# Non-murine models to investigate tumor-immune interactions in head and neck cancer

**Carlos Rossa Junior**[1,2] and **Nisha J. D'Silva**[2,3]

[1]Department of Diagnosis and Surgery, School of Dentistry at Araraquara, UNESP- Univ Estadual Paulista, Araraquara, SP, Brazil

[2]Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, 48109, USA.

[3]Pathology, Medical School; University of Michigan, Ann Arbor, 48109, USA.

## Abstract

The immune response has important roles in the biology of solid tumors, including oncogenesis, tumor growth, invasion and metastasis, and response to treatment. Improved understanding of tumor-immune system interactions has provided promising therapeutic options that are based on the rescue and enhancement of the anti-tumoral host response. Immune-based treatments have been approved for clinical use in various types of cancer, including head and neck cancer (HNC); other strategies involving combination therapies are currently in development. These novel therapies were developed based on knowledge derived from in vitro, in silico, and in vivo pre-clinical studies. However, clinical trials seldom replicate the efficacy observed in pre-clinical animal studies. This lack of correlation between pre-clinical studies and clinical trials may be related to limitations of the models used; which highlights the relevance of considering immune-related aspects of different pre-clinical models. Murine models are the most frequently used pre-clinical models of HNC and are discussed elsewhere. Non-murine models have characteristics that offer unique opportunities for the study of HNC etiology, therapeutic strategies, and tumor-immune system interactions. The current review focuses on immune-related aspects of non-murine models, including dog, cat, pig, zebrafish, and frog, that could be used to investigate tumor-immune interactions in HNC.

## Keywords

head and neck cancer; experimental models; immunology

**Correspondence should be addressed to:** Dr. Carlos Rossa Junior, UNESP- Univ Estadual Paulista, School of Dentistry in Araraquara, Department of Diagnosis and Surgery, CEP 14801-903, Araraquara, SP, Brazil. c.rossa@unesp.br or Dr. Nisha J D'Silva, Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, 1011 North University Ave, Room G018, Ann Arbor, MI 48109-1078, USA, Phone: (734) 764-1543, Fax: (734) 764-2469, njdsilva@umich.edu.

**Conflict of interest**: The authors have declared that no conflict of interest exists.

## Introduction

The relationship between solid tumors and the host immune response is a hallmark of cancer (1) and an important aspect of tumor biology. The immune response may affect oncogenesis, tumor progression, and response to treatment. In the last two decades, therapeutic strategies aimed at harnessing the anti-tumoral immune response have been developed and introduced into clinical practice; the impact has been somewhat limited in tumors with reduced immunogenicity and transient in some tumors (2).

Head and neck cancer (HNC) is an immunosuppressive tumor (3). Increased prevalence of alternatively-activated macrophages (M2 phenotype) and of Th2- or $T_{reg}$-polarized T cells are correlated with more aggressive disease and poorer outcome (4–6). Conversely, increased numbers of CD8+ T cells are associated with better outcomes (7). Therefore, alleviating the immunosuppression associated with HNC to promote the endogenous anti-tumoral response by the immune system was a promising therapeutic strategy. Indeed, reducing the immunosuppression associated with the PD1/PD-L1 immunoinhibitory checkpoint has nearly tripled the 2-year survival rates of recurrent and metastatic HNC in comparison with other treatments (8). However, this 16.9% 2-year survival rate does not correspond to the dramatic efficacy observed in pre-clinical studies (9–11).

Pre-clinical studies are necessary for investigation of mechanisms of tumorigenesis, tumor progression/metastasis, and for the assessment of therapeutic strategies. Selection of models that recapitulate the dynamic contribution of the immune system in tumor progression will likely enhance the correlation between pre-clinical models and clinical trials. Interpretation of published research and selection of the appropriate model for future studies, should take into consideration the immune environment of the pre-clinical model. The majority of pre-clinical studies in HNC use murine models (reviewed recently in Rossa & D'Silva(12)); however non-murine models may provide unique features to investigate HNC, such as greater similarity with human physiology and anatomy than rodents, spontaneously-occurring rather than experimentally-induced or genetically-triggered HNC, improved visualization of cells in vivo, and opportunities for therapeutic trials in advanced stages of disease. Moreover, similarly to mice, some of these non-murine models are amenable to genetic manipulation. The drawbacks of using non-murine models include limited availability of reagents, higher cost, and increased housing space (Figure 1). Common limitations to all murine and non-murine pre-clinical models include the difficulty in adequately replicating the presence and long-term influence of conditions directly related with the etiology and progress of HNC in humans such as, HPV infection, alcohol and tobacco use, both independently and combined.

This review discusses immune-related aspects of selected non-murine models that can be used to study HNC. This information may help with interpretation of published data and design of new studies.

## Domestic animal models

Cats and dogs, the most common household pets, spontaneously develop cancer in the oral cavity. Since these are immunocompetent animals, their tumors may be more representative of HNC in humans than tumors induced in murine models in terms of initiation, genomic instability, heterogeneity, tumor microenvironment, and host immunity-tumor interactions (13). Moreover, the complete genome of the dog (14) and cat are published (15). In contrast to laboratory-based animals, which have standardized strains/ breed, housing conditions, treatment, possibility of programming sample collection time points, and defined criteria for euthanasia, these conditions may vary with household pets. The utility of household cat and dog models in tumorigenesis and mechanistic studies is reduced since diagnosis usually occurs in advanced stages. Procurement of these naturally occurring cases may also increase the timeline for study completion. However, these models are appropriate in therapeutic trials aimed at medical outcomes such as reducing tumor burden, and improving survival and quality of life. Moreover, owners, veterinary clinicians, and the public community, are very amenable to novel experimental treatments since there is no standard-of-care for HNC in dogs or cats; and also considering that these tumors are usually diagnosed at advanced stages, with limited treatment options and poor prognosis. Also, most clinical trials provide a financial incentive or at least do not involve out-of-pocket expenses to the owner, which is important given the paucity of health insurance coverage in veterinary medicine (16). Importantly, relative to translation to clinical studies in humans, many of the variations observed in household pets (e.g., living/ housing conditions, genetic heterogeneity of individuals and of neoplastic cells, diagnosis in advanced stages) are similar to those observed in humans (16–18). Therefore, household pets may be an appropriate intermediate model between pre-clinical studies in mice and clinical studies in humans.

## Feline spontaneous oral squamous cell carcinoma (FOSCC) model

Oral cancer represents approximately 10% of all neoplasms affecting aged cats (19), which is comparable to HNC prevalence among all cancers in humans (3–8%, depending on the population) (20). The most common oral cancer in aged cats (12 – 13 years old) is FOSCC, with primary locations in the gingiva, floor-of-mouth, and dorsum of the tongue (21). As in humans, FOSCC is usually diagnosed in advanced stages, and is associated with poor prognosis and reduced overall survival. For in-depth specific reviews of FOSCC as a study model in comparative oncology, please see (16, 22).

Molecular similarities between FOSCC and human HNC include increased expression of EGFR (23), 5-lipoxygenase (24), as well as aberrant expression of p53 (25). Also, FOSCC presents increased vascularization and cell proliferation (23) though these characteristics may vary with the primary tumor site (26). FOSCC is similar to HPV-negative human HNC (27). Metastases to lymph nodes (31% of cases) and lung (10% of cases) (28), and loco-regional invasion of maxilla or mandible (21) are similarities between FOSCC and human HNC.

Cell lines derived from FOSCC have been characterized as models to study human HNC (29), including etiopathogenesis and treatment (30, 31). However, most studies on FOSCC

have been published in the veterinary literature, which may limit visibility and access to researchers focused on human HNC (Table 1).

The normal oral mucosa of cats is parakeratinized stratified squamous epithelium with shallow rete ridges. The subjacent tissue is composed of loose connective tissue, minor salivary glands, striated muscle, blood vessels, lymphatics with a few clusters of lymphocytes and plasma cells, and sparse metachromatic mast cells (32). MHC II-positive cells with dendritic cell morphology cluster beneath the basal epithelial layer in association with CD3+ cells. Interestingly, CD8+ cells predominate over CD4+ cells both in the connective tissue and in intraepithelial sites (32). Feline chronic gingivostomatitis, an inflammatory condition of undefined etiology that may affect gingival and non-gingival oral mucosa, is characterized by epithelial hyperplasia and greater cellularity in the subjacent stroma. The numbers of CD3+ (T lymphocytes), CD79+ (plasma cells), and MHC II-positive (macrophages, dendritic cells) cells increase with severity of inflammation. Together these findings suggest a functional immune response in the oral mucosa of cats that is largely similar to that of humans. Overall, the cellular and soluble components of the cat's immune system are similar to those of humans; however only three IgG subclasses were identified in cats. Moreover, cats lack one locus of the class II MHC gene cluster (33), which may affect antigen presentation and ultimately the adaptive immune response (Table 2). Similarity with the immune system of humans is also supported by similar susceptibility to infections (viral, fungal, bacterial), particularly affecting the respiratory tract, as well as asthma (34). However, a study focusing on the immune response to FOSCC in the cat model was not identified in a literature search.

In FOSCC, administration of anti-inflammatory agents as adjuvant to chemotherapy provides an added benefit in terms of survival (35) and the increased expression of COX-1, COX-2 (36) and 5-lipoxygenase (24). This suggests that the host response is a relevant factor in the initiation and progression of these tumors. Interestingly, studies assessing tumor-host response interactions in the FOSCC were not identified. However, mammary tumors in cats are notoriously aggressive and similar to breast cancer in humans with respect to histopathology, and biology, including pattern of metastasis (37), suggesting comparable tumor-immune interactions.

## Canine spontaneous oral squamous cell carcinoma (COSCC) model

Neoplasms of the mouth and pharynx represent 5.4% of all malignant tumors in dogs, which is similar to the incidence of HNC in humans (20). The oral cavity is the most common site of COSCC. Canine HNC may present as a rare, aggressive, invasive, and frequently metastatic tonsillar SCC (38), or a more common (second most common oral tumor in dogs), less aggressive non-tonsillar SCC, of which 80% have a good prognosis and 19% develop metastases (39).

The most frequent site of primary oral SCC in dogs is the gingiva, whereas the tongue (a common site in humans) is rarely affected. In general, dogs are more frequently taken for preventive health care visits by their owners, which may facilitate procurement of cases for research (34).

Constituents of the immune system in dogs are comparable to the human immune system. Dogs present the same range of lymphoid cell subsets including CD8 cytotoxic T cells, NK cells, monocytes/macrophages, neutrophils, CD4 T helper cells and the polarized subsets Th1, Th2, Th17 and Treg that are characterized by expression of the same cytokines as in humans. Innate immune cells express a similar range of pathogen-recognition receptors, including cytosolic NOD-like receptors, as well as the same spectrum of antigen-presenting cells (40). There are slight differences in the humoral response, as dogs present four subclasses of IgG that are functionally equivalent to those of humans (41, 42) (Table 2).

In general, dogs have lower genetic diversity and higher linkage disequilibrium than humans because of intensive selective breeding (43). This may affect the functionality of the immune system, which may be related to their susceptibility to arthropod-borne infections, cutaneous allergies, and autoimmune conditions linked to genetic inheritance of susceptibility haplotypes of MHC genes (34). Dogs are more prone to genetically-associated (or breed-specific) tumors, such as mast cell tumors in Boxers and Labradors, and hemangiosarcomas in German shepherds (44).

Higher grade COSCCs are usually observed in the tonsillar region, whereas the gingiva may present both low- and high-grade tumors (45). Tonsillar SCC are more frequently associated with lymph node metastasis and with increased expression of Ki-67 (46). Non-tonsillar SCC is associated with loco-regional invasion, spread into adjacent bone, and, in more advanced stages, lymph node metastasis; pulmonary metastases are rare. Interestingly, tumors with greater inflammation are associated with worse prognosis in non-tonsillar SCC (39). Non-tonsillar COSCC presents heterogeneous copy number abnormalities, mostly amplifications involving similar genes affected in human HNC (of 63 amplified genes, 42 are also amplified in human HNC), such as *IKBKB, MYC, FGFR1, ADAM9.* Similar to human HNC, cell cycle *CDKN2A* is frequently deleted in COSCC. Moreover RNAseq analysis showed similarities between dog and human HNC in increased expression of genes associated with cell cycle (*CDK4, CDK6, E2F1*), protein kinase activity (*EGFR, AKT1, PIK3CA*) and TGFβ-related genes (*TGFB1, TGFB2, TGFBR1, TGFBR2, SMAD3*). Overall, COSCC is similar to human primary HNC in regard to molecular heterogeneity and complexity, tumor microenvironment, biology, and histopathology (47). Mutation of *HRAS* is observed in COSCC (and in approximately 4% of human HNC (48, 49) and correlates with increased activation of MAPK and PI3K signaling (50). Also similar to human HNC, COSCC presents increased angiogenesis and VEGF expression (51), as well as Cox-2 expression (52).

There was no correlation between positivity for canine papilloma virus DNA and expression of p16 tumor suppressor protein (53), suggesting that papilloma virus infection may not have a significant role in the development of COSCC. Both human and canine HNC are associated with increased expression of high mobility group A2 protein (HMGA2), which is considered a negative prognostic marker in human HNC (54). In humans, upregulation of HMGA2 protein is related with altered post-transcriptional regulation by let-7 miRNA (55).

Moderate to severe tumor-associated inflammation was observed in approximately 70% of well- and moderately-differentiated COSCC (56). Reduced survival of dogs has been

correlated with increased inflammation in non-tonsillar OSCC (39). However, no reports were identified showing characterization of inflammatory cell types or of interaction between inflammatory phenotype and tumor aggressiveness or clinical outcome. Similar to the feline model, most canine studies including clinical trials are published in veterinarian journals (Table1) and the clinical trials usually involve non SCC tumors. Together these factors reduce the visibility of information to researchers focused on human HNC and may be related to the limited use of the canine model to assess tumor-immune interactions.

## Porcine model

The anatomy, body mass, and tissue responses in pigs have greater similarity with humans in comparison to rodents, cats, or dogs. This makes the pig an interesting model for surgery, chemotherapy, radiation therapy, and imaging studies (57). Moreover, pigs have greater physiologic and genomic similarities with humans than rodents, cats or dogs (58), which is supported by the use of porcine-derived insulin (until the introduction of recombinant human insulin), porcine-derived heparin (only FDA-approved source), and porcine heart valves in humans (59). Additionally, pigs have a relatively short gestational period (<3 months) and produce a large offspring (approximately 12 piglets), are relatively easy to maintain, and have dosing and pharmacokinetic characteristics similar to humans, which is useful in therapeutic drug trials. However, the initial cost of experimental pigs is much greater than rodents.

The immune system of pigs is similar in composition to other mammals, including man (Table 2). Innate immunity includes neutrophils, macrophages, dendritic cells, NK cells, $\gamma\delta$T cells, and also similar expression of pattern-recognition receptors, cytokines, chemokines, complement factors, and antimicrobial peptides (60). Interestingly, porcine NK cells express MHC class II and costimulatory CD80/CD86, which allows them to stimulate CD4+ T cells (61). Similar to humans, in pig fetuses, B cells develop in the liver and T cells mature/develop in the spleen. In adult humans and pigs, B cells form in the bone marrow and T cells mature in the thymus. B cells produce the same five immunoglobulin isotypes as other mammals. Maturation of $\alpha\beta$T cells in the thymus is similar to humans, resulting in CD3^{high} single positive CD4/T-helper or CD8/cytotoxic T cells. However, pigs have a much higher proportion of $\gamma\delta$T cells, which predominate in the peripheral blood, in contrast to humans in whom $\alpha\beta$T cells are predominant. Also, in contrast to humans, porcine $\gamma\delta$ and $\alpha\beta$T cells maintain expression of CD8$\alpha$ and MHC class II after activation. Even activation of CD4+CD8- T helper cells leads to the expression of CD8$\alpha$ and peripheral CD4/CD8 double-positive T cells in the effector/memory pool (62). The lymph nodes of pigs are also distinct, as they are characterized by an inverted structure different from that of humans and mice, comprised mostly of paracortical and cortical areas without a larger medullary region. Also, in pigs, lymphocytes exit the lymph nodes directly in the blood, via high endothelial venules and not via efferent lymph vessels (63). On the other hand, pigs present a lymphoid structure that is similar to the Waldeyer's ring in humans, including palatine and pharyngeal tonsils, which are absent in mice. Functionally, the MHC complex in pigs is comprised of three major gene clusters, which is smaller than other mammal MHC gene complexes. Also, the pig is the only mammal in which the MHC gene complex spans the centromere (64). Despite these peculiarities of the pig immune system, it is functionally similar to human

immune responses in 80% of analyzed parameters, as opposed to 10% in a human-mouse comparison using the same parameters (65, 66).

There are lines of mini-pigs (with reduced space and food requirements), specifically bred for biomedical research, including inbred and clonal lines of genetically identical animals. The publication of the pig genome (67) allowed the identification of natural mutations that are similar to those observed in human diseases and provided a fundamental resource for the generation of genetically-engineered lines. These include shRNA-induced stable gene knockdown combined with somatic cell nuclear transfer to generate clonal pig lines harboring the genetic modification (68), as well as the use of zinc finger nucleases and transcription activator-like effector nucleases (TALEN) directly in pig zygotes to edit the pig genome independently of cloning (somatic cell nuclear transfer) (69), CRISPr and transposon systems (70).

Similar to human cells and in contrast to murine cells, porcine cells are resistant to oncogenic transformation by genetic manipulation and chemical carcinogens and require simultaneous changes in 6 genes (induced by retroviral-mediated expression of cDNAs for mutated forms of hTERT, p53, Cyclin D1, CDK4, c-Myc and H-ras) for malignant transformation (71). Transgene expression of activated *v-Ha-ras* is not sufficient to induce tumors in pigs (72), whereas the same transgene expressed under the same promoter is carcinogenic in mice (73). Some porcine cancer models have been developed, including cutaneous skin lesions and melanomas associated with inherited mutations propagated by selective breeding (74), and a model of chemically-induced liver tumors (75). Transgenic pigs harboring mutations of tumor suppressor *TP53* develop lymphomas and osteogenic tumors (76). Transgenic animals expressing a Cre-induced constitutively-active mutated form of oncogenic *K-ras* were also developed (77). A genetically-engineered animal with Cre recombinase-induced simultaneous expression of mutated tumor suppressor *TP53* and active *K-ras* oncogene, called 'oncopig', has been successfully used as a model for soft-tissue sarcoma (78, 79) and hepatocellular carcinoma (80). Another transgenic pig line with simultaneous transgene expression (active *Kras* and *cMyc,* and a repressor of p53) has been described as a conditional intestinal cancer model (81). The transgene is under the control of Flp recombinase, which is conditionally-induced by tamoxifen under the control of an intestinal epithelium-specific promoter.

Transplantation models are also possible in pigs, but allogeneic transplantation requires immunosuppression (71), which limits the utility of the model to study tumor-immune interactions. In fact, there are genetically-engineered lines of immunosuppressed pigs that are amenable to xenografts. *IL2rg*-deficient pigs have X chromosome-linked heritability and display a T-B+NK- phenotype that better resembles X-SCID syndrome in humans in comparison to *IL2rg*-targeted mice. Moreover, these animals are amenable to allogeneic immune system reconstitution (82). *Rag1/Rag2*-targeted transgenic pigs have a T-B-NK+ phenotype (83–85), with NK cells having an unaltered phenotype and functionality, as opposed to the overactive, proliferation-deficient NK cells that are characterized by a different surface marker profile in *Rag*-targeted mice (84, 86). The immune phenotype in these model animals may be exploited to study the relevance of B cells or NK cells in tumor-immune system interactions. *Rag/IL2rg*-targeted pigs present a T-B-NK- immune phenotype

(87), and although attempts of human immune reconstitution (humanization) in pigs have not been published, similarity with the human immune system, and progress in genetic manipulations and conditions for successful engraftment of hematopoietic stem cells (84, 88), are promising. One study was identified with the human xenograft pig model of cancer (glioblastoma) (89) using human cell lines, and not primary cells or tissues (patient-derived xenograft, PDX). Most interestingly, nearly 20 years ago a study describing a laparoscopic approach for sigmoid colectomy used a xenograft of HeLa cells into the peritoneal cavity of fully immunocompetent young pigs (90). This study reported human cells in 63% of port sites and mini-laparotomies, suggesting that implantation of human cancer cells may be successful in young animals.

A domestic mini-pig model for the study of radiation-induced tissue changes in the oral cavity was recently described (91). An earlier study assessed the effect of radiation on parotid salivary glands in mini-pigs (92). Mouthwash formulations aimed at preventing chemo- or radiation-induced oral mucositis have been tested in the pig model (93, 94). In relation to HNC, because of the similarities in size and anatomy, porcine models are also used for training and assessment of novel surgical biopsy techniques (95, 96). Interestingly, reports of the pig model for investigations of molecular mechanisms of HNC pathogenesis, progression, or treatment, were not identified.

## Zebrafish model

This non-mammal vertebrate model has unique characteristics that are attractive for investigations of tumor-immune system interactions. High fecundity, short generation time, large number of offspring that reduces turnaround time for experiments, and external embryonic development that allows genetic manipulation in early developmental stages, are some advantages. Moreover small size that makes it amenable to therapeutic drug trials with low cost, optical clarity that allows in vivo visualization of fluorescent-tagged cells, and monitoring processes including angiogenesis, tumor growth, and cell-cell interaction, are additional practical and scientific aspects of zebrafish biology that are valuable for an in vivo model of cancer. All these features, in combination with the possibility of genetic manipulation and the current knowledge of the zebrafish genome (97) may be used for the development of novel models in an immunocompetent animal. Macrophages and neutrophils develop in the first two days of zebrafish embryogenesis, and there is no adaptive immune system in the first 30 days of development (98). This temporal segregation of immune development allows investigations of tumor-innate immunity interactions in the absence of adaptive immunity in young zebrafish, and tumor-innate/adaptive immune system interactions in adult zebrafish over 30 days of age.

The adult zebrafish has both innate and adaptive immunity (Table 2). Innate immune cells, complement factors, and anti-microbial enzymes are present soon after fecundation (99). Various putative orthologs of mammalian pattern recognition receptors, including TLRs and NODs, have been identified (99), as well as macrophages, neutrophils, mast cells, and antigen-presenting dendritic cells (100, 101*). There are T cells expressing αβ- and γδ-T cell receptors and B cells producing three types of immunoglobulins: IgM, IgT, which is an evolutionary ortholog of IgA (102*), and IgD. T cell subpopulations express co-receptors

and similar membrane markers found in mammals (CD3, CD28, CTLA4) and include CD4+, CD8+, Treg, and Th17 cells (103*, 104*). These cells are functionally active in vitro and in vivo, and express and respond to similar lymphocyte-related cytokines as mammals (105*). Fish cells also express MHC class I and II. Cytotoxic responses mediated by CD8 T cells and NK cells are also similar to mammals and involve granule exocytosis and interaction with death receptors (FasL/Fas) (104*).

Zebrafish can be used in models of spontaneous cancers, relying on genetic pathways of cancer that are conserved between fish and humans (106*). Genetic signature profiles and histopathologic characteristics of human and zebrafish cancers have a high degree of similarity (107*). The first cancer models established in zebrafish were lymphoid (B and T cell) leukemia models (108*) and are based on the overexpression of a mutated proto-oncogene under the control of a lymphoid-specific *Rag2* promoter. Some solid tumor models (e.g., liver, pancreatic, rhabdomyosarcoma, melanoma) were developed by genetic screens of mutations induced by the carcinogen N-ethyl-N-nitrosourea (ENU) (109*), but may also be generated by forward and reverse genetic manipulation approaches (110*, 111*). Expression of mutated oncogenes that are observed in human primary cancers can be induced by direct DNA injection (112*), retroviral vectors (113*), transposon-mediated integration (114*), or in a cell/tissue-specific targeted (i.e., promoter-specific) and inducible manner (heat-shock Cre-LoxP, Tetracycline-regulated, a transposon/gene trap method, a synthetic steroid hormone-regulated system, or a GAL4/UAS system) (115*–119*). Site-specific mutagenesis (using approaches such as CRISPr/Cas9, zinc finger nucleases or TALENs) (120*–122*) is also used to generate transgenic zebrafish lines.

Established transgenic zebrafish lines (123*) may be selectively crossed to generate compound lines harboring two or more transgenes and to study gene interaction in tumor development (124*, 125*). Some transgenic zebrafish lines are of particular interest in HNC as they harbor mutations that are frequently detected in human tumors, such as *p53* (126*), *PTEN* (127*) and *Ras* (125*). Genetically-altered immunosuppressed lines of zebrafish can be used in xenograft studies, such as the *Rag1* mutant that presents an underdeveloped thymus and lacks B and T cell activity, including a complete absence of alloantigen-induced cytotoxic T cell response. Although the lack of alloantigen rejection may allow for xenograft transplants, the *Rag1* mutant zebrafish do not develop SCID syndrome and are able to survive in non-specific pathogen-free conditions. This indicates that their innate immunity is activated in a compensatory manner, with increase in the monocyte/granulocyte cell population and shift in the immune response from the spleen to the hepatopancreas (128*).

Homozygous diploid clonal zebrafish lines allow the transplantation of tumors (usually chemically induced) in a syngeneic immunocompetent model, without the need for sub-lethal irradiation or drug-induced immunosuppression (129*).

Allogeneic transplantation of tumor cells from genetically-altered animals and xenograft transplants of mammalian tumor cells are possible (130*). However, these transplantation strategies require immunosuppression by sub-lethal irradiation in adult fish or the use of juvenile fish (less than 30 days-old) that lack adaptive immunity combined with high dose corticosteroid-induced immunosuppression (131*). Mammalian tumor cells can be grafted

into early embryos (<24 post-fertilization), which lack mature immune cells. This approach allows for the study of angiogenesis, proliferation, and migration of tumor cells (132*, 133*). However the experimental time is limited to a few days and the underdeveloped organs restrict the possibility of studying tumor-host interactions (129*). More recently, patient-derived xenograft (PDX) models of breast (134*) and gastric (135*) cancers have been described though all the considerations regarding tumor-immune interactions remain relevant, since these models use immunodeficient zebrafish larvae. Nevertheless, these novel PDX models allow assessment of proliferation, angiogenesis, invasive potential of primary tumor cells, and drug sensitivity (136*). Interestingly, only 8 studies of HNC in zebrafish were identified. All but one of these studies focused on tumor cell biology without assessment of tumor-immune system interactions (Table 1).

Limitations of this model include greater evolutionary dissimilarity in comparison to mammalian models, and lower temperature (28°C) required by zebrafish that may affect behavior of mammalian tumor cells and pharmacological properties of chemotherapeutics. Nevertheless, zebrafish is a powerful model for large-scale screens of novel water-soluble chemotherapeutics (137*, 138*). Allogenic and xenograft models also require immunosuppression of the host animal, which limits their use in the study of tumor-immune system interactions.

## Frog (*Xenopus*) model

*Xenopus tropicalis* (western clawed frog) is the only species in the genus *Xenopus* to have a diploid genome. Its genome sequence has been published (139*), which facilitates the use of this model in research. Additional advantages are the small size (4–6 cm) and shorter generation time (< 5 weeks), and a large number of eggs per spawn. Both in vitro and in vivo experimental approaches have been used in cancer research, from oocytes to egg extracts, from cell cultures to whole embryos (140*). The *Xenopus* oocyte has been described as a 'living test tube' to study DNA biology and metabolic regulation because it is large enough to allow easy manipulation (microinjection), and single-cell biochemical measurements (141*). Cell free extracts of *Xenopus* eggs have been used to study DNA replication, repair, and damage response (142*), processes that are directly implicated in oncogenesis and resistance to treatment. Molecular mechanisms controlling tissue differentiation are highly conserved between amphibians and mammals. In fact, developmental biology studies in the *Xenopus* model have uncovered and enhanced understanding of conserved signaling pathways (e.g., Wnt, Shh, Notch, Smad) that are frequently dysregulated in cancer (143*– 145*). Also elucidated from *Xenopus* studies are cellular processes associated with tumor invasion and metastasis, such as proliferation, apoptosis, epithelial-mesenchymal transition, angiogenesis and lymphangiogenesis (140*, 146*, 147*).

Embryos of *Xenopus* have been used in cancer research using genome editing (148*–152*). A model of thymic lymphoid tumors transplanted into isogenic *Xenopus laevis* (African clawed frog), tadpoles, and young adult post-metamorphic froglets (T-cell deficient adults may also be used) allows investigations of tumor growth, neovascularization, immune infiltrate, and microenvironment (153*). Similar to the zebrafish model, genetic engineering in *Xenopus* is facilitated by the extra-uterine development of large and optically clear

embryos. Also, similar to zebrafish, *Xenopus* embryos are usually derived from outbred founders, which is more closely related to disease in humans (152*). Outbreeding reduces influences associated with genetic background that could occur with inbred mice (154*), such as passenger mutations and phenotypic biases (155*). However, zebrafish undergo full genome duplication (156*) that may result in redundant gene duplicates (Ohnologs), as opposed to the true diploid genome of *Xenopus* that facilitates the identification of orthologues. Also, in contrast to zebrafish, some organ systems in *Xenopus* (e.g., lungs, limbs) are evolutionarily closer to humans (157*). Knowledge of *Xenopus* developmental biology allows targeted nuclease approaches (CRISPR/Cas9 or TALEN) by site-specific injections to induce tissue-restricted mutations (roughly similar to Cre-LoxP system in mice, but bypassing the need for cross-breeding), avoiding embryonic lethality and excessive toxicity. Moreover, direct injection of targeted nucleases is a straightforward technique that permits large samples of genetically-modified frogs (152*).

Similar to mice, it is possible to obtain homozygous or heterozygous genetically modified adult frogs that harbor deficiencies in tumor suppressor genes (158*, 159*). Genetically engineered tumors that develop in situ can be used to investigate tumor-stromal interactions. Interestingly, tumor-like structures will develop in F0 animals after injection of targeted nucleases into developing embryos and it is possible to simultaneously (i.e., single-step) edit three genes using a multiplex approach of genome engineering. In addition, unilateral injections of targeted nucleases can generate animals with wild-type and mutant organs (158*). It is also possible to breed genetically-modified animals to heterozygosity and assess changes in the tumor phenotype (152*).

*Xenopus* models can also be used for validation of pharmacological activity of compounds on established tumors, with the advantage of adding water-soluble compounds directly to the rearing water (similar to zebrafish). From the water, compounds may be taken up by different routes, avoiding injections or other procedures that involve additional manipulation and time (151*, 160*).

Spontaneous tumors are relatively rare in amphibians (161*) and this resistance to tumor development provides an interesting model to study tumor immunology. In fact, studies in *Xenopus* have contributed to the understanding of the role of T cells in immune-surveillance and immune-editing, as well as the role of reduced expression of class I MHC in immunoescape by tumors (161*, 162*).

Development of some amphibians, including *Xenopus tropicalis*, is unique because of the intermediary larval stage (tadpole) from fertilized egg to adult. Metamorphosis is the process by which the developing animal transitions from exclusively aquatic to air-breathing life. This involves external/anatomic changes (loss of the tail and external gills, growth of limbs, changes in the skin) and internal changes involving nearly all systems (digestive, circulatory and respiratory systems, ossification and remodeling of bones) (163*). The immune system also differs at these stages of development, as T helper-like cells are only present in the adult. A full allograft rejection response and expression of class I MHC and class II MHC in thymocytes and T cells also develops with completion of metamorphosis (164*). In the adult frog, a functional histocompatibility complex analog to HLA (called 'XLA') mediates graft

rejection and mixed leukocyte response (MLR). Thymectomy impairs MLR and allograft rejection, as well as proliferation in response to T cell antigens and T cell-dependent antibody responses. However, it does not affect rejection of xenografts or production of antibodies induced by T cell-independent antigens. This suggests that the thymus generates and contains T helper-like cells, although helper cells are reported to be more abundant and efficient in the spleen (164*). Adult animals present MHC-restricted cytotoxic and helper T-cell responses (165*, 166*) (Table 2). Moreover, important anti-tumor mechanisms are conserved and can be assessed in this model. This includes NK and CD8+ T cell-mediated anti-tumor responses (class I MHC-restricted and also unrestricted and non-classical class I MHC cytotoxic responses) that are enhanced by heat shock proteins (167*).

The humoral response is also present and mediated by B cells. IgM is the most abundant of the five isotypes of antibodies, that include another polymeric molecule called IgX (analog to IgA), IgY (analog to IgG), IgD and IgF (168*, 169*). Antibody responses in *Xenopus* are slower than in mammals and involve initial production of IgM, followed by IgY. Interestingly, many B cells produce IgM and IgY isotypes simultaneously, in an incomplete class switch.

In general, the immune system of *Xenopus* is less complex than that of mammals, with no lymph nodes and a smaller number of lymphocytes (164*) (Table 2). However, *Xenopus* can be used to study lymphangiogenesis, as lymphatic vessels are present in both tadpoles and adult animals (170*). The lymphatic system is composed of subcutaneous spaces that are interconnected but separated by one-way valves. There are no lymph nodes, and lymph is pumped into the circulatory system by specialized structures called lymph hearts (171*). Although structurally different from the mammalian lymphatic system, it is possible to use computer tomography to visualize and track lymph movement in *Xenopus* (172*); which may provide an interesting opportunity to study the immune system and tumor metastasis (153*).

Despite the interesting practical and biological characteristics, studies on HNC in the *Xenopus* model were not identified. Investigations of tumor-immune interactions are mostly limited to the spontaneous thymic lymphoid tumor model; which has significant biological differences with SCC. Nevertheless, the *Xenopus* model offers unique opportunities to study tumor-immune interactions that can be explored.

## Concluding remarks

HNC is a highly heterogeneous disease, associated with multiple genetic and epigenetic changes. In addition to the higher risk associated with particular demographic profiles (males, >50 years, tobacco), affected patients present unique genetic and epigenetic characteristics and co-morbidities that influence oncogenesis, and the host response. There has been an increasing realization that understanding and modulating the host response in HNC is critical to early diagnosis, prognosis, and development of improved therapeutic strategies. Pre-clinical models have a fundamental role in understanding biological mechanisms associated with HNC progression and in evaluating therapeutic strategies. Traditionally, most in vivo studies in HNC, including studies on tumor-immune interactions,

involve rodents (hamsters, mice, rats). This review is a summary of information on non-murine models with respect to immune characteristics and applicability in HNC research. These alternative in vivo models provide unique characteristics that may be exploited in the study of HNC-immune interactions (Figure 1). These characteristics include spontaneous development of orthotopic tumors in aged animals; physiology and anatomy similar to humans, segregating the influence of innate and adaptive immunity, and improved in vivo visualization of tumor cells. Similarly to rodents, some of these models allow for targeted genetic manipulation and immunosuppression. It is important to note that all pre-clinical models have limitations, and mimicking dormant HPV viral infection status, and alcohol and tobacco use, are particularly difficult as these conditions may be present independently and combined, influencing oncogenesis and tumor progression over an extended period. Moreover, each of these non-murine models has its own challenges including cost, housing space, and for spontaneous HNC in dogs and cats, greater difficulty in procuring affected animals. These characteristics and the immune peculiarities of the alternative in vivo models should be considered in the design of studies exploring HNC-immune interactions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements:

## References

References that are followed by an asterisk (*) in the manuscript (text and table) can be found in the appendix.

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–674. [PubMed: 21376230]

2. Popovic A, Jaffee EM, Zaidi N. Emerging strategies for combination checkpoint modulators in cancer immunotherapy. The Journal of clinical investigation. 2018;128(8):3209–3218. [PubMed: 30067248]

3. Ferris RL. Immunology and Immunotherapy of Head and Neck Cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33(29):3293–3304. [PubMed: 26351330]

4. Alves AM, Diel LF, Lamers ML. Macrophages and prognosis of oral squamous cell carcinoma: A systematic review. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2018;47(5):460–467.

5. Gooden MJ, de Bock GH, Leffers N, Daemen T, Nijman HW. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. British journal of cancer. 2011;105(1):93–103. [PubMed: 21629244]

6. Hanna GJ, Liu H, Jones RE, Bacay AF, Lizotte PH, Ivanova EV, et al. Defining an inflamed tumor immunophenotype in recurrent, metastatic squamous cell carcinoma of the head and neck. Oral oncology. 2017;67:61–69. [PubMed: 28351582]

7. de Ruiter EJ, Ooft ML, Devriese LA, Willems SM. The prognostic role of tumor infiltrating T-lymphocytes in squamous cell carcinoma of the head and neck: A systematic review and meta-analysis. Oncoimmunology. 2017;6(11):e1356148. [PubMed: 29147608]

8. Ferris RL, Blumenschein G Jr., Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. Oral oncology. 2018;81:45–51. [PubMed: 29884413]

9. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(19):12293–12297. [PubMed: 12218188]

10. Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. Cancer research. 2005;65(3): 1089–1096. [PubMed: 15705911]

11. Zhang C, Wu S, Xue X, Li M, Qin X, Li W, et al. Anti-tumor immunotherapy by blockade of the PD-1/PD-L1 pathway with recombinant human PD-1-IgV. Cytotherapy. 2008;10(7):711–719. [PubMed: 18841514]

12. Rossa C Jr., D'Silva NJ. Immune-relevant aspects of murine models of head and neck cancer. Oncogene. 2019.

13. Supsavhad W, Dirksen WP, Martin CK, Rosol TJ. Animal models of head and neck squamous cell carcinoma. Veterinary journal. 2016;210:7–16.

14. Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature. 2005;438(7069):803–819. [PubMed: 16341006]

15. Montague MJ, Li G, Gandolfi B, Khan R, Aken BL, Searle SM, et al. Comparative analysis of the domestic cat genome reveals genetic signatures underlying feline biology and domestication. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(48): 17230–17235. [PubMed: 25385592]

16. Wypij JM. A naturally occurring feline model of head and neck squamous cell carcinoma. Pathology research international. 2013;2013:502197. [PubMed: 23970998]

17. Khanna C, Lindblad-Toh K, Vail D, London C, Bergman P, Barber L, et al. The dog as a cancer model. Nature biotechnology. 2006;24(9):1065–1066.

18. Kamb A What's wrong with our cancer models? Nature reviews Drug discovery. 2005;4(2):161–165. [PubMed: 15688078]

19. Stebbins KE, Morse CC, Goldschmidt MH. Feline oral neoplasia: a ten-year survey. Veterinary pathology. 1989;26(2):121–128. [PubMed: 2711569]

20. Shield KD, Ferlay J, Jemal A, Sankaranarayanan R, Chaturvedi AK, Bray F, et al. The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012. CA: a cancer journal for clinicians. 2017;67(1):51–64. [PubMed: 28076666]

21. Martin CK, Tannehill-Gregg SH, Wolfe TD, Rosol TJ. Bone-invasive oral squamous cell carcinoma in cats: pathology and expression of parathyroid hormone-related protein. Veterinary pathology. 2011;48(1):302–312. [PubMed: 20940448]

22. Cannon CM. Cats, Cancer and Comparative Oncology. Veterinary sciences. 2015;2(3):111–126. [PubMed: 29061935]

23. Bergkvist GT, Argyle DJ, Morrison L, MacIntyre N, Hayes A, Yool DA. Expression of epidermal growth factor receptor (EGFR) and Ki67 in feline oral squamous cell carcinomas (FOSCC). Veterinary and comparative oncology. 2011;9(2):106–117. [PubMed: 21569196]

24. Wakshlag JJ, Peters-Kennedy J, Bushey JJ, Loftus JP. 5-lipoxygenase expression and tepoxalin-induced cell death in squamous cell carcinomas in cats. American journal of veterinary research. 2011;72(10):1369–1377. [PubMed: 21962280]

25. Snyder LA, Bertone ER, Jakowski RM, Dooner MS, Jennings-Ritchie J, Moore AS. p53 expression and environmental tobacco smoke exposure in feline oral squamous cell carcinoma. Veterinary pathology. 2004;41(3):209–214. [PubMed: 15133168]

26. Yoshikawa H, Ehrhart EJ, Charles JB, Thamm DH, Larue SM. Immunohistochemical characterization of feline oral squamous cell carcinoma. American journal of veterinary research. 2012;73(11):1801–1806. [PubMed: 23106467]

27. Munday JS, Knight CG, French AF. Evaluation of feline oral squamous cell carcinomas for p16CDKN2A protein immunoreactivity and the presence of papillomaviral DNA. Research in veterinary science. 2011;90(2):280–283. [PubMed: 20621320]

28. Soltero-Rivera MM, Krick EL, Reiter AM, Brown DC, Lewis JR. Prevalence of regional and distant metastasis in cats with advanced oral squamous cell carcinoma: 49 cases (2005–2011). Journal of feline medicine and surgery. 2014;16(2):164–169. [PubMed: 24027053]

29. Rathore K, Alexander M, Cekanova M. Piroxicam inhibits Masitinib-induced cyclooxygenase 2 expression in oral squamous cell carcinoma cells in vitro. Translational research : the journal of laboratory and clinical medicine. 2014;164(2):158–168. [PubMed: 24631063]

30. Klobukowska HJ, Munday JS. High Numbers of Stromal Cancer-Associated Fibroblasts Are Associated With a Shorter Survival Time in Cats With Oral Squamous Cell Carcinoma. Veterinary pathology. 2016;53(6):1124–1130. [PubMed: 26940838]

31. Supsavhad W, Dirksen WP, Hildreth BE, Rosol TJ. p16, pRb, and p53 in Feline Oral Squamous Cell Carcinoma. Veterinary sciences. 2016;3(3).

32. Harley R, Gruffydd-Jones TJ, Day MJ. Immunohistochemical characterization of oral mucosal lesions in cats with chronic gingivostomatitis. Journal of comparative pathology. 2011;144(4):239–250. [PubMed: 21084098]

33. Yuhki N, Beck T, Stephens R, Neelam B, O'Brien SJ. Comparative genomic structure of human, dog, and cat MHC: HLA, DLA, and FLA. The Journal of heredity. 2007;98(5):390–399. [PubMed: 17675392]

34. Day MJ. Cats are not small dogs: is there an immunological explanation for why cats are less affected by arthropod-borne disease than dogs? Parasites & vectors. 2016;9(1):507. [PubMed: 27646278]

35. Wiles V, Hohenhaus A, Lamb K, Zaidi B, Camps-Palau M, Leibman N. Retrospective evaluation of toceranib phosphate (Palladia) in cats with oral squamous cell carcinoma. Journal of feline medicine and surgery. 2017;19(2):185–193. [PubMed: 26755491]

36. Hayes A, Scase T, Miller J, Murphy S, Sparkes A, Adams V. COX-1 and COX-2 expression in feline oral squamous cell carcinoma. Journal of comparative pathology. 2006;135(2–3):93–99. [PubMed: 16952367]

37. Hassan BB, Elshafae SM, Supsavhad W, Simmons JK, Dirksen WP, Sokkar SM, et al. Feline Mammary Cancer. Veterinary pathology. 2017;54(1):32–43. [PubMed: 27281014]

38. Mas A, Blackwood L, Cripps P, Murphy S, De Vos J, Dervisis N, et al. Canine tonsillar squamous cell carcinoma -- a multi-centre retrospective review of 44 clinical cases. The Journal of small animal practice. 2011;52(7):359–364. [PubMed: 21726228]

39. Fulton AJ, Nemec A, Murphy BG, Kass PH, Verstraete FJ. Risk factors associated with survival in dogs with nontonsillar oral squamous cell carcinoma 31 cases (1990–2010). Journal of the American Veterinary Medical Association. 2013;243(5):696–702. [PubMed: 23971850]

40. Day MJ, Schultz RD. Veterinary immunology : principles and practice. Second edition. ed. Boca Raton, Florida; London, England; New York: CRC Press; 2014 1 online resource (329 pages) p.

41. Bergeron LM, McCandless EE, Dunham S, Dunkle B, Zhu Y, Shelly J, et al. Comparative functional characterization of canine IgG subclasses. Veterinary immunology and immunopathology. 2014;157(1–2):31–41. [PubMed: 24268690]

42. Strietzel CJ, Bergeron LM, Oliphant T, Mutchler VT, Choromanski LJ, Bainbridge G. In vitro functional characterization of feline IgGs. Veterinary immunology and immunopathology. 2014;158(3–4):214–223. [PubMed: 24560097]

43. Leroy G, Verrier E, Meriaux JC, Rognon X. Genetic diversity of dog breeds: between-breed diversity, breed assignation and conservation approaches. Animal genetics. 2009;40(3):333–343. [PubMed: 19222436]

44. Dobson JM. Breed-predispositions to cancer in pedigree dogs. ISRN veterinary science. 2013;2013:941275. [PubMed: 23738139]

45. Todoroff RJ, Brodey RS. Oral and pharyngeal neoplasia in the dog: a retrospective survey of 361 cases. Journal of the American Veterinary Medical Association. 1979;175(6):567–571. [PubMed: 511751]

46. Mestrinho LA, Pissarra H, Carvalho S, Peleteiro MC, Gawor J, Niza M. Comparison of Histological and Proliferation Features of Canine Oral Squamous Cell Carcinoma Based on Intraoral Location: 36 Cases. Journal of veterinary dentistry. 2017;34(2):92–99. [PubMed: 28631549]

47. Liu D, Xiong H, Ellis AE, Northrup NC, Dobbin KK, Shin DM, et al. Canine spontaneous head and neck squamous cell carcinomas represent their human counterparts at the molecular level. PLoS genetics. 2015;11(6):e1005277. [PubMed: 26030765]

48. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333(6046):1154–1157. [PubMed: 21798897]

49. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157–1160. [PubMed: 21798893]

50. Mochizuki H, Breen M. Sequence analysis of RAS and RAF mutation hot spots in canine carcinoma. Veterinary and comparative oncology. 2017;15(4):1598–1605. [PubMed: 27714944]

51. Martano M, Restucci B, Ceccarelli DM, Lo Muzio L, Maiolino P. Immunohistochemical expression of vascular endothelial growth factor in canine oral squamous cell carcinomas. Oncology letters. 2016;11(1):399–404. [PubMed: 26870224]

52. Pestili de Almeida EM, Piche C, Sirois J, Dore M. Expression of cyclo-oxygenase-2 in naturally occurring squamous cell carcinomas in dogs. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2001;49(7):867–875. [PubMed: 11410611]

53. Munday JS, French A, Harvey CJ. Molecular and immunohistochemical studies do not support a role for papillomaviruses in canine oral squamous cell carcinoma development. Veterinary journal. 2015;204(2):223–225.

54. Miyazawa J, Mitoro A, Kawashiri S, Chada KK, Imai K. Expression of mesenchyme-specific gene HMGA2 in squamous cell carcinomas of the oral cavity. Cancer research. 2004;64(6):2024–2029. [PubMed: 15026339]

55. Sterenczak KA, Eckardt A, Kampmann A, Willenbrock S, Eberle N, Langer F, et al. HMGA1 and HMGA2 expression and comparative analyses of HMGA2, Lin28 and let-7 miRNAs in oral squamous cell carcinoma. BMC cancer. 2014;14:694. [PubMed: 25245141]

56. Nemec A, Murphy B, Kass PH, Verstraete FJ. Histological subtypes of oral non-tonsillar squamous cell carcinoma in dogs. Journal of comparative pathology. 2012;147(2–3):111–120. [PubMed: 22300705]

57. Rothschild MF, Ruvinsky A, C.A.B. International. The genetics of the pig. Second edition. ed. Wallingford, Oxfordshire, UK: CABI; 2011 x, 507 pages p.

58. Walters EM, Prather RS. Advancing swine models for human health and diseases. Missouri medicine. 2013;110(3):212–215. [PubMed: 23829105]

59. Schook LB, Collares TV, Darfour-Oduro KA, De AK, Rund LA, Schachtschneider KM, et al. Unraveling the swine genome: implications for human health. Annual review of animal biosciences. 2015;3:219–244. [PubMed: 25689318]

60. Mair KH, Sedlak C, Kaser T, Pasternak A, Levast B, Gerner W, et al. The porcine innate immune system: an update. Developmental and comparative immunology. 2014;45(2):321–343. [PubMed: 24709051]

61. De Pelsmaeker S, Devriendt B, Leclercq G, Favoreel HW. Porcine NK cells display features associated with antigen-presenting cells. Journal of leukocyte biology. 2018;103(1):129–140. [PubMed: 29345060]

62. Sinkora M, Butler JE. The ontogeny of the porcine immune system. Developmental and comparative immunology. 2009;33(3):273–283. [PubMed: 18762210]

63. Rothkotter HJ. Anatomical particularities of the porcine immune system--a physician's view. Developmental and comparative immunology. 2009;33(3):267–272. [PubMed: 18775744]

64. Lunney JK, Ho CS, Wysocki M, Smith DM. Molecular genetics of the swine major histocompatibility complex, the SLA complex. Developmental and comparative immunology. 2009;33(3):362–374. [PubMed: 18760302]

65. McAnulty PA. The minipig in biomedical research. Boca Raton: CRC Press/Taylor & Francis,; 2012 Available from: http://ezproxy.msu.edu/login?url=http://search.ebscohost.com/login.aspx?direct=true&scope=site&db=nlebk&db=nlabk&AN=411945.

66. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. Trends in microbiology. 2012;20(1):50–57. [PubMed: 22153753]

67. Groenen MA, Archibald AL, Uenishi H, Tuggle CK, Takeuchi Y, Rothschild MF, et al. Analyses of pig genomes provide insight into porcine demography and evolution. Nature. 2012;491(7424): 393–398. [PubMed: 23151582]

68. Bordignon V, El-Beirouthi N, Gasperin BG, Albornoz MS, Martinez-Diaz MA, Schneider C, et al. Production of cloned pigs with targeted attenuation of gene expression. PloS one. 2013;8(5):e64613. [PubMed: 23737990]

69. Lillico SG, Proudfoot C, Carlson DF, Stverakova D, Neil C, Blain C, et al. Live pigs produced from genome edited zygotes. Scientific reports. 2013;3:2847. [PubMed: 24108318]

70. Watson AL, Carlson DF, Largaespada DA, Hackett PB, Fahrenkrug SC. Engineered Swine Models of Cancer. Frontiers in genetics. 2016;7:78. [PubMed: 27242889]

71. Adam SJ, Rund LA, Kuzmuk KN, Zachary JF, Schook LB, Counter CM. Genetic induction of tumorigenesis in swine. Oncogene. 2007;26(7):1038–1045. [PubMed: 16964292]

72. Yamakawa H, Nagai T, Harasawa R, Yamagami T, Takahashi J, Ishikawa K-i, et al. Production of Transgenic Pig Carrying MMTV/v-Ha-ras. Journal of Reproduction and Development. 1999;45(2):111–118.

73. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell. 1987;49(4):465–475. [PubMed: 3032456]

74. Borovansky J, Horak V, Elleder M, Fortyn K, Smit NP, Kolb AM. Biochemical characterization of a new melanoma model--the minipig MeLiM strain. Melanoma research. 2003;13(6):543–548. [PubMed: 14646615]

75. Li X, Zhou X, Guan Y, Wang YX, Scutt D, Gong QY. N-nitrosodiethylamine-induced pig liver hepatocellular carcinoma model: radiological and histopathological studies. Cardiovascular and interventional radiology. 2006;29(3):420–428. [PubMed: 16502159]

76. Sieren JC, Meyerholz DK, Wang XJ, Davis BT, Newell JD Jr., Hammond E, et al. Development and translational imaging of a TP53 porcine tumorigenesis model. The Journal of clinical investigation. 2014;124(9):4052–4066. [PubMed: 25105366]

77. Li S, Edlinger M, Saalfrank A, Flisikowski K, Tschukes A, Kurome M, et al. Viable pigs with a conditionally-activated oncogenic KRAS mutation. Transgenic research. 2015;24(3):509–517. [PubMed: 25716163]

78. Schook LB, Collares TV, Hu W, Liang Y, Rodrigues FM, Rund LA, et al. A Genetic Porcine Model of Cancer. PloS one. 2015;10(7):e0128864. [PubMed: 26132737]

79. Schachtschneider KM, Liu Y, Makelainen S, Madsen O, Rund LA, Groenen MAM, et al. Oncopig Soft-Tissue Sarcomas Recapitulate Key Transcriptional Features of Human Sarcomas. Scientific reports. 2017;7(1):2624. [PubMed: 28572589]

80. Schachtschneider KM, Schwind RM, Darfour-Oduro KA, De AK, Rund LA, Singh K, et al. A validated, transitional and translational porcine model of hepatocellular carcinoma. Oncotarget. 2017;8(38):63620–63634. [PubMed: 28969016]

81. Callesen MM, Arnadottir SS, Lyskjaer I, Orntoft MW, Hoyer S, Dagnaes-Hansen F, et al. A genetically inducible porcine model of intestinal cancer. Molecular oncology. 2017;11(11):1616–1629. [PubMed: 28881081]

82. Suzuki S, Iwamoto M, Saito Y, Fuchimoto D, Sembon S, Suzuki M, et al. Il2rg gene-targeted severe combined immunodeficiency pigs. Cell stem cell. 2012;10(6):753–758. [PubMed: 22704516]

83. Ito T, Sendai Y, Yamazaki S, Seki-Soma M, Hirose K, Watanabe M, et al. Generation of recombination activating gene-1-deficient neonatal piglets: a model of T and B cell deficient severe combined immune deficiency. PloS one. 2014;9(12):e113833. [PubMed: 25437445]

84. Huang J, Guo X, Fan N, Song J, Zhao B, Ouyang Z, et al. RAG1/2 knockout pigs with severe combined immunodeficiency. Journal of immunology. 2014;193(3):1496–1503.

85. Suzuki S, Iwamoto M, Hashimoto M, Suzuki M, Nakai M, Fuchimoto D, et al. Generation and characterization of RAG2 knockout pigs as animal model for severe combined immunodeficiency. Veterinary immunology and immunopathology. 2016;178:37–49. [PubMed: 27496741]

86. Karo JM, Schatz DG, Sun JC. The RAG recombinase dictates functional heterogeneity and cellular fitness in natural killer cells. Cell. 2014;159(1):94–107. [PubMed: 25259923]

87. Lei S, Ryu J, Wen K, Twitchell E, Bui T, Ramesh A, et al. Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency. Scientific reports. 2016;6:25222. [PubMed: 27118081]

88. Choi YJ, Kim E, Reza A, Hong K, Song H, Park C, et al. Recombination activating gene-2(null) severe combined immunodeficient pigs and mice engraft human induced pluripotent stem cells differently. Oncotarget. 2017;8(41):69398–69407. [PubMed: 29050212]

89. Khoshnevis M, Carozzo C, Bonnefont-Rebeix C, Belluco S, Leveneur O, Chuzel T, et al. Development of induced glioblastoma by implantation of a human xenograft in Yucatan minipig as a large animal model. Journal of neuroscience methods. 2017;282:61–68. [PubMed: 28284687]

90. Reymond MA, Tannapfel A, Schneider C, Scheidbach H, Kover S, Jung A, et al. Description of an intraperitoneal tumour xenograft survival model in the pig. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2000;26(4):393–397.

91. Arnold CR, Kloss F, Singh S, Vasiljevic D, Stigler R, Auberger T, et al. A domestic porcine model for studying the effects of radiation on head and neck cancers. Oral surgery, oral medicine, oral pathology and oral radiology. 2017;123(5):536–543.

92. Stramandinoli-Zanicotti RT, Sassi LM, Schussel JL, Torres MF, Funchal M, Smaniotto GH, et al. Effect of fractionated radiotherapy on the parotid gland: an experimental study in Brazilian minipigs. International archives of otorhinolaryngology. 2013;17(2):163–167. [PubMed: 25992008]

93. Sanz R, Calpena AC, Mallandrich M, Gimeno A, Halbaut L, Clares B. Development of a buccal doxepin platform for pain in oral mucositis derived from head and neck cancer treatment. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2017;117:203–211.

94. Hu L, Wang Y, Cotrim AP, Zhu Z, Gao R, Zheng C, et al. Effect of Tempol on the prevention of irradiation-induced mucositis in miniature pigs. Oral diseases. 2017;23(6):801–808. [PubMed: 28326646]

95. Curry JM, Bloedon E, Malloy KM, Cognetti DM, Merton DA, Goldberg BB, et al. Ultrasound-guided contrast-enhanced sentinel node biopsy of the head and neck in a porcine model. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery. 2007;137(5):735–741. [PubMed: 17967637]

96. Seoane J, Varela-Centelles P, Esparza-Gomez G, Cerero-Lapiedra R, Seoane-Romero JM, Diz P. Simulation for training in oral cancer biopsy: a surgical model and feedback from GDPs. Medicina oral, patologia oral y cirugia bucal. 2013;18(2):e246–250.

97. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013;496(7446):498–503. [PubMed: 23594743]

98. Lam SH, Chua HL, Gong Z, Lam TJ, Sin YM. Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. Developmental and comparative immunology. 2004;28(1):9–28. [PubMed: 12962979]

99. Li Y, Li Y, Cao X, Jin X, Jin T. Pattern recognition receptors in zebrafish provide functional and evolutionary insight into innate immune signaling pathways. Cellular & molecular immunology. 2017;14(1):80–89. [PubMed: 27721456]

100. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. Blood. 2001;98(10):3087–3096. [PubMed: 11698295]

**Frog**

**Advantages**

Number of offspring

Cell-free model, larvae and adult

External embryonic development

Developmental segregation of T cell functionality

Study of immune-mediated resistance

Screening of chemical compounds

**Disadvantages**

More evolutionary distance than mammals.

Induced tumors

No lymph nodes

**Pig**

**Advantages**

Anatomical/physiological similarity to humans

Immune competent or deficient

Transgenic available

**Disadvantages**

Cost

Induced tumors

No established oral SCC models

**Zebrafish**

**Advantages**

Size, number of offspring, external embryonic development.

Screening of chemical compounds

Optical clarity

Transgenic available

**Disadvantages:**

Induced tumors

Greater evolutionary dissimilarity

Lower optimal temperature for maintenance

**Cat**

**Advantages**

Immune competent

Spontaneous tumors

Orthotopic

Therapeutic trials

Genetic and phenotypic heterogeneity

**Disadvantages**

Procurement of cases

Limited availability of reagents

No transgenic models

**Dog**

**Advantages**

Immune competent

Spontaneous tumors

Orthotopic

Genetic trait

Therapeutic trials

**Disadvantages**

Procurement of cases

Limited availability of reagents
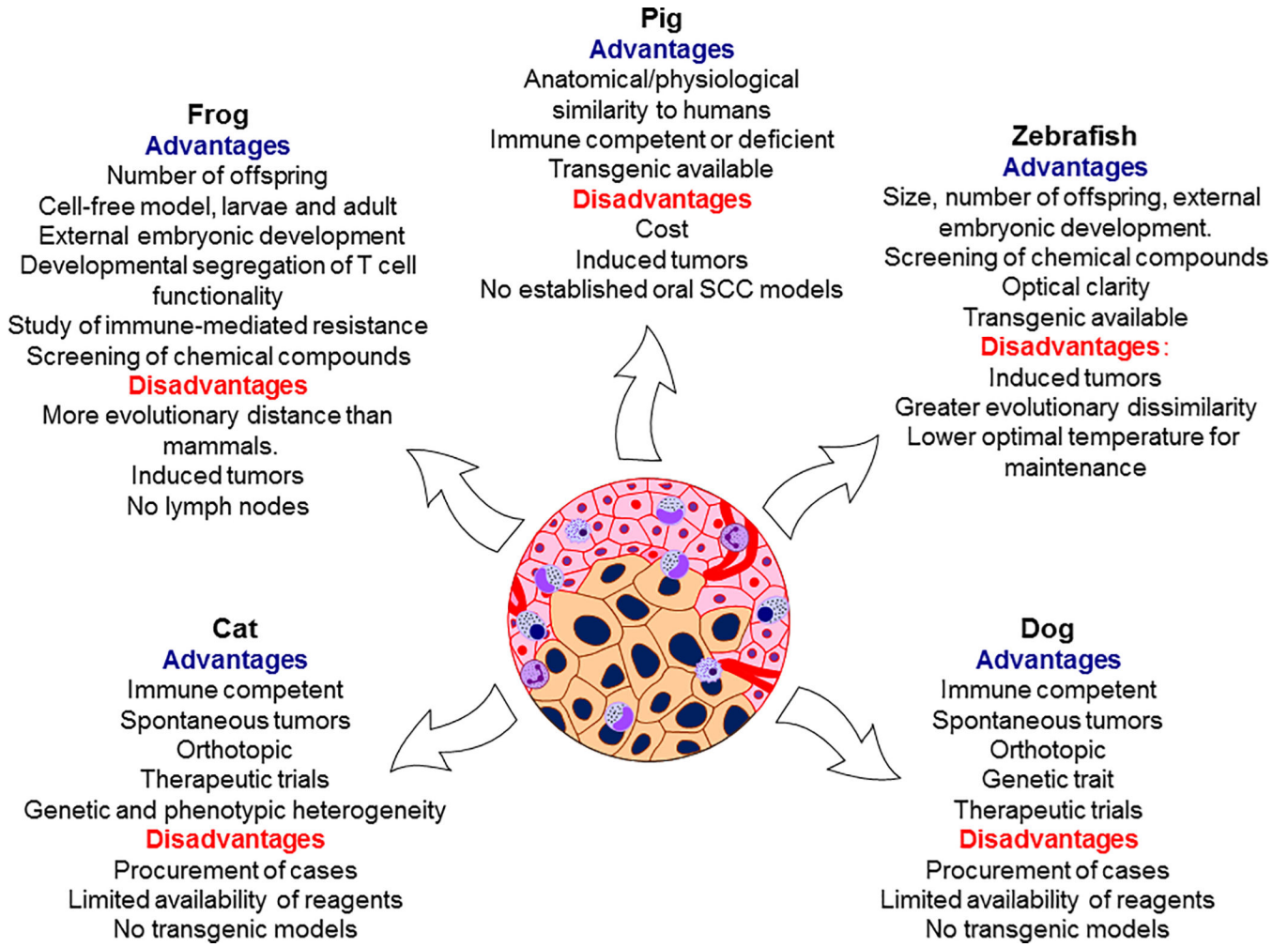
No transgenic models

**Figure 1 –.**

Summary of characteristics of select non-murine models that could be used to investigate tumor-immune interactions in head and neck cancer.

**Table 1 –**

Summary of HNC studies using non-murine models. References followed by an asterisk (*) can be consulted in the Appendix. Note that the *Xenopus* and porcine models are not included in this table because HNC studies specifically using this model were not identified.

| Model | Aim | Outcome | Annotation | Reference |
|---|---|---|---|---|
| **In vitro Canine/ Feline HNC cell lines** | Biological mechanism | Paraneoplastic humoral hypercalcemia | Canine HNC cell line was used to study the regulation of PTHrP in response to various stimuli of relevance in HNC. The canine HNC cell line is an adequate model to study the regulation of PTHrP in HNC. | (173*) |
| | Therapy | Cytotoxicity of doxorubicin and a synthetic analog | Canine and feline HNC cell lines were treated with doxorubicin and a synthetic analogue. Cell proliferation, apoptosis and production of ROS were increased by treatment, especially when combined with PI3K/Akt inhibitors | (174*) |
| | Therapy | Association of receptor tyrosine kinase and COX-2 inhibitors | Canine and feline cell lines were treated with RTK and COX-2 inhibitors. Cell proliferation and expression of cancer-related markers were reduced via c-kit and Akt signaling pathways. The response was comparable to that of a human HNC cell line. | (29) |
| **Feline** | Therapy | Pharmacokinetics of tested drug | Expression of NAD(P)H:quinone oxidoreductase (NQO1) in TMAs of human and feline OSCC. In vitro cytotoxicity of the synthetic NQO1 substrate and pharmacokinetics of the drug in vivo. | (175*) |
| | Therapy | Efficacy of nanoparticle-delivered siRNA | Tumor-targeted nanoparticles carrying protein kinase C2 (CK2) siRNA. 30% of the animals showed reduction in CK2 expression in the tumors by IHC. Adverse effects of treatment were recorded. | (176*) |
| | Therapy | Safety and efficacy of micro brachytherapy | Intra-tumoral injections of $^{166}$Holmium-loaded microspheres induced a partial response in 55% of cats, allowing for surgical resection. Improved overall survival time in animals showing a partial response. | (177*) |
| | Etiology | Detection of intratumoral hypoxia | Hypoxia was assessed in HNC and non-neoplastic tissues by PET/CT using iodinated sensor and fluorescent probe. FOSCC is an adequate model for the investigation of intratumoral hypoxia. | (178*) |
| **Canine** | Therapy | Dose escalation and tumor response | Animals presenting various spontaneous tumors including OSCC were treated with sequential systemic administration of TNF-alpha and IL-2. Below or at maximum tolerated dose, treatment caused only mild adverse effects. Tumor regression was observed in 75% of SCC. | (179*) |
| | Therapy | Safety and efficacy of anti-malarial drug artesunate and its main metabolite | Animals were treated with the tested compounds for varying periods of time (7–385 days), presenting mild and transient adverse effects. 30% of the animals had short-term (4 weeks) stabilization of disease progression. | (174*) |
| | Therapy | Efficacy of photodynamic therapy | Animals with OSCC of varying sizes were treated by systemic administration of a photosensitizer followed by laser | (180*) |

| Model | Aim | Outcome | Annotation | Reference |
|-------|-----|---------|------------|-----------|
| | | | irradiation. Surgical reduction was performed initially for tumors with surface to base depth greater than 1 cm. 70% of animals were considered cured, with no recurrence 17 months after treatment. | |
| | Therapy | Efficacy of a novel photosensitizer used with photodynamic therapy | Both dogs and cats with various types of tumors including intra- and extra-oral HNC were systemically treated with a novel photosensitizer and subjected to PDT. 70% of tumors showed partial response or complete remission, but the efficacy in intra-oral cancer was lower. Adverse reactions to the novel photosensitizer were mild. | (181*) |
| **Zebrafish** | Mechanism | Cell proliferation, invasion | Silencing of glucose-regulated protein 94 reduced proliferation of a human HNC cell line in a xenotransplantation model by impaired mitochondrial function. | (182*) |
| | Therapy | Cell migration, invasion | Biochemical inhibitor of Hsp90 reduces tumor cell migration in a xenotransplantation model. | (183*) |
| | Therapy | Cell migration, invasion | Two strategies for the inhibition of podoplanin receptor reduced the dissemination of HNC cells in the xenotransplantation model | (184*) |
| | Mechanism | Cell migration, invasion | Inhibition of lipid raft-associated Flotillin-1 by shRNA reduced NF-kB activation and dissemination of HNC cells in a xenotransplantation model | (185*) |
| | Mechanism | Cell proliferation, migration, invasion | Overexpression of receptor tyrosine kinase DDR2 increased migration and invasion, but not proliferation of human HNC cells in the xenotransplantation model | (186*) |
| | Mechanism | Cetuximab resistance in HNC cells | Cetuximab-resistant human HNC cells were used in a xenotransplantation model. Association of cetuximab and NF-kB inhibitor effectively suppressed cetuximab-resistant cells | (187*) |
| | Therapy | Toxicity of a marine microbial extract with anti-tumoral effects | In the cytotoxic concentrations used to induce autophagic cell death in HNC cells, the extract was not toxic | (188*) |
| | Mechanism | Tumor-immune system interaction | Extracellular vesicles from human HNC cells reduced the expression of IL-13 mRNA by innate immune cells of zebrafish larvae | (189*) |

**Table 2 –**

Summary of relevant characteristics of the immune system in the non-murine models reviewed (Information in this table was derived from studies cited in the text corresponding to each model).

| Characteristic | Model | | | | |
|---|---|---|---|---|---|
| | Cat | Dog | Pig | Zebrafish | Frog |
| Segregation of innate and adaptive immunity | No | No | No | Yes | Yes (reduced T cell activity in embryos and tadpoles) |
| Experimental immunosuppression | No | No | Yes | Yes | Yes |
| MHC-analog system | Yes | Yes | Yes | Yes | Yes |
| T cells and analog phenotypes (T helper, CTL) | Yes (lack one locus of class II MHC) | Yes | Yes | Yes | Yes |
| B cells | Yes | Yes | Yes | Yes | Yes |
| Antibody isotypes | 4 | 4 | 5 | 3 | 5 |
| NK cells | Yes | Yes | Yes | Yes | Yes |
| Macrophages | Yes | Yes | Yes | Yes | Yes |
| Neutrophils | Yes | Yes | Yes | Yes | Yes |
| Dendritic cells | Yes | Yes | Yes | Yes | Yes |
| Lymph nodes | Yes | Yes | Yes (structure similar to the Waldeyer's ring of humans) | No | No |
| Allografts | No | No | Yes | Yes | Yes |
| Xenografts | No | No | Yes | Yes | No |
| Orthotopic tumors | Yes | Yes | Yes | No | No |