also reside as part of mucosal tissues at sites such as the lower bowel, anus, vulva, mouth, and upper aero-digestive tract. Melanocytes are also found in the uvea/iris of the eye and in the inner ear.

Genetic predisposition to melanoma in humans

Cutaneous melanoma is largely a malignancy of fair-skinned people with familial and sporadic genetic risk factors. Population-based genome-wide association studies (GWAS) have been particularly informative at defining the relevant melanoma risk regions in the sporadic disease, and to date, around 20 genome-wide significant loci have been identified [1,2]. These include regions surrounding the melanocortin 1 receptor (*MC1R*) and tyrosinase (*TYR*) genes. MC1R is a G-protein coupled receptor located in the plasma membrane that plays an important role in controlling the microphthalmia-associated transcription factor (*MITF*) gene (Figure 1). Disruptive mutations of *MC1R* are associated with red hair, freckling, and sun sensitivity due to a failure in the processing of red/yellow pheomelanin to brown/black eumelanin. Importantly, the function of *MC1R* is highly conserved across species and contributes to skin colouration in a range of higher vertebrates and in fish [1,3]. Tyrosinase is the rate-limiting enzyme in the production of melanin (variants of which include eumelanin and pheomelanin, described above); *TYR* is transcriptionally regulated by MITF binding to its promoter. The oxidase activity of tyrosinase converts dopa to dopaquinone, a precursor of melanin. Mutations in tyrosinase result in a rare disorder called oculocutaneous albinism [4,5], which is associated with ultraviolet (UV) sensitivity, while common variants associated with blue eyes have been linked to melanoma predisposition by GWAS [2]. In addition to the *MC1R* and *TYR* genes, a variant (E318K) in the aforementioned *MITF* gene is associated with increased melanoma risk in sporadic and familial cases, and is an intermediate genetic risk factor [6]. Genes linked to naevus density have also been implicated in disease development (such as *PLA2G6* and *IRF4*), with common variants in or near these genes being revealed by GWAS [2,7]. Likewise, melanoma GWAS have identified variants linked to the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene [1,2]. Importantly, loss-of-function

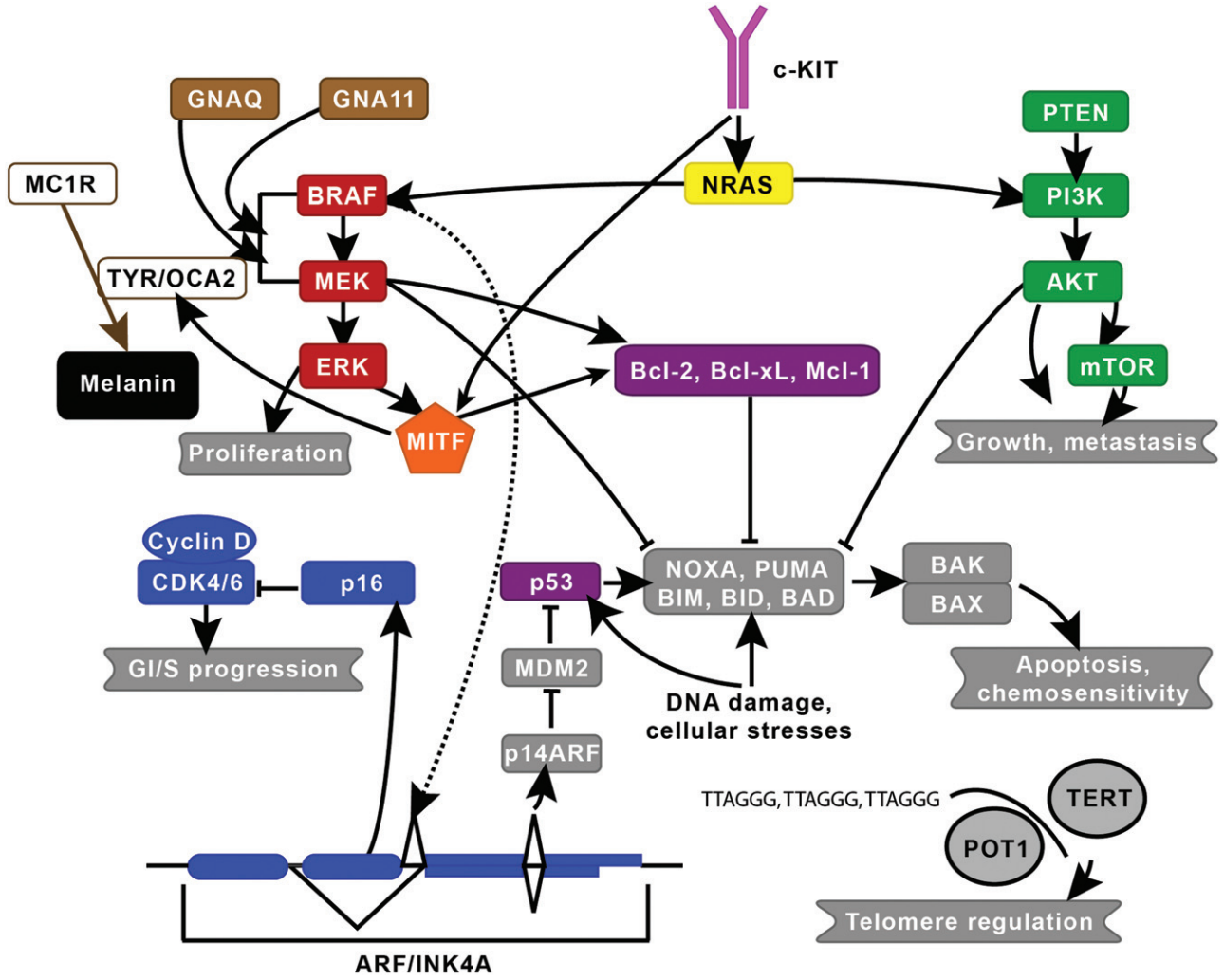

Figure 1. Established melanoma pathways. The two major signalling pathways implicated in melanoma are the mitogen-activated protein kinase and the phosphatidylinositol-4,5-bisphosphate 3-kinase pathways, which are in red and green, respectively. Key genes include c-KIT (pink), CDK (blue), GNAQ/GNA11 (brown), MITF (orange), NRAS (yellow), and P53/BCL (purple). MC1R, which is involved in skin pigmentation, and TERT and POT1, which are involved in telomere regulation, are also shown. This figure was modified from Vidwans *et al* [142] under the Creative Commons Attribution License. The lines shown indicate known interactions between pathways or molecules.

mutations in *CDKN2A*, and its binding partner, the product of the cyclin-dependent kinase 4 (*CDK4*) gene, have been identified in highly melanoma-prone families [8–10], firmly linking disruption of cell cycle control and melanoma risk. In addition to the abovementioned genes, recent work has implicated components of the telomere regulation machinery as playing important roles in melanomagenesis. First, the telomerase reverse transcriptase gene (*TERT*) was identified in GWAS studies, and by the analysis of a familial melanoma pedigree, a −57 bp mutation creating an ETS transcription factor binding site that activates the *TERT* promoter was identified [11]. More recently, melanoma family studies have identified the protection of telomeres 1 gene (*POT1*), with mutations in the DNA binding domain of POT1 dramatically increasing telomere length and promoting telomere fragility [12,13]. POT1 is a component of the shelterin complex, which is a key regulator of telomere end-processing. Other components of this complex have been shown to be truncated in melanoma-prone families including *ACD* and *TERF2IP*, but the exact consequences of these mutations on telomere function are yet to be defined [14].

While these genetic susceptibility studies have defined the landscape of predisposition to cutaneous melanoma, less is known about the germline genetic contribution to other forms of melanoma. At present, we know that rare loss-of-function variants in the *BAP1* gene, encoding the BRCA1-associated protein-1 (ubiquitin carboxy-terminal hydrolase), a deubiquitinating enzyme, predispose to ocular melanoma, with some patients also developing cutaneous melanomas and tumours of other sites [15,16], yet these mutations account for less than 10% of uveal melanoma families. Little is known about the germline genetics of acral and mucosal melanoma, due largely to the rarity of these forms of the disease, and no association with pigmentation genes has been observed [17]. Some reports have suggested that patients with Werner syndrome, a

DNA repair and ageing syndrome, have an increased incidence of these cancers [17].

Genetic predisposition to melanoma in animals

With the exception of mutations in *MC1R*, few studies have addressed the genetics of predisposition to melanoma development in animal models. The studies that have been performed have again revealing an important role for genes that influence pigmentation in the cutaneous disease. In horses, for example, a 4.6 kb intronic mutation in the *STX17* (syntaxin-17) gene was found to be associated with a vitiligo-like depigmentation phenotype and susceptibility to melanoma [18]. Cutaneous melanoma also occurs in dogs, with some reports of differences in breed susceptibility suggesting a genetic basis for melanoma risk [19]. Whether melanoma susceptibility in dogs and horses, or indeed in mice and fish, is mediated via the same genes and pathways as those in humans is not known. Thus, there is a significant opportunity to use genetic studies in animal models to define new genes and loci that influence melanoma risk, and to use these data to guide genetic studies in humans.

Somatic mutations in human melanoma

Most of what has been learnt about the somatic genetics of cutaneous melanoma in humans has been revealed in the last 15 years. The family studies that identified germline mutations in *CDKN2A* led to the identification of somatic mutation in this gene, and also deletions of the entire gene locus [9,20]. Likewise, studies that identified the phosphatase and tensin homolog (*PTEN*) gene as being mutated in glioma led to the identification of mutations and deletions of this locus in melanoma [21]. Studies in mouse melanoma models have contributed significantly to our understanding of melanoma and have established a key role for the mitogen-activated protein kinase (MAPK) pathway in melanoma development. In early studies, a $HRAS^{G12V}$ transgene was expressed in melanocytes, resulting in highly penetrant melanoma formation [22]. Several years later, activating mutations of the neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*) gene were identified in human melanomas at a frequency of 20% [23–25], and in 2002, amplicon sequencing studies of melanoma cell lines resulted in the identification of activating *BRAF* mutations, most converting a valine at position 600 to a glutamic acid ($BRAF^{V600E}$), generating a constitutively active kinase in around 50% of cases [26]. The discovery of this somatic mutation resulting in constitutive activation of the MAPK pathway has revolutionized the field of melanoma genetics and has recently facilitated a revolution in new therapeutics, with clinically approved agents targeting mutant BRAF, and MEK and ERK now in use [27]. More recently, next-generation sequencing of melanomas and matched germline DNA has identified as many as 20 genes as being statistically significantly mutated in human melanoma [28–30]. Many of these genes are components of the MAPK and phosphoinositide 3-kinase (PI3K) pathways, or cell cycle regulatory genes such as the protein phosphatase 6, catalytic subunit (*PPP6C*), in addition to regulators of the chromatin landscape (*ARID2*, *ARID1A*, and *ARID1B*) [31–33]. While these studies have defined the complexity of melanoma, they have also challenged the one-size-fits-all approach to disease management. Less is known about the genetics of the non-cutaneous forms of melanoma but acral, uveal, and mucosal melanomas tend to harbour mutations in the guanine nucleotide-binding protein G(q) subunit alpha (*GNAQ*), the guanine nucleotide binding protein (G protein), alpha 11 (Gq class) (*GNA11*), and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*c-KIT*) genes [17]. Figure 1 provides an overview of the established melanoma pathways.

Next-generation sequencing of human melanomas brings 'the end of the beginning' for melanoma gene discovery

To date, around 500 human melanoma germline/tumour pairs have been sequenced, with these tumours being largely of cutaneous origin and a limited number of acral and mucosal origin [28–30,34,35]. Likewise, a limited number of uveal melanomas have been sequenced [28,30], although The Cancer Genome Atlas (TCGA) is currently collecting tumours for sequencing and analysis. So what have these sequencing studies actually taught us? First, the sequence has given us a clearer view of the frequency of mutations in known driver genes. Prior to these studies, we knew, for example, that *BRAF* and *NRAS* mutations occurred but tumour sequencing studies have helped us to resolve their absolute prevalence, particularly at positions outside of the canonical $BRAF^{V600}$ and $NRAS^{Q61}$ residues. Secondly, sequencing has identified new genes. For example, hotspot mutations in *PPP6C* and *RAC1* have been identified, and represent potential sites for therapeutic targeting. We knew previously that RAC GTPase activity could contribute to melanoma development [36], but we had no clear way of grappling with it mechanistically, or for exploiting this knowledge in the clinic. The sequence of human melanomas has also taught us something of the constellations of mutations that occur within individual tumours. We know now, for example, that the RAC1 P29S hotspot mutation is mutually exclusive from mutations in other components of the Rho family, a result that would be predicted, but is clarifying nonetheless [30]. We have also learnt that there is a third subtype of cutaneous disease called *NRAS/BRAF* wild-type melanoma, which is characterized by a high C > T mutation burden, amplifications and mutation of *c-KIT*, and alterations of *NF1* [28,30]. More recently, a fourth subclass called *NRAS/BRAF/NF1* wild type has been proposed [29]. While these studies have been broadly informative, melanomas rarely contain the same complement of mutations, so how specific mutations co-operate to promote tumour formation remains unanswered.

Sequencing of human tumours has also informed us that the major mutagen in cutaneous melanoma is UV light which drives a C > T mutation pattern. It has also revealed the potential role of other processes such as oxidative stress [30]. Despite the apparent clarity that sequencing has provided, there is still much to learn, and integrating the sequence data with functional studies, and studies in model systems will be key.

Using the mouse to model melanoma

There are multiple ways to model melanoma in the mouse, to allow the identification of key oncogenes/tumour suppressor genes involved in melanoma initiation, progression, and metastasis, as well as for the preclinical testing of therapeutics. Here we will outline the use of patient-derived xenografts, genetically engineered mice, and melanoma cell lines.

Melanoma cell lines

Established human and mouse melanoma cell lines continue to be workhorses for mechanistic studies, as much can be gained from their analysis. The B16 mouse melanoma model was created in the 1970s from a melanoma that developed spontaneously in a C57BL/6 mouse and was then passaged *in vivo* through ten rounds of tail vein injection and collection of subsequent pulmonary metastases to create the B16-F10 line [37]. This cell line has been used in a plethora of tumour immunology and metastasis studies [37,38].

In vitro work has its limitations (such as a lack of extracellular matrix and 3D growth), and thus, the use of human cell lines *in vivo* is frequently used to model melanoma, usually by subcutaneous injection/engraftment into immunodeficient mice [typically non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice] that do not produce lymphocytes or NK cells. These cell line xenograft models allow melanoma cells to directly establish interactions with the stroma, the lymphatic system, and blood vessels. Cell line xenografts have been widely used in determining drug responses [39]; however, cell lines are undeniably altered during adaptation to *in vitro* conditions and long-term culture, limiting their usefulness in certain aspects of modelling human melanoma. In particular, their ability to predict clinical drug responses has been widely questioned and critiqued [40].

Patient-derived xenograft (PDX) models

Patient-derived xenograft (PDX) or 'tumourgraft' samples are collected under ethical approval as fresh biopsy tissue or fine needle aspirates and within hours implanted subcutaneously into immunodeficient mice [41]. A high degree of similarity, at the level of expression and DNA sequence, has been demonstrated between PDXs and donor tumours, with human melanoma PDXs found to be predictive of metastasis [42]. Excitingly, the utility of PDXs for informing patient care was recently demonstrated, with PDXs found to be predictive of patient drug response [43].

PDX models, however, do have several important limitations – in particular, the absence of a fully functional immune system – although it is possible to generate partially 'humanized mice' using patient-derived CD34+ haematopoietic stem cells capturing some elements of the human immune system [44].

Genetically engineered mice (GEM) models

Although mice rarely develop melanoma spontaneously, they can do so when genetically engineered to carry defined mutations that mimic the genetic lesions (or their consequences) found in human melanomas. These engineered mutations can result in activation of oncogenes (such as mutant $Braf^{V600E}$ or $Nras^{Q61R}$) and/or inactivation of key tumour suppressor genes (such as *Cdkn2a* or *Pten*). Examples of GEM models are listed in Table 1 and shown in Figure 2. In mice, genetic modification of the germline melanoma susceptibility gene *Cdkn2a* (*p16Ink4a* null mice) does not result in melanoma, with these mice typically developing soft-tissue sarcomas and lymphomas [45]. One way around this is to use of compound GEM models. For example, mice carrying melanocyte-specific (tyrosinase promoter-controlled) expression of activated $HRAS^{G12V}$ on an *Ink4a*-deficient background develop spontaneous cutaneous melanomas after a short latency and with a high penetrance [22]. Interestingly, in addition to cutaneous melanomas, these mice also developed ocular melanomas, as has been reported for mice carrying melanocyte-specific expression of activated $NRAS^{Q61K}$ on an *Ink4a*-deficient background (Figure 2) [46]. Mutational activation of BRAF in mice carrying conditional melanocyte-specific expression of $Braf^{V600E}$ (or the mouse equivalent, $Braf^{V618E}$) has also been used to model melanoma development, tumour progression, and drug resistance [47–49].

Given the importance of UV light as a key mutagen in the initiation of melanoma, GEM models of UV-induced melanoma have been developed, such as *HGF/SF* mice, which were used to show that a single dose of burning UV radiation to neonates, but not adults, is necessary and sufficient to induce melanomas with high penetrance, thus providing experimental support for epidemiological evidence that suggests that childhood sunburn poses a significant risk for developing melanoma [50]. More recently, neonatal UVB exposure was shown to accelerate melanoma growth and enhance distant metastases in $Hgf\text{-}Cdk4^{R24C}$ mice [51], and pulmonary metastasis of melanomas in adult $Hgf\text{-}Cdk4^{R24C}$ mice was found to occur after treatment with the mutagen DMBA and repeated UVB exposure [52]. The $Hgf\text{-}Cdk4^{R24C}$ model has also been used to generate metastatic melanoma by introducing *p16-null* and *Nme23*-null alleles [53,54].

GEM models have the advantage over other systems of autochthonous tumour development in an environment where the tumour cells interact reciprocally

Table 1. Examples of genetically engineered mouse (GEM) models of melanoma

GEM name	Genes involved	Phenotype	Reference
MT/ret	Transgenic mice with the *RET* proto-oncogene fused to the mouse metallothionein promoter-enhancer	• Mice develop hyperpigmented skin due to aberrant melanogenesis and melanocytic tumours develop but do not metastasize • The transgenic line '304/B6' (which has been back-crossed to C57BL/6 for ten generations) spontaneously develops systemic skin melanosis, benign melanocytic tumours, and melanoma that undergoes metastasis to distant organs • On a background of *Ednrb* heterozygosity, these mice show late-onset melanoma development with a high percentage of metastasis, and poor prognosis after tumour development	Iwamoto *et al* [136] Kato *et al* [137] Kumasaka *et al* [138]
HGF/SF	Transgenic mice with the metallothionein promoter driving overexpression of hepatocyte growth factor/scatter factor (HGF/SF)	• The skin of these mice has melanocytes in the dermis, epidermis, and dermal–epidermal junction, and thus this model is more akin to human skin • Aged *HGF/SF*-transgenic mice develop sporadic melanoma with metastasis • Cutaneous melanomas arise in UV-irradiated *HGF/SF*-transgenic mice in distinct stages that resemble human disease, including grossly identifiable premalignant lesions, intermediate radial and vertical growth stages of heterogeneous histopathologies, and late metastatic spread to a variety of distant organs	Takayama *et al* [139] Noonan *et al* [50,140]
$p16^{Ink4a-/-}$	A $p16^{Ink4a}$-specific knockout mouse that retains normal $p19^{Arf}$ function	• Although these mice develop melanomas, they more preferentially develop soft-tissue sarcoma and/or splenic lymphoma	Sharpless *et al* [45]
Tyr-HRAS	Transgenic mice with mouse tyrosinase gene promoter driving overexpression of an oncogenic form of *HRAS* ($HRAS^{G12V}$)	• These mice spontaneously developed cutaneous and ocular tumours that are locally invasive and do not undergo metastasis • The incidence and latency of melanoma development are accelerated on an *Ink4a*-deficient background	Chin *et al* [22]
$Tyr::NRAS^{Q61K}$	Transgenic mice with mouse tyrosinase gene promoter driving overexpression of a dominant-active human *NRAS* ($NRAS^{Q61K}$)	• Mice showed hyperpigmented skin and develop cutaneous metastasizing melanoma • On an *Ink4a*-deficient background, > 90% of the mice developed melanomas that at 6 months micro-invade the epidermis and disseminate to lymph nodes, lung, and liver	Ackermann *et al* [135]
$Hgf\text{-}Cdk4^{R24C}$	Overexpression of the hepatocyte growth factor (HGF) and an oncogenic mutation in cyclin-dependent kinase 4 ($CDK4)^{R24C}$	• These mice rapidly develop multiple invasive melanomas in the skin following neonatal or adult carcinogen treatment (UV and/or DMBA), which spontaneously metastasize to lymph nodes and lungs • Primary DMBA-induced melanomas have been used to derive cell lines that when subcutaneously administered to C57BL/6 immunocompetent mice, spontaneously develop lung metastases	Tormo *et al* [141] Gaffal *et al* [51] Bald *et al* [52]
$LSL\text{-}Braf^{V600E}$	Conditional expression of $Braf^{V600E}$ from the endogenous *Braf* locus	• When crossed with *Tyr::CreER* mice and tamoxifen was rubbed on their skin, these mice showed skin hyperpigmentation and the appearance of naevi harbouring senescent melanocytes, with ~70% developing melanomas • On a $p16^{INK4a}$ null background, these mice developed melanoma with increased penetrance and decreased latency	Dhomen *et al* [47]
$Braf^{CA}$	Conditional expression of $Braf^{V600E}$ from the endogenous *Braf* gene	• When crossed with *Tyr::CreER* mice and tamoxifen is rubbed on their skin, these mice develop benign melanocytic hyperplasias that fail to progress to melanoma over 15–20 months • On a *Pten* null background, these mice developed melanoma with 100% penetrance, short latency, and metastases (lymph nodes and lungs) • Melanoma development was prevented by inhibitors of mTorc1 (rapamycin) or MEK1/2 (PD325901) but only whilst the drug was being administered (cessation of administration led to melanoma development). Combined rapamycin and PD325901 treatment led to shrinkage of established melanomas	Dankort *et al* [49]

Table 1. Continued

GEM name	Genes involved	Phenotype	Reference
LSL-Braf^{V618E}	Conditional expression of Braf^{V618E} from the endogenous murine *Braf* gene (Braf^{V618E} is analogous to BRAF^{V600E} in humans).	• When crossed with *Tyr::CreER* mice and tamoxifen was rubbed on their skin, these mice showed skin hyperpigmentation and naevi with ~80% developing melanoma • *Sleeping Beauty* insertional mutagenesis in this model accelerated melanoma latency and penetrance. Treatment with the BRAF inhibitor PLX4720 resulted in tumour regression followed by relapse, and analysis of transposon insertion sites in these melanomas identified putative mediators of resistance	Perna *et al* [48]

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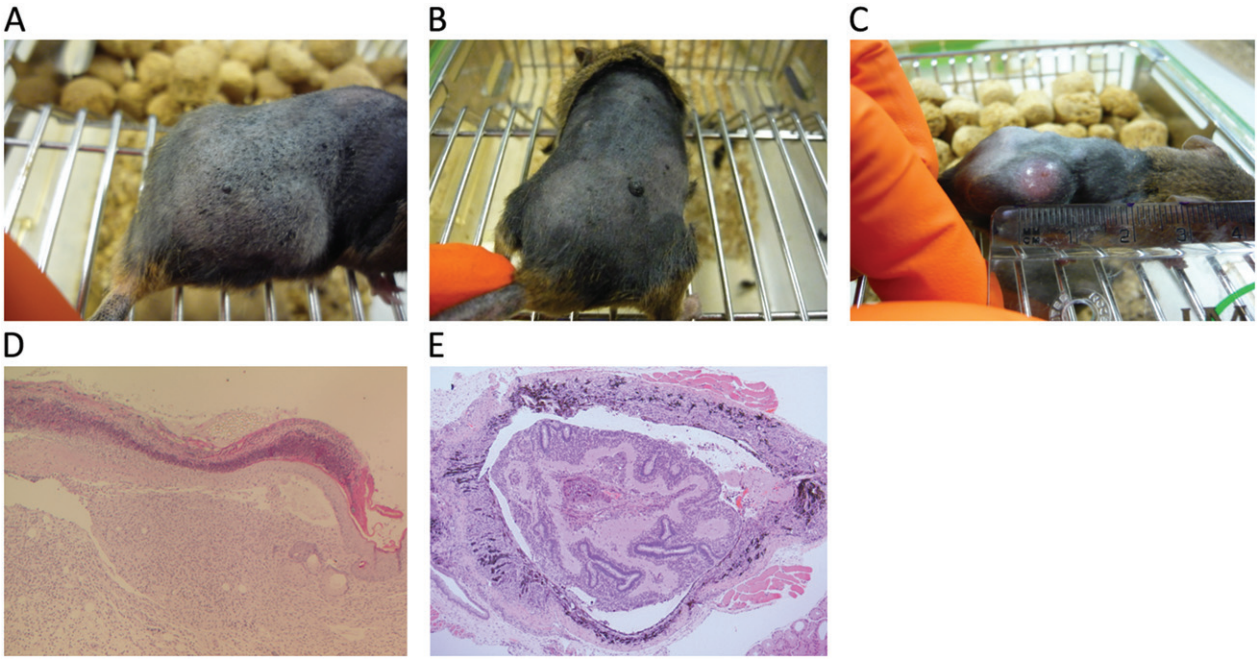

Figure 2. Naevi and melanomas driven by oncogenic forms of *Braf* and *NRAS*. (A, B) Naevi developing in adult *Braf*^{V618E} mice [48]. Naevi generally became visible 6–8 weeks after the induction of *Braf*^{V618E} expression. (C, D) Melanoma from the same *Braf*^{V618E} model. D shows an invasive malignant melanoma with evidence of infiltration and destruction of the overlying surface epithelium and invasion into the subcutaneous adipose tissue. The average latency to melanoma formation was 426 days in this model. (E) H&E-stained section of an ocular melanoma, with melanoma cell infiltration of the lens and the subretinal tissues, that developed in a 13-week-old *Tyr::NRAS*^{Q61K} mouse [135]. Original magnification × 50.

with the immune system and other components of the microenvironment. Disadvantages of GEM models include their expense and the fact that tumours often arise after a long latency (9–12 months) and generally do not carry the mutagenic load found in human tumours. Regardless of these factors, these models have made a fundamental contribution to our understanding of melanoma development.

Using the dog to model melanoma

Dogs as spontaneous models of melanoma

Malignant melanoma is a relatively common cancer in domestic dogs and represents a unique model of human melanoma that is highly heterogeneous and arises and metastasizes spontaneously in an immunocompetent animal. There is potential to relate the molecular character of individual tumours to clinical outcome, as pet dogs receive therapy ranging from surgery, radiation, and cytotoxic chemotherapy through to molecularly targeted therapy and immunotherapy. Here we will review the utility of canine melanoma as a comparative model and as a preclinical model of human melanoma.

Incidence, anatomic location, and clinical progression of melanoma in dogs

Many domestic animals develop spontaneous melanocytic neoplasms, including dogs, cats, horses, and pigs. Malignant melanoma is more common in the dog compared with other species and the majority of cases arise in the oral cavity (mucosal), with haired skin (cutaneous), nailbed epithelium and footpad (subungual and acral), and ocular (uveal) locations being less common [55] (Figure 3). Canine oral melanoma is highly aggressive with frequent metastases, especially to

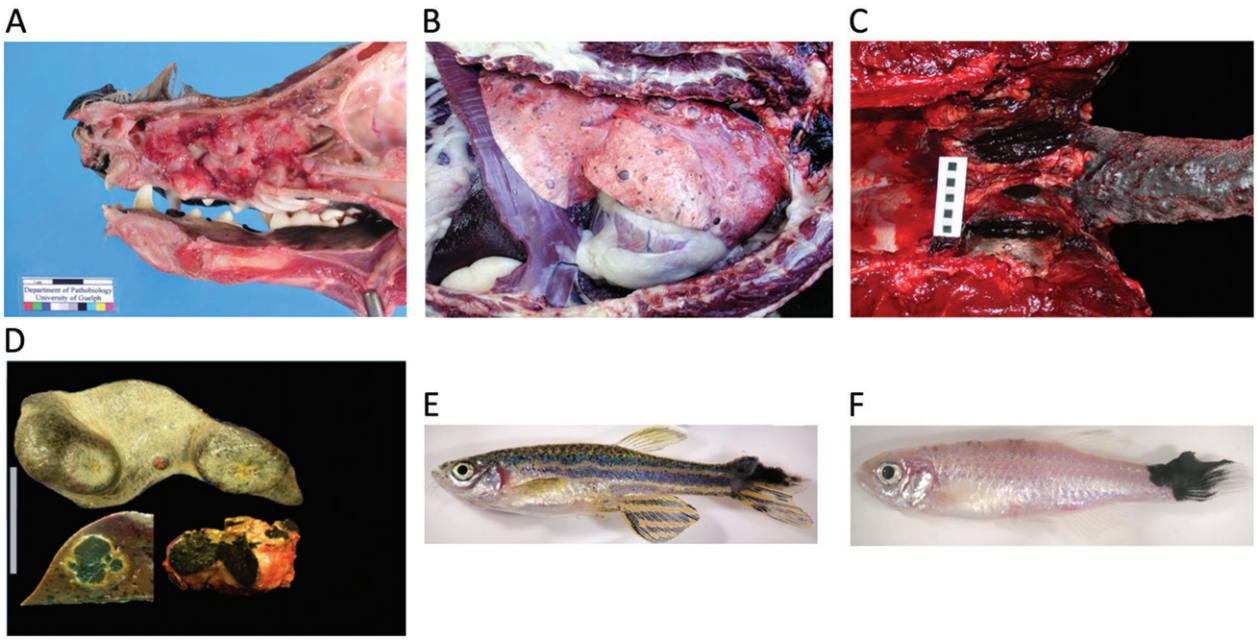

Figure 3. Canine, equine, and zebrafish melanoma. (A) A canine melanoma developing in the nasal cavity and (B) spreading to the viscera, particularly the liver. (C) An equine melanoma showing multinodular dermal lesions around the tail base and masses expanding into the pelvic canal and regional nodes. (D) An equine spleen with multiple malignant melanomas, and liver and lymph node from the same case. (E) Melanomas arising in a *BRAF*^{V600E}*; mitf* zebrafish and (F) in a *BRAF*^{V600E}*; p53* zebrafish. The photographs in A and B were kindly provided by Jeff Caswell, Department of Pathobiology, University of Guelph, Guelph, Ontario, Canada N1G2W1.

local lymph nodes and lungs. In contrast to human cutaneous melanomas, dog cutaneous melanomas are most often benign [55]. Some dog breeds are over-represented in oral melanoma studies and may be predisposed to developing the disease. In a study of 2350 dogs with melanocytic tumours, poodles, Beauce shepherds, rottweilers, schnauzers, Scottish terriers, and Labrador retrievers had a higher percentage of these tumours than other breeds [56]. In general, this study also found that black-coated breeds were over-represented and that pale or white-coated breeds were under-represented in terms of developing melanocytic tumours. Conventional treatment for oral melanoma in dogs involves surgical resection and/or radiation of the primary tumour to control local disease [57], while treatment of metastatic disease is much less successful. Most metastases are resistant to chemotherapy and a variety of immunotherapeutic approaches have been attempted [58]. A commercially produced melanoma vaccine (ONCEPT, Sanofi) is available and has shown efficacy in canine melanoma compared with historical controls [59], but there is controversy as to the level of effect when there has not been a randomized trial [60], and some studies have failed to show an effect on clinical outcome [61]. Other immunomodulatory approaches have been applied experimentally in small groups of dogs and show some potential [62,63]. As described in more detail below, there are a number of receptor tyrosine kinase genes that are mutated in canine melanomas and tyrosine kinase inhibitor drugs are already commercially available and used clinically in canine cancer patients [64], but to date no trials have been published using these compounds in canine melanoma.

Genetics of canine melanoma

The complete canine genome sequence was first released in 2005 [65], revealing a significant shared ancestral sequence in common with humans. Indeed, canine DNA and protein sequences are more similar to humans than are those of mice [65]. Due to this similarity, molecular tools for studying canine diseases are quite advanced, especially since a large proportion of antibodies raised against human antigens work equally well against canine proteins. Canine oral melanoma does not have UV radiation as a risk factor, so it is not surprising that the spectrum of mutations differs from human cutaneous melanoma. The BRAF^{V600E} mutation is found in about 6% of canine oral melanomas [66], as are non-canonical *BRAF* mutations. *NRAS* mutations have also been found at the same location as those in human melanoma (the residue corresponding to Q61), and loss-of-function mutations in *PTEN* have been reported [56]. Also similar to humans, loss of *PTEN* expression, and *c-KIT* mutation, and/or overexpression of *c-KIT* are common in the canine disease. Importantly, comparative copy number studies have been performed between dog and human melanomas of mucosal and acral origin, suggesting that in concordance with what is known for human melanomas, canine melanomas of the oral mucosa and cutaneous epithelium are discrete and initiated by different molecular pathways [67].

Canine melanoma as a preclinical model

The overall success rate of translating cancer therapies from murine preclinical models to treatments with clinical utility in humans is estimated to be around 5%

[68]. Although the increasingly sophisticated mouse *in vivo* modelling systems described above are more likely to capture the complexity of human cancer than the less refined systems used in the past, a complementary approach is to include dogs with spontaneous cancers [69]. Until recently, humans could be considered a preclinical model for dogs; the majority of drugs used in veterinary medicine are derived from drugs initially designed and tested for efficacy in humans. There is an organized network for conducting clinical trials in dogs with cancer across the United States and Canada organized through the National Cancer Institute, called the Comparative Oncology Trials Consortium [70,71]. This allows for multi-centre clinical trials with defined inclusion and exclusion criteria, much like human clinical trials [71]. From the perspective of the veterinary patients and their owners, the access to investigational therapies is a clinical trial; from the perspective of human patients, these can be thought of as preclinical trials. This has led to the concept of co-clinical trials, where both human and canine patients with the same tumour type, or mutation spectrum, receive the same drugs [72]. In addition to mirroring the heterogeneity and complexity of spontaneously arising cancer, dog trials have other practical advantages; the contracted disease timeline allows earlier assessment of effects on disease progression and overall survival, since the lifespan of dogs is far shorter than that of humans and canine cancers progress more quickly in general. Furthermore, new drugs are commonly tested for toxicity in laboratory beagles, so the initial safety and sometimes the pharmacodynamics and pharmacokinetics are already known for dogs.

A recent, excellent study by Simpson *et al* [73] explored the utility of canine melanoma as a model of the human disease, and readers are referred there for a more in-depth review. The consensus of that group was that there are substantial clinical and histopathological similarities between mucosal melanomas in the two species. The Simpson study leveraged the Canine Comparative Oncology and Genomics Consortium (http://www.CCOGC.net), which contains a large collection of canine tumours including matched dog melanoma/normal pairs. To date, there have been relatively few large clinical trials in canine melanoma. The development of the canine melanoma vaccine introduced above involved only 58 patients [59]. As in humans, the majority of melanoma therapies tried in dogs have failed, which although discouraging, might be considered evidence for the utility of canine melanoma as a model. As canine melanoma is a heterogeneous cancer, has developed in the context of an intact immune system, and occurs in a genetically heterogeneous population of animals, only the most robust investigational drugs will be able to show efficacy in a clinical trial. Thus, although the majority of melanomas forming in dogs are of mucosal origin, and thus rarer in frequency than common melanomas in humans which are cutaneous, there are significant opportunities in studying dog melanomas alongside those of human and other species.

Using the horse to model melanoma

Incidence, anatomic location, and clinical progression of melanocytic tumours in horses

Melanocytic tumours are common tumours of horses, representing approximately 4–8% of all tumours [74] and up to 19% of cutaneous tumours [75,76].

As in other species, the terminology and classification of melanocytic tumours in horses has been inconsistent over time and has led to confusion between clinicians and pathologists [77]. Four clinical syndromes are currently recognized in horses: melanocytic naevi (sometimes referred to as melanocytoma); dermal melanomas; dermal melanomatosis; and anaplastic malignant melanoma [78]. Some of the melanocytic naevi resemble human naevi [79], and these occur in both grey and non-grey horses, usually on the legs, body or neck rather than the perineal region. Equine dermal melanomas and dermal melanomatosis are histopathologically similar, distinguished by their clinical presentation; the former tend to be solitary discrete tumours, whereas dermal melanomatosis presents as multifocal dermal lesions, often coalescing and usually occurring in typical locations (most commonly the genital or tail base/perineal region, and less commonly periocular and perioral). Dermal melanomatosis is a disease of grey and white horses, and beyond the age of 15 years, at least 80% of grey horses will have melanomas at some location [78,80]. While they usually have a benign initial presentation, they often develop multi-centrically and are often associated with blood vessels [for example, in the wall of the guttural pouches (paired air-filled chambers formed from outpouching of the Eustacian tube), around the parotid salivary glands and lymph nodes, paralumbar, peri-aortic and neck/carotid region]. In addition, many will progress to true malignant forms with lymphatic and visceral metastases [81]. Malignant forms occur in both grey and non-grey horses, although the risk of malignant transformation may be greater in non-grey horses [75]. At least in grey horses, histopathological features do not reliably predict malignant behaviour [82], although application of new biomarkers, such as RACK1, may show promise [83]. Ocular [84] and mucosal melanomas [85,86] are far less common in horses than in other domestic species.

Equine melanoma as a comparative model

As in canine melanomas, equine melanomas are not thought to be associated with exposure to UV light. Development of the grey hair coat colour in horses with age is an autosomal dominant trait associated with a high incidence of melanoma, and also vitiligo-like depigmentation [18]. The causative mutation for this phenotype is a 4.6-kb intronic duplication in the *STX17* (syntaxin 17) gene, which constitutes a *cis*-acting regulatory mutation. Both *STX17* and the neighbouring *NR4A3* gene are overexpressed in melanomas from grey horses. It is known that the duplication in *STX17* is strongly

associated with constitutive activation of the ERK pathway in melanocytic cells from grey horses, highlighting the universal importance of the MAPK/ERK pathway in melanomagenesis [87]. Further, experimental models using reporter constructs in transgenic zebrafish have demonstrated that the duplicated *STX17* sequence acts as a strong enhancer in neural crest cells and has subsequent melanophore-specific activity during embryonic development, consistent with the phenotypic manifestation of the mutation in horses [88]. This study went on to demonstrate that one region of the construct up-regulated the reporter gene expression in a melanocyte-specific manner and contained two microphthalmia-associated transcription factor (MITF) binding sites, which are good candidates for mediating the melanocyte-specific activity of the duplication.

As in other species, tumour subtype and breed/individual variation (germline genetics) are likely to influence the phenotype of the melanomas formed [89]. Indeed, grey horses that possess a loss-of-function mutation in the *ASIP* (agouti signalling protein) gene have a higher incidence of melanoma, implicating melanocortin-1 receptor signalling in melanoma development in these animals [18].

In terms of biological behaviour, grey horse melanomas usually have an extended period of benign growth, prior to malignant transformation and metastasis, in contrast to most human melanomas, which metastasize early. *In vitro* cell lines of primary and metastatic horse melanomas revealed expression of p53, while expression of the tumour suppressors p16 and PTEN was absent from the metastatic line [90], potentially implicating the latter pathways in disease progression.

In terms of histopathology, animal-type melanoma in humans represents a rare distinct melanoma subtype, characterized by proliferation of heavily pigmented epithelioid and spindled melanocytes, that resembles the heavily pigmented melanomas seen in grey horses [91,92]. In humans, the disease has a young age of onset (median 35 years old) and is considered to be more indolent than conventional melanoma; it has a tendency for regional lymphatic metastasis but infrequently progresses to disseminated metastatic disease and death. Direct comparison of the genetic and molecular alterations in human and equine melanomas will provide fascinating insights into the mechanisms of melanomagenesis [93].

Using zebrafish to model melanoma

The translational impact of zebrafish models of melanoma

Modelling melanoma in zebrafish provides important opportunities for *in vivo* imaging, chemical screens, and genetics. Zebrafish cancers, including melanoma, share many histopathological features with human cancers, and molecular signatures closely align with those of human cancer. Here we outline the use of genetically engineered zebrafish and xenograft models, and discuss how zebrafish have become instrumental for chemical screens for drug leads and repurposing for melanoma.

Genetically engineered zebrafish (GEZ) models

The zebrafish genome shares over 70% similarity with the human genome, and over 80% of human disease genes – including oncogenes and tumour suppressors – have orthologs in zebrafish [94]. Zebrafish cancer models have primarily depended on transgenic expression of oncogenes and *N*-ethyl-*N*-nitrosourea (ENU)-induced genetic mutations in tumour suppressor genes. However, the advent of genome editing with the clustered regularly interspaced short palindromic repeats (CRISPR) system now enables precise and tissue-specific genetic editing that will enable more refined genetic modelling of human melanoma [95–99].

In the first zebrafish melanoma model, human $BRAF^{V600E}$ protein expressed from the melanocyte *mitfa* promoter led to the generation of naevi, and a mutation in *p53* ($p53^{-/-}$) was required for progression to melanoma [100]. This was the first animal model of the $BRAF^{V600E}$ mutation and was consistent with genetics in human patients whereby expression of $BRAF^{V600E}$ is sufficient to drive naevi, but requires additional mutations for progression of melanoma from naevi [26]. Building on the $BRAF^{V600E}$;$p53^{-/-}$ model, Zon and colleagues generated a modified zebrafish whereby the $BRAF^{V600E}$ transgene was co-expressed with one of 17 candidate genes from a recurrently amplified region in human melanoma on chromosome 1q21 [101]. Screening for genes that promoted the rapid onset of melanoma, they discovered that overexpression of the histone methyltransferase SETDB1 can accelerate the onset and invasion of melanoma. High expression levels of SETDB1 are common in human melanoma and indicate that changes in chromatin factors may be critical in melanoma progression through changes in gene regulation, such as the *hox* genes [101].

An important feature of zebrafish melanoma is the ability to study melanocyte development genes and how the lineage can become misregulated in melanoma [102]. The master melanocyte transcription factor MITF is a melanoma oncogene and has been implicated in melanoma drug resistance, but until recently it had not been modelled in an animal. A unique temperature-sensitive *mitf* mutation in zebrafish ($mitfa^{vc7}$) has recently been used to study MITF activity in the control of melanocyte proliferation and differentiation in embryogenesis, and as a cancer gene in the development and survival of melanoma [103–105].

RAS mutations have also been modelled in zebrafish. Expression of $HRAS^{G12V}$ ($HRAS^{12V}$) protein in *kit*-expressing melanocyte progenitors is sufficient to drive rapid expansion of melanocyte numbers in the larval form and melanoma in the adult, and this is dependent on PI3K signalling [106]. Co-operation studies have also demonstrated that elevated RAC activity, often associated with melanoma in humans, can accelerate the

progression of $HRAS^{V12}$-driven malignant melanoma [107]. While $HRAS^{V12}$ melanoma studies have helped to establish melanoma models important for drug screens and cell biology studies [108], *NRAS* mutations are the common RAS family melanoma mutation, and genetic models in zebrafish indicate that $NRAS^{Q61K}$ mutations in melanocytes require co-operation with loss of *p53* to promote melanoma [109].

As with mice, limitations of the zebrafish $BRAF^{V600E}$ models include the lengthy time for spontaneous tumour formation and that genetically engineered animals do not seem to have the diversity and number of mutations found in human melanomas [110]. Accelerating tumour formation with $HRAS^{V12}$ mutations enables melanoma to be visualized at the earliest stages in the zebrafish [106,108]. Zebrafish embryos and larvae are transparent, enabling details of cell biology and the lineage to be visualized in living animals. An important example of this is the interactions of the immune system with $HRAS^{V12}$ oncogene-expressing melanocytes at the very earliest stages of neoplasia. Immune cells provide trophic support to $HRAS^{V12}$ oncogene-expressing melanocytes [111,112].

Transplantation models of melanoma in zebrafish

Transplantation assays are fundamental to understanding cancer cell malignancy, migration, and cancer-initiating cells. Zebrafish provide transplantation studies at three stages: the early embryo, the larvae, and the adult animal [113]. Transplantation into the early embryo (prior to gastrulation) has been used to identify important melanoma pathways, such as nodal via the generation of an ectopic developmental axis [114–117]. Transplantation of human cancer cells into the larval stage can lead to melanoma masses within a few days, and enables the study of tumour-induced vascularization and cancer cell metastatic spread. The availability of lines with fluorescently labelled vasculature, such as fli-GFP, allows for angiogenesis or lymphoangiogenesis to be visualized in living animals [118,119]. Fluorescently labelled melanoma cells can also be visualized in the process of co-operative behaviours during invasion in zebrafish embryos [120]. An advantage to these early-stage transplantation studies is the large number of zebrafish that can easily be injected and that can be coupled to live confocal imaging [121]. The zebrafish immune system in these early stages primarily consists of innate immune cells, and the adult immune system is not fully functional until 28 days of development [113]. In some cases, transplanted melanoma cells have capitalized on neutrophil migration routes to new metastatic niches [122].

Adult transplantation studies in zebrafish have been important for assessing tumour potential, visualizing cancer homing and metastasis, and in competitive assays for tumourigenicity. Important considerations in adult transplantation studies include the need to suppress the immune system. To get around these issues, immunosuppression can be induced by gamma irradiation prior to transplantation (eg 20–25 Gy), or dexamethasone in larval/juvenile fish, and isogenic strains have recently become available [113]. Adult zebrafish are no longer transparent, preventing detailed visualization of engrafted tumours in living animals. Recently, a transparent adult fish, called *casper*, has been generated that enables visualization of transplanted melanoma cells – either by their endogenous black pigmentation or via a fluorescent transgene – at the single cell level [102,123,124]. Limitations of the adult transplantation studies are that human cancer cells do not engraft due to immunogenicity and that most cells are injected via intraperitoneal injection rather than orthotopically [113].

Small molecule and drug screening in zebrafish

A unique feature of the zebrafish system is the ability to treat the whole organism with drug treatments by administering chemical compounds to the water [125]. This approach can be used to directly test the function of a targetable pathway in transplantation studies, to screen for new drug leads during early embryogenesis, and for testing compounds in adult zebrafish cancer models [126]. Examples include small molecule screens on the melanocyte lineage that identified 5-nitrofuran compounds, which are also effective in human melanoma [127], and the changes caused by $BRAF^{V600E}$*;p53* at the embryonic level that identified leflunomide, which is currently in clinical trials for melanoma [126] (Clinical trials.gov identifier NCT01611675). Overall, phenotypic small molecule screening in zebrafish is proving effective at multiple stages of the drug discovery pipeline including hit identification, target identification, lead optimization, and preclinical animal modelling [128,129].

Other models of melanoma not discussed here include the Sinclair swine model [130], which shows spontaneous regression; the Libechov minipig model [131]; and 3D human to mouse transplant models [132]. There are also *Xiphophorus* models [133] and an opossum melanoma model [134].

Conclusion

Perspectives and relevance of animal models to melanoma in humans

Animal model studies in a range of species confirm the 'naevus–melanoma' pathway as the major sequence of pathological progression to melanocytic malignancy. They also establish naevi as neoplasms that have mutations in oncogenes and tumour suppressor genes, as opposed to the previously held pathological view of naevi as non-neoplastic hamartomas. The experimental animal model studies demonstrate that some cancer genes (such as *BRAF*, *NRAS*, *MITF*, *TP53*, *P16/CDKN2A*, *BAP1*, *PTEN*, *C-KIT*, etc) can drive naevus formation and/or progression to melanoma in

various combinations, while sequencing studies of human melanomas emphasize the genetic heterogeneity of the disease with potential for reclassification based on the genetic phenotype in the future. Multi-species comparative pathology and genomics (human, mouse, zebrafish, dog, horse, other) help to identify new melanoma genes for cutaneous melanoma, mucosal melanoma, and less common melanomas at other sites, including the study of rare subtypes of melanoma. These molecular studies also shed light on melanoma progression genes that influence the stage or aggressive behaviour of the melanoma, potentially contributing to an improved molecular and mechanistic understanding of melanoma progression to metastasis in patients and serving as predictors of outcome or potential therapeutic targets. Small animal models (such as mouse or zebrafish) are informative for preclinical drug testing and investigation of mechanisms of drug resistance, as well as providing new insights into the melanoma–immune system interactions, which are of increasing relevance to patient therapy.

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Author contribution statement

LvdW, EEP, GAW, and AF wrote sections of the manuscript on mouse, zebrafish, dog, and horse melanoma, respectively. TB, MJA, and DJA wrote on the genetics and pathology of human melanoma. All authors contributed to revision of the manuscript and the final published paper. All authors contributed equally.

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