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I am submitting herewith a dissertation written by Carmen Maria Helena Colitz, D.V.M. entitled "Characterization of the Tumor and Ocular Phenotype in Transgenic Line TgN3261Rpw." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

John Erby Wilkinson, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

CHARACTERIZATION OF THE TUMOR AND OCULAR PHENOTYPE IN TRANSGENIC LINE TgN3261Rpw

A Dissertation

Presented for the

Doctor of Philosophy Degree

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Carmen Maria Helena Colitz, D.V.M.

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DEDICATION

This dissertation is dedicated to my parents
and to my grandmother

Michael John Colitz,

Clara Ilse Colitz

and

Carmen Julia Vergel

who have given me invaluable educational opportunities,
unconditional love and endless support.

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Lastly, but certainly not least, to my husband Federico I owe immense gratitude for his support and belief in me. He has taught me more about myself than anyone.

ABSTRACT

TgN3261Rpw is a line of transgenic mice generated at the Oak Ridge National Laboratory as part of a large scale insertional mutagenesis program under the direction of Richard P. Woychik, Ph.D. When originally screened for phenotypic abnormalities, TgN3261Rpw mice were found to have multiple ocular anomalies. The abnormalities evident on ophthalmic examination were absence of a pupillary light response, posterior synechiae, and posterior polar cataracts. As the transgenic mice aged, 28% of the mice developed abdominal distension and became moribund due to the development of histiocytic sarcomas. A small percentage of transgenic mice developed neoplastic disease in their thoracic cavities and dermis. None of the wild type mice developed these diseases. On necropsy, the mice affected by histiocytic sarcomas had hepatomegaly, splenomegaly, and lymphadenopathy, the smaller group had thickened pleurae and pericardia, and another small group had firm small nodules, large rapidly growing masses in their dermis. The tumors were diagnosed as histiocytic sarcoma or reticulum cell sarcoma, type A. Techniques used to specifically identify the cell of origin of this tumor were immunohistochemistry, enzyme cytochemistry, flow cytometry, and molecular analysis of the immunoglobulin and T-cell receptor genes. The intradermal tumors required transplant experiments into SCID mice to confirm the histiocyte as the cell of origin. The primary ocular abnormalities include anterior segment dysgenesis, persistent hyperplastic primary vitreous and persistent hyperplastic tunica vasculosa lentis, and persistent pupillary membranes. Sequella to these anomalies include posterior synechiae, detached retinas, posterior lenticonus, and

posterior polar cataracts. The tumor phenotype and the ocular phenotype can hypothetically be united as an abnormality in the growth and terminal differentiation of the resident tissue macrophages due to the transgene insertion.

PREFACE

During the second year of veterinary school at the University of Tennessee College of Veterinary Medicine, the author became interested in ophthalmology. She received a summer externship at the veterinary school in Dr. J. Erby Wilkinson's laboratory and Dr. Wilkinson allowed her to pursue her interest in ophthalmology. For three months, the author worked to culture canine retinal pigment epithelial cells and performed experiments on these cells such as mitogen assays. During her senior year of veterinary school, the author applied for and received a Post-Doctoral Research Training Fellowship which began after graduation. Since then, the author has pursued a Ph.D. in the Comparative and Experimental Medicine program through the College of Veterinary Medicine at the University of Tennessee. This dissertation is a complete representation of her work over the past three years.

The first part reviews the literature and brings together what seems to be a very broad project. The project, the characterization of the ocular and tumor phenotypes, which transgenic mouse line, TgN3261Rpw, develops was generated at the Oak Ridge National Laboratory by Richard Woychik, Ph.D. The following three parts fully characterize the three phenotypes in detail.

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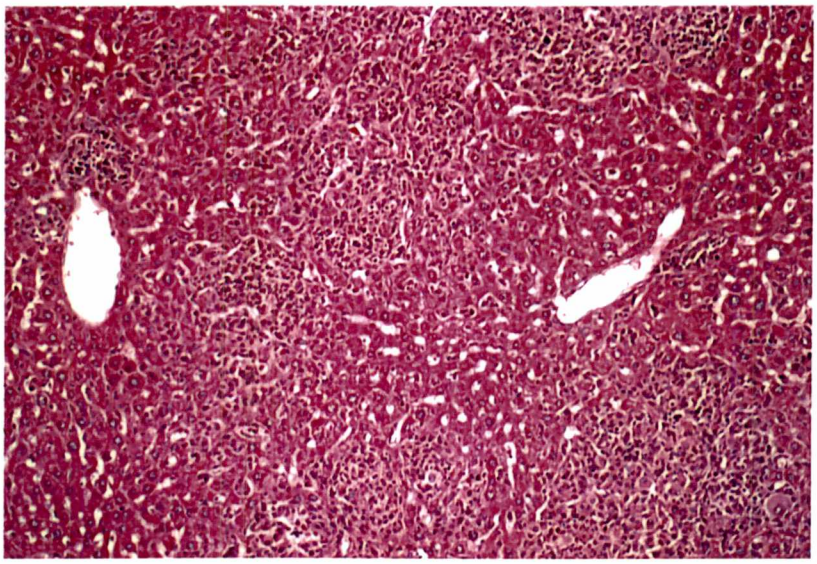

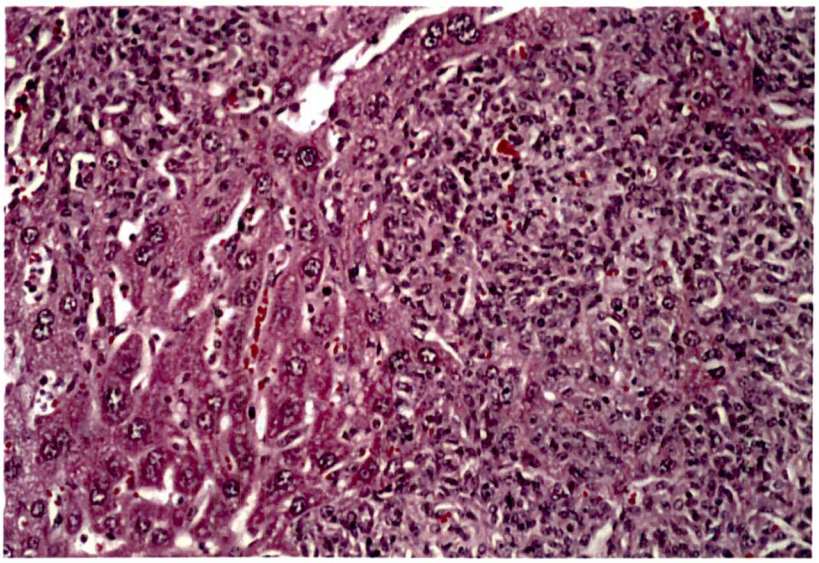

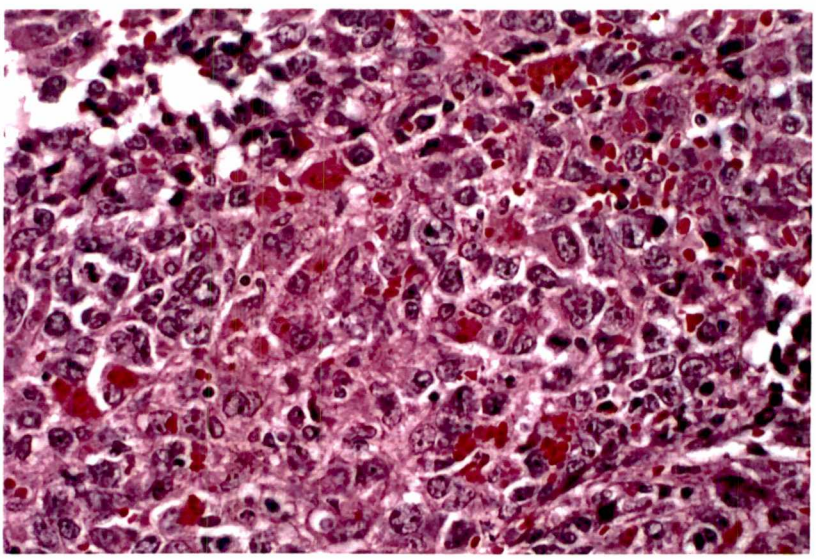

PART 1:

INTRODUCTION AND REVIEW OF THE LITERATURE

I) INSERTIONAL MUTAGENESIS

The analysis of random insertional mutations in mice allows a direct comparison of an altered phenotype with the underlying genetic defect. Random insertional mutants can be generated using either of two methods. The first method utilizes standard methods for the production of transgenic mice and requires the microinjection of a foreign piece of DNA into the pronucleus of a one-cell fertilized egg. The source of the DNA is unimportant except as a molecular marker for the insertion site. The DNA can code for another protein (such as tyrosinase in albino mice) or be noncoding. The expression of the transgene is variable and is unrelated to the phenotype-associated insertional mutation. The second method involves the retroviral infection of either a pre-implantation or post-implantation embryo. The microinjected foreign DNA or the retrovirus inserts randomly in the genome of that organism and may cause a disruption of a gene. The vast majority of random insertions that cause a phenotype result in embryonic lethality in the homozygous state; but , with a reduced frequency, an overt phenotype will result in postnatal animals. If the insertional mutations causing overt phenotypes are similar to diseases which occur either in the human or domestic animal populations, they can then be studied as potential models for human or domestic animal diseases.

Meisler has noted that a significant percentage of insertional mutations represent re-mutations of classical loci such as the twirler, hairless and ld mutations, which has simplified the cloning of these classical mutations. Insertional mutagenesis is also an important method for identifying previously unknown loci. Access to the site of insertion is possible by using the foreign piece of DNA, known as the transgene, as a molecular

tag to screen a library of genomic DNA from the transgenic mice and to identify the sequences flanking the transgene. This flanking DNA is then used as a probe to screen a wild-type genomic library in order to clone the wild-type locus. Once the wild-type locus is cloned, techniques such as exon trapping and zoo blotting can be used to identify exons of the wild-type gene. Following identification of exons, the screening of cDNA libraries is used to identify the mutant gene.

This procedure for identifying the mutated gene is straightforward, but there can be complications. Translocations, duplications, and deletions can occur at the site of insertion. However, these changes can be analyzed at the molecular level and the relationship to the phenotype can be determined. Transmission of the foreign DNA is generally stable for generations.[1]

An important consideration when initially characterizing a new insertional mutant involves determining whether the mutation segregates with the transgene. In general, if the transgene insertion alters a functional gene, a heterozygous mouse will develop the abnormality at a higher frequency in the case of a dominant trait, a homozygous mouse will develop the abnormality if the trait is recessive, and wild type mice should rarely, if ever, develop the disease trait.

Insertion of a transgene can alter gene function in many ways depending upon where in the gene it inserts. Insertion near endogenous enhancers or promoters may cause inappropriate expression of the gene, usually in underexpression, expression at inappropriate times and places, or null mutations. Disruption of the coding sequence can result in null mutations and dominant negative mutations. These examples can result in

inappropriate expression and protein production, a null mutation, or overexpression (especially in retroviral insertion which transmit as a dominant trait versus recessive for other types of insertions). Hypomorphs are also a possible outcome.

II) TgN3261Rpw

The TyBS minigene consists of the promoter and the cDNA of the tyrosinase gene. This has been used to make transgenic animals in at least two well established insertional mutagenesis programs.[2] The two programs are headed by Richard P. Woychik at the Oak Ridge National Laboratory (ORNL) and Paul Overbeek at the Baylor College of Medicine. The strain of mice used to make insertional mutants at ORNL is FVB/N, an albino stock. When the TyBS minigene is injected into the pronucleus of a fertilized egg and randomly inserts into the genome of that animal, the independent expression of the tyrosinase minigene can correct the albino phenotype and the animal will be pigmented. In many instances, the heterozygous mouse has a coat color that is half as dark as the homozygous animal because it only has one copy of the TyBS minigene. The homozygous animal has two copies, therefore its fur is twice as dark. This method was created to make identifying the transgenic animals simpler.[2] One need only look at the litter at birth and notice the color of their fur to know which animals are transgenic. With some frequency, the minigene will not be expressed (as a pigment change in the case of TyBS) and standard methods such as Southern blot analysis must be used to identify the transgenic animals.

TgN3261RPW (Tg3261) is one line of insertional mutant mice generated by random insertional mutagenesis at the Oak Ridge National Laboratory under the direction of Richard P. Woychik, Ph.D. These mice have multiple ocular abnormalities and a predisposition to tumor development.

III) MALIGNANT NEOPLASIA

A. General information

The development of cancer is a multistep process where the end result is unrestrained proliferation of altered cells which fail to differentiate appropriately. This growth harms the host due to tissue invasion and metastasis to other sites. Neoplasia is a multi-hit process which involves mutations in multiple genes. Cells must first undergo transformation, a process which involves mutations in proto-oncogenes of mammalian cells. Once activated by mutations, proto-oncogenes become oncogenes which affect the ability of cells to control proliferation. Alterations in oncogenes encode proteins that function inappropriately, abnormally, or at improper concentrations to promote cell proliferation. One or more cellular oncogenes are involved in the transformation processes of spontaneous cancers.[3] Additional mutations that lead to the malignant phenotype occur in proto-oncogenes, tumor suppressor genes, cell cycle regulatory genes and other genes associated with metastasis.

Proto-oncogenes are the cellular counterparts of viral genes responsible for inducing transformation.[4] Mutations in proto-oncogenes are usually gain-of-function mutations which are dominant to the wild-type allele. These mutations may occur in the regulatory elements of a gene, leading to excessive amounts of normal protein, or the mutations may occur in the structural gene itself. An oncogene can be defined as a gene whose activity leads to enhanced cell growth.[5] Since oncogene mutations are dominant, a mutation in a proto-oncogene only requires one mutational event for activation so that it can contribute to abnormal growth. Oncogene mutations

are not usually inherited through the germ line because their expression would disrupt development and cause death to the fetus. Therefore, oncogenes do not usually play a part in inherited predisposition to cancer.

Tumor suppressor genes are dominantly-acting negative regulators of cell proliferation, often referred to as recessive oncogenes. Both copies of a tumor suppressor gene must acquire independent mutations in order to contribute to abnormal growth of cells. This usually occurs by a mutational event in one allele, followed by a reduction to homozygosity in the other allele, which is called loss of heterozygosity (LOH). These genetic alterations are loss-of-function mutations and they are recessive to the wild-type allele.[6] Mutations in tumor suppressor genes affect the structural gene, or they shut down regulatory elements; therefore, either no gene product is synthesized or dominant negative mutations cause the production of an abnormal protein. In contrast to oncogenes, altered tumor suppressor genes can readily be inherited through the germ line. When one mutated allele is inherited, there is a high frequency of the second mutational event causing a reduction to homozygosity and an increased risk of cancer.[6]

Once tumor cells have growth autonomy and begin to proliferate, the growing tumor, if larger than 2 μm in diameter, must acquire a blood supply for continued proliferation.[7] Tumor angiogenesis is defined by the directional sprouting of new vessels toward a solid tumor. Either the tumor cells themselves or host inflammatory cells attracted to the tumor site promote tumor angiogenesis.[8] Angiogenesis requires that a critical number of cells in the tumor switch to the angiogenic phenotype. This switch is highly

associated with the transition to neoplasia with metastasis. Angiogenesis is a necessary but not sufficient trait for metastasis.

The initial response to the tumor angiogenic stimulus is the dissolution of the basement membrane surrounding a preexisting vessel. Endothelial cells then migrate out of the preexisting vessel and towards the tumor. Migration of endothelial cells is required for angiogenesis, but division of endothelial cells is not.[8] The process of angiogenesis depends on a net balance between positive and negative angiogenic factors,[9-11] such as vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), the integrin $\alpha_v\beta_3$, interleukin-8, angiotropin and angiogenin.[8,9,12] Up-regulation of these pro-angiogenic factors breaks down intercellular barriers and stimulates endothelial cell migration and proliferation. These pro-angiogenic factors can be made by the tumor cells, transferred from the extracellular matrix or released by nearby macrophages. However, the up-regulation of positive factors must be balanced by down-regulation of anti-angiogenic factors. Thrombospondin-1, cartilage derived inhibitor, tissue inhibitors of metalloproteinase 1 and 2 (TIMP-1 and 2), platelet factor 4 and angiostatin are examples of naturally occurring inhibitors of angiogenesis that are down-regulated during tumorigenesis.[8,10,11,13,14]

Once angiogenesis is established, tissue invasion can begin. This is an active process that involves tumor cell adhesion and digestion of intercellular substances so that tumor cells can pass through them. One of the most important proteases involved in tissue invasion is the

metalloprotease, collagenase IV. Other nonspecific proteases are also involved, including cathepsin B and D, trypsins, heparinases and tissue plasminogen activator.[13] Cell-cell attachment foci are down-regulated, leading to increased motility and invasiveness, while cell-substrate adhesion molecules are up-regulated. Other proteins produced are autocrine motility factors, such as scatter factor/hepatocyte growth factor (SF/HGF), which selectively stimulates cell migration with minimal effect on cell proliferation. Also, proteases are produced that degrade extracellular matrices.[15]

The tumor cells generally gain entry to the bloodstream by passing through tumor-induced neocapillaries or through existing small veins and venules. This process is called intravasation. Once in the blood vessels, the tumor cells must have the ability to survive. The forces working against them include turbulence, sheer and distortion pressures, attack from the immune system, high oxygen concentration, and serum factors.[16] Most tumor cells are unable to survive in an environment of 100% serum or plasma; therefore, their half-lives in blood vessels are shortened. The tumor cells themselves must also be able to change shape in order to pass through small capillaries.[8] Tumor cells survive such forces by homotypic and heterotypic clumping. Homotypic clumping is cell-cell clumping of the tumor cells; heterotypic clumping involves the aggregation of tumor cells with platelets. The platelet/tumor embolus forms a "cocoon" around the tumors cells, protecting them from cytotoxic T-lymphocytes and natural killer cells.[8,16] The tumor cells can also evade immune attack by down-regulating MHC antigens and by tumor-specific immunosuppression.

Endothelial receptors help target metastatic cells in predetermined locations. If the tumor cells survive in the bloodstream, they must extravasate and colonize the new tissues when they arrest in a site suitable to their growth. One of the first and most important steps in metastasis is adhesion of tumor cells to the vascular endothelium at the secondary site. It has been proven that tumor cells preferentially adhere to the capillary endothelial cells from the secondary site of metastasis.[16-18] Once tumor cells have adhered to the vascular endothelium, the endothelial cells retract, exposing the subendothelial basement membrane. The subendothelial basement membrane contains adhesive molecules such as laminin, types IV and V collagen, vitronectin, and heparan sulfate proteoglycan.[8] The extracellular matrices of different organs vary and the tumor cells show preferences for the extracellular matrix components from their preferred secondary sites. Once exposed to the extracellular matrix, the tumor cells must invade the tissue parenchyma, which is accomplished by breaking down the subendothelial membrane and the interstitial connective tissue matrix. Tumor cells, without the help of accessory cells recruited in the initial tumor site, break down these barriers using the degradative enzymes that they produce.[8] Tumor cells which minimize their time in the bloodstream by rapid transit through the vascular endothelium and subendothelial basement membrane, and can extravasate increase their chances of successful metastasis.

There are two theories about sites of tumor cell metastastasis. Ewing theorized that various tissues are passive receptacles for tumor cells.[19] Passive arrest occurs due to the size of the tumor cells and the size of the

capillaries through which they are passing. Some tumor cells become trapped in small capillary beds and if conditions are acceptable, they establish a metastatic focus. The majority of tumor cells which lodge at the first vascular bed encountered will not form a metastasis; instead, they are more likely to form metastases downstream from that first vascular bed.[8,16] It is hypothesized that there is a specific cellular adhesive interaction present at the secondary site.[16] Paget hypothesized that certain tumors were predisposed to spread to particular tissues based on the tissue's ability to support the growth of those tumors.[20]

In either case, colonization is accomplished by clonally dominant cell populations, which become progressively independent from exogenous growth factors, produce their own growth factors, and respond aberrantly to certain cytokines.[13] A candidate metastasis suppressor gene, Nm23, is associated with the development of metastasis. In transfection experiments with murine cancers, overexpression of nm23 protein resulted in a significant decrease in the tumor's metastatic potential when injected into mice.[13] Once metastasis has occurred, angiogenesis is required *de novo* for continued tumor growth at the distant sites. All of these events leading to metastasis, (invasion, intravasation and extravasation, colonization, and *de novo* angiogenesis at a distant site) require the tumor cells to be heterogeneous, unstable, and redundant. These are the hallmarks of the metastatic process. Many of the traits of metastatic tumor cells including cell adhesion, motility, and invasion are also seen in embryonic development and wound healing.[4,13,21]

Metastatic tumor cells can undergo a dormant period before rapid growth. Though unproven, it is hypothesized that tumor cells can enter G0, the resting phase of the cell cycle, during dormancy. Other ways that tumor cells may remain dormant include control of tumor size by the immune system or by hormone depletion in hormone-dependent tumors. Micrometastases may also be kept in check by inhibition of angiogenesis or by inhibition of the angiogenic phenotype.[21] One study found that inhibition of angiogenesis restricted tumor growth by increasing the incidence of apoptosis.[21] The dormant period of micrometastases does not last indefinitely; either through conversion to the angiogenic phenotype or by removal of circulating angiogenesis inhibitors, the micrometastases escape dormancy, up-regulate angiogenesis and continue to grow.[21]

B. Tumor suppressor genes

The Retinoblastoma susceptibility gene (Rb) was the first tumor suppressor gene characterized. Knudson noted that 40% of cases of familial retinoblastoma occurred in infants with a mean age of 14 months and they were usually bilateral with an average of three tumors per patient.[6] Some of these patients had a family history of this tumor. If the patients were surgically treated, then they would likely develop independent neoplasms later in life, generally osteosarcomas. The other 60% of cases of retinoblastoma had no family history of cancer; the tumors were detected later (average age 30 months) and the patients developed a single unilateral tumor.[6] Knudson hypothesized correctly that the children in the first group

were born with one mutant allele in a gene later known as the retinoblastoma susceptibility gene (Rb). Soon after, the second allele would mutate and this would give rise to early onset retinoblastoma. The children in the second group were not, however, born with such a mutation. Two independent somatic mutations in the Rb gene had to occur in order to give rise to the later singular tumor. Mutations in Rb have also been detected in osteosarcomas, small-cell lung carcinomas, bladder carcinomas, prostate carcinomas, breast carcinomas, cervical carcinomas, and some leukemias.[6]

The product of the retinoblastoma susceptibility gene (pRb) is a negative regulator of G1 cell cycle progression. The function of pRb is controlled by cell cycle-specific phosphorylation/dephosphorylation reactions. The hypophosphorylated form of pRb is found in G0 and early G1 and is growth suppressive by inactivating members of the E2F family of transcriptional transactivators.[22,23] The E2F family is a group of positive transcription factors with oncogenic activities which is involved in the expression of genes such as N-myc, c-myc, dihydrofolate reductase (DHFR), and other early response genes necessary for S-phase control.[22] In middle to late G1, pRb complexes with the D cyclins and their cyclin dependent kinase partners, CDK4/6, which hyperphosphorylate pRb on threonine and serine residues.[22,24,25] This phosphorylation disrupts complex formation with E2F so that E2F is activated and the cell enters S-phase.[23] Phosphorylation results from complex-mediated targeting by CDK4 [22] and pRb remains phosphorylated throughout S, G2, and M. pRb becomes unphosphorylated once again late in M phase by phosphatase activity.[24] In

addition to cell cycle control, pRb is also critical in development and differentiation of erythroid and central nervous system tissues. Targeted mutant mice homozygous for mutations at Rb die by day 16 of embryonic development due to these abnormalities.

The p53 gene is another important tumor suppressor gene. Like Rb, p53 mutations can be carried through the germline as in the case of human Li-Fraumeni syndrome. This syndrome is characterized by a family member developing a sarcoma at an early age with at least two close relatives also having developed cancer. Alterations in p53 are the most common genetic mutations occurring in human cancers, and they have been reported to occur in at least 50% of all types of tumors.[26]

The p53 gene has been called "guardian of the genome" because it plays a role in G1 growth arrest and apoptosis in response to DNA-damaging agents, and it is a component of a spindle checkpoint that ensures the maintenance of diploidy.[26,27] p53 is a 393 amino acid nuclear phosphoprotein that is expressed in all cells.[6] p53 expression can be induced by ionizing radiation or DNA-damaging agents which in turn induce $p21^{waf1/cip1}$ and GADD45 expression.[28,29] $p21^{waf1/cip1}$ is a potent inhibitor of cyclins and cyclin-dependent-kinases (Cdk) and may block the action of proliferating cell nuclear antigen (PCNA).[26,28,29] It has been shown that when p53 induces $p21^{waf1/cip1}$, cyclin complex-mediated phosphorylation of pRb is inhibited.[26,30] This implies that p53 indirectly regulates pRb phosphorylation and thereby induces cell cycle arrest at the

G1 checkpoint.[31,32] Arrest at G1 allows the cells that are irreparably damaged to undergo apoptosis, or allows repair of damaged DNA, possibly by multiple DNA repair genes, such as GADD45.[29] The molecular mechanisms by which p53 functions at the spindle checkpoint are presently not understood. As a checkpoint regulator, p53 may prevent transmission of oncogenic mutations and indirectly suppress tumorigenesis.[33] In many tumors, p53 mutation occurs as a late event suggesting that loss of p53-dependent apoptosis is as important to tumor progression as the loss of proliferation control.[33] The cellular oncogene, c-myc, is a positive regulator of the cell cycle and can induce cell cycle re-entry despite the presence of large amounts of p53 protein. The activation of c-myc and inappropriate G1-S checkpoint bypass result in p53-induced apoptosis[34,35] once again proving p53's tumor suppressive mechanism. Interestingly, it has been found that active pRb can antagonize p53-dependent apoptosis. If pRb is inactive, p53 induces apoptosis.[36] Unlike Rb, p53 is not necessary for embryonic development, but 90% of targeted mutant mice homozygous for a p53 knockout mutation develop one or more tumors before six months of age.[37,38] Other mutational events enhance the chances for cancer to progress by affecting the DNA repair system or causing expression of genes that determine cell longevity. Bcl-2, BAG-1, bcl-X_L, and BHRF-1 are genes that can prolong cell life by inhibiting apoptosis leading to tumor growth by inhibition of programmed cell death (e.g. as in lymphoma).[39-41]

Tumor cells have developed another method of self-immortalization

through reactivated telomerase activity. Telomeres are specialized structures at the ends of eukaryotic chromosomes that consist of tandem repeats of the sequence TTAGGG. All normal adult somatic cells lose 50 to 200 nucleotides of telomeric sequence per cell division. This may be the "mitotic clock" by which cells count their divisions and once they have shortened to a critical length, the cells undergo crisis and die.[42] Telomerase activity is present in human and chicken fetal development.[43] This activity is down-regulated just before or just after birth and remains passive unless cancer develops.[43] At least 90% of primary human tumors exhibit telomerase activity. This reinstates telomere repeat units, stabilizes the telomeres and immortalizes the tumor cells.[44] Telomerase RNA expression has been found in several normal human cells and tissues that lack telomerase activity as well as in preneoplastic and early stage tumors. RNA expression increases further in late stage tumors and telomerase activity is evident only in end stage tumors. This implies that telomerase activity is not necessary for primary tumor formation but is involved in later stages of tumor progression.[44]

C. Reticulum cells and histiocytic sarcoma

In her in-depth study from 1954, Thelma Dunn references Marshall's definition of reticular tissues which includes the lymph nodes, thymus, bone marrow and spleen, and additional elements of this tissue that exist scattered throughout the body. These include histiocytes and fibroblasts, cells of the peripheral blood, and primitive elements that can proliferate to produce any

of the cells found in the lymphatic or blood-forming tissue.[45] According to Dunn, reticulum cells are made up of three types of mature cells which include 1) reticulum cells, solitary fixed cells which form the cellular elements of the reticular framework of the main reticular organs; 2) histiocytes, the ameboid free cells lying in the tissues and capable of phagocytosis, and 3) monocytes, cells circulating in the peripheral blood.[45] In more recent reports, the phrase 'reticulum cells' includes 1) histiocytic reticulum cells, the phagocytic cells seen in germinal centers; 2) fibroblastic reticulum cells, the supporting stromal elements of the lymph nodes thought to be related to fibroblasts; 3) dendritic reticulum cells which are antigen-presenting cells also believed to interact with B lymphocytes, and 4) interdigitating reticulum cells, immune accessory cells involved in antigen presentation to T lymphocytes.[46-49] The mononuclear phagocyte system (MPS) includes promonocytes from the bone marrow, blood monocytes and tissue macrophages.[50,51]

Tissue macrophages, or histiocytes, are "professional phagocytes" that arise from the colony forming unit granulocyte-macrophage (CFU-GM) in the bone marrow, under the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor(M-CSF)/CSF-1.[48,52-54] GM-CSF and M-CSF/CSF-1 directly regulate proliferation and differentiation of myeloid progenitor cell into different stages of macrophage populations. CSF-1 is essential for inducing terminal differentiation in the MPS and GM-CSF acts primarily on mature myeloid progenitor cells.[53,55] The stem cell, CFU-GM, generates promonocytes that

mature in the bone marrow to monocytes which circulate in the peripheral blood.[52] Adherence to endothelium and then extracellular matrix is an important initial step in the transition of the blood monocyte to the histiocyte.[56] Once in the tissues, histiocytes complete their maturation and are ready to function in wound healing, in defense against microorganisms, in antigen presentation, and in eliminating undesirable particulate debris.[52,56] It is hypothesized that one population of histiocytes are under the control of CSF-1 and make up the steady-state population of resident tissue macrophages, while a second population of tissue macrophages is under the control of GM-CSF and these respond to inflammatory stimuli.[57]

Neoplastic disorders of histiocytic origin are extremely rare and difficult to diagnose.[50,51,58] Cell morphology is not a reliable means of diagnosing histiocytic sarcomas because of their marked pleiomorphic mononuclear phenotype.[59,60] Enzyme histochemistry and immunohistochemistry have been used extensively in an attempt to identify the set of enzymes and antibodies that characterize these tumors, but the tumors can express different antigens and contain different enzymes depending on their stage of differentiation and their local environment.[47,50,51,59-64] Gene rearrangement studies are the most reliable method to rule out lymphocytes as the cell of origin. During the development of B and T lymphocytes, immunoglobulin and T cell receptor genes, respectively, undergo multiple rearrangements in order to prepare them for their roles in the immune system.[65-67] Cells of

monocyte/macrophage origin do not undergo such rearrangements and maintain their germline configuration.[62,68,69] Therefore, the defining characteristics of a true histiocytic sarcoma are erythrophagocytosis, positive results for multiple histiocytic markers and negative results for B- and T-cell markers and antigen receptor rearrangements.[50,68]

Though histiocytic sarcomas are rare, their diagnosis is important due to their aggressive course with rapid and widespread dissemination. Histiocytic sarcomas are not related to sarcomas of follicular dendritic cells, which stain positively for R4/23, or interdigitating cells, which stain positively for ATPase and S-100; histiocytic sarcomas do not stain for these markers.[47,48,59] Erythrophagocytosis is a characteristic feature of histiocytic sarcomas which sarcomas of follicular dendritic cells and interdigitating cells do not possess.[50] Understanding the mutations that occur in histiocytes which ultimately result in histiocytic sarcoma is difficult because of the lack of an animal model of this disease, although true histiocytic sarcomas have been reported in an epidemic in the Northern Pike, *Esox lucius* L., in the Aland Island of Finland.[70]

D. Heterogeneity of macrophages

Macrophages are an ubiquitously distributed population of mononuclear phagocytes responsible for clearance of pathogens and damaged tissue cells, production of cytokines, and response to immunomodulatory stimuli.[71,72] With such a large range of biological roles, macrophages must have the capabilities to adapt and function quickly and

correctly. Heterogeneity of macrophages has long been known, but the manner in which macrophages acquire this heterogeneity is not as well understood. Conceivably, it may originate through various pathways. The first way in which macrophages may acquire heterogeneity is at the level of the bone marrow precursor cells. Such cells would be preprogrammed and selectively expanded under different stimuli resulting in functionally different populations in various systemic environments. On the other hand, phenotypically identical cells could differ functionally due to local tissue constraints. Growth factors have also been shown to affect macrophage progenitors by influencing gene expression and behavior of mature cells. Finally, the transient expression of various functions during macrophage differentiation has been proposed as the mechanism for macrophage heterogeneity.[73] These functions, alone, or in combination, may explain why macrophages exhibit heterogeneity which may be dependent on the specific situation and site.

It has been suggested that heterogeneity is genetically predetermined prior to release of the monocyte from the bone marrow.[72,73] Witsell, *et al*[73] reported a developmental mechanism for the generation of macrophage heterogeneity that supports a controlled developmental pathway of differentiation resulting in distinct phenotypes.[73] A heterogeneous population of monocytes leaves the bone marrow and these cells are capable of responding to the environmental stimuli they encounter, providing a mechanism for tissue-related heterogeneity.[73] The microenvironment in which the macrophage precursor differentiates affects directly the mature cell

phenotype. Reverse transcriptase-polymerase chain reaction (RT-PCR) experiments have shown that phenotypes differ with growth factors used to propagate the colony, the period of time allowed for differentiation, and the addition of various other exogenous stimuli.[73]

Heterogeneous monocytes are growth factor dependent. Colony-stimulating factors (CSF) regulate granulocyte and macrophage formation *in vivo*.[74] Colony stimulating factors can induce non-dividing granulocyte-macrophage precursors to begin dividing within three hours.[75] Stimulation by CSFs is required throughout the cell cycle and higher concentrations of CSFs shorten cell cycle time and delay maturation of cells by preventing them from entering the post-mitotic state.[72] This indicates that there is not a set number of mitotic divisions that precursor cells must undergo before entering terminal differentiation. Experiments *in vitro* have shown that CSFs have four additional actions besides inducing proliferation in responsive cells. These include maintaining the functional integrity of the cell membrane (which enhances the survival of precursors and mature cells), inducing irreversible commitment in differentiation, inducing cellular maturation, and finally, stimulating functional activity of mature cells (e.g. phagocytosis or synthesis of various biologically active molecules).[72]

Colony stimulating factors include granulocyte-macrophage (GM-CSF), granulocyte (G-CSF), macrophage (M-CSF), multipotential (multi-CSF) and CSF-1; interleukin-6 has also been shown to have colony-stimulating activity.[54,72] Numerous cell types including fibroblasts, stromal cells, endothelial cells, macrophages, and lymphocytes are known to

produce one or more of the CSFs.[72] Each CSF has a unique membrane receptor and more than one type of receptor may be found on granulocyte-macrophage progenitors and their maturing progeny.[76] Responsive progenitor cells are initially able to respond to the first growth factor that binds its appropriate receptor. This binding, in turn, influences the behavior of the other CSF receptors on the same cell and begins signal cascades specific to the particular type of receptor.[76]

Witsell, *et al*[57] demonstrated that there may be two macrophage populations comprised of an inflammatory GM-CSF-derived population and a steady-state CSF-1-derived population. The GM-CSF-derived population is produced in the bone marrow during immunological challenge.[77] Tumor necrosis factor-alpha (TNF-alpha) released from the tissues induces differentiation of GM-CSF-derived cells and inhibits differentiation of CSF-1-derived cells. As the challenge abates, GM-CSF decreases to normal levels and allows the steady-state macrophage population to re-equilibrate.[57] The CSF-1 steady state cells may comprise the macrophage progenitor cells dispersed in the peripheral tissues which are maintained by a self-renewal mechanism.[78] As previously mentioned, CSFs are produced by many types of cells in the periphery where they may either encourage self-renewal or at least postpone maturation into terminally differentiated tissue macrophages. These same CSFs are capable of acting differently on other cells by suppressing their self-renewal and inducing differentiation.[72] The target cells themselves may posses a genetic control element which dictates

whether they will self-renew or generate committed progeny, or the mechanisms of self-renewal or differentiation, themselves, may be concentration dependent.

Macrophage differentiation is difficult to follow due in part to the various phenotypes present in any given macrophage population. Cell surface antigens are differentially expressed as myeloid cells develop into macrophages. Specific functions also change based on the stage of differentiation. The immediate microenvironment (e.g. oxygen consumption)[79] likely determines the functional phenotype of its resident macrophages. Surface antigens expressed on peritoneal macrophages are unique and not expressed on macrophages from liver, spleen, lymph nodes, or thymus or on monocytes.[71] It is known that by production of metalloproteases, peripheral blood monocytes are able to migrate into tissues where they differentiate into functional macrophages. Renewal of tissue macrophages occurs through local proliferation of progenitor cells in the tissues when necessary for maintenance of populations. The heterogeneity which they acquire is likely based on the type(s) of CSFs produced in the given tissue and the other growth factors present.[74,80] This normal population renewal has little or no dependence on monocyte influx.[80]

Under abnormal conditions, such as neoplasia, the cells would likely self-renew rather that differentiate in order to propagate more cells with a higher probability of mutations. In a study by Just, *et al*[81], macrophage progenitor cells were "immortalized" in the presence of IL-3 or when co-

cultured with stromal cells. This moved more cells toward self-renewal rather than differentiation thus increasing the chances of mutations due to rapid, continual proliferation. If more mutations occur in conjunction with cells that may already be transformed (as possibly occurs in the Tg3261 mutant mice), this would lead to the histiocytic sarcomas which occur in Tg3261. If the resident tissue macrophages are the cells of origin of histiocytic sarcoma occurring in Tg3261, then we may assume that these are under the control of CSF-1, not GM-CSF (according to Witsell, *et al*'s results)[57] since they appear to arise in interstitial tissues, not bone marrow. If the GM-CSF-derived macrophages were the cell of origin for the histiocytic sarcomas arising in Tg3261, then there might be neoplastic cells in the peripheral blood, but none have been found.

E. Immunohistochemistry

Immunohistochemistry and immunocytochemistry use antibodies labeled with chromogenic substances that permit visualization of specific cellular components (antigens or enzymes) in tissue sections or individual cells. This technique requires that specific antibodies find and bind to their corresponding antigens on or in the cells. The enzymatic reaction between horseradish peroxidase, hydrogen peroxide, and a chromogen (usually diaminobenzidine) result in a brown, granular pigment at the site of antigen-antibody reaction. One of the most commonly used methods of immunochemistry is the avidin-biotin peroxidase technique.

The intermediate filaments are components of the cellular cytoskeleton. They include cytokeratin, vimentin, desmin, and glial fibrillary

acidic protein (GFAP). Neoplastic cells maintain the intermediate filament of their parent cell so that antibodies to these intermediate filaments are important diagnostic tools when characterizing a tumor. Cytokeratins are found only in epithelial cells. The keratins are a family of heterogeneous proteins ranging in molecular weight from 40 to 68 kDa. Vimentin is a single peptide of 58 kDa present in all cells of mesenchymal origin. Desmin is found in smooth, skeletal and cardiac muscle and has a molecular mass of 55 kDa. GFAP is characteristic of glial and neural tissue and it is the major protein component of glial filaments which is characteristic of astrocytes.

Antibodies specific for cell surface markers on cells or cytoplasmic markers in cells are used to characterize cells of interest. The tumor cells in Tg3261 mice have the morphology of histiocytes, but other tumors (e.g. large B-cell lymphoma)[64] can resemble histiocytes. For this reason, it is important to use reagents that will rule out lymphoid cells as the cell of origin. Antibodies against lysozyme, alpha-1-antichymotrypsin, alpha-1-antitrypsin, and anti-CD68 have been shown to stain neoplastic cells in some histiocytic tumors as well as the lectins, peanut agglutinin and soy bean agglutinin.[59,62,64,68,82] Antibodies which consistently stained neoplastic histiocytes in previous studies are anti-CD68 antibody and the lectins.[59,62,64,68] Lysozyme, alpha-1-antichymotrypsin and alpha-1-antitrypsin were far less consistent when staining human histiocytic neoplasias but they showed positive staining in other cell types including lymphomas, sarcomas, melanomas and carcinomas.[51,59,62,64,68,82]

IV) OCULAR EMBRYOLOGY AND DEVELOPMENT

A. Prenatal ocular development in the mouse

Morphologic development of the eye in the mouse begins at day 9 post-coitum and can be broken down into several stages (reviewed in reference 84):

Stage one includes day 9 and day 10 of embryonic development. The optic vesicles form on the lateral walls of the prosencephalon just before the prosencephalon divides into the telencephalon and the diencephalon. The optic pit invaginates to form the optic vesicle and optic stalk, which will form the future exit of the optic nerve. The neuroectoderm of the optic vesicle comes into close contact with the surface ectoderm. The surface ectoderm and the optic vesicles thicken and form the lens placode and the retinal disc, respectively.

Stage two includes days 10 and 11 of embryonic development. The optic vesicle invaginates to form the optic cup. The lens placode, which forms from surface ectoderm, invaginates to form the lens pit. The optic stalk continues to develop, progressing from inferior to superior so the sides of the optic cup and stalk meet inferiorly at the embryonic ocular fissure (also called the optic fissure). The inner layer of the optic cup (the future neurosensory retina) undergoes intense proliferation at the this time, but the underlying layer of neuroectoderm (the future retinal pigment epithelium) does not.[83]

Stage three extends from day 11 to day 13 of embryonic development. The lens pit closes and the lens vesicle separates from the surface ectoderm. An intact lens capsule surrounds the lens vesicle. The posterior lens cells begin to elongate and fill the lens space forming the primary lens fibers. The

anterior cuboidal cells of the lens do not transform. As the lips of the embryonic ocular fissure meet, they fuse anterior to the optic stalk and fusion progresses anteriorly and posteriorly.[83] The inner layer of the optic cup is now comprised of two layers, the inner marginal zone and the outer nuclear zone. The hyaloid artery, a branch of the internal ophthalmic artery, enters through the embryonic ocular fissure along with numerous mesenchymal cells which move toward the posterior chamber.[84]

Days 13 and 14 of embryonic development make up stage four. The eyelids begin to form as invaginations of surface ectoderm into the head mesenchyme.[85] The corneal epithelium develops from surface ectoderm and at this stage is associated with the developing retina and lens.[85] Anterior chamber formation commences. The lens space is obliterated by primary lens fibers. The tunica vasculosa lentis and primary vitreous, the transient vasculature that nourishes the embryonic lens, begin to envelop the lens. The retina is now composed of the inner and outer neuroblastic layers. The future retinal pigment epithelium is still quiescent.[84]

Stage five includes days 14 and 15 of embryonic development. The eyelids cover more than half of the cornea. The mesenchymal cells (of neural crest origin) produce the corneal stroma. The tunica vasculosa lentis and the primary vitreous have enveloped the entire lens. Nerve fibers in the retina begin to form. The sclera develops around the optic vesicle; and the vessels form just inside the sclera and will become the future choriocapillaris.[84]

Stage six extends from day 15 to day 17 of embryonic development. The eyelids now cover the entire cornea; this organizes the conjunctiva into bulbar, fornix and palpebral epithelium. The corneal epithelium, bulbar epithelium, fornix epithelium and palpebral epithelium are now well defined.[85] The cornea is organized into stroma covered by corneal epithelium, but the completion of corneal maturity occurs after birth in the mouse. Secondary lens fibers begin to form and the lens sutures are evident. The anterior aspects of the optic cup are growing forward indicating the future anterior uvea and iridocorneal angle. The retina has lightly staining nuclei in its inner portion and more densely staining nuclei in its outer portion. The extraocular muscles were formed earlier in development but are just now evident. The tunica vasculosa lentis, pupillary membrane, and primary vitreous consolidate and are visible around the lens and along the inner retina.[84]

Stage seven is comprised of days 17 through 19 and is the final stage of embryonic ocular development. The lens has lost most of the cell nuclei in its embryonic nucleus. The sutures are more complex in structure. The anterior uvea and iridocorneal angle are further developed. The ciliary body has two layers of epithelia, the outer pigmented epithelium which is continuous with the retinal pigment epithelium and the inner nonpigmented layer which is continuous with the inner neuroblastic layer of the retina.

Ocular development ceases for a period of time and development continues after birth.

B. Postnatal development of the anterior segment structures and the retina

At birth, the cornea is still composed of organized stroma and overlying corneal epithelium. The corneal epithelium is not fully differentiated at this stage and it is hypothesized that these cells do not lose their 'stem' cell characteristics until the corneal basement membrane is mature. Development of the corneal basement membrane occurs early in post-natal life.[86] The development of the ciliary body and iris occur after birth in the mouse. Neural crest cells use the basal lamina of the lens vesicle as a substrate to migrate between the surface ectoderm and optic cup anterior to the lens. These mesenchymal cells give rise to the anterior iridial stroma, the ciliary muscle, and the iridocorneal angle.[83] This results in a solid sheet of mesenchymal tissue which eventually remodels to form the anterior chamber, and the segment which covers the future pupil is the pupillary membrane.

The retina is also immature at the time of birth in the mouse. An apparently homogeneous, mitotically active population of ventricular cells produces the cells that compose the neural retina. Each mitotic division occurs at the outer retinal surface and produces two daughter cells. These two daughter cells then migrate back into the retina where their DNA undergoes replication. This back and forth movement occurs numerous times until the cells differentiate into the appropriate cell types which constitute the mature retina.[87] The pattern of apoptosis in the postnatal development of the murine retina was found to be under genetic control and part of the normal ontogenetic process.[88] Histogenetic cell death refers to

the deletion of cells during tissue and organ differentiation.[88] Most histogenetic cell death in the retina occurs during the first 14 days postpartum (D 1-14) and is complete by D 21. The incidence of degenerating cells follows a differentiation gradient occurring centrally first, then progressing toward the periphery.[88] This same gradient is seen during retinal cell division. Cell division arrests by D 5-6 in the center of the retina and by D 11 in the periphery of the retina.[87] A significant number of apoptotic cells is evident in the ganglion cell layer in the immediate postnatal period with a peak at D 2-5 and terminating by D 11. It is difficult to identify which cell type is undergoing apoptosis in the ganglion cell layer at this time because there is a mixture of amacrine cells and ganglion cells. The inner plexiform and inner nuclear layers show degeneration of numerous cells, probably amacrine cells, between D 3-8. The outer plexiform layer forms at D 5 and segregates the inner nuclear layer. This coincides with an increase in ventricular cell death. The most noticeable increase in ventricular cell death occurs in the innermost ventricular cell layers. These cells later become Muller and bipolar cells and they reach a degeneration peak between D 8-10 and cease by D 18. Interestingly, synaptic contacts between horizontal, bipolar, and photoreceptor cells occur in the outer plexiform layer between D 5 and 14 in the mouse.[89] This parallels the maximum death of bipolar and ganglion cells and inner rods. Development of the outer plexiform layer bisects the rod population with up to 40% of differentiating rod nuclei located on the inner side of this plexiform layer. These nuclei rapidly traverse the synaptic layer to the outer side, but most die in the migration

between D 5-11.[88]

C. Normal primary vitreous and tunica vasculosa lentis development

The primary vitreous forms the space between the developing lens and retina. The predominant source of the primary vitreous is the primitive, mesodermal, hyaloid vascular system. The embryonic lens and retina also contribute. The hyaloid vasculature enters through the embryonic ocular fissure and extends to the posterior surface of the lens. The tunica vasculosa lentis arises from the mesenchymal tissue at the anterior leading edge of the optic cup. The blood vessels of the tunica vasculosa lentis and the hyaloid capillary network anastomose to form the perilenticular mesodermal vascular network. The purpose of the hyaloid vasculature system is to nourish the lens during development.[90] When the tunica vasculosa lentis and hyaloid vasculature have completely surrounded the lens, thickening of the lens capsule accelerates, suggesting that this vascular network promotes capsule development.[91] Once the nonpigmented epithelium of the ciliary body begins to produce aqueous humor, this vasculature begins to regress. In the mouse, the hyaloid capillary network and the tunica vasculosa lentis regress between 5 and 21 days postpartum.[92] For decades it has been postulated that macrophages induce regression of the hyaloid vascular system and its associated tunica vasculosa lentis by occluding the capillary lumens.[83,93,94] More recently, Lang and Bishop[92] have shown that macrophages, specifically hyalocytes, are responsible for inducing apoptosis

in capillary endothelial cells, thereby mediating developmentally programmed tissue remodeling. Hyalocytes are tissue macrophages that reside primarily in the vitreous cortex.[95,96] In a 1994 report, Lang, *et al*,[97] suggested that hyalocytes induce apoptosis sporadically throughout the regression phase and for any one capillary segment, a critical point occurs where cell killing results in a lumen too narrow for blood flow. Synchronous apoptosis ensues along the capillary segment.

D. Persistence of the hyperplastic primary vitreous and persistence of the tunica vasculosa lentis

Persistent hyperplastic tunica vasculosa lentis (PHTVL) and persistent hyperplastic primary vitreous (PHPV) refer to the failure of regression of the tunica vasculosa lentis and hyaloid vasculature. PHTVL/PHPV is a congenital ocular anomaly that occurs in dogs[96,98-102], humans[103-105] and a line of transgenic mice.[92,106,107] Sequellae resulting from this anomaly include posterior capsular cataract, retrolental fibrovascular membrane causing leukocoria, and secondary retinal detachment.[98,99,102,108-110] The retrolental membrane is sometimes pigmented, which is reportedly due to migration of uveal melanocytes to the membrane.[103] Contraction of this membrane causes wrinkling of the posterior lens capsule, producing a tear in the capsule which subsequently leads to a posterior polar cataract.[104] In 1935, Ida Mann proposed that abnormal adhesion of persistent hyaloid vessels prevents normal development of the secondary vitreous.[111]

In humans, PHTVL/PHPV is usually a unilateral non-hereditary condition.[112] In only 10% of cases, the condition is bilateral and is associated with severe malformations as in trisomy 13/15.[103,113] In the Doberman Pinscher, PHTVL/PHPV occurs as a bilateral hereditary condition possibly due to inbreeding.[108] Conventional karyotyping studies have not found any chromosomal aberrations in this breed.[108]

An occasional finding in humans with PHTVL/PHPV is an immature iridocorneal angle due to abnormal cleavage.[103] It is possible that the initial abnormality, that is, abnormal regression of the perilenticular vasculature is due to malfunction of the hyalocytes. This in turn leads to abnormal development of the anterior and posterior chambers including the iris, cornea, and iridocorneal angle, and abnormal development of the vitreous chamber, including lack of production of secondary vitreous. In order to more fully understand the abnormal interactions of the tissue layers that contribute to these anomalies, it is necessary to understand how the ocular structures normally develop.

E. Ocular development on the cellular and molecular level

i. Neural crest:

The neural crest is transiently formed at the apical edge of the neural fold. When the neural fold is about to close to form the neural tube, neural crest cells begin to migrate to their specified locations. The mechanism of neural crest cell migration involves three major classes of cell surface adhesion receptors. Cadherins, which are calcium-dependent cell-cell

adhesion molecules, mediate cell-cell homophilic adhesions. Members of the immunoglobulin superfamily (e.g. N-CAM) are calcium-independent cell adhesion molecules. Integrins are the third class of cell adhesion receptors.[114] The extracellular matrix (ECM) is also important for neural crest cell migration. Some components of the ECM are promoters of cell migration while others are inhibitors of cell migration. A careful balance of positive and negative signals guides the neural crest cells to their correct destinations.[114] Growth factors are also involved in neural crest cell migration. TGF-ß is indirectly involved by regulating the production of ECM components including collagens, proteoglycans and fibronectin.[114] Neural crest cells originating in the anterior midbrain migrate to the eye rudiments where they form most of the ocular structures including corneal stoma and endothelium, the iris and ciliary stroma, iris and ciliary musculature, endothelial cells which cover the trabecular meshwork, choroidal melanocytes and the sclera.

Retinoic acid receptors are nuclear transcription factors and are localized to neural crest cells and their derivatives which migrate into and around the optic cup during normal development.[114] This suggests that retinoic acid receptors are vital to the entire process of neural crest cell migration from beginning to end at their final locations. Retinoic acids, the ligand for retinoic acid receptors, therefore, likely play a role in the chemotaxis of the migrating cells.[114] Retinoic acids are synthesized asymmetrically in the dorsoventral axis of the developing eye and have been shown to regulate the growth and differentiation of retinal pigment epithelial

cells, lens epithelial cells, corneal endothelial cells, and stromal cells.

ii. Growth factors

Fibroblast growth factor receptors (FGFR) are expressed in the developing eye. FGF-R1 is expressed in the outer neuroepithelial layer of the optic vesicle and cup which gives rise to the retinal pigment epithelium. The lens epithelium expresses FGF-R3 to a greater extent than FGFR1. FGF-R2 is expressed in mesenchymal cells which surround the optic cup, but not in the neural crest itself. These cells differentiate to form the sclera and also become melanocytes in the choroid. Acidic and basic FGF, the ligands for FGF-R2, are probably necessary for controlling differentiation of cells into their specific cell types.[114] Experiments in chick embryos have established that FGF-R1 and FGF-R2 are coexpressed in developing retinas. During development, neural retina cells and retinal pigment epithelial cells differ in their responsiveness to the mitogenic effects of FGF.[115] These differences may affect regulation of the cells' proliferation.

Transforming growth factor beta (TGF-ß) is a 25 kDa peptide growth factor which occurs in five isoforms. The pre-pro form has a binding protein that is cleaved to a latent secreted form upon processing. Most mammalian cells secrete latent TGF-ß that is processed to the final biologically active form.[116] Both the latent and the secreted form of TGF-ß1 appear to be produced and processed, respectively, by the hyalocytes. Hyalocytes are the only cellular occupants of normal vitreous, and they may potentially control the production and the rate of TGF-ß1 activation in the vitreous.[116]

Hyalocytes are also immunoreactive for TGF-ß2 and ß3.[116] Previous studies have shown vitreous to possess angiogenic inhibitory substances[117,118] and endothelium and smooth muscle cell inhibitors.[119] TGF-ß may be a candidate for a vitreous inhibitor of angiogenesis. TGF-ß has the same properties (i.e. acid and heat stability and activation, and extreme hydrophobicity) as the inhibitory substance discovered by Lutty, *et al.*[119] These results imply that hyalocytes are able to produce and degrade most of the constituents of vitreous, and they help maintain the avascularity of the vitreous as well.[116]

iii. Important genes involved in ocular development

The Pax-6 gene encodes a paired domain and a paired-like homeodomain which are DNA-binding motifs.[120] Pax-6 is essential for normal eye development and is expressed in the optic vesicle and the surface ectoderm. The optic vesicle is involved in correct growth and positioning of the lens[121] which is important for development of the retina. At day 8 of embryonic development (E 8) in the mouse, Pax-6 transcripts are evident in the optic pit and diencephalon. By E 9, expression in the head surface ectoderm is down-regulated and is restricted to the lens placode, the nasal placode, and the adjacent tissues. Between E 8 and E 9.5, Pax-6 must act in order for normal lens formation to proceed. Structures derived from surface ectoderm (i.e. the lens pit, the lens vesicle, the lens, and the developing cornea) express Pax-6 until at least E 15.5.[122] Expression in

neural ectoderm derived structures beginning on E 9.5 is localized strongly around the rim of the developing optic cup and more weakly expressed posterior to the optic cup and in the proximal optic vesicle structures. In the early optic cup, the future retinal pigment epithelium (RPE) and the future neural retina express Pax-6, but by E 15.5, expression was only seen in the anterior regions of the RPE.[122]

iv. Lens

The anterior lens surface is covered by a single layer of simple cuboidal epithelium; the rest of the lens is made up of elongated lens fiber cells. During lens development, the equatorial epithelial cells differentiate into fiber cells. Differentiation of lens fiber cells involves cell elongation, synthesis and accumulation of large amounts of lens crystallin proteins, suspension of DNA synthesis and cell division, and final degradation of most membrane-bound organelles.[123] The factor(s) necessary for perpetual replication of lens epithelial cells in both adults and embryos is still unknown. In a recent study in chick eyes,[124] a lens mitogen was identified in the anterior chamber of the embryonic eye. It is hypothesized that this mitogen is derived from the vascular system of the embryo. An inhibitory factor of lens cell elongation was also found. It is believed that it prevents serum IGF-1 from causing anterior lens cells to differentiate into fibers, thus maintaining lens polarity.[124] IGF-1 is a potent inducer of fiber differentiation.[125] Vitreous humor in the chick embryo inhibited proliferation of lens epithelial cells.[124] It was suggested in a study by McAvoy, *et al*, [126] that variations in

FGF concentration in the anterior chamber, the posterior chamber and the vitreous chamber maintained lens polarity. It is more likely that inhibitory and stimulatory factors in the different compartments of the eye determine lens polarity rather than just one factor.[124]

v. Cornea

A stem cell is any cell with a high capacity for self-renewal throughout adult life. Stem cells also have long cell cycles and infrequently divide. It is believed that stem cells can undergo asymmetric cell division which gives rise to daughter cells which are further differentiated. These daughter cells are known as "transient cells" or TA cells.[127] In the adult cornea, stem cells are present in the limbus, but in the developing cornea, all of the cells are in a stem or stem-like state.[128] The cornea does not attain full maturation until sometime between birth and eyelid opening in the rat[128] and presumably so in the mouse. Alpha-enolase is a glycolytic enzyme and a marker of stem and early TA cells that is expressed at high levels in the developing cornea until D 7.[129] Epidermal growth factor receptor (EGF-R) is also expressed at high levels in the developing cornea.[128] Alpha-enolase and EGF-R may be linked by c-myc, whereby, EGF, acting through EGF-R, stimulates c-myc production which, in turn, stimulates synthesis of alpha-enolase.[130] As the corneal epithelium terminally differentiates and changes cell shape from flattened ovoid to columnar, alpha-enolase and EGF-R are down-regulated. The decrease in EGF-R occurs concurrently with a proliferation surge

between D 7 and eyelid opening (D 12-14).[131] This surge in cell proliferation provides the eye with a protective layer of mature epithelium. Differentiation of the ocular surface is believed to begin at the eyelid margin and spreads to the cornea.

The ROSG antibody identifies a sugar epitope on a high molecular weight, highly glycosylated molecule that is a component of the glycocalyx of the ocular surface. ROSG is expressed in apical cells of the differentiated ocular surface epithelium.[131] The ROSG antibody has been shown to bind the palpebral conjunctiva near the lid margin before eyelid opening and then extends to the ocular surface and corneal epithelium within 24 hours of eyelid opening in the rat.[131] ROSG antigen expression coincides with mucus production by goblet cells. Before eyelid opening, mucin is not evident on the ocular surface. It is possible that ROSG antigen is required for the spread of mucus and the subsequent prevention of invading pathogens.[131]

Insulin-like growth factor-II (IGF-II) is a peptide homologous to insulin. IGF-II is expressed prenatally and probably functions in an autocrine and/or paracrine manner in the differentiation of some mesodermally-derived tissues. IGF-II expression follows an anteroposterior pattern in the developing eye with corneal expression rising and declining initially followed by scleral expression of IGF-II. Anterior cornea differentiates earlier than the posterior sclera.[132] At E18, mouse corneal stromal cells are highly differentiated and express little IGF-II in comparison to the high levels expressed in the immature sclera and extraocular muscles.[133] This

anteroposterior pattern of IGF-II expression parallels the morphological scleral differentiation that occurs in humans.[134] Developing scleral cells also have significant levels of the type 2 IGF receptor protein which follows the developmental progression of IGF-II mRNA production.[133] Cells expressing the IGF-II gene are in close proximity to those responsive to type 2 IGF receptor which may suggest an important regulatory role for IGF-II in ocular development, specifically the determination of the ultimate size and shape of the developing eye.[133]

CONCLUDING REMARKS

Tg3261 is an interesting insertional mutant with two seemingly separate phenotypes. But, they are not as different as one may believe. The tumors are of histiocytic origin possibly due to a lack of terminal differentiation which allows uncontrolled proliferation and further mutations. The ocular phenotype may stem back to an abnormally functioning hyalocyte, the histiocyte which resides in the eye. Though the ocular hyalocytes do not undergo uncontrolled proliferation, the abnormalities evident initially and their sequella may be due to a lack of terminal differentiation of these cells as well. The random insertion of the TyBS minigene into the genome of this mouse line may have occurred in a gene controlling the maturation and terminal differentiation of histiocytes. Another possibility is that the insertion of the transgene is causing the abnormal function of the disrupted gene at a time that is crucial to ocular development.

FUTURE DIRECTIONS

TgN3261Rpw is an interesting mouse with phenotypes which lend themselves to an endless number of experiments. The basic tumor biology of the histiocytic sarcomas is a project that can stand alone. Necessary experiments include determining whether these cells are truly under CSF-1 control, as hypothesized. Preliminary results of a study examining the wound healing in these mice do show differences between wild type mice and their transgenic counterparts. More numbers of mice and an established set of criteria will determine whether these results are valid. If they are repeatable, then a trial to investigate the cause is warranted since macrophages called into action following such a stimulus would require the GM-CSF-controlled macrophages. Another experiment would involve growing non-neoplastic histiocytes in culture from wild type mice and transgenic mice and comparing their phagocytic abilities and their growth rates to each other and to neoplastic histiocytes in culture.

The ocular phenotype in this mouse also has the potential for future experiments involving embryological development. The post-natal development of the anterior segment has not been studied to the extent that the retina has in terms of differentiation, proliferation and occurrence of apoptosis.[87,88] The wild type and unaffected transgenic mice are the perfect model for this type of investigation, and the affected transgenic mice will give valuable information involving the fate of the neural crest cells in abnormal anterior chamber development.

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PART 2:

CHARACTERIZATION OF TUMOR PHENOTYPE IN TRANSGENIC LINE TgN3261Rpw

I) ABSTRACT

TgN3261Rpw is a line of transgenic mice generated using insertional mutagenesis. A significant percentage (28%) of the transgenic animals developed histiocytic sarcomas before 13 months of age, and none of the wild type litter mate controls or FVB/N strain controls examined developed the disease. The affected mice developed hepatomegaly, splenomegaly, lymphadenopathy, dyspnea and ascites due to infiltrating neoplastic histiocytes. The tumor cells infiltrated the organs and effaced the tissue architecture. The neoplastic cells exhibited erythrophagocytosis, they stained positively for markers characteristic of histiocytes (anti-CD68 and peanut agglutinin), and they maintained germline configuration of T cell receptor and immunoglobulin genes.

II) INTRODUCTION

Tissue macrophages, or histiocytes, are "professional phagocytes" that arise from the colony forming unit granulocyte-macrophage (CFU-GM) in the bone marrow, under the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor(M-CSF)/CSF-1.[1-3] GM-CSF and M-CSF/CSF-1 directly regulate proliferation and differentiation of myeloid progenitor cell into different stages of macrophage populations. CSF-1 is essential for inducing terminal differentiation in the mononuclear phagocyte system (MPS) and GM-CSF acts primarily on mature myeloid progenitor cells.[3,4] The stem cell, CFU-GM, generates promonocytes that mature in the bone marrow to monocytes which circulate in the peripheral blood.[1] Adherence to endothelium and then extracellular matrix is an important initial step in the transition of blood monocyte to histiocyte.[5] Once in the tissues, histiocytes complete their maturation and are ready to function in wound healing, in defense against microorganisms, in antigen presentation and in eliminating undesirable particulate debris.[1,5]

Neoplastic disorders of histiocytic origin are extremely rare and difficult to diagnose.[6-8] Cell morphology is not a reliable means of diagnosing histiocytic sarcomas because of their markedly pleiomorphic mononuclear phenotype.[9,10] Enzyme histochemistry and immunohistochemistry have been used extensively in an attempt to find the appropriate enzymes and antibodies which characterize these tumors, but

the tumors can express different antigens and contain different enzymes depending on their stage of differentiation and their local environment.[6,8-14] Gene rearrangement studies are the most reliable method to rule out lymphocytes as the cell of origin. During the development of B and T lymphocytes, immunoglobulin and T cell receptor genes, respectively, undergo multiple rearrangements in order to prepare them for their roles in the immune system.[15-17] Cells of monocyte/macrophage origin do not undergo such rearrangements and maintain their germline configuration.[13,18,19] Therefore, the defining characteristics of a true histiocytic sarcoma are erythrophagocytosis, positive results for multiple histiocytic markers and negative results for B- and T-cell markers and antigen receptor gene rearrangements.[6,18]

TgN3261Rpw (Tg3261) is a line of transgenic mice generated in a large scale insertional mutagenesis program at the Oak Ridge National Laboratory (figure 1). When initially screened for obvious abnormalities, homozygous mice exhibited cataracts and abnormal irides. In addition, up to 28% of Tg3261 mice developed histiocytic sarcomas before 13 months of age.

III) MATERIALS AND METHODS

A. Animals, necropsy and histopathology

Tg3261 mice were maintained at the Oak Ridge National Laboratory. Tg mice were identified by coat color and confirmed to be transgenic by Southern blot analysis (see section H). Mice were sacrificed by inhalant anesthesia using Metophane (Pitmann-Moore) followed by cervical dislocation when the animals exhibited signs of illness (dyspnea, abdominal distension, cachexia) or upon reaching approximately 13 months of age. Full necropsies were performed and tissues were fixed in 10% buffered neutral formalin, or 4% paraformaldehyde and Bouin's fixatives. Tissues were then paraffin-embedded, sectioned at 4 μm, stained with hematoxylin and eosin, and examined via light microscopy.

Liver, spleen, kidney, and thymus were frozen immediately at -80°C or were snap-frozen in liquid nitrogen prior to standard phenol-chloroform extraction of DNA.[20]

B. Immunohistochemistry

Immunohistochemical staining was performed on tissues fixed in 10% buffered neutral formalin, 4% paraformaldehyde, or Bouin's fixatives which were then embedded in paraffin and sectioned at 4 μm. Standard avidin-biotin peroxidase complex (ABC) immunohistochemistry was performed using diaminobenzidine as the chromogen. Antibodies to the following antigens were used as primary antibodies: pan-cytokeratin, vimentin, S-100, lysozyme, myosin, smooth muscle-specific actin, desmin, alpha-1-antitrypsin, alpha-1-antichymotrypsin (Biogenex Laboratories), anti-mouse polyvalent

immunoglobulins (Sigma Immunochemicals), and CD68 (DAKO). Standard lectin staining protocols were followed to stain with peanut agglutinin (Sigma Immunochemicals). Tissues were considered positive for that antibody if unequivocal labeling of the neoplastic cells was seen. Controls used no primary antibody or lectin.

C. Enzyme cytochemistry

Cytospin preparations of tumor cells were stained for alpha-naphthyl acetate esterase (Sigma Immunochemicals) following kit directions. Briefly, slides were dehydrated through decreasing concentrations of ethanols, fixed in citrate-acetate formaldehyde for 30 seconds followed by a 60 second rinse. Slides were placed in a Coplin jar filled with naphthol AS-D chloroacetate solution for 15 minutes at 37°C in the dark. Slides were then rinsed thoroughly in double distilled water for at least two minutes, counterstained in hematoxylin, rinsed in tap water and allowed to air dry. Slides were evaluated by light microscopy.

Cytospin preparations of tumor cells were stained for leucocyte acid phosphatase (Sigma Immunochemicals) following kit instructions. Slides were fixed for 30 seconds, rinsed thoroughly and placed in Coplin jars containing premixed solution for one hour in the dark in a 37°C water bath. Slides were rinsed in double distilled water, counterstained with hematoxylin, air dried, and evaluated by light microscopy.

D. Flow cytometry

Cells from fresh tissues were minced, counted and suspended in PBS-1 % bovine serum albumin (BSA)-Na azide (NaN_3) solution. Cells were centrifuged, the supernatant was discarded leaving 200 μl and incubated with the primary antibody on ice for 45 minutes. Samples were washed twice in PBS-BSA-NaN_3, incubated with the secondary antibody on ice for 45 minutes and washed twice. Following centrifugation, pellets were resuspended in PBS-BSA-NaN_3 and subjected to FACS-SCAN analysis. Primary antibodies used were anti-B220 (PE), anti-Ly-5 (FITC), anti-Mac-1/CD11 (PE), anti-CD3 (FITC) (all, Pharmingen) and anti-CD68 (indirect FITC) (DAKO).

E. Gene rearrangement studies

Analysis of rearrangement of T cell receptor and immunoglobulins were performed as previously described.[16] DNA was extracted from tissues, purified by a standard phenol-chloroform ethanol precipitation technique, and digested with restriction endonucleases (Promega) for 2 hours at 37°F. The resulting fragments were separated by electrophoresis in 0.8% agarose gels and transferred to nylon membranes (NEN Research Products) for Southern blot analysis. The membranes were hybridized with alpha-^{32}P-radiolabeled DNA probe fragments (Amersham),washed, and subjected to autoradiography (Kodak) or phosphorimaging (Molecular Dynamics). The four DNA probes used in this study were an immunoglobulin heavy chain joining region J_H probe; an immunoglobulin kappa-chain

constant region Cß probe; and the beta-1 and beta-2 T-cell receptor (TCR) genes (donated by Dr. Monica Justice, ORNL).

T-Cell Receptor Beta-Chain Gene: Approximately 10 μg of DNA from each tissue was digested with Pvu II before hybridization with the TCR beta-1 probe; or with Hind III before hybridization with the TCR beta-2 probe.

Immunoglobulin Genes: Approximately 10 μg of DNA from each tissue was digested with EcoRI before hybridization with the immunoglobulin heavy chain joining region J_H probe; or with Bgl II before hybridization with the immunoglobulin kappa-chain constant region Cß probe.

F. Electron microscopy

Tumors were examined by electron microscopy following fixation, dehydration, and embedding by routine methods.[21]

G. Cytogenetic analysis

i. Meiotic study-testis

The protocol for testes preparation followed that of Evans, *et al.*[22] In brief, the contents of the tubules from a whole testis were suspended in isotonic (2.2%) sodium citrate solution. The germinal cells were sedimented by centrifuging at 500 rpm for 5 minutes, leaving most of the sperm in the supernatant fluid which was discarded. The cells were resuspended in hypotonic (1%) sodium citrate solution and left at room temperature for 12 minutes. They were then resedimented and fixed as a concentrated suspension in a mixture of 3 parts absolute ethyl alcohol to 1 part glacial acetic acid plus chloroform. This was followed by two quick changes into

fresh fixative. A small amount of final fixed suspension was placed on a clean glass slide, allowed to air dry and stained with lactic-acetic-orcein.[22]

ii. Mitotic study-spleen

The spleens were removed and placed in a petri dish (60 X 15 mm) with 5 ml of Hank's balanced salt solution. The cells were dispersed with forceps and a 5 ml syringe with a 20 gauge or 21 gauge needle. Using the same syringe, the cell suspension was collected and transferred to a 15 ml sterile centrifuge tube, and centrifuged for 5 minutes at 1200 rpm. The cells were resuspended with culture media (RPMI 1640) (Gibco), 25% heat inactivated fetal bovine serum (JRH), 2 mM L-Glutamate 100x (Gibco), 2 mM penicillin-streptomycin 100x, 0.72 mM Concavalin A (Sigma Immunochemicals), and 0.144 mM B-Mercaptoethanol (Sigma Immunochemicals)). Three to five millions cells/ml were transferred to a 75 cm^2 tissue culture flask with up to 15 ml of media. Flasks were incubated, vertically, in a 37°C incubator for 48 hours. One hour prior to harvest 150 μl ethidium bromide (2.5 mM solution) was added to each flask. Thirty minutes prior to harvest 50 μl of Colcemid (10 mg/ml) was added. At harvest, cell suspension was collected in a 15 ml centrifuge tube and centrifuged for 5 minutes at 1200 rpm. Supernatant was discarded and cells were resuspended in 8 ml hypotonic (1%) sodium citrate solution (0.075 M) for 8 minutes 37°C. Tubes were centrifuged for 5 minutes at 1200 rpm, 1/2 to 2/3 of supernatant was discarded and the tube was flicked to gently resuspend pellet. Carnoy's fixative (1 part glacial acetic acid to 3 parts methanol) was added carefully up to 5 ml. Cells were allowed to stand at room temperature

for 15 minutes, gently resuspended and centrifuged for 5 minutes at 1200 rpm. The supernatant was discarded and fresh fixative added to resuspend cells followed by centrifugation. The previous step was repeated 4 or 5 times. Finally, fixative was removed and 0.5 - 1 ml of fresh fixative was added to resuspend cells and to make slide spreads.

H. Southern blot analysis

Southern blot analyses to identify transgenic animals were run with 10 μg of genomic mouse DNA prepared from tail samples following standard procedure.[23] DNAs were digested with *Hind* III or *Eco* RI using conditions specified by the enzyme manufacturer (Promega). Digested DNAs were run on a 0.8% agarose gel and transferred to nylon membrane (NEN Research Products) for Southern blot analysis. The membranes were hybridized with alpha-^{32}P-radiolabeled DNA probe fragments (Amersham), washed, and subjected to autoradiography (Kodak) or phosphorimaging (Molecular Dynamics).[24,25] The probe DNA used was the full TyBS transgene (4.1 kB).

IV) RESULTS

A. Disease progression, necropsy and histopathology

One hundred twenty seven transgenic, sixty non-transgenic littermates, and twenty one FVB/N stock controls were examined in these studies. All of the animals were genotyped by coat color which was confirmed by Southern blot analysis (see section H). Twenty-eight percent of the transgenic animals necropsied developed histiocytic sarcomas by 13 months of age and none of the wild type or FVB/N control animals developed the disease. Table 1 shows the tumor locations broken down into locations and age of animals. The abdominal form was the most common and involved the liver, mesenteric and other regional lymph nodes, and the spleen resulting in abdominal distension (figures 2, 3, 5, and 6). The mice developed a serosanguinous ascites free of tumor cells (figure 4). Histiocytic sarcomas in the thoracic cavity involved the thoracic and pulmonic pleura; the ribs had white round firm nodules on their pleural surface; the pericardium and pleura were invaded and thickened, and the trancheobronchial lymph nodes were enlarged. Skin tumors occurred over the scapula and abdomen, at the base of the tail, and around the tarsus.

The enlarged livers of the animals with histiocytic sarcomas contained a moderate to intense, diffuse and/or nodular infiltrate of moderately pleiomorphic neoplastic round cells. The infiltrating cells were often centered around portal areas, but were present throughout the parenchyma as well. In some cases, large nodular foci completely replaced the normal liver architecture (figure 26).

TABLE 1. Numbers of mice necropsied and total numbers and percentages of those with histiocytic sarcoma.

	n	Liver (%)	Spleen (%)	Thorax (%)	Skin (%)
< 6 months	14	3 (21)	7 (50)	2 (14)	2 (14)
6 - 9 months	25	7 (28)	7 (28)	2 (8)	2 (8)
9-13 months	88	28 (32)	24 (27)	10 (11)	3 (3)
WT	60	0 (0)	0 (0)	0 (0)	0 (0)
FVB/N	21	0 (0)	0 (0)	0 (0)	0 (0)

The neoplastic histiocytes were variable in size and shape, ranging from large round cells with moderate amounts of pale, finely vacuolated cytoplasm to small oval to elongate cells with scant pale cytoplasm. The nuclei were round to oval, with coarsely clumped and peripheral chromatin and prominent nucleoli. Cell margins were indistinct. Mitotic figures were common. Occasionally, the small neoplastic cells formed nests and large nodules in which many of the cells were moderately spindle shaped forming palisades and interlacing bundles and nests surrounded by a delicate connective tissue framework.

In the spleens, the morphology and degree of infiltration were also variable. In some cases, the spleens were massively enlarged and the normal structures were completely replaced by sheets of neoplastic cells (figure 6). In other spleens, the infiltrate was more limited, with remnants of

periarteriolar sheaths and some red pulp remaining. In a few cases, the infiltrate was limited to areas of the non-lymphoid white pulp. Similar to the liver, the morphology of neoplastic cells in the spleens was variable, ranging from a uniform population of round histiocytic cells, to small, spindle shaped cells (figures 7 - 9). In many cases, both types of cells were present in the same spleen.

The neoplastic histiocytic infiltrate in the lymph nodes was also variable. In many cases, the entire lymph node architecture was effaced and replaced by an infiltrate of homogeneous small neoplastic histiocytes (figure 10 - 15). However, in many of the lymph nodes, the cortical and medullary architecture was maintained. In these lymph nodes, there was a striking difference in the appearance of the neoplastic cells in the cortex versus the medulla. The neoplastic cells in the cortex were a mixed population of the small, round to oval to slightly spindle-shaped cells. In the medulla, the neoplastic histiocytes were large, with abundant eosinophilic cytoplasm and distinct cell margins.

In other organs (pericardium, pleura, meninges, mesentery) the neoplastic infiltrates were associated with moderate amounts of fibrous connective tissue reaction.

In the skin, the tumors were poorly differentiated with many oval to spindle cells forming palisades and interlacing facicles. There was an admixture of round, histiocytic cells throughout the tumor and these formed the predominant cell type in some areas (figures 16 - 18).

B. Immunohistochemistry

The neoplastic histiocytes in the liver, spleen, lymph nodes, and pleura stained positively with antibodies to vimentin, leucocyte common antigen (LCA), CD68, lysozyme and with the lectin, peanut agglutinin (PNA) (figures 19 - 21). Tumor cells did not stain with antibodies to pan-cytokeratin, S-100, alpha-1-antitrypsin, alpha-1-antichymotrypsin, Mac-1, Mac-2, desmin, smooth muscle-specific actin, myosin, and immunoglobulins. In the skin tumors, the neoplastic cells were negative for all markers except anti-vimentin antibody.

C. Enzyme cytochemistry

Neoplastic histiocytes were positive for alpha-naphthyl acetate esterase and for leucocyte acid phosphatase. Cells had diffuse staining in the cytoplasm.

D. Flow cytometry

The tumor cells examined reacted only with anti-CD68 antibody. They failed to stain with antibodies to anti-CD4, anti-B220, anti-Mac-1, and anti-Ly-5.

E. Gene rearrangement studies

Both unaffected control tissues and neoplastic tissues studied showed germline configuration of the immunoglobulin loci (J_H and kappa) and T-cell receptor loci (beta-1 and 2).

F. Electron microscopy

Ultrastructurally, the neoplastic histiocytes formed sheets and nests of round to oval to slightly spindle shaped cells with numerous interdigitating microvillus projections. The nuclei were round to oval and often indented. The cytoplasm contained few organelles.

G. Cytogenetic Analysis

The epididymis contained abundant sperm with 70% motility. Twenty cells in diakinesis/metaphase I were examined and all were normal (20 bivalents).

Fourteen spleen metaphase cells were scored. Twelve had 40XY and 2 with 39 XY (autosomal). Three karyotypes were analyzed and all were normal (figure 22).

H. Southern blot analysis

Mice with pigmented coat colors were confirmed transgenic by Southern blot analysis.

V. DISCUSSION

Though histiocytic sarcomas are rare, their diagnosis is important due to their aggressive course with rapid and widespread dissemination. Histiocytic sarcomas are not related to sarcomas of follicular dendritic cells, which stain positively for R4/23, or interdigitating cells, which stain positively for ATPase and S-100; histiocytic sarcomas do not stain for these markers.[2,9,12] Erythrophagocytosis is another characteristic feature of histiocytic sarcomas which sarcomas of follicular dendritic cells and interdigitating cells do not possess.[6]

Immunohistochemistry results of the tumors identify the cell of origin as the histiocyte. Anti-CD68 antibody has been used extensively as a marker for human histiocytic sarcomas.[1,6,9,12-14,18] The lectins, peanut agglutinin and soy bean agglutinin, are also reliable markers of both normal and malignant histiocytes.[14,26] Alpha-1-antichymotrypsin (AACT) is believed to be a marker for histiocytes, but it is not as specific as once believed. Soini, *et al*,[27] showed that over 80% of different carcinomas, sarcomas, melanomas and lymphomas were positive for AACT and thus ruled it out as a specific marker for histiocytes. Another group also found AACT to be positive in some lymphomas and in metastatic carcinomas.[8] Interestingly, the histiocytic sarcomas arising in Tg3261 did not react with AACT or with alpha-1-antitrypsin. This may be a result of their stage of differentiation. Perhaps, these tumors are too immature to express these markers, or the antigens are not accessible to the antibody due to processing or the need for enzyme pretreatment.

The alpha-naphthyl acetate esterase reaction is a valuable marker of monocyte/macrophages or MPS system.[11] The positive reaction of the tumor cells with this, combined with the leucocyte acid phosphatase assay, further substantiate the cell of origin of the tumors in question. That is, they are of myeloid origin, more specifically, tissue macrophages.

Erythrophagocytosis and the results of the immunohistochemistry, enzyme cytochemistry, histopathology, electron microscopy and gene rearrangement studies fulfill all the requirements possible for definite diagnosis of histiocytic sarcoma.

Understanding of the mutations that occur in histiocytes that ultimately result in histiocytic sarcoma is difficult because of the lack of an animal model of this disease, though true histiocytic sarcomas have been reported in an epidemic in the Northern Pike, *Esox lucius* L., in the Aland Island of Finland.[28] The use of a fish as a model of human disease is an unfavorable choice; but a mouse model for histiocytic sarcoma, especially with the consistent occurrence of this tumor in Tg3261, is a possibility. Mouse models of human disease have been used for years for diseases such as diabetes mellitus and polycystic kidney disease.[29,30] Since Tg3261 is an insertional mutant, it will be possible to find the location of the mutation using molecular biology techniques and hopefully to find the human homologue of the gene.

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PART 3:

DIAGNOSIS AND CHARACTERIZATION OF INTRADERMAL TUMORS IN TRANSGENIC LINE TgN3261Rpw: MYSTERY SOLVED

I) ABSTRACT

TgN3261Rpw is a line of transgenic mice generated by insertional mutagenesis at the Oak Ridge National Laboratory in a large scale insertional mutagenesis program. A significant percentage (28%) of the mutant mice developed histiocytic sarcomas in the liver, spleen, lymph nodes, thoracic pleura, and pericardium. A small percentage of mice developed intradermal tumors which are highly heterogeneous and do not resemble the typical histiocytic sarcomas nor do they possess the same immunohistochemical markers as the histiocytic sarcomas except for vimentin. These intradermal tumors posed a diagnostic challenge. In transplant experiments using SCID mice the intradermal tumors were proven to be of the same cell of origin as the tumors arising within the abdomen and the thorax of TgN3261Rpw mice.

II) INTRODUCTION

Spindle cell tumors are a highly heterogeneous group of tumors and they pose a diagnostic challenge. These tumors are diagnosed on a histogenetic basis according to the tissue they resemble; but, depending on the stage of differentiation of the cells, conclusive diagnosis on morphologic criteria is not possible. Cases such as these require further techniques including immunohistochemical staining, flow cytometry, enzyme cytochemistry, gene rearrangement studies, and transplant experiments.

TgN3261Rpw (Tg3261) is a line of transgenic mice generated in a large scale insertional mutagenesis program at the Oak Ridge National Laboratory. When screened for obvious abnormalities, a small percentage of mutant mice had cataracts and abnormal irides. As the mice aged, 28% of the mutant mice developed histiocytic sarcomas before 13 months of age while none of the wild type and FVB/N controls developed this tumor. Two forms of this disease were observed. The visceral phenotype occurred either in the abdominal cavity wherein malignant histiocytes invaded the liver, spleen (figures 2 - 6), and regional lymph nodes, or they occurred in the thoracic cavity where malignant histiocytes invaded the pulmonary and thoracic pleura, pericardium and regional lymph nodes. The visceral tumor cells were rounded to ovoid, exhibited erythrophagocytosis, stained positively for histiocytic markers and lacked immunoglobulin and T-cell receptor gene rearrangements. The second form consisted of intradermal tumors (figures 16 - 18) which posed a diagnostic challenge because they did not resemble the visceral tumors, nor did they possess any of the visceral tumor's characteristics. The intradermal tumor cells were mixed, with spindle shaped cells and round cells intermingled or segregated and stained only for

vimentin.

Tg3261 was generated by insertional mutagenesis and we believe that only one gene was disrupted, therefore, both tumors could have come from the same cell lineage. Our objective was to determine if the intradermal tumor occurring in the abdomen and thorax was of the same lineage as the tumor arising in the skin.

III) MATERIALS AND METHODS

A. Tumor cell transplants

Tumors from either mesenteric lymph node or dermal sarcomas were minced, the cells resuspended, and counted with a Coulter counter. One million cells from either the dermal tumors or the abdominal tumors were inoculated either subcutaneously between the scapulae in 6 SCID mice, or directly into the peritoneum of 6 SCID mice for a total of 24 SCID mice. After approximately 4-6 weeks, all SCID mice developed tumors, and were sacrificed. The tumors were fixed in 10% buffered neutral formalin, paraffin embedded, sectioned, stained with standard hematoxylin and eosin stains, and evaluated by light microscopy.

B. Immunohistochemistry

Immunohistochemical staining was performed on these tissues and on the tissues from the transgenic mice used for this experiment. Standard avidin-biotin peroxidase complex (ABC) immunohistochemistry was performed with anti-CD68 antibody (DAKO) and anti-vimentin antibody (Biogenex) using diaminobenzidine as the chromogen. Standard lectin staining protocol was followed to stain for peanut agglutinin (PNA) (Sigma Immunochemicals).

IV) RESULTS

A. Tumor cell transplants

The abdominal and thoracic organs and lymph nodes of all twelve mice (figure 23 and 24) inoculated intraperitoneally with the TgN3261Rpw intradermal histiocytic sarcoma were infiltrated with histiocytic sarcoma cells. Within 4-6 weeks, the mice developed hepatomegaly, splenomegaly, ascites, thickened peritoneums, pleural and pericardial metastases, and all lymph nodes were affected . Tumor cells transplanted subcutaneously between the scapulae from both the dermal histiocytic sarcoma and the abdominal histiocytic sarcoma formed large (2.5 cm diameter), rapidly growing aggressive tumors within 6 weeks (figures 25 and 28). The tumors, if allowed to grow larger than 2.5 cm in diameter, expanded down over the triceps musculature and had a large necrotic center. Subcutaneous tumors in two of the SCID mice metastasized to the spleen, one mouse had been inoculated with dermal tumor cells and the other with abdominal tumor cells.

Histiocytic sarcomas in the livers of the SCID mice had a moderate to intense, diffuse and/or nodular infiltrate of moderately pleiomorphic neoplastic cells (figures 29 and 32). The infiltrating cells were often centered around portal areas, but were also present throughout the parenchyma. In some cases, large nodular foci completely replaced the normal liver architecture. The neoplastic histiocytes were variable in size and shape, ranging from large round cells with moderate amounts of pale, finely vacuolated cytoplasm to small oval to elongate cells with scant pale cytoplasm. The nuclei were round to oval, with coarsely clumped and peripheral chromatin and prominent nucleoli. Cell margins were indistinct. Occasionally, the small neoplastic cells formed nests and large nodules.

The subcutaneous histiocytic sarcoma cells in the SCID mice were moderately spindle-shaped and pleiomorphic. They formed palisades and interlacing bundles and nests surrounded by a delicate connective tissue framework (figures 30 and 33). The intradermal histiocytic sarcomas which metastasized to the spleen ranged from a uniform population of round histiocytic cells, to small, spindle-shaped cells (figures 31 and 34). In both cases, both types of infiltrate were present in the same spleen.

The neoplastic histiocytic infiltrate in the lymph nodes was also variable. In many cases, the entire lymph node architecture was effaced and replaced by an infiltrate of homogeneous small neoplastic histiocytes. However, in many of the lymph nodes, the cortical and medullary architecture was accentuated. In these lymph nodes, there was a striking difference in the appearance of the neoplastic cells in the cortex versus the medulla. The neoplastic cells in the cortex were a mixed population of the small, round to oval to slightly spindle shaped cells. In the medulla, the neoplastic histiocytes were large, with abundant eosinophilic cytoplasm.

In other organs (pericardium, pleura and mesentery), the neoplastic infiltrates were associated with moderate amounts of fibrous connective reaction.

The histiocytic sarcomas which developed in SCID mice due to transplantation from the affected transgenic mesenteric lymph node and the skin tumor were histopathologically similar to those in the transgenic mice. Both phenotypes were recapitulated entirely.

B. Immunohistochemistry

All resulting tumors were positive for anti-vimentin antibody. The original abdominal tumor cells stained positively for anti-vimentin antibody, anti-CD68 antibody (figures 19, 20, and 35) and the lectin, PNA (figure 21). The original skin tumor did not stain with either anti-CD68 antibody nor with PNA. However, when either the skin or abdominal tumor were transplanted intraperitoneally into SCID mice, the resulting histiocytic sarcomas stained positively for anti-CD68 antibody and PNA as did the resulting subcutaneous histiocytic sarcomas which originated from the abdominal tumor (figures 36 and 37). Conversely, when the skin tumor was transplanted subcutaneously into SCID mice, the resulting histiocytic sarcomas were negative for anti-CD68 antibody and positive for PNA. Splenic metastases from the subcutaneous tumors originating from the skin and the lymph node tumors both stained positively for anti-CD68 antibody and PNA (figure 38). Positive controls used in the experiment (besides the tumors of origin) were histiocytic sarcomas from Tg3261 previously proven to be positive for these antibodies. Results are summarized in table 1.

TABLE 1. Immunohistochemical staining results, comparing tumors arising in Tg3261 mice and used in transplant experiments versus the resulting tumors in SCID mice.

	vimentin	CD68	PNA
Tg liver (106)	+	+	+
Tg ID (114)	+	-	-
SCID liver (106)	+	+	+
SCID ID (106)	+	+	+
SCID spl (106)	+	+	+
SCID liver (114)	+	+	+
SCID ID (114)	+	-	+
SCID spl (114)	+	+	+

Number in parentheses refers to the Tg3261 mouse's number; spl=spleen, ID=intradermal tumor, PNA=peanut agglutinin antibody.

V) DISCUSSION

Tumor cells from Tg3261 skin and the mesenteric lymph node inoculated into the peritoneum of SCID mice recapitulated the histiocytic sarcoma phenotype seen in Tg3261. In fact, both the abdominal and the thoracic forms occurred together in the SCID mice. Likewise, when transplanted subcutaneously, the neoplastic histiocytes from visceral origin acquired the morphologic and immunohistochemical features of the dermis-derived histiocytes. These results support our hypothesis that all of the tumors in Tg3261 mice arise from the same cell of origin, that is, the tissue macrophage or histiocyte. The histological differences and immunohistochemical staining differences which occurred in Tg3261 mice between the dermal and the visceral tumors were likely due to heterogeneity of macrophages, growth factors, and local tissue constraints.

Heterogeneity of macrophages has long been known, but the manner in which macrophages acquire this heterogeneity is not as well understood. Conceivably, it may originate through various pathways. The first way in which macrophages may acquire heterogeneity is at the level of the bone marrow precursor cells. Such cells would be preprogrammed and selectively expanded under different stimuli resulting in functionally different populations in various systemic environments. On the other hand, phenotypically identical cells could differ functionally due to local tissue constraints. Growth factors have also been shown to affect macrophage progenitors by influencing gene expression and behavior of mature cells. Lastly, the transient expression of various functions during macrophage differentiation has been proposed as the mechanism for macrophage heterogeneity.[2] These functions, alone, or in combination, may explain why

macrophages exhibit heterogeneity which may be dependent on the specific situation and site.

It has been suggested that heterogeneity is genetically predetermined prior to release of the granulocyte-macrophage (GM) precursor from the bone marrow.[2,3] Witsell, *et al*[2] reported a developmental mechanism for the generation of macrophage heterogeneity that supports a controlled developmental pathway of differentiation resulting in distinct phenotypes.[2] A heterogeneous population of monocytes leaves the bone marrow and these cells are capable of responding to the environmental stimuli they encounter, providing a mechanism for tissue-related heterogeneity.[2] The microenvironment in which the macrophage precursor differentiates affects directly the mature cell phenotype. Reverse transcriptase-polymerase chain reaction (RT-PCR) experiments have shown that phenotypes differ with growth factors used to propagate the colony, the period of time allowed for differentiation, and the addition of various other exogenous stimuli.[2]

Heterogeneous monocytes are growth factor dependent. Colony-stimulating factors (CSF) regulate granulocyte and macrophage formation *in vivo*.[4] Colony stimulating factors can induce non-dividing granulocyte-macrophage precursors to begin dividing within three hours.[5] Stimulation by CSFs is required throughout the cell cycle and higher concentrations of CSFs shorten cell cycle time and delay maturation of cells by preventing them from entering the post-mitotic state.[3] This indicates that there is not a set number of mitotic divisions that precursor cells must undergo before

entering terminal differentiation. Experiments *in vitro* have shown that CSFs have four additional actions besides inducing proliferation in responsive cells. These include maintaining the functional integrity of the cell membrane which enhances the survival of precursors and mature cells; inducing irreversible commitment in differentiation; inducing cellular maturation; and finally, stimulating functional activity of mature cells (e.g. phagocytosis or synthesis of various biologically active molecules).[3]

Colony stimulating factors include granulocyte-macrophage (GM-CSF), granulocyte (G-CSF), macrophage (M-CSF) and multipotential (multi-CSF); interleukin-6 has also been shown to have colony-stimulating activity.[3] Numerous cell types including fibroblasts, stromal cells, endothelial cells, macrophages, and lymphocytes are known to produce one or more of the CSFs.[3] Each CSF has a unique membrane receptor and more than one type of receptor may be found on granulocyte-macrophage progenitors and their maturing progeny.[6] Responsive progenitor cells are initially able to respond to the first growth factor that binds its appropriate receptor. This binding, in turn, influences the behavior of the other CSF receptors on the same cell and begins signal cascades specific to the particular type of receptor.[6]

Witsell, *et al*[7] demonstrated that there may be two macrophage populations comprised of an inflammatory GM-CSF-derived population and a steady-state CSF-1-derived population. The GM-CSF-derived population is produced in the bone marrow during immunological challenge.[8] Tumor

necrosis factor-alpha (TNF-alpha) released from the tissues induces differentiation of GM-CSF-derived cells and inhibits differentiation of CSF-1-derived cells. As the challenge abates, GM-CSF decreases to normal levels and allows the steady-state macrophage population to re-equilibrate.[7] The CSF-1 steady state cells may comprise the macrophage progenitor cells dispersed in the peripheral tissues which are maintained by a self-renewal mechanism.[9] As previously mentioned, CSFs are produced by many types of cells in the periphery where they may either encourage self-renewal or at least postpone maturation into terminally differentiated tissue macrophages. These same CSFs are capable of acting differently on other cells by suppressing their self-renewal and inducing differentiation.[3] The macrophage progenitor cells themselves may posses a genetic control element which dictates whether they will self-renew or generate committed progeny.

In cases of abnormal conditions, such as neoplasia, cells would likely self-renew rather that differentiate in order to propagate more cells with increased possibility of mutations. In a study by Just, *et al*[10], progenitor cells were "immortalized" in the presence of IL-3 or when co-cultured with stromal cells. This moved the cells toward self-renewal rather than differentiation thus increasing the chances of mutations due to rapid, continual proliferation. If this occurs in conjunction with cells that may already be transformed as possibly occurs in Tg3261 mice, this may pave the groundwork for the histiocytic sarcomas which occur in Tg3261. If we are to assume that the resident tissue macrophages are the cells of origin of histiocytic sarcoma

occurring in Tg3261, then we may also assume that these are under the control of CSF-1, not GM-CSF (according to Witsell, *et al* 's results).

The transgene used to generate Tg3261 likely inserted into a gene responsible for differentiation of tissue macrophages. Because two distinct tissue macrophage populations are affected, the skin histiocytes and the visceral histiocytes, it is likely that the mutated gene is expressed (or not expressed) while the cells are in the marrow, which transforms the cells before they reach their final destination and become resident tissue macrophages, and the behavior of the tumors differs due to the local microenvironment. Another phenotype not mentioned, but relevant, is the ocular phenotype (manuscript in preparation). A small percentage of transgenic mice have persistence of the hyperplastic primary vitreous and tunica vasculosa lentis and anterior segment dysgenesis. These congenital anomalies may develop due to an abnormality in the hyalocytes, which are the tissue macrophages which reside in the vitreous cortex. If there is an abnormality in this cell population as well, then three different populations of resident tissue macrophages have been affected by the insertion of the transgene.

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PART 4:

CHARACTERIZATION OF THE OCULAR PHENOTYPE IN TRANSGENIC LINE TgN3261Rpw

I) ABSTRACT

We have characterized a line of insertional mutant mice, TgN3261Rpw, which was generated at the Oak Ridge National Laboratory in a large-scale insertional mutagenesis program. This line of mice has two phenotypes, multiple developmental ocular abnormalities and a high incidence of histiocytic sarcomas. Here we will discuss the ocular abnormalities in detail. Briefly, the anomalies include persistent hyperplastic primary vitreous, persistent hyperplastic tunica vasculosa lentis, anterior segment dysgenesis, retrolental fibrovascular membrane, posterior polar cataract, and secondary detached retina.

II) INTRODUCTION

The primary vitreous forms the space between the developing lens and retina. The predominant source of the primary vitreous is the primitive, mesodermal, hyaloid vascular system; the embryonic lens and retina also contribute. The hyaloid vasculature enters through the embryonic ocular fissure and extends to the posterior surface of the lens. The tunica vasculosa lentis arises from the mesenchymal tissue at the anterior leading edge of the optic cup. The blood vessels of the tunica vasculosa lentis and the hyaloid capillary network anastomose to form the perilenticular mesodermal vascular network. The purpose of the hyaloid vasculature system is to nourish the lens during development.[1] When the tunica vasculosa lentis and hyaloid vasculature have completely surrounded the lens, thickening of the lens capsule accelerates, suggesting that this vascular network promotes capsule development.[2] Once the nonpigmented epithelium of the ciliary body begins to produce aqueous humor, this vasculature begins to regress.

In the mouse, the hyaloid capillary network and the tunica vasculosa lentis regress between 5 and 21 days postpartum.[3] Persistent hyperplastic tunica vasculosa lentis (PHTVL) and persistent hyperplastic primary vitreous (PHPV) refer to the failure of regression of the tunica vasculosa lentis and the hyaloid vasculature. PHTVL/PHPV is a congenital ocular anomaly occurring in dogs[4-9], humans[10-12] and a line of transgenic mice.[3,13,14] Sequellae resulting from this anomaly include posterior polar cataract, retrolental fibrovascular membrane causing leukocoria, and secondary retinal detachment.[4,6,9,15-17] The retrolental membrane is sometimes pigmented;

this has been reported to be due to migration of uveal melanocytes to the membrane.[10] Contraction of this membrane causes a tear in the lens capsule which subsequently leads to a posterior polar cataract.[11]

TgN3261Rpw (Tg3261) is a line of transgenic mice generated in a large scale insertional mutagenesis program at the Oak Ridge National Laboratory. When screened for obvious abnormalities, a small percentage of the transgenic mice had cataracts and abnormal irides. Another interesting phenotype exhibited by Tg3261 was that 28% of transgenic mice developed histiocytic sarcomas before 13 months of age and none of the wild type littermates exhibited either phenotype. Tg3261 was generated using the FVB/N strain of albino mice. This strain is one of the many strains which exhibit retinal degeneration due to a mutation in the *rd* gene which causes a defect in the beta subunit of rod cGMP-phosphodiesterase. This defect results in a loss of rod photoreceptors beginning at post-natal day 8 and by day 28, all rods and 97% of cones have degenerated. The mice still have normal pupillary light responses and do not seem blind in their actions, probably due to the gradual decrease in vision. Ocular changes noted in the transgenic mice do not include this retinal abnormality.

III) MATERIALS AND METHODS

A. Southern blot analysis

Southern blot analyses to identify transgenic animals were run with 10 μg of genomic mouse DNA prepared from tail samples following standard procedure.[18] DNAs were digested with *Hind* III or *Eco* RI using conditions specified by the enzyme manufacturer (Promega). Digested DNAs were run on a 0.8% agarose gel and transferred to nylon membrane (NEN Research Products) for Southern blot analysis. The membranes were hybridized with alpha-^{32}P-radiolabeled DNA probe fragments (Amersham), washed, and subjected to autoradiography (Kodak) or phosphorimaging (Molecular Dynamics).[19,20] The probe DNA used was the full TyBS transgene (4.1 kB). Southern blot analysis was used to confirm the coat color phenotype resulting from the insertion of the transgene.

B. Ophthalmic examination

One drop of 1% tropicamide (Akorn) was instilled in each eye 30 minutes before each mouse was examined. A direct ophthalmoscope was used to assess the presence of a fundic reflex. The mice were then examined using a Top Con slit lamp biomicroscope. Physical structures evaluated were the cornea, depth of the anterior chamber, amount of iris dilatation, and the lens.

C. Histopathology

Mice were euthanatized either when they exhibited signs of disease (dyspnea, abdominal distension, or cachexia) or when they reached

approximately 12 months of age. The method of euthanasia was inhalant anesthesia using Metophane (Pitman Moore) prior to cervical dislocation.

Eyes were enucleated and placed in 10% buffered neutral formalin. Following fixation, eyes were embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined by light microscopy. Abnormal eyes were also stained with Jones-methenamine silver stain for basement membranes.

D. Examination of primary vitreous

Normal wild type and heterozygous mice of 10, 16, and 21 days of age were euthanized as previously specified. Eyes were enucleated and placed in phosphate buffered saline pH 7.2 (PBS). Dissection was begun by making a puncture adjacent to the optic nerve using a 27 G needle. Scissors were used to cut from the needle hole to the limbus. The cut was then continued equatorially around the limbus, with care not to puncture the lens capsule, until the eye was in two pieces . Eyes were then placed in hematoxylin (Biogenex) for 5 minutes. Eyes were fixed in 4% formalin for 30 minutes and stored in PBS pH 7.2 for future research.

IV) RESULTS

A. Southern blot analysis

Southern blot analysis confirmed that the mice genotyped as transgenic by coat color pigmentation were in fact transgenic.

B. Ophthalmic examination

Eyes from adult unaffected transgenic and wild type mice were all positive for fundic reflection when examined with a direct ophthalmoscope. A positive fundic reflection was seen as a red reflection of the choriocapillaris in response to focal light. Both eyes from unaffected transgenic and wild type mice were fully dilated 30 minutes following one drop of topical tropicamide. Unaffected transgenic mice and wild type mice showed no evidence of cataracts in either eye.

Following one drop of tropicamide, eyes from affected transgenic mice were either partially dilated leaving dyscoria or they could not be dilated at all. The fundic reflex was not visible. Younger (2-4 months) affected transgenic mice had immature posterior polar cataracts. Older (6-8 months) affected transgenic mice had mature or hypermature cataracts. The mice behaved differently from their unaffected transgenic and wild type littermates: the affected transgenic mice seemed disoriented and were cautious when ambulating in their cages.

C. Histopathology

Unaffected transgenic and wild type mice showed no abnormalities in structures of the eyes except photoreceptor degeneration, which occurs in the FVB/N background stock, and was discussed earlier.

Affected transgenic mice showed numerous abnormalities. These abnormalities included persistent hyperplastic primary vitreous with or without posterior lens capsule rupture, posterior polar cataract, and retrolental fibrous metaplasia with or without pigment (figure 39 - 42). Also observed was anterior segment dysgenesis involving the cornea and anterior uvea (figure 43 and 44). Extensive posterior synechiae involved most of the posterior irides in miotic pupils which lacked pupillary light responses (figure 45 - 47).

Jones-methenamine silver stain of eyes affected with anterior segment dysgenesis syndrome allowed better assessment of the degree of disorganization in the mutant eyes because the stain delineates the abnormal lens capsule and Descemet's membrane (figures 48 - 50).

D. Examination of the primary vitreous

The tunica vasculosa lentis in ten day old unaffected transgenic and wild type mice had already regressed. The primary vitreous was still present with vessels coursing over the posterior lens capsule. The primary vitreous in sixteen day old unaffected transgenic and wild type mice was still evident; but in 21 day old mice, the primary vitreous vasculature was mostly absent except for a few non-patent strands.

The affected transgenic mouse examined was 3 months old. There was no secondary vitreous present nor was there a space for it. The primary vitreous was thickened and attached to the retina. A posterior polar cataract was evident in association with the primary vitreous. Examination of the anterior segment of the eye revealed a shallow anterior chamber. Complete posterior synechia was also present as evidenced by the total attachment of the iris to the anterior lens surface.

V) DISCUSSION

A small percentage of Tg3261 mice have an interesting developmental ocular anomaly. The anterior segment does not form properly, and the hyaloid vasculature fails to regress. The abnormal behavior, i.e. cautious movement, may be explained due to total blindness caused by the interference in light reaching their retinas. The retrolental fibrovascular membrane present in the transgenic mice does not allow light to reach the retina and their visual pathways are unlikely to develop (bilateral amblyopia). It must be noted that all of the mice are mutants at the rd locus, that is, they have a form of retinal degeneration in which all rods and 97% of cones degenerate by one month of age, the same timeframe during which normal murine retinas mature.[19,21] This mutation is common in many mouse strains, including FVB/N, and is due to a mutation in the ß-subunit of the phosphodiesterase gene.[19,21] Though this makes the unaffected transgenic and wild type mice blind by 28 days of age, light is able to reach their retinas at all times and the visual pathways develop properly. They also retain their pupillary light responses, another indication of viable photoreceptors and of normal optic nerve and oculomotor nerve development.

For decades it has been postulated that macrophages induce regression of the hyaloid vascular system and its associated tunica vasculosa lentis by occluding the capillary lumens.[22-24] More recently, Lang and Bishop[3] have shown that macrophages, specifically hyalocytes, are responsible for this regression and that they mediate developmentally

programmed tissue remodeling.

Hyalocytes are tissue macrophages that reside primarily in the vitreous cortex.[5,25] In a 1994 report using rats, Lang, *et al*,[26] suggested that hyalocytes induce apoptosis sporadically throughout the regression phase in capillary endothelial cells, and for any one capillary segment, a critical point occurs where cell killing results in a lumen too narrow for blood flow. Synchronous apoptosis ensues along the capillary segment.

As aforementioned, if this transient embryonic vasculature fails to regress, sequellae including cataracts and detached retinas occur which contribute to blindness. In humans, PHTVL/PHPV is usually a unilateral non-hereditary condition.[27] In only 10% of cases, the condition is bilateral and is associated with severe malformations as in trisomy 13/15.[10,28] In the Doberman Pinscher, PHTVL/PHPV occurs as a bilateral hereditary condition without other malformations and is possibly due to inbreeding.[15] Conventional karyotyping studies have not found any chromosomal aberrations in this breed.[15] It is possible that PHPV is occurring in the Doberman Pinscher due to a small mutation which is not evident in karyotype studies.

In 1935, Ida Mann[29] hypothesized that abnormal adhesion of persistent hyaloid vessels prevents normal development of the secondary vitreous. If this is the case, it would explain the lack of secondary vitreous in affected Tg3261 mice. It is also possible that the posterior synechiae and abnormal anterior segment development also limit the formation of the anterior and posterior chambers and of aqueous humor production. One can

hypothesize that without normal chamber cleavage there is abnormal aqueous humor production which in turn inhibits regression of the transient embryonic vasculature. We hypothesize that one or more components of aqueous humor induces the terminal differentiation of hyalocytes and without aqueous humor, the hyalocytes are unable to function properly.

An occasional finding in humans with PHTVL/PHPV is an immature iridocorneal angle due to abnormal cleavage.[10] Perhaps the initial abnormality, that is, abnormal regression of the perilenticular vasculature may be due in part to malfunction of the hyalocytes. This in turn leads to all of the previously mentioned abnormalities as well as abnormal development of the vitreous chamber, including lack of production of secondary vitreous. In order to more fully understand the abnormal interactions of the tissue layers that contribute to these anomalies, it is necessary to understand the precise migration and interplay of the neural crest cells, the neuroepithelial cells and the mesenchymal cells.

In conclusion, Tg3261 is an interesting insertional mutant that requires further embryological studies that will concentrate not only on the normal interaction of the hyalocytes and the other cells involved in normal ocular development but also on the abnormal migration of ocular cells occurring in the affected transgenic mice. Also important is cloning the disrupted gene using molecular biology techniques.

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APPENDIX

Figure 1: TgN3261Rpw mice.

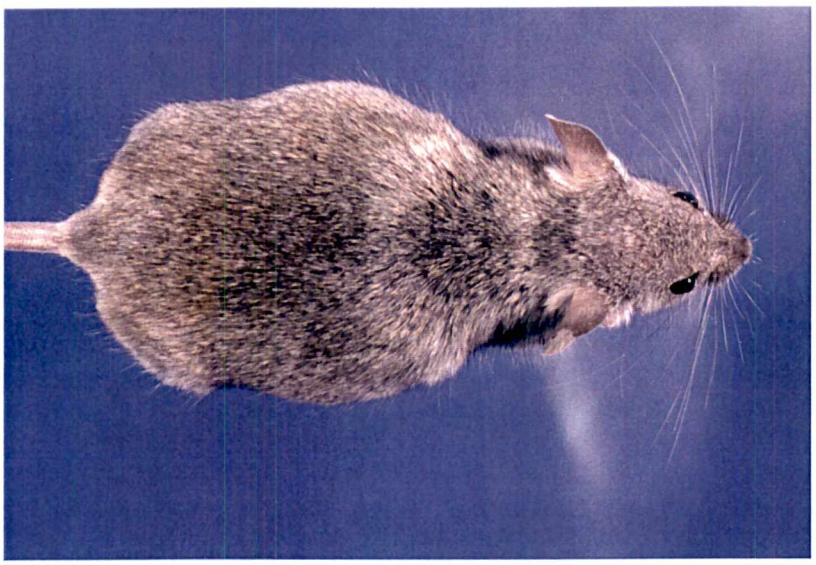
Three mice from the same litter; agouti colored mouse on the left is darker and more runted than the agouti colored mouse on the right; and the albino mouse is wild type.

Figure 2: Abdominal distension.

TgN3261Rpw mouse showing a distended abdomen due to accumulation of ascites secondary to histiocytic sarcoma infiltrating the liver.

Figure 3: In situ hepato-splenomegaly.

TgN3261Rpw mouse with hepatomegaly, splenomegaly, and lymphdenopathy due to histiocytic sarcoma. (L=liver, S=spleen, arrow points to an enlarged lymph node)



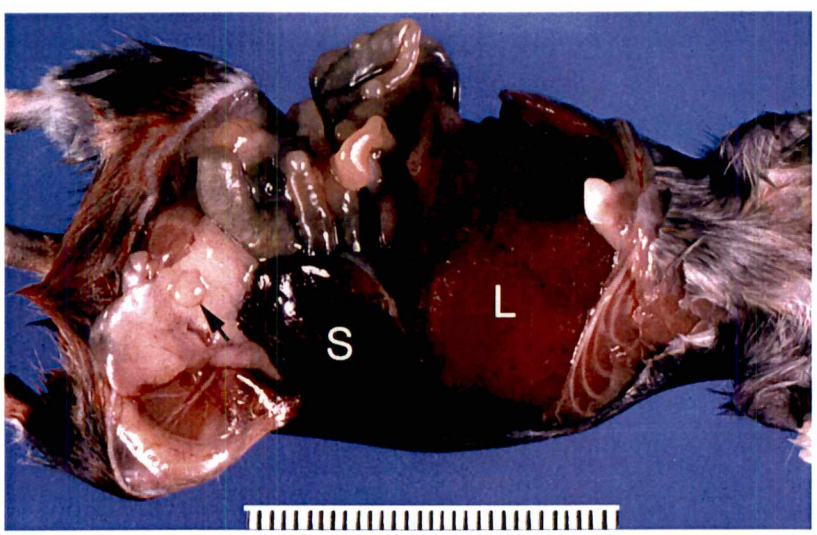

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Figure 4: Ascites with intact peritoneum.

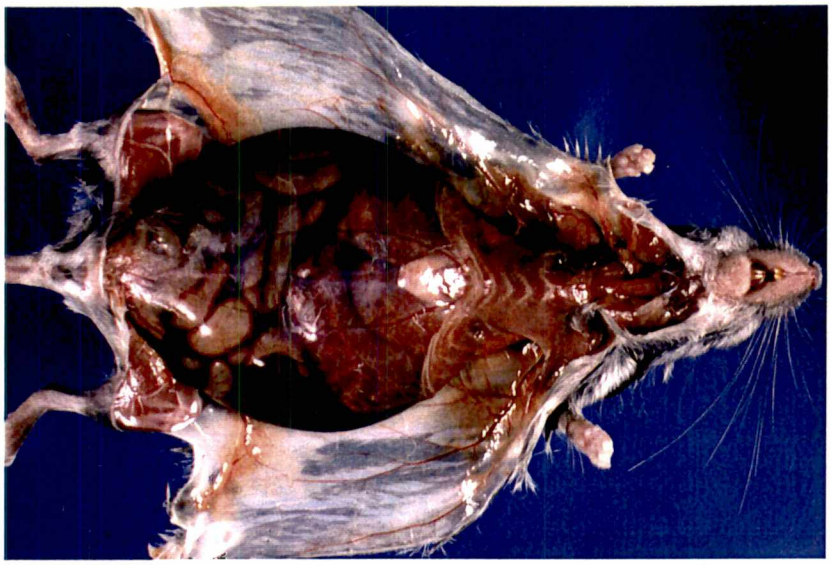
TgN3261Rpw mouse with serosanguinous ascites, hepatomegaly, splenomegaly, and lymphadenopathy due to histiocytic sarcoma as seen through an intact peritoneum.

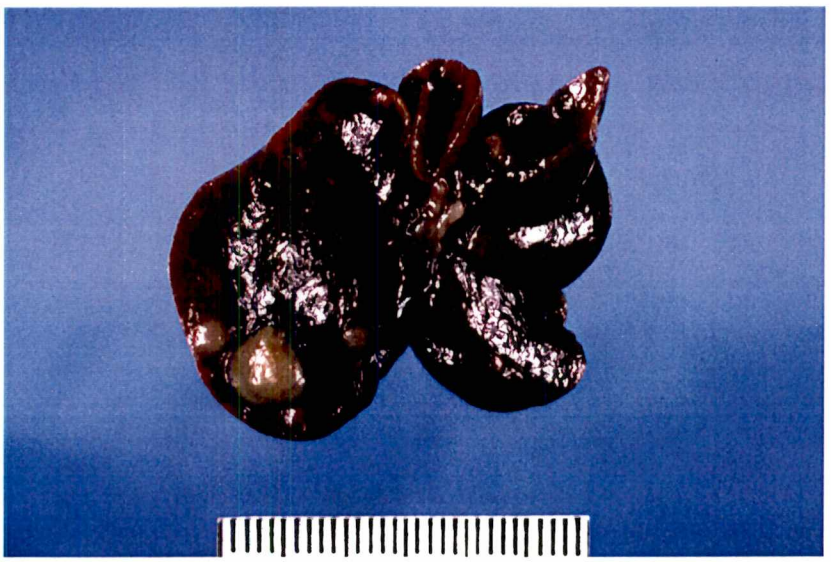
Figure 5: Gross hepatomegaly.

Hepatomegaly and large, focal nodule due to histiocytic sarcoma from in TgN3261Rpw mouse.

Figure 6: Gross hepatomegaly and splenomegaly.

Hepatomegaly and massive splenomegaly due to histiocytic sarcoma in a TgN3261Rpw mouse.





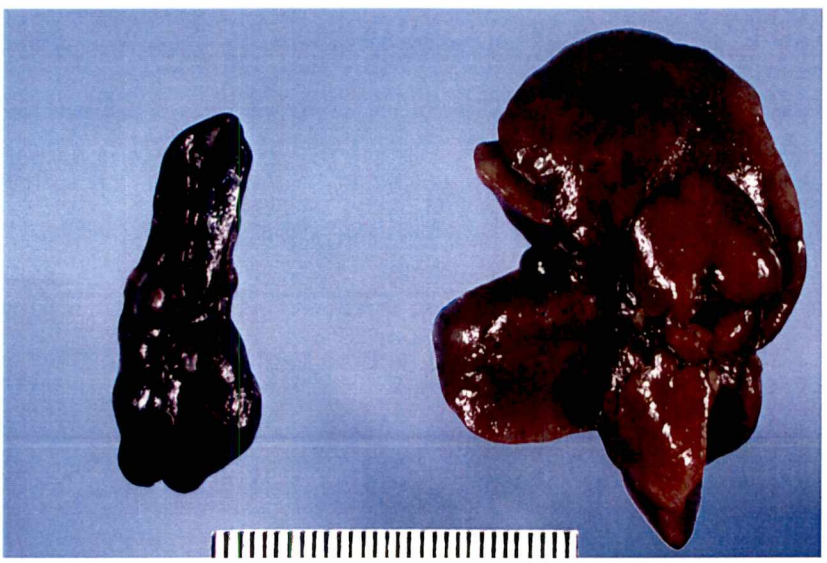


Figure 7: Histiocytic sarcoma in a spleen. (10X)

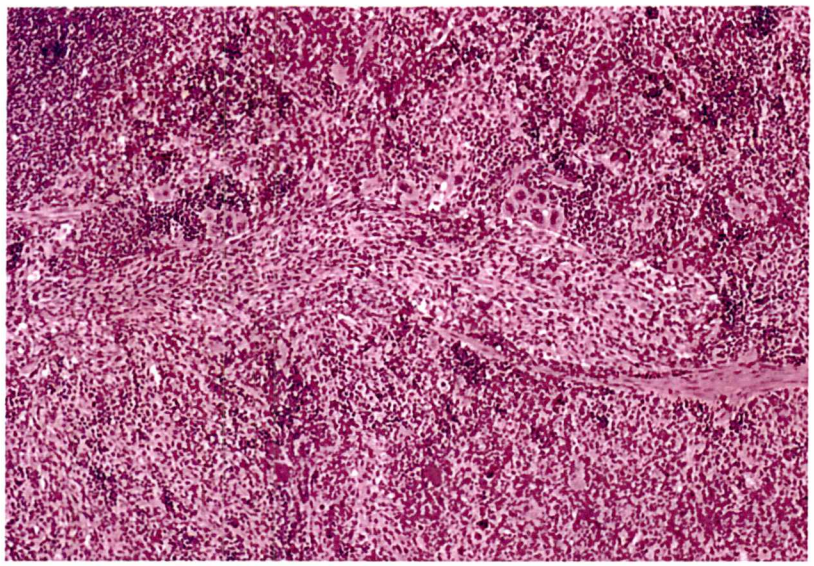
Histiocytic sarcoma cells streaming through the white pulp of the spleen in a TgN3261Rpw mouse.

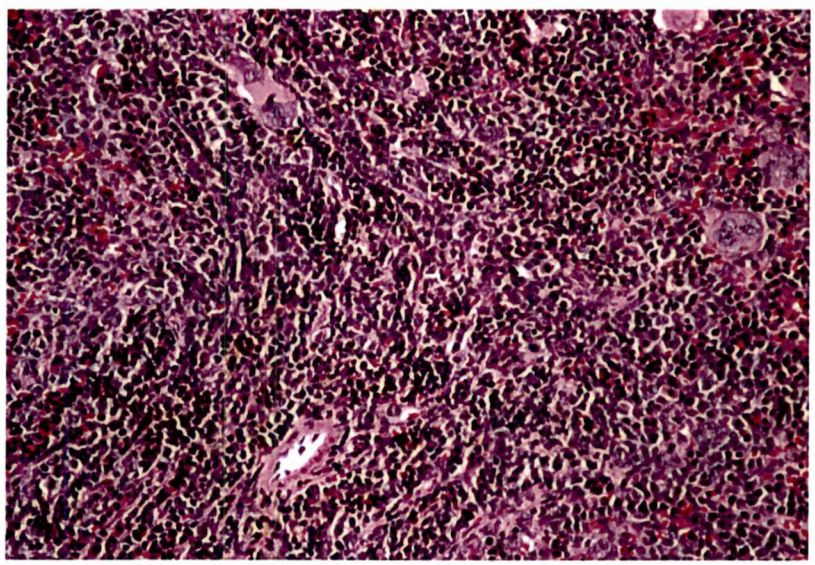
Figure 8: Histiocytic sarcoma in a spleen. (20X)

Histiocytic sarcoma cells infiltrating the spleen of a TgN3261Rpw mouse.

Figure 9: Histiocytic sarcoma in a spleen. (40X)

Histiocytic sarcoma cells infiltrating the spleen of a TgN3261Rpw mouse. (40X)





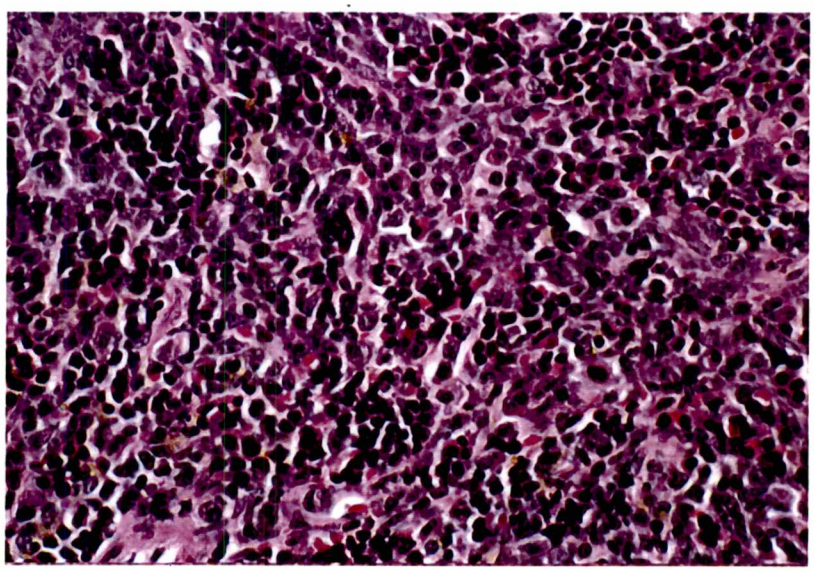


Figure 10: Histiocytic sarcoma in a lymph node. (20X)

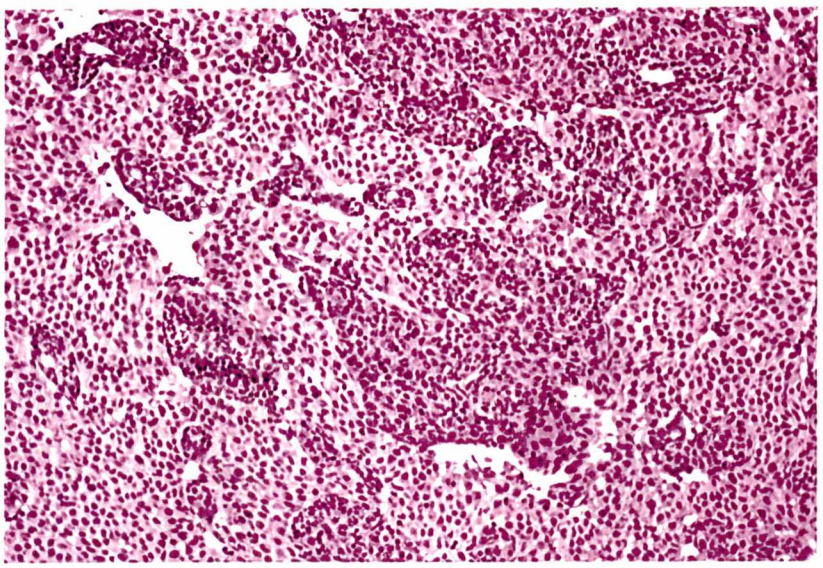
Lymph node infiltrated with histiocytic sarcoma in an 8 month old TgN3261Rpw mouse.

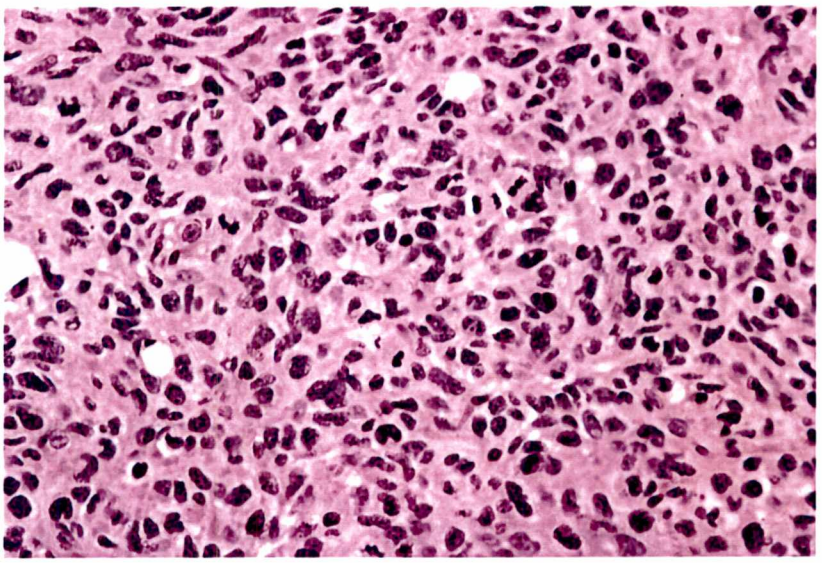
Figure 11: Histiocytic sarcoma in a lymph node. (40X)

Lymph node infiltrated with histiocytic sarcoma in a TgN3261Rpw mouse. Neoplastic cells have numerous mitotic figures.

Figure 12: Histiocytic sarcoma in a lymph node. (40X)

Mesenteric lymph node infiltrated with histiocytic sarcoma in a 10 month old TgN3261Rpw mouse.





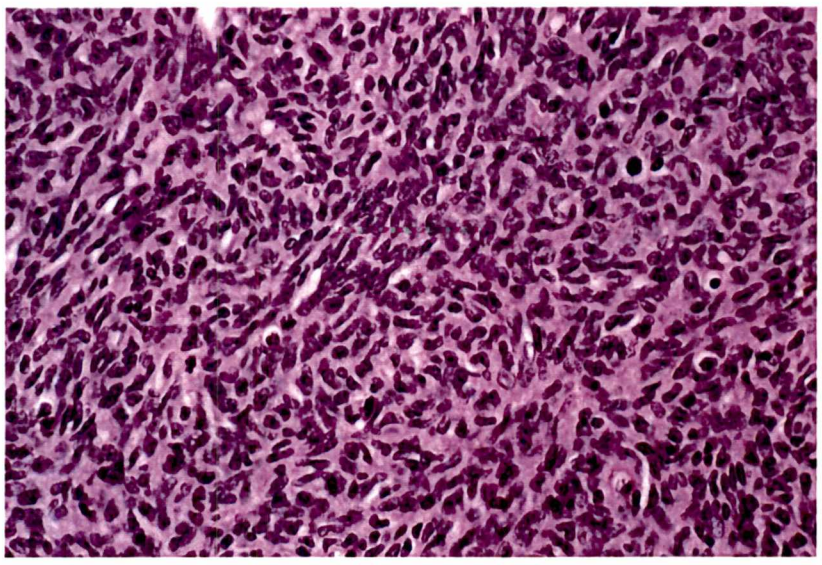


Figure 13: Histiocytic sarcoma in a lymph node. (10X)

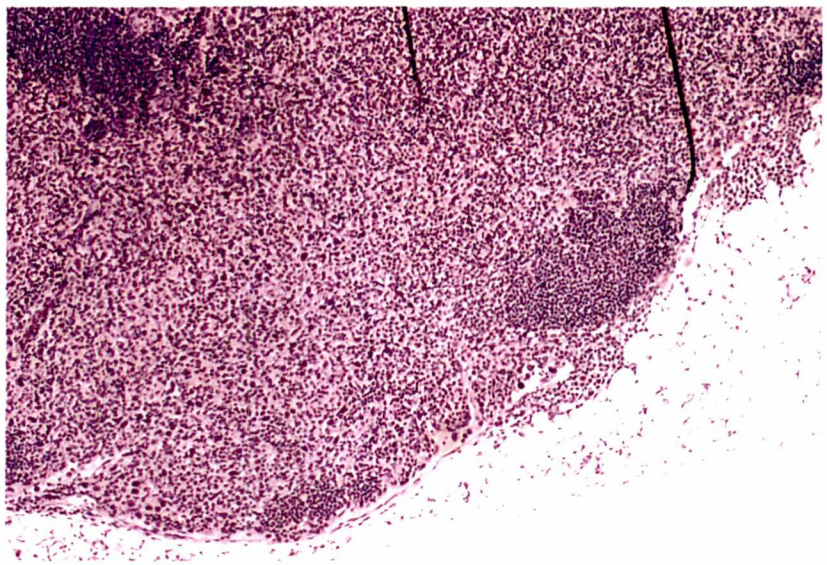
Lymph node architecture accentuated by histiocytic sarcoma cells in a TgN3261Rpw mouse. The neoplastic cells in the cortex are a mixed population of the small, round to oval to slightly spindle-shaped cells. In the medulla, the neoplastic histiocytes are large, with abundant eosinophilic cytoplasm.

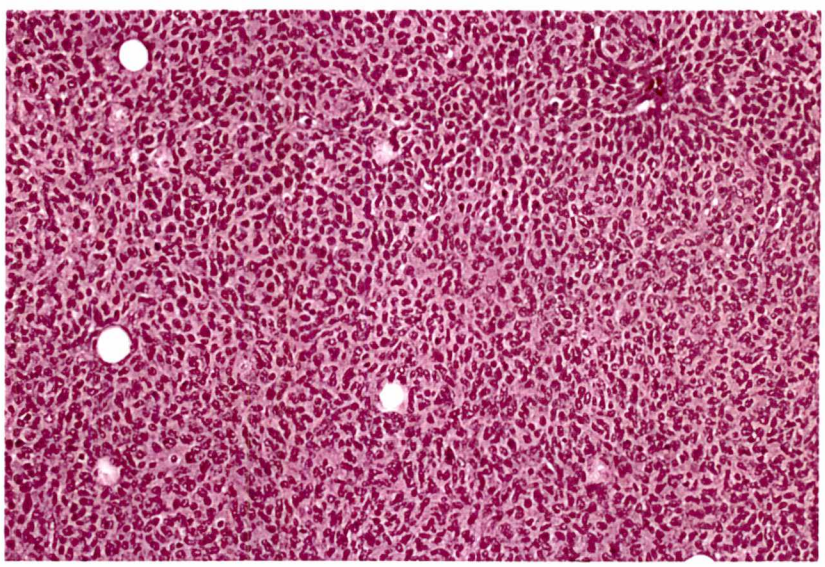
Figure 14: Histiocytic sarcoma in a lymph node. (20X)

Lymph node infiltrated by histiocytic sarcoma cells in a 12 month old mouse. Numerous mitotic figures are evident.

Figure 15: Histiocytic sarcoma in a lymph node. (40X)

Lymph node infiltrated by histiocytic sarcoma cells in a 12 month old mouse. Cells have numerous mitotic figures.





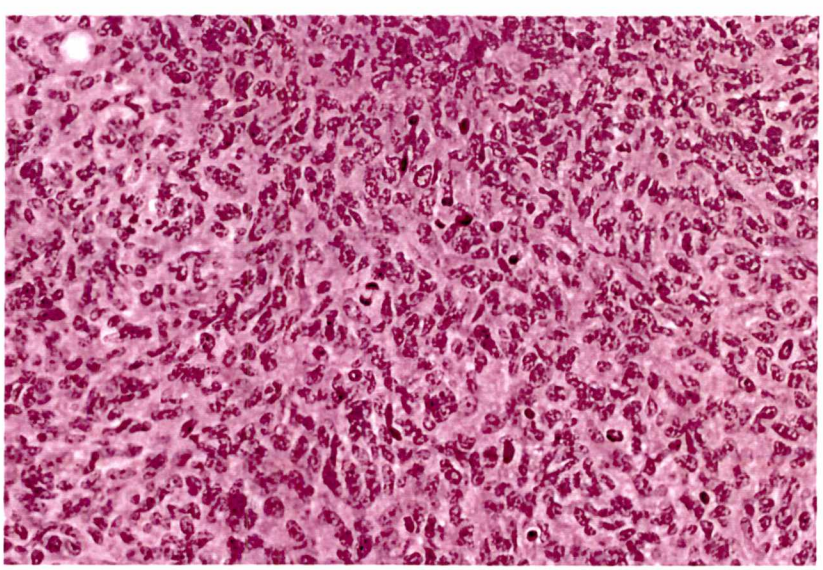


Figure 16: Intradermal histiocytic sarcoma. (10X)

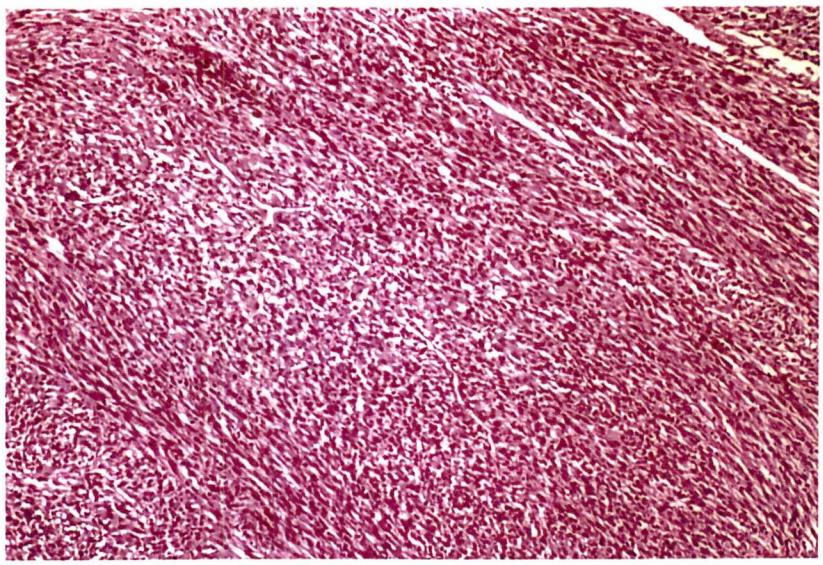
Histiocytic sarcoma aggressively invading the preputial dermis in a TgN3261Rpw mouse.

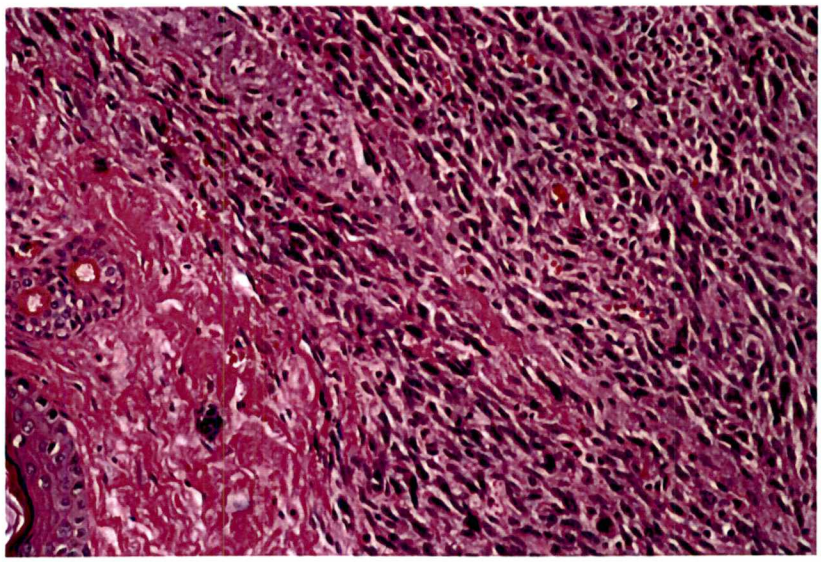
Figure 17: Intradermal histiocytic sarcoma. (20X)

Histiocytic sarcoma aggressively invading the dermis overlying the abdomen in a TgN3261Rpw mouse.

Figure 18: Intradermal histiocytic sarcoma. (40X)

Histiocytic sarcoma aggressively invading the dermis overlying the abdomen in a TgN3261Rpw mouse.





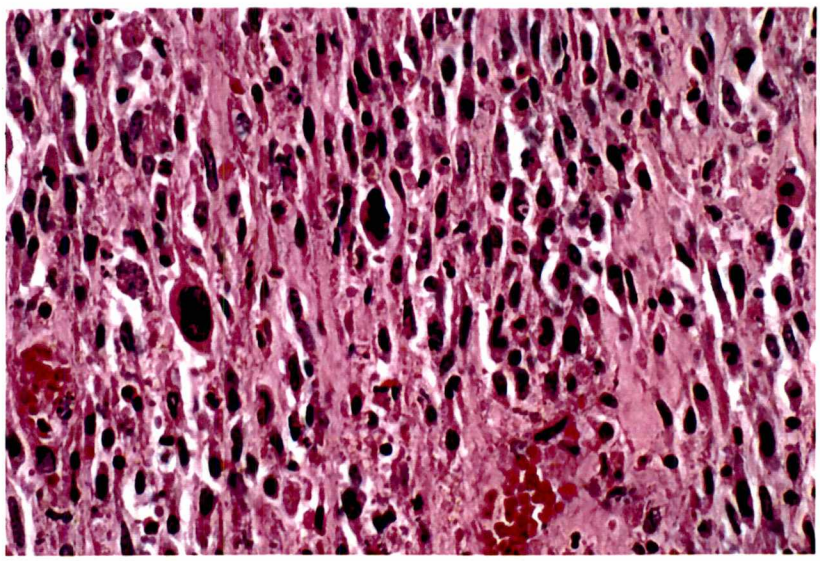


Figure 19: Immunohistochemistry - anti-vimentin antibody.

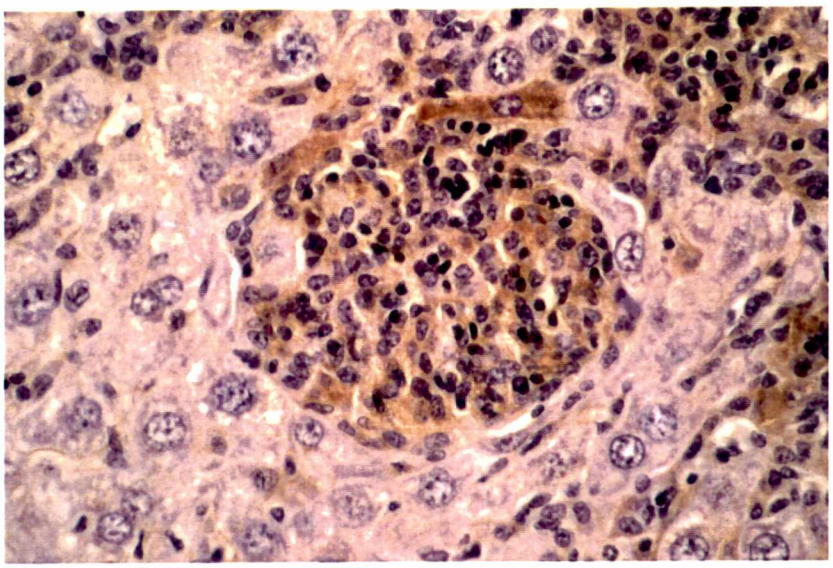
Immunohistochemical staining for anti-vimentin antibody in a liver from a TgN3261Rpw mouse affected with histiocytic sarcoma. The cluster of neoplastic histiocytes stain positively for anti-vimentin antibody, while the hepatocytes do not react.

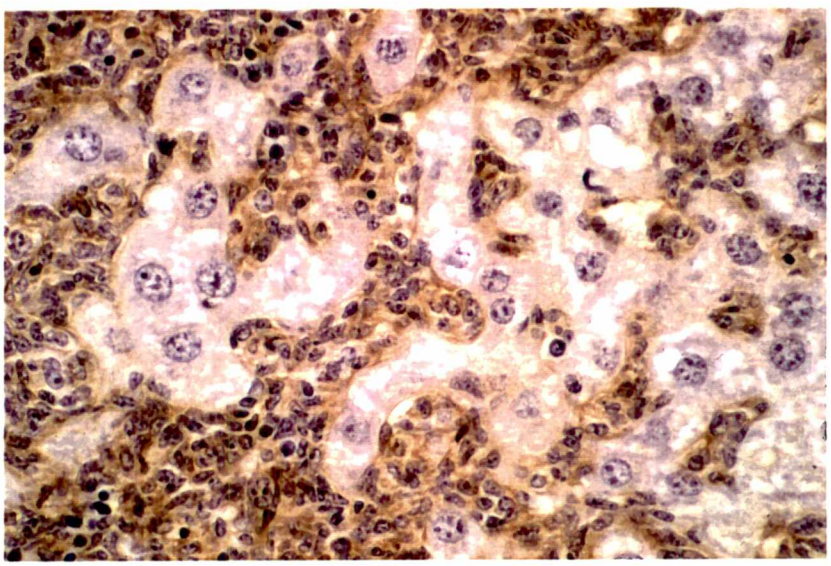
Figure 20: Immunohistochemistry - anti-CD68 antibody.

Immunohistochemical staining for anti-CD68 antibody in a liver from a TgN3261Rpw mouse affected with histiocytic sarcoma. The neoplastic histiocytes surrounding the hepatocytes stain positively, while the hepatocytes do not react.

Figure 21: Immunohistochemistry - lectin, peanut agglutinin (PNA).

Immunohistochemical staining for PNA in a liver from a TgN3261Rpw mouse affected with histiocytic sarcoma. The neoplastic histiocytes surrounding the hepatocytes stain positively, while the hepatocytes do not stain.





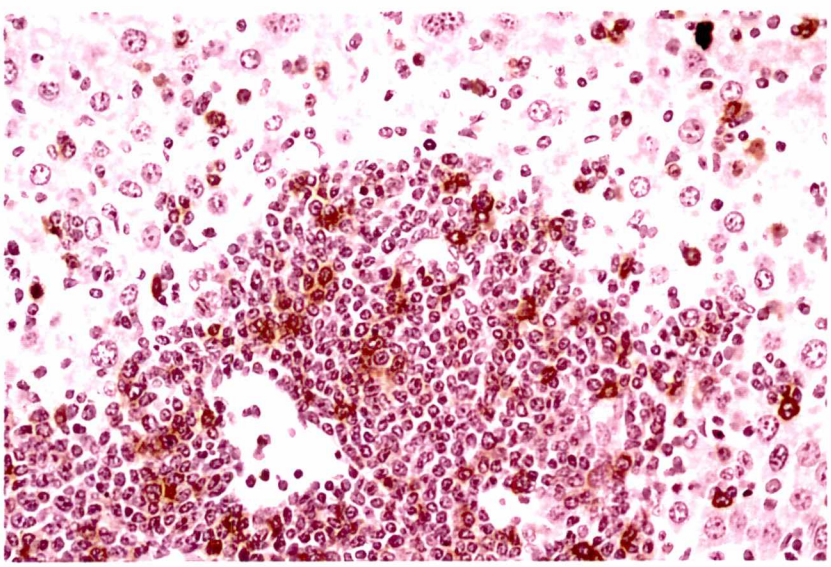


Figure 22: Karyotype of TgN3261Rpw.

This is one of the three karyotype experiments performed on TgN3261Rpw mice. All were normal.

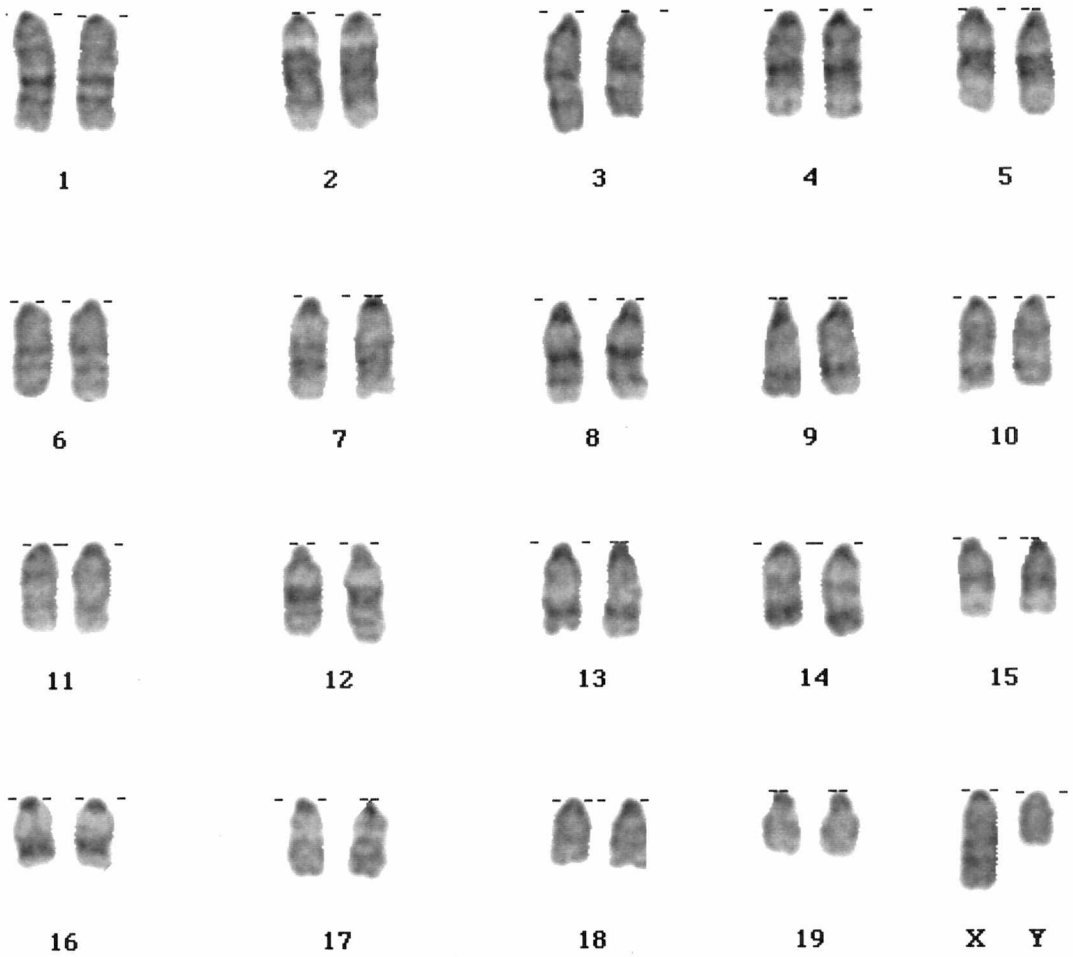


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Figure 23: Gross SCID mouse-peritoneum intact.

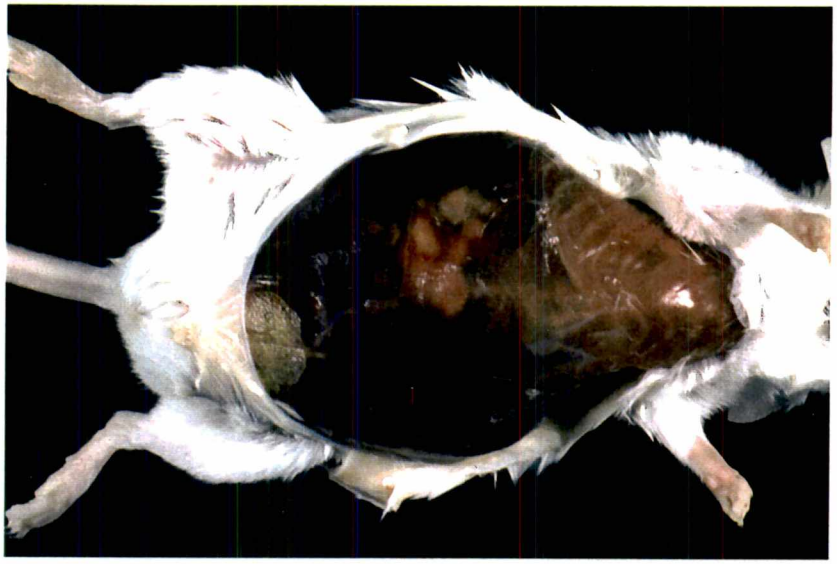
SCID mouse with peritoneum intact inoculated intraperitoneally ~6 weeks prior with histiocytic sarcoma cells from an intradermal histiocytic sarcoma which developed in a TgN3261Rpw mouse. The SCID mouse developed serosanguinous ascites, hepatomegaly, splenomegaly, and lymphadenopathy.

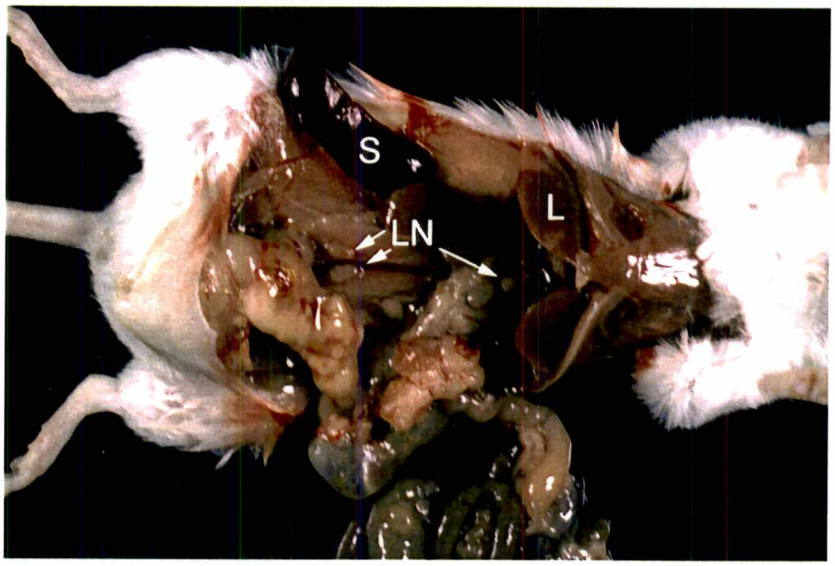
Figure 24: Gross SCID mouse-peritoneum removed.

SCID mouse with peritoneum removed; hepatomegaly, splenomegaly, and lymphadenopathy are evident. (L=liver, S=spleen, LN=lymph nodes)

Figure 25: SCID mouse-shoulder tumor.

SCID mouse was inoculated subcutaneously between the scapulae with intradermal histiocytic sarcoma cells which developed in a TgN3261Rpw mouse. The tumor was rapidly growing, aggressive and invasive.




S
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L

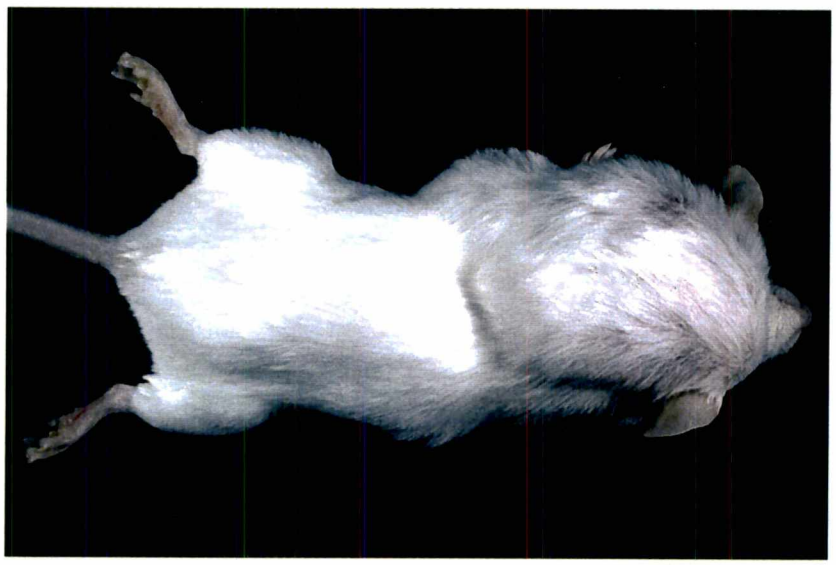
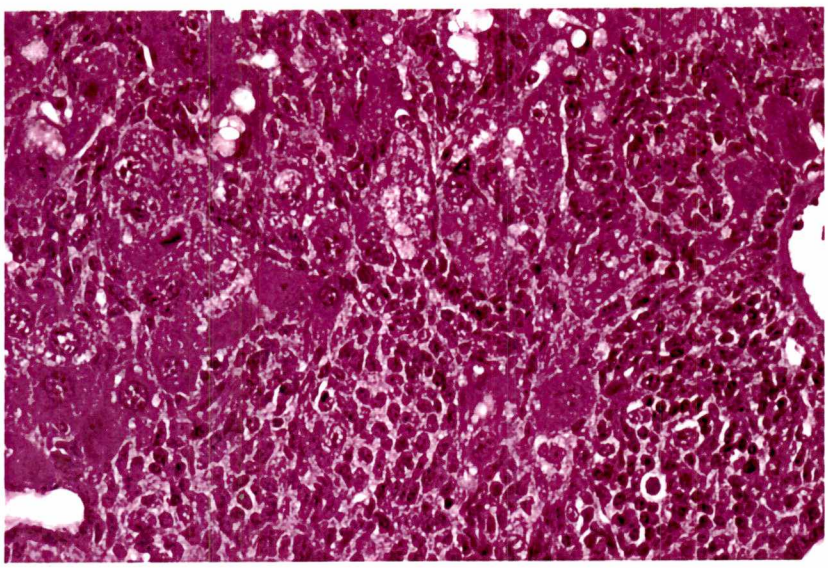
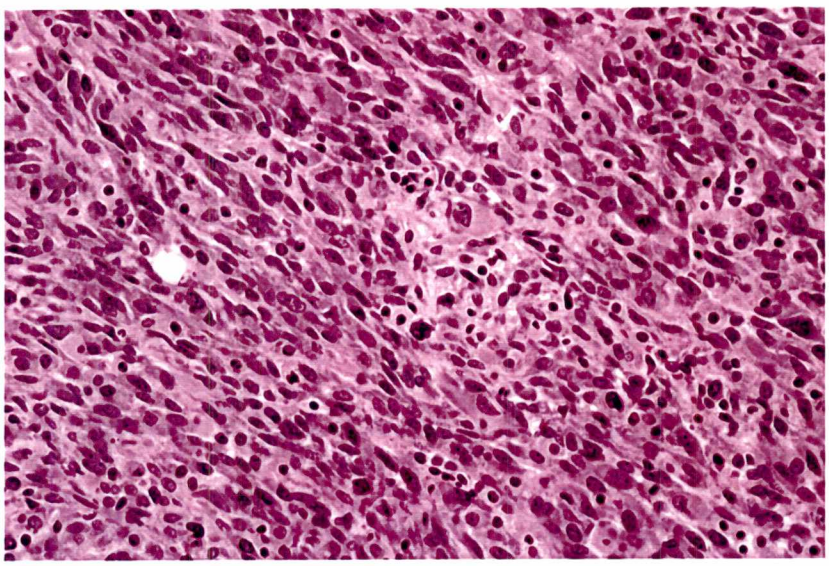


Figure 26: Histiocytic sarcoma in the liver of a TgN3261Rpw mouse. (40X)
Liver infiltrated with histiocytic sarcoma from TgN3261Rpw mouse. These cells were inoculated into SCID mice in an attempt to recapitulate both tumor phenotypes.

Figure 27: Intradermal histiocytic sarcoma in a TgN3261Rpw mouse. (40X)
Highly undifferentiated histiocytic sarcoma in the dermis of a TgN3261Rpw mouse.

Figure 28: Subcutaneous histiocytic sarcoma in a SCID mouse.
Tumor removed from SCID mouse in figure 24 approximately 6 weeks following subcutaneous inoculation with histiocytic sarcoma cells.





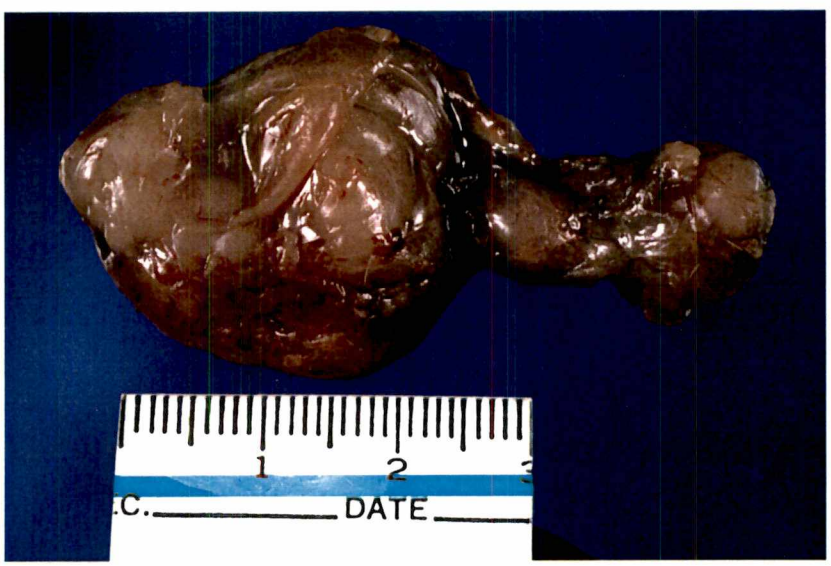

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Figure 29: SCID mouse-histiocytic sarcoma in liver. (40X)

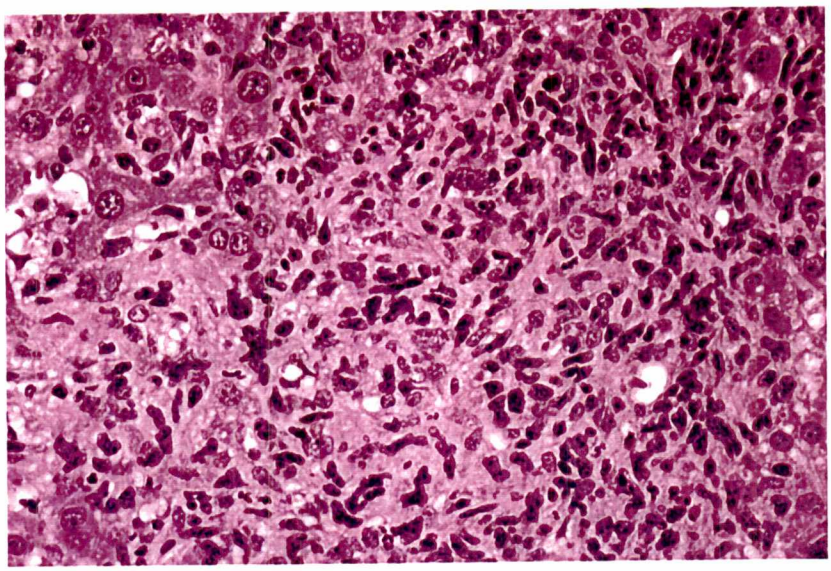
Liver architecture effaced due to infiltration of histiocytic sarcoma cells in a SCID mouse inoculated intraperitoneally with tumor cells from an intradermal histiocytic sarcoma which developed in a TgN3261Rpw mouse.

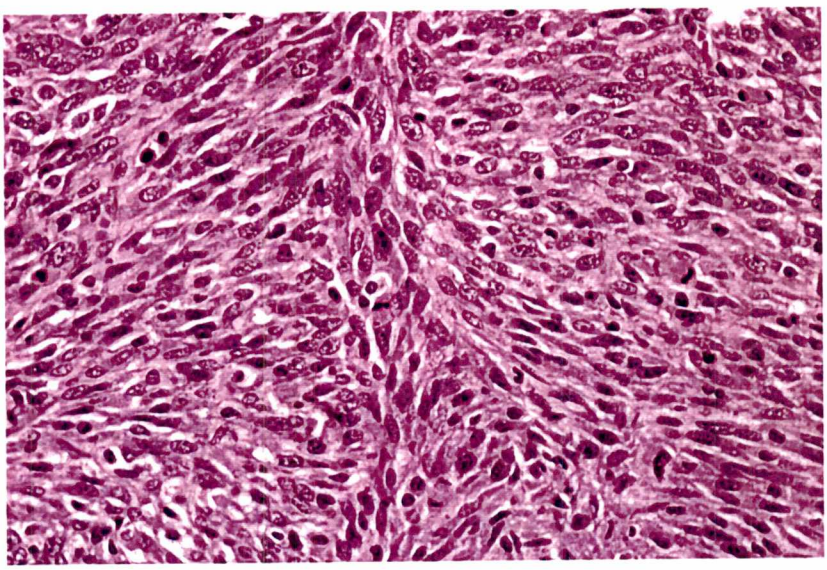
Figure 30: SCID mouse-subcutaneous histiocytic sarcoma. (40X)

Tumor infiltrating the dermis between the scapulae in a SCID mouse inoculated with histiocytic sarcoma cells from an intradermal histiocytic sarcoma which developed in a TgN3261Rpw mouse.

Figure 31: SCID mouse-metastasis to spleen from subcutaneous tumor. (40X)

Histiocytic sarcoma which has metastasized to the spleen following inoculation between the scapulae in a SCID mouse with tumor cells from a subcutaneous tumor which developed in a TgN3261Rpw mouse.





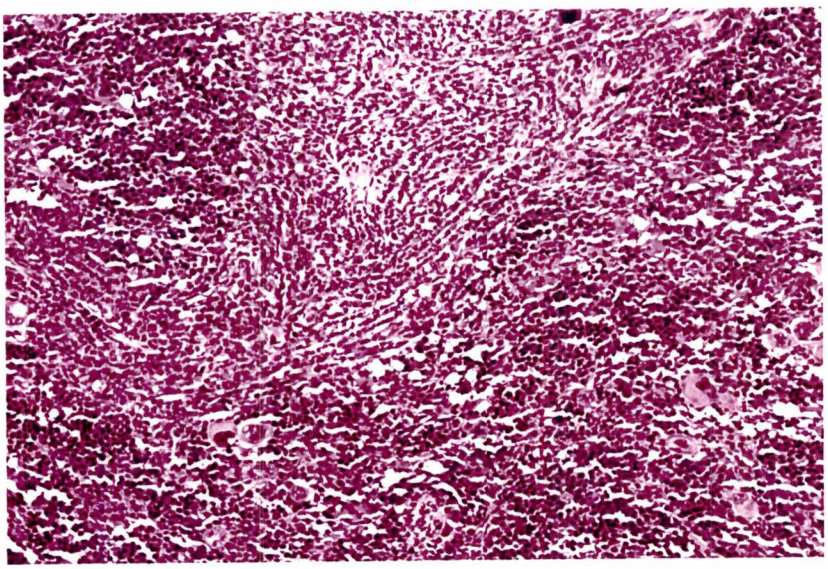


Figure 32: SCID mouse-histiocytic sarcoma in the liver. (40X)

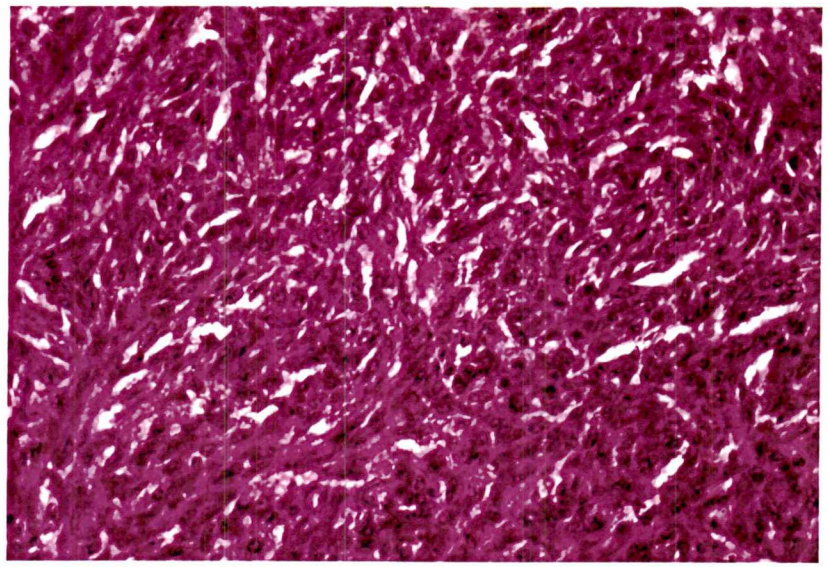
Histiocytic sarcoma infiltrating the liver of a SCID mouse. The tumor cells are from an affected mesenteric lymph node in a TgN3261Rpw mouse.

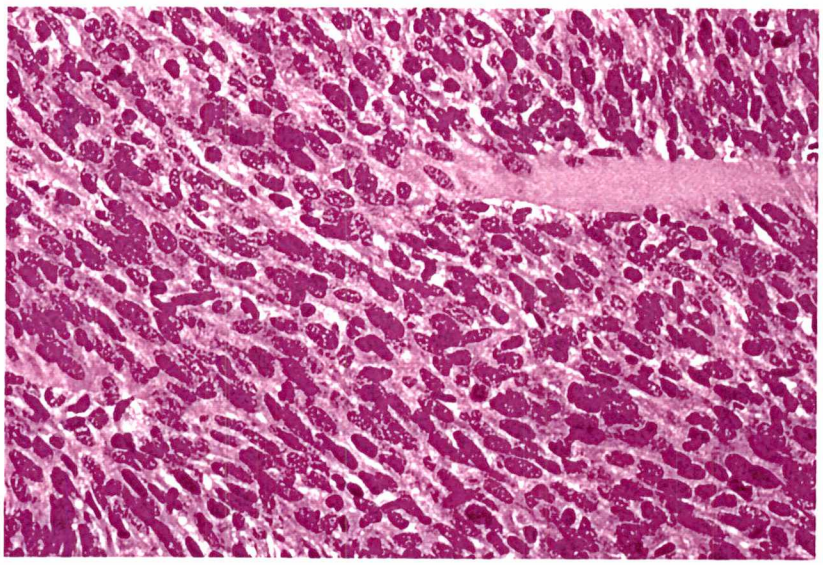
Figure 33: SCID mouse-subcutaneous histiocytic sarcoma. (40X)

Tumor infiltrating the subcutis between the scapulae in a SCID mouse inoculated with histiocytic sarcoma cells from a mesenteric lymph node infiltrated with histiocytic sarcoma in a TgN3261Rpw mouse.

Figure 34: SCID mouse-metastasis to spleen from subcutaneous tumor. (40X)

Histiocytic sarcoma which has metastasized to the spleen following inoculation between the scapulae in a SCID mouse with tumor cells from a mesenteric lymph node infiltrated with histiocytic sarcoma in a TgN3261Rpw mouse.





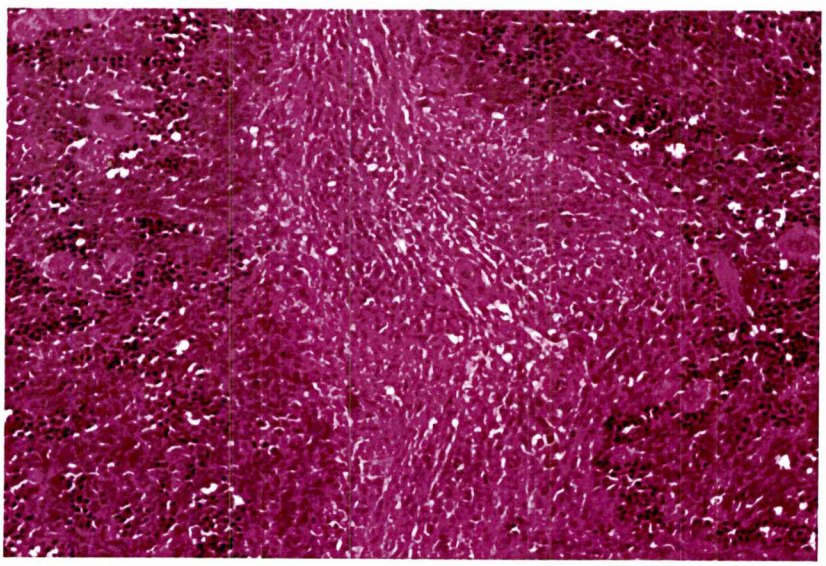
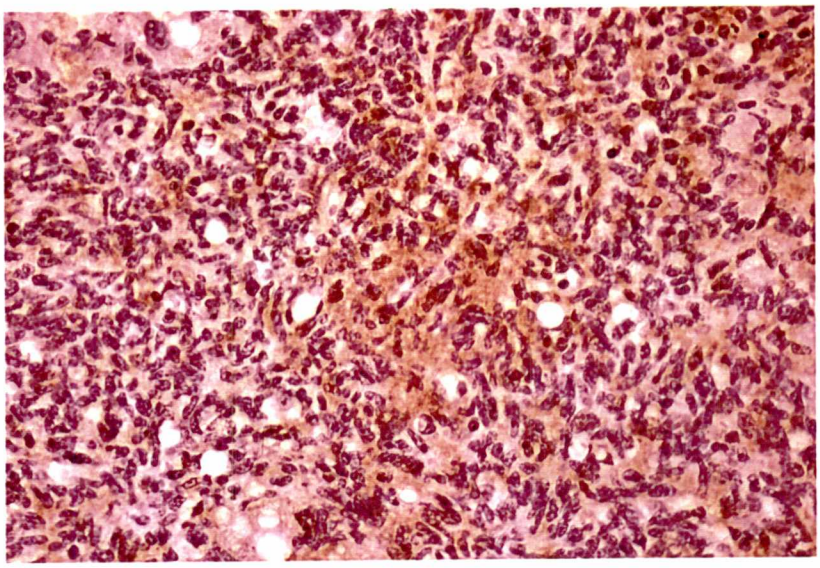


Figure 35: Immunohistochemistry-anti-CD68 antibody. (40X)
Histiocytic sarcoma cells in the liver of a TgN3261Rpw mouse which stained positively for anti-CD68 antibody.

Figure 36: (SCID) Immunohistochemistry-anti-CD68 antibody. (20X)
Subcutaneous histiocytic sarcoma in a SCID mouse previously inoculated with tumor cells from an affected TgN3261Rpw mesenteric lymph node which stained positively for anti-CD68 antibody.



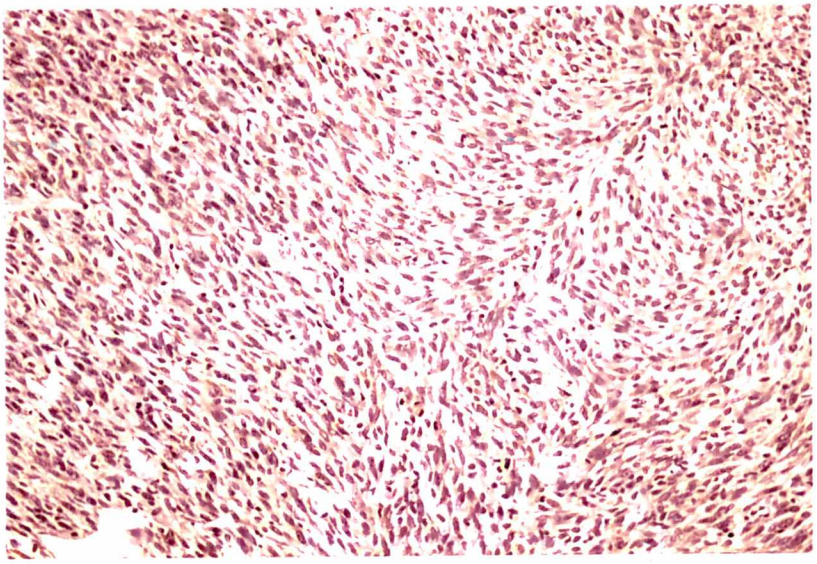
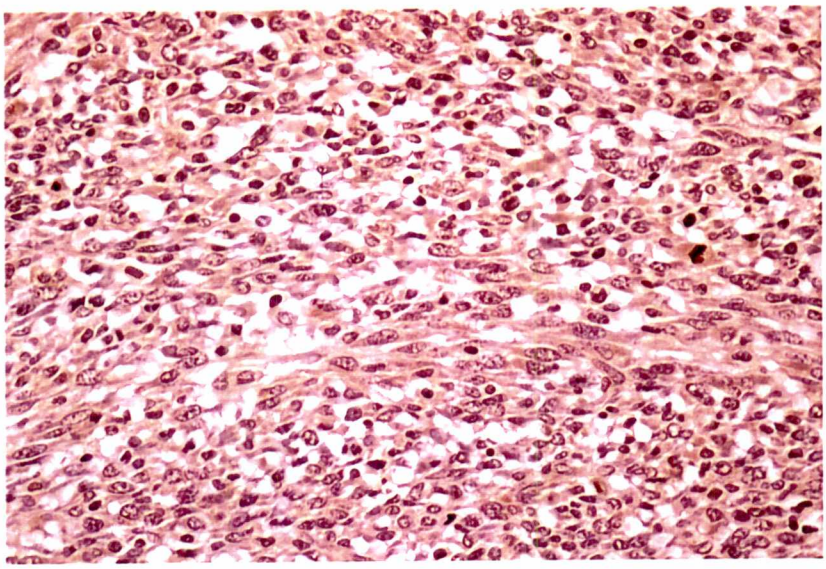


Figure 37: (SCID) Immunohistochemistry-lectin, peanut agglutinin (PNA). (40X)

Subcutaneous histiocytic sarcoma in a SCID mouse previously inoculated with tumor cells from an affected TgN3261Rpw mesenteric lymph node which stained positively for PNA.

Figure 38: (SCID) Immunohistochemistry- lectin, peanut agglutinin (PNA). (20X)

Histiocytic sarcoma in the spleen of a SCID mouse which has metastasized from the intradermal tumor and stained positively for PNA.



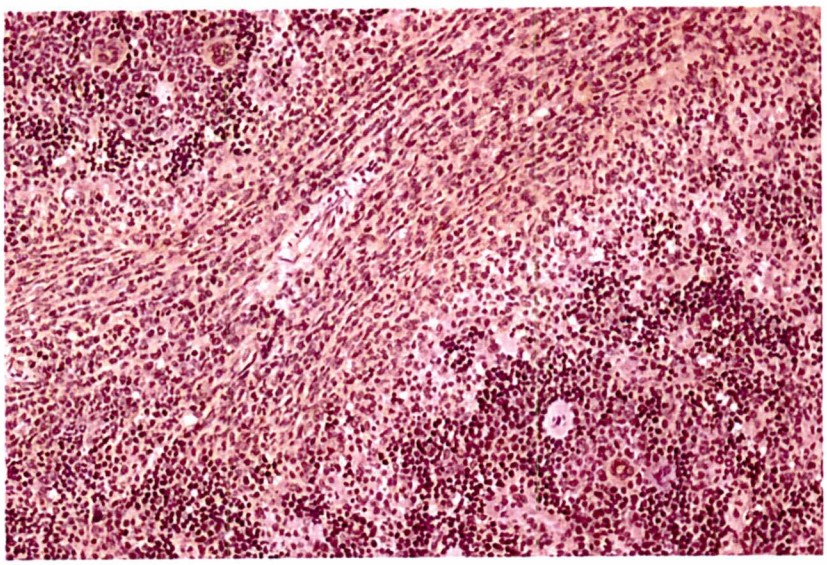


Figure 39: Persistent hyperplastic primary vitreous. (4X)

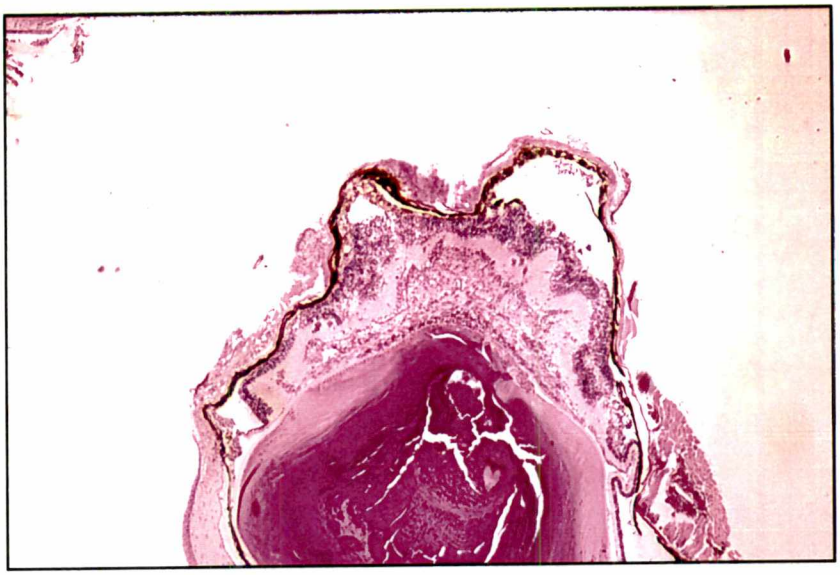
Left eye of an affected TgN3261Rpw mouse with persistent hyperplastic primary vitreous, posterior lenticonus, posterior polar cataract, nonpigmented retrolental fibrovascular membrane, and retinal detachment.

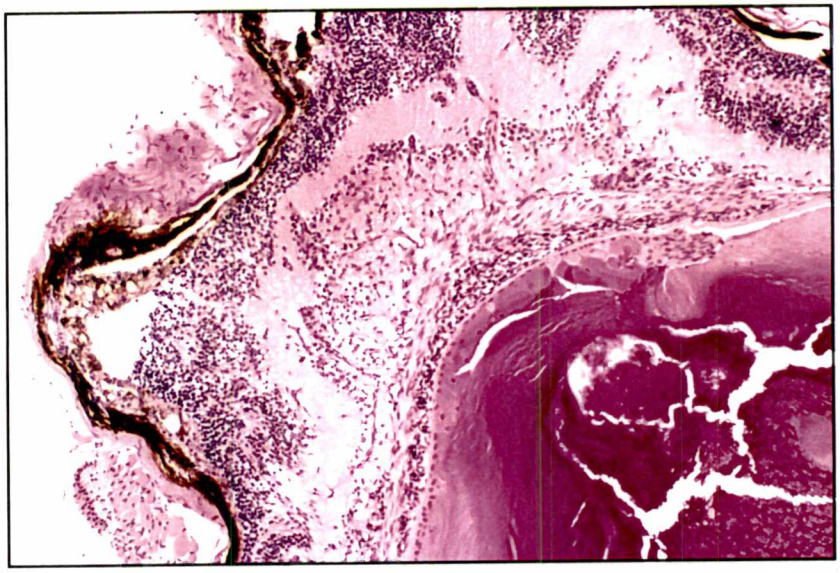
Figure 40: Persistent hyperplastic primary vitreous. (10X)

A more magnified view of the eye in figure 39. There is severe nonpigmented retrolental fibrovascular metaplasia, retinal cell disorganization, retinal detachment, posterior polar cataract, and posterior lenticonus due to persistent hyperplastic primary vitreous.

Figure 41: Persistent hyperplastic primary vitreous. (10X)

Right eye of the same affected TgN3261Rpw mouse. The retina is not as disorganized as in the left eye, but nonpigmented retrolental fibrovascular membrane, retinal detachment, and persistent hyperplastic primary vitreous are evident.





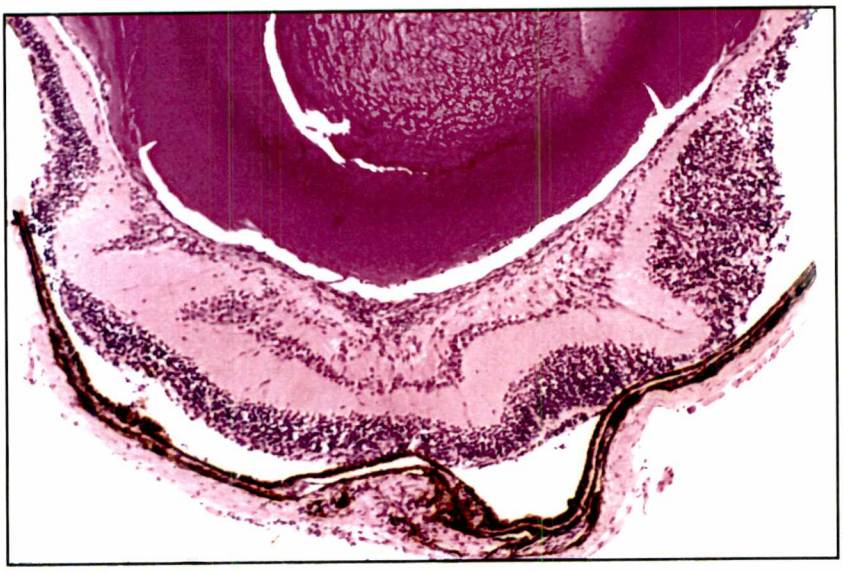


Figure 42: Persistent hyperplastic primary vitreous. (10X)

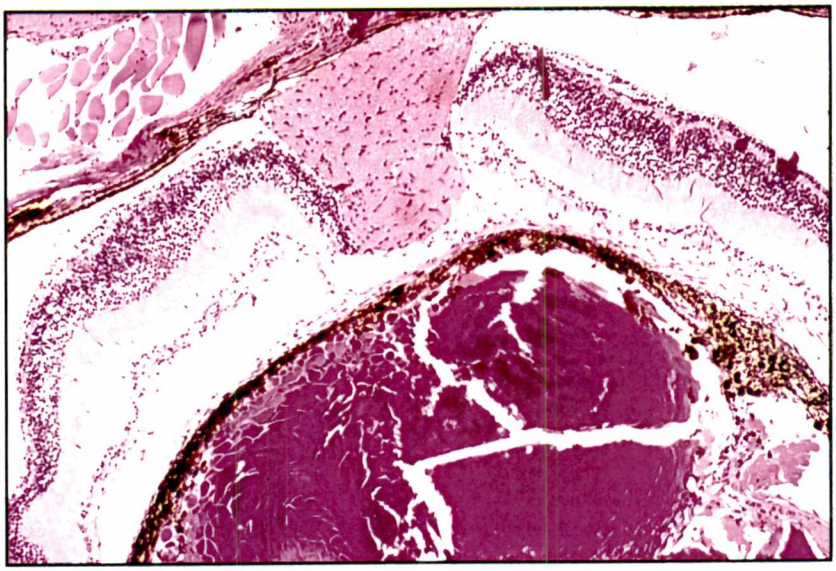
Posterior aspect of the left eye in an affected TgN3261Rpw mouse. The eye displays persistent hyperplastic primary vitreous, pigmented retrolental fibrovascular metaplasia invading the posterior aspect of the lens, posterior polar cataract, retinal detachment, and an optic nerve which is protruding through the retina and into the vitreous chamber.

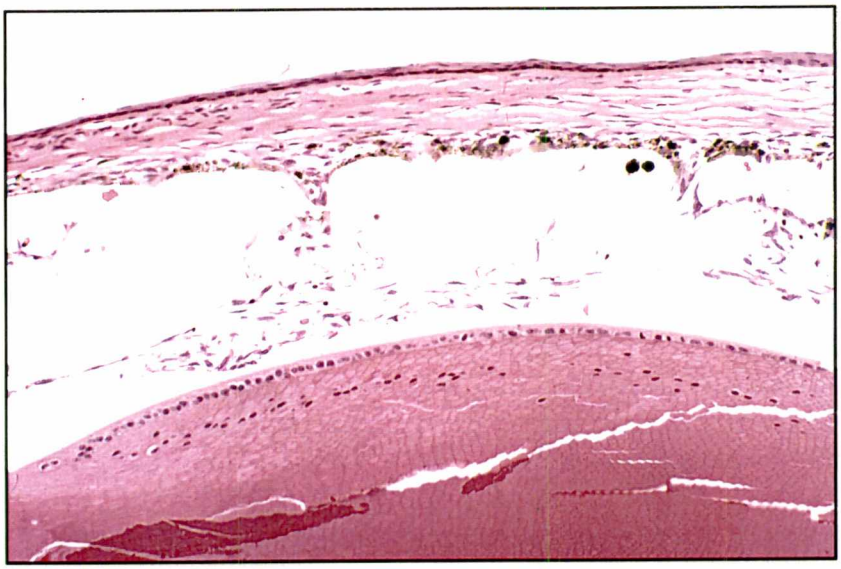
Figure 43: Anterior segment dysgenesis. (20X)

Affected TgN3261Rpw mouse with anterior segment dysgenesis. Persistence of the tunica vasculosa lentis is evident and there are pigmented cells throughout the posterior cornea, possibly disorganized uveal melanocytes.

Figure 44: Anterior segment dysgenesis. (10X)

Anterior segment dysgenesis in a 4 month old mouse. The anterior chamber is filled with presumably neural crest derived cells which are highly disorganized. There is no evidence of an anterior nor a posterior chamber. The lens has cataractous changes.





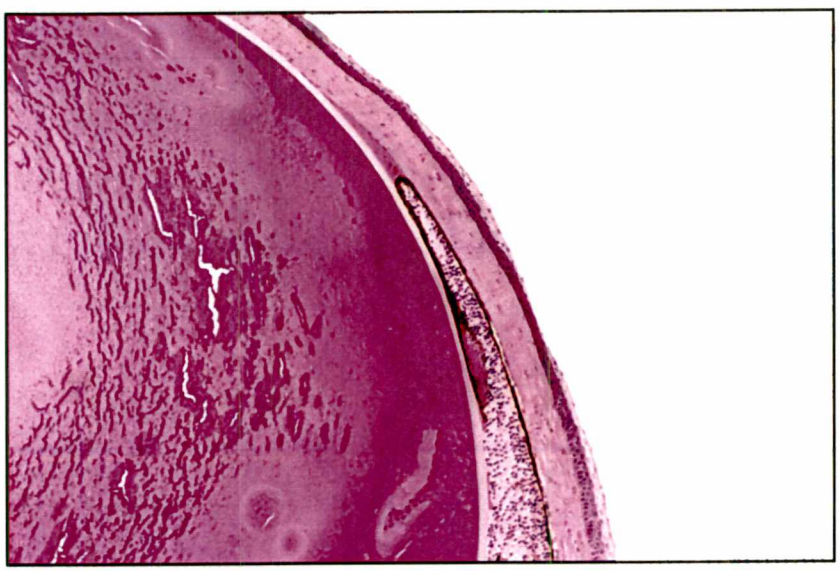


Figure 45: Persistent pupillary membrane. (4X)

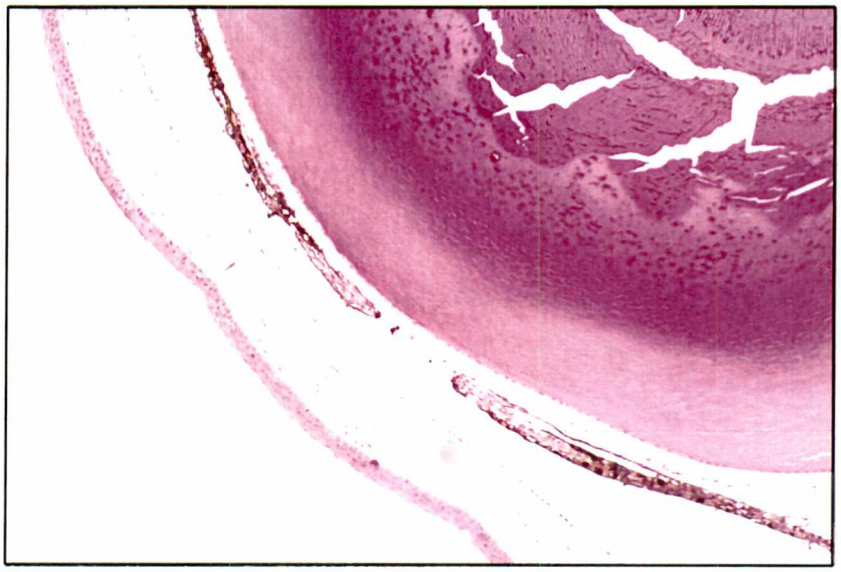
Anterior segment of the left eye from TgN3261Rpw mouse. The iris is hypoplastic, pupil is slightly covered by a persistent pupillary membrane and it is extremely miotic.

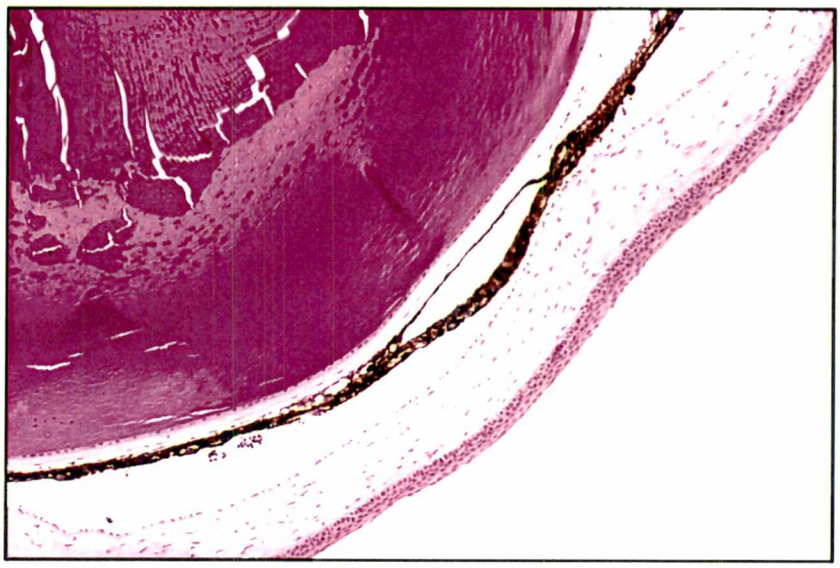
Figure 46: Persistent pupillary membrane. (10X)

The pupil has not developed properly and a thin remnant of the pupillary membrane is attached to the anterior lens capsule.

Figure 47: Persistent pupillary membrane. (10X)

This eye has severe posterior synechiae involving most of the iris.





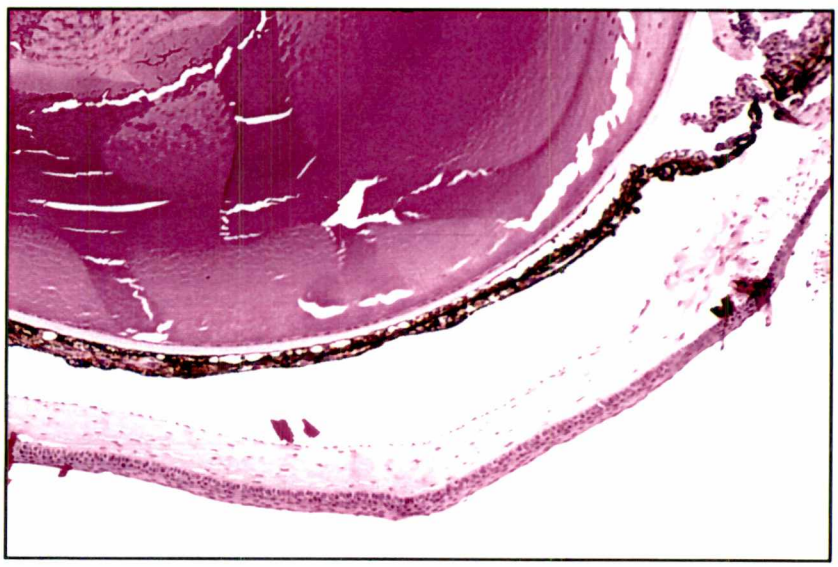


Figure 48: Jones-methenamine stain. (10X)

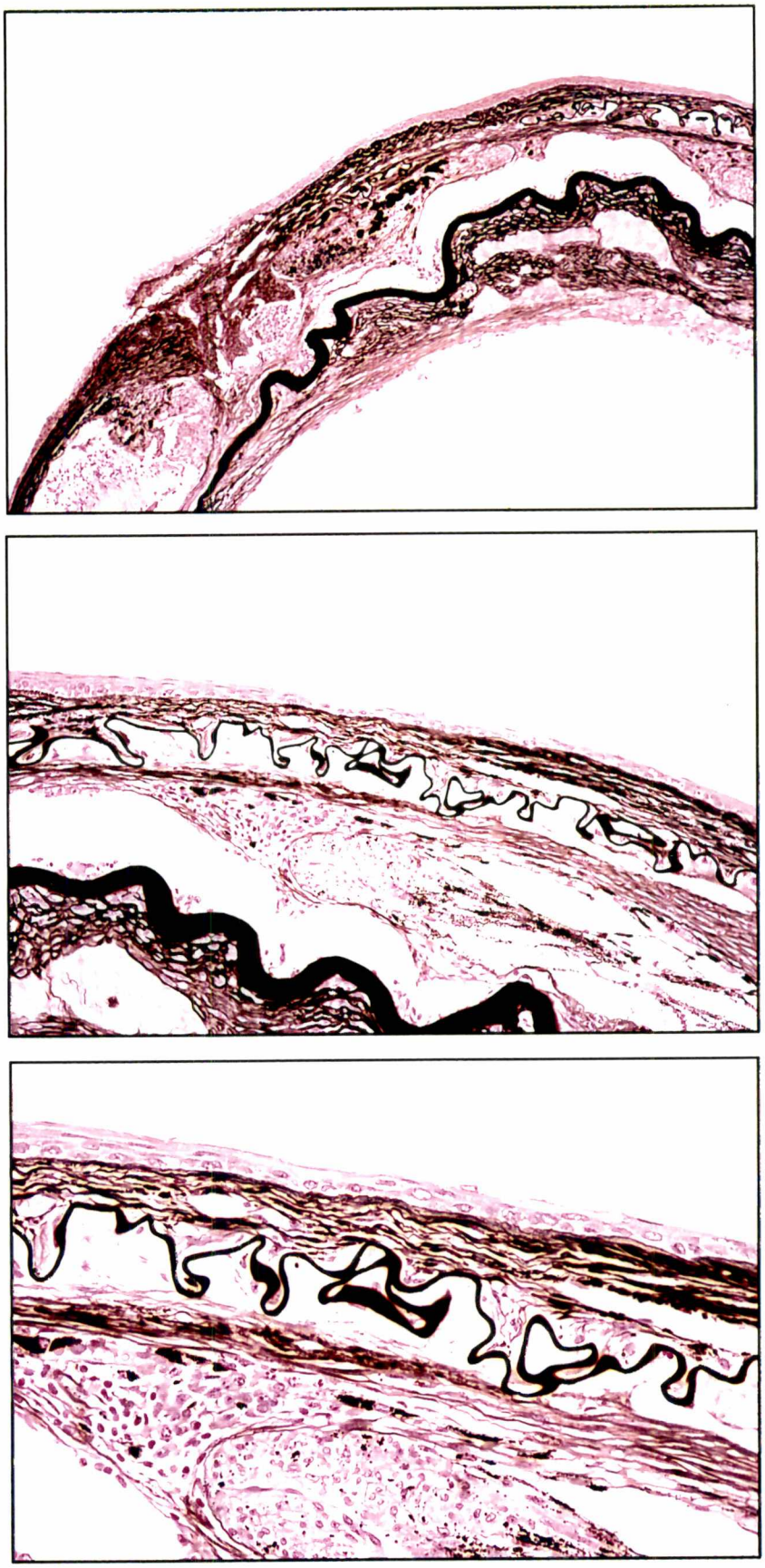
TgN3261Rpw mouse with anterior segment dysgenesis. The basement membranes are stained with Jones-methenamine silver stain. The thicker stained membrane is the anterior lens capsule and the thinner membrane which resembles a folded ribbon is Descemet's membrane.

Figure 49: Jones-methenamine stain. (20X)

The same TgN3261Rpw mouse with anterior segment dysgenesis syndrome. Descemet's membrane is highly convoluted anterior to the persistent tunica vasculosa lentis and its fibrovascular membrane.

Figure 50: Jones-methenamine stain. (40X)

Magnified view of the same TgN3261Rpw mouse with anterior chamber cleavage syndrome. Descemet's membrane is highly convoluted anterior to the persistent tunica vasculosa lentis and its fibrovascular membrane.



VITA

Carmen Maria Helena Colitz was born in Miami, Florida on April 3, 1968. She attended grade school at Saint James parochial school and high school at Madonna Academy in Hollywood, Florida where she graduated in May, 1985. She attended La Sorbonne, the University of Paris during the summer of 1985 and entered the University of Florida in the fall of that year. She received an Associate of Arts in 1986 and transferred to the University of Tennessee, Knoxville in the spring of 1989. In August of 1989, she entered the University of Tennessee College of Veterinary Medicine. Following graduation from veterinary school, she received a Post-Doctoral Research Training Fellowship and began graduate studies in the Comparative and Experimental Medicine Program at the University of Tennessee College of Veterinary Medicine. The doctoral degree was received August 1996.

She is presently in a comparative ophthalmology residency training program at North Carolina State University College of Veterinary Medicine which will be completed June 1999.