DISSERTATION

SINUS TUMORS OF ROCKY MOUNTAIN BIGHORN SHEEP: INVESTIGATION OF AN INFECTIOUS ETIOLOGY

Submitted by

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ABSTRACT

SINUS TUMORS OF ROCKY MOUNTAIN BIGHORN SHEEP: INVESTIGATION OF AN INFECTIOUS ETIOLOGY

Rocky Mountain bighorn sheep are an icon in Colorado. As our state animal, bighorn sheep are a well-recognized symbol of the wildlife, wildlands, and wilderness-centric people that Colorado is famous for. Efforts to manage and conserve this species are a priority in Colorado and throughout western North America. As part of those efforts a great deal of research has been conducted to understand bighorn sheep respiratory disease, the leading infectious cause of death in these animals. In the process of investigating respiratory disease in bighorn sheep in Colorado, we discovered a surprisingly high occurrence of sinus tumors within the upper respiratory tracts of many animals. This disease had not been described previously and became the focus of work for this dissertation. Here, I have compiled our findings regarding the characterization of bighorn sheep sinus tumors and the results of our efforts to identify an infectious etiology for this disease.

Through the examination of naturally-occurring cases, we identified characteristic histologic and gross features of bighorn sheep sinus tumors to define this disease. We also analyzed factors associated with sinus tumors at a population level. The results of this study suggest that bighorn sheep sinus tumors are an infectious disease, maintained within specific geographic areas corresponding to distinct populations of animals. Our results also suggest a role for bighorn sheep sinus tumors in predisposing animals to secondary infections by bacterial agents that can cause pneumonia.

To specifically test the hypothesis that bighorn sheep sinus tumors are a transmissible disease, we experimentally inoculated bighorn sheep and domestic sheep lambs with a cell-free filtrate derived from a naturally-occurring bighorn sheep sinus tumor and its associated exudates. Within 18 months post-inoculation we demonstrated transmission of the disease to both bighorn sheep and domestic sheep species, supporting our hypothesis that bighorn sheep sinus tumors represent an infectious process. This experiment also provided an opportunity to examine tumors early in development, further characterize the cells comprising the tumors, and suggest mechanisms for pathogenesis.

With evidence that bighorn sheep sinus tumors are caused by an infectious agent, we also attempted to identify a specific etiology for this disease. We primarily used PCR methods with degenerate PCR primers to evaluate samples from bighorn sheep sinus lining tissues for the presence of herpesviruses and retroviruses, which are well-known causes of infectious tumors. We successfully identified the presence of herpesviral and (likely endogenous) retroviral sequences in our samples, but we were unable to find an association between these viruses and the occurrence of sinus tumors.

Based on similarities between bighorn sheep sinus tumors and oncogenic retroviral diseases of domestic sheep and goats, we specifically screened our samples for the presence of Jaagsiekte sheep retrovirus (JSRV), and enzootic nasal tumor viruses (ENTV-1 and ENTV-2). We successfully identified ENTV-2-specific sequences from some of our samples, but an association between this virus and bighorn sheep sinus tumors was not clear. We found an association between ENTV-2 and early tumor cases, but not well-defined tumors.

While our PCR data alone did not definitively identify ENTV-2 as the cause of bighorn sheep sinus tumors, our histologic, histochemical, and immunohistochemical results have helped

us to develop a hypothesis for the pathogenesis of bighorn sheep sinus tumors, and provided additional support for the hypothesis that this disease is caused by ENTV-2. Our working hypothesis for the pathogenesis of bighorn sheep sinus tumors is that epithelial cells of the sinus lining are infected by ENTV-2, but that uninfected periosteal pluripotent cells are stimulated to replicate, resulting in predominantly stromal tumors. This hypothesis is based on histologic observations, histochemical stains used to differentiate cell types, and IHC results specifically identifying the presence of ENTV antigen within surface epithelial cells of experimentally-induced tumors, but not within the predominating stromal cells of the tumors. These results help to explain why detection of the virus is uncommon in well-developed stromal tumors, but more easily detected in early tumor cases with less stromal proliferation.

Additional research will help to further elucidate the pathogenesis of bighorn sheep sinus tumors, and the potential role that tumors may play in predisposing bighorn sheep to fatal respiratory disease. The definitive identification of an etiologic agent for bighorn sheep sinus tumors, and the development of an antemortem diagnostic assay will greatly enhance efforts to understand and manage this disease.

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Bighorn Sheep Natural History

Evolutionary History of Sheep

The evolutionary history of sheep is surprisingly complicated, with many contradictions and uncertainty in the literature. Some of this uncertainty can be attributed to a poor fossil record. The mountainous habitat of early sheep contributed few fossil imprints, most of which were ground away by multiple glaciations. Because of the poor fossil record, recent attempts to reconstruct the evolutionary history of sheep have relied on genetic studies to determine the divergence of species based on "molecular clock" analyses. These genetic studies yield inconsistent data, likely based on differences including computational strategies, sample sizes, and ultimately on interpretation of other data including the fossil record, and previously published studies, both of which are routinely used to constrain estimates of species divergence.

Taking into account the very wide range of estimates in the literature, the history of sheep likely began in Asia about 2.5 million years ago, when sheep (genus *Ovis*) first split from a common ancestor with goats. This is based on the first sheep-like fossils which appeared in China approximately 2.42 million years ago[83], although the origin of the sheep species was estimated by one well-cited study to be 6.8 million years ago based on "morphological, ethological and molecular information"[63].

Genetic estimates of the separation between modern-day North American wild sheep and European domestic sheep are inconsistent, in part because these studies root their analyses using either the 2.5 or 6.8 million year history of the genus *Ovis*. Based on fossil evidence, it is certain that, at the latest, separation of these species occurred when sheep migrated from Asia (Siberia) across the Beringia land bridge into North America (Alaska). This migration is estimated to

have occurred during the late Pleistocene Ice Age, around 300,000 years ago during the Illinoian glaciation[54] based on bighorn sheep-like fossils found in Alaska dating to this time period[62,135]. Therefore, based on fossil records, North American wild sheep and European domestic sheep are separated by between 300,000 and 2.42 million years of evolutionary time.

Genetic studies do tend to agree that the most recent common ancestor to bighorn sheep and domestic sheep appeared shortly after the appearance of the genus *Ovis*[20,63,113], so it is likely that the separation of bighorn and domestic sheep is closer to 2 million years ago rather than 300,000 years ago, with a great deal of evolution between the species occurring before sheep crossed into North America (Figure 1.1). Bighorn sheep cluster phylogenetically with Siberian snow sheep, the closest relative to the primitive sheep which crossed Beringia to North America. The split between the predecessor to Siberian snow sheep/North American wild sheep and the predecessor to modern European wild sheep appears to have occurred shortly after the appearance of the genus *Ovis.* (Table 1.1, Figure 1.1).

Cowan hypothesized that once sheep arrived in North America, the divergence of North American wild sheep species was driven by subsequent glaciations within the Pleistocene age[25]. Korobitsyna proposed a timeline for these speciations based on "paleontological, biogeographical, and chromosomal data"[72]. According to this interpretation of events, after the Illinoian glacial period, the interglacial Sangamon period saw a rise of ocean waters, flooding the Bering Sea land bridge and likely isolating the ancestors of modern Siberian snow sheep (*Ovis nivicola*) from sheep in North America. During this interglacial period, an ice-less corridor also formed in the western United States. This allowed for migration of sheep southward from Alaska and Canada and into new habitat. When a new glaciation period (the Wisconsin

Table 1.1: Classification of the wild species of the genus *Ovis*. Revised from Rezaei (2010)[113], according to Nadler et. al (1973)[93]

Common Name	**Scientific Name**
Dall Sheep	*Ovis dalli*
Bighorn Sheep	*Ovis canadensis*
Snow Sheep	*Ovis nivicola*
Argali	*Ovis ammon*
Asiatic Mouflon	*Ovis orientalis*
Urial	*Ovis vignei*
European Mouflon	*Ovis musimon*

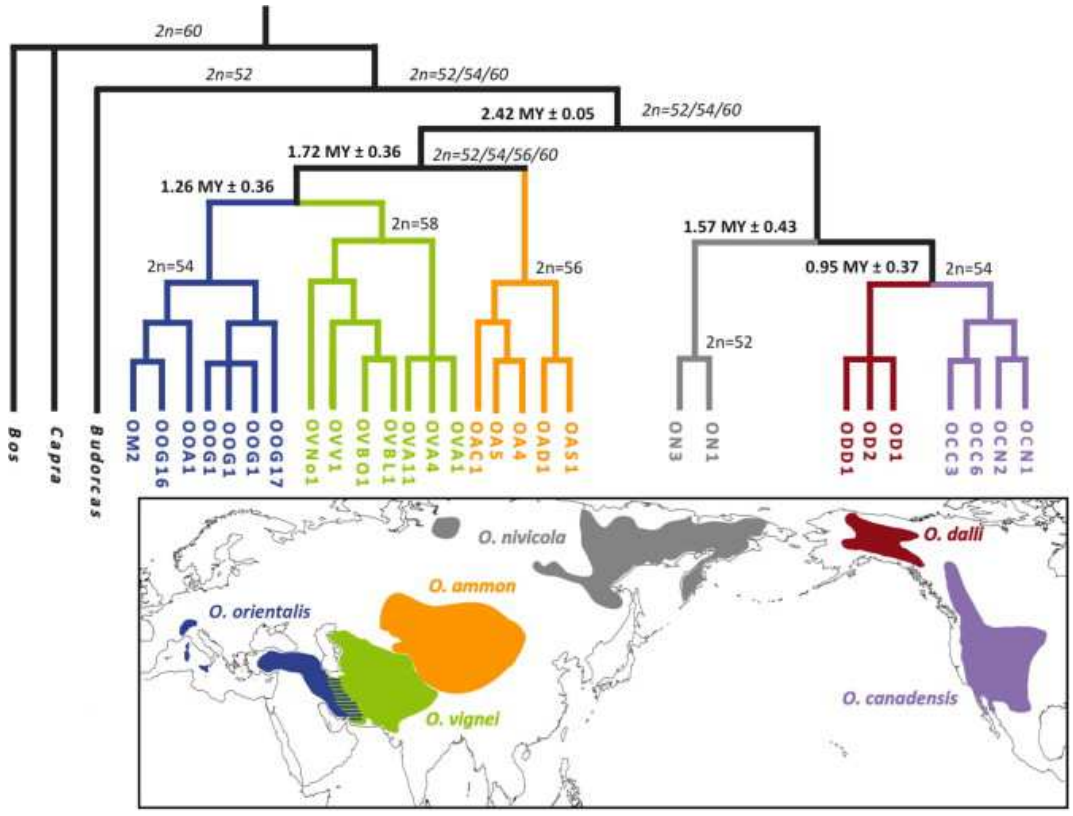

Figure 1.1: Phylogeography of the wild *Ovis* species. Original work by Rezaei et. al (2010)[113]. The map shows the geographic distribution of the seven wild *Ovis* species according to the classification by Nadler et. al (1973)[93], Table 1. The chronogram is assembled based on a history of genus *Ovis* as 2.42 million years.

glaciation) commenced around 30,000 years ago[54], a sheet of ice would have separated these northern and southern sheep populations. The northern population evolved to become thinhorn sheep (*Ovis dalli*), which currently range from Alaska to northern Canada, and have 2 recognized subspecies, Dall's sheep (*Ovis dalli dalli*), and Stone's sheep (*Ovis dalli stonei*). The southern population evolved to become bighorn sheep (*Ovis canadensis*), which currently range from southern Canada to Mexico, and have three recognized subspecies: Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*), desert bighorn sheep (*Ovis canadensis nelsoni*), and Sierra Nevada bighorn sheep (*Ovis canadensis sierrae*)[138,140].

While the timeline proposed by Korobitsyna is supported by fossil evidence, even the most conservative genetic analyses, presuming the origin of *Ovis* as only 2.42 million years ago, estimates that bighorn and thinhorn sheep split from Siberian snow sheep 1.57 million years ago (Figure 1.1), which would indicate that sheep crossed into North America long before the Illinoian glaciation. Keeping all of these factors in mind, it is probably safe to conclude that the genus *Ovis* is at least 2.4 million years old, that a common ancestor to bighorn and domestic sheep lies close to the origin of the genus *Ovis*, and that wild sheep in North America developed unique characteristics of independent species based on geographic separation by successive glaciations.

Perhaps the most important perspective gained by a discussion of sheep evolutionary history is the definitive evolutionary distance between bighorn sheep (*Ovis canadensis*) and domestic sheep (*Ovis aries*). These species are separated by perhaps millions of years of evolutionary time, during which each species has evolved along with a distinct set of pathogens. Subsequent human activity has brought domestic sheep and bighorn sheep species into contact. In the process, we have exposed these animals to pathogens for which each species has not had

millions of years to evolve an immune response. This fact should be considered when evaluating any pathogen transmissible between these species.

Biology of Rocky Mountain Bighorn Sheep

This literature review focuses on Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*), which currently range from the mountains of southern Canada to New Mexico, and account for the majority of the bighorn sheep currently found in Colorado. Rocky Mountain bighorn sheep are the largest of the bighorn sheep; an average-sized adult ram weighs approximately 175 pounds and up to 320 pounds[124], with his horns accounting for some 10% of that body weight. This gives bighorn sheep rams the largest horns among ruminants[56]. Adult ewes weigh closer to 120 pounds[124], and have small, slender horns, not nearly to the rams' proportions.

The majority of the year bighorns are found in small groups, or bands, of animals. Adult ($\geq$ 2yrs) bighorn rams form male-only groups while ewes, lambs, and juvenile rams range together in ewe-lamb bands[124,143]. The sizes of bighorn sheep bands vary, particularly with resource availability and time of year, but band sizes are most frequently in the 2-9 animal range[124]. Foraging groups numbering at least 5 animals are suggested as adequate to balance time spent foraging versus time spent being alert for predators[11], and a study in Rocky Mountain National Park in 1970 demonstrated ram bands averaging about 6 animals/band, and ewes 8 animals/band[143]. Several bands of bighorn sheep in a given geographic area may have overlapping ranges, interact regularly, and come together during the winter (breeding) months. These larger groups of sheep with fairly regular and direct interactions are considered a herd[58], and are likely to share habitat needs, genetics, diseases, and other factors important for management. Herds of bighorns may then be further connected (primarily by long-distance

movements by rams) in a geographic region such as a mountain range. These groups of herds that are more loosely connected, but still maintain factors such as gene flow and transfer of pathogens are considered a population[58], and are managed in such a way that acknowledges this larger-scale connectivity.

Seasonal migration patterns vary, but in general the winter season (approximately October through May) is spent at low elevations, allowing animals to take advantage of the availability of better forage as compared to higher elevations[124]. The summer season (approximately May through October) is spent at higher elevations as snow melt retreats and grazing resources are available further away from lower elevation predators[49,124]. Some bighorn herds do not migrate from summer to winter ranges, but rather use the same range yearlong[124,129,143].

Breeding occurs during the winter season, typically in late November and continuing through December[90,124]. At this time, ram and ewe bands mix together, forming larger herds that exist through all or part of the winter[124]. Rams may migrate to winter ranges one to two months earlier than ewes to establish dominance relationships via spectacular displays and horn clashes. These ram clashes are nearly completed and dominance relationships established by the time actual breeding begins[124].

The pre-breeding and breeding seasons are stressful for both rams and ewes. Firsthand observations of rams fighting with each other and harassing ewes are dramatic[90,129]. These descriptions not only document the pre-rut clashes between rams that bighorn sheep are famous for, but also on the brutality that befalls the ewes during the breeding season – “chased all over the country to the point of exhaustion… not only tak[ing] a severe beating but knocked off rocks fifteen or twenty feet high...I have watched them crawl into crevices…but the minute they get

onto their feet and come out they must race for their lives"[129]. Once breeding is completed and typically all of the ewes capable of bearing lambs are impregnated, the ewe and ram groups again separate, but remain on winter ranges until the end of the winter season.

Following breeding in November/December, gestation is estimated at 180 days, with lambing occurring in late May through June[30,124]. In early to mid-May, ewes migrate to higher summer ranges in preparation for lambing. Ewe groups tend to re-use the same area for lambing year after year[9,124], suggesting that familiarity with summer ranges is important to the survival of young lambs. Based on typical lambing ranges, an ideal lambing area is a high, rocky area that affords protection from coyotes, mountain lions, and other terrestrial predators[49]. During this time in the early summer, the threat of predation appears to outweigh the benefits of staying in lower ranges for the ewes and young lambs[49]. The rams in contrast (with no further interest in the ewes or their new progeny) are still enjoying the comparatively better resources at lower elevations. The ram herds will eventually migrate up to higher summer ranges, following the greening up of forage as the snow pack retreats[124].

Ewes typically give birth to a single lamb, although twinning has been reported[124,129]. Unlike deer and elk, bighorn ewe mothers do not hide their lambs and wander away, but rather stay close and call the lambs to them in any instance of suspected danger[124,129]. Supposedly, the rambunctious lambs have been known to "fake" an alarm to their mothers, just to delight in seeing them respond[129]. Once lambs are weaned, and winter weather begins to set in, ewe-lamb bands again migrate down to lower elevations for protection from the elements, and to again form breeding herds, completing this seasonal cycle.

Both groups of sexes, but most dramatically females, have high fidelity to small home ranges[57]. This distribution of small bands of bighorn sheep with small home ranges reflects their

fragmented habitat, as well as the previously mentioned importance of ewe familiarity with a small summer range. Given their high fidelity to small home ranges, bighorn sheep ewes have limited potential for dispersal and colonization of new habitat[36]. Bighorn sheep rams, however, are more apt to disperse than ewes and movements of these rams beyond maternal home ranges likely account for gene flow between fragmented herds[36].

This brief description of bighorn sheep natural history is a superficial look at basic sheep biology, and does not address the many factors that contribute to population dynamics of the species. Additionally, this discussion has focused on Rocky Mountain bighorn sheep, and has not touched on the differences between the subspecies of bighorn sheep. However, one can still appreciate the features of bighorn sheep ecology which contribute to disease transmission. These include: Stressful time periods, particularly associated with the breeding season; gregarious behaviors, again associated with the breeding season between ewes and rams, and between ewes year-round; the intensity of direct interactions between ewes and lambs within the perinatal period; and finally the propensity of young rams to disperse great distances, spreading not only genes but also potentially diseases within populations of bighorn sheep.

Population Declines and Respiratory Disease

The historical numbers of bighorn sheep in North America are uncertain. Previously, accepted population estimates of bighorn sheep numbers prior to invasion of habitat by early white settlers were up to 2 million animals[17,123], although more recently it has been suggested that this number may be 10-fold too high[135]. Regardless, the number of wild sheep in North America decreased dramatically as a result of the extensive hunting and loss of habitat that accompanied the settlement of this region[17,46]. The arrival of domestic sheep in mountain ranges in the late 1800's added to competition for range with the already-depleted populations of

bighorn sheep, and in addition the arrival of domestic sheep brought new pathogens. Steep declines of bighorn sheep populations during this period have been attributed to continued hunting and loss of habitat[17,46,102], as well as epidemics of Scabies mites (*Psoroptes spp*) which were likely acquired from domestic sheep, although transmission from domestic sheep has not been definitively proven[67,102,127]. The termination of the scabies epidemic in bighorn sheep near the turn of the 20th century was followed by moderate recovery in bighorn populations[17,102].

By the 1920's another decline in bighorn sheep populations was noticed, and bighorn sheep populations plummeted in Glacier National Park, Yellowstone National Park, and Rocky Mountain National Park[17,81,102]. Deaths were attributed primarily to outbreaks of bronchopneumonia. In 1939, Marsh wrote a review of 13 years' history of disease losses in bighorn sheep from the Sun River Game Preserve in Montana, Glacier National Park, Yellowstone National Park, and the National Bison Range[81]. Marsh recognized two disease syndromes: chronic bronchopneumonia in adult sheep, and acute pneumonia in 2 to 3 month old lambs. This report included excellent descriptions of clinical symptoms, gross pathology, and histopathology.

Marsh reported that for the adult syndrome, in affected bighorn sheep herds the sheep were thin, weak, and coughing, with difficulty breathing particularly after exertion. At necropsy, Marsh noted subacute or chronic bronchopneumonia with consolidation, primarily affecting the cranioventral and cardiac lobes of the lung, usually accompanied by pleuritis and adhesions. Histologically, Marsh noted congestion of alveolar capillaries, peribronchiolar infiltrates of chronic inflammatory cells, and filling of alveoli with "leukocytes" suggesting predominantly neutrophils. Marsh reports similar necropsy findings of bronchopneumonia in dead lambs; consolidation, suppuration, and necrosis of the right cranioventral and cardiac lung lobes, with

adhesions to the pericardium and chest wall[81].

These early descriptions match remarkably well with subsequent descriptions of bighorn sheep pneumonia[8,23,98,133] and with outbreaks of bronchopneumonia that still occur sporadically in bighorn sheep herds throughout western North America. All-age die-offs in adult bighorn sheep typically occur in the winter months and are likely associated with increased density of sheep, the stress of the breeding season, and decreased nutrition on winter ranges[87,91,102,133].

A second type of outbreak occurs in 2-3 month old lambs, which has been termed "summer lamb mortality". While the pneumonias of adults and lambs have distinct similarities based on gross and microscopic pathology, a link between all-age die-offs and summer lamb mortality has not been proven. Summer lamb mortality often follows all-age winter die-offs; lambs are born in normal abundance in the spring, but are decreased to a small percentage by the summer[23,48,91]. This summer lamb mortality can decimate bighorn sheep populations, already depleted from the preceding die-off and unable to recruit new lambs into the population. Summer lamb mortality can continue for an additional five years or more[91], making significant recruitment into affected herds nearly impossible.

Outbreaks of bighorn sheep pneumonia (all-age die-offs and summer lamb mortality) have been observed in Colorado bighorns for decades [8,50,59,110]. In Colorado, populations of bighorn sheep fell from approximately 8,000 in 1922 to 2,235 in 1941[102]. While a general increase in the Colorado bighorn sheep herd has been seen more recently (6,045 in 1988 and 7,040 in 2007[58], these increases have been attributed mainly to management activities including extensive trapping and relocation of sheep[58]. While these transplants appear to be augmenting bighorn sheep populations based on an overall uptrend in the total population, transplanted herds are small and show limited potential for sustainability or growth without continued intervention

[12,58,128]. The overall decline of bighorn sheep noted in the 1920's has indeed continued, with many populations of Colorado bighorns resisting extirpation by way of human intervention, primarily transplants.

Proposed Causes of Bighorn Sheep Pneumonia

The cause of bighorn sheep pneumonia has been, and remains, a debated topic. Early investigations into die-offs of bighorn sheep, such as those described above by Marsh, indicated that pneumonia was primarily due to lungworm infections, allowing secondary bacterial invasion by *Pasteurella* bacteria. These bacteria were isolated from both lung and blood, suggesting that the ultimate cause of death in these cases was terminal septicemia[81,110]. Based on these findings, Potts made the diagnosis of "hemorrhagic septicemia", a recognized disease in domestic animals characterized by fibrinous pneumonia and isolation of *Pasteurella* bacteria from the lungs and blood[110].

At the time of these early investigations, the bacterial component of the pneumonia was considered secondary to lungworm infection. Packard, studying the decline of bighorns in Rocky Mountain National Park concluded "Apparently debility caused by parasites, increasingly activated by dietary deficiencies, and particularly the irritation and congestion caused by lungworms, permit the deadly pneumonic bacteria to enter the bloodstream"[102]. This hypothesis has been supported by others over the period of continued decline of bighorn sheep populations[64,65,121,131]. In the late 1970's, Spraker investigated summer lamb mortality in the Pikes Peak herd in Colorado and determined that pneumonia was associated with severe cases of lungworm infection. In these lambs, very high loads of lungworm in ewes were transmitted transplacentally to lambs via placental veins which delivered third-stage larvae to the fetal liver[131]. These larvae migrated to newborn lamb lungs during the first week of life, causing

physical damage to the lungs that continued as lungworms reached maturity and began to reproduce. Final maturation of lungworms and release of first stage larvae coincided with the onset of pneumonia at approximately 6-8 weeks of age[131]. The timing of this event correlated well with the onset of summer lamb mortality at 2-3 months of age, and was thought to predispose the lungs to bacterial infection.

Treatment of the Pikes Peak herd with anthelmintics was followed by increased lamb survival[121], further supporting the hypothesis that lungworm was a significant factor contributing to summer lamb mortality. Subsequent studies investigating the effects of lungworm treatment on lamb mortality, however, have not shown this direct association in other herds[88], and lungworm infection has not been shown to successfully induce pneumonia in bighorn sheep lambs experimentally[120]. It is clear that lungworm is one factor with the potential to contribute to pneumonia in bighorn sheep, particularly in regards to summer mortality of lambs in herds where populations are heavily concentrated and lungworm loads are very high. However, it is likely that lungworm is acting as a predisposing factor in bacterial bronchopneumonia.

While research has shown that lungworm infection can predispose bighorn sheep to pneumonia, it seems that ultimately bighorn sheep adults and lambs are dying from severe bacterial bronchopneumonia and sepsis. In 1962, George Post provided a review of bighorn sheep pneumonia, suggesting that the characteristics of the pneumonia outbreaks and the isolation of *Pasteurella* bacteria indicated a greater role for bacteria than was previously considered. Like Potts, Post observed that the characteristics of the disease in bighorn sheep were similar to outbreaks of hemorrhagic septicemia (also called shipping fever) in domestic ruminants, caused by *Pasteurella* bacteria, with possible contributing factors including viruses and stress[109]. To differentiate the disease in wild ruminants, Post referred to bighorn sheep

disease as “pasteurellosis”, so as not to be confused with the more well-established disease of hemorrhagic septicemia/shipping fever.

Post defined pasteurellosis as “an acute, infectious respiratory disease which terminates by septicemic invasion”. Comparing the disease in bighorn sheep to diseases in domestic animals, Post suggested that the course of the disease in bighorns was consistent with domestic animal pasteurellosis, and not lungworm infection. He felt that the rapid courses of the described outbreaks were most consistent with “…a virulent organism and not the slow, debilitating disease which usually results from parasitism”[109]. To support his hypothesis that transmission of lungworms was not the primary cause of bighorn sheep pneumonia, Post also cited the normal occurrence of lungworms in healthy bighorn sheep[109].

However, like his observations of lungworms in normal bighorn sheep, Post also concluded that *Pasteurella* bacteria could be isolated from normal bighorn sheep, and he recognized this as a problematic factor in interpreting the bacteria as being the primary cause of pneumonia. In addition, Post commented on the multiple strains of *Pasteurella* isolated from dead bighorns, and suspected that perhaps more than one strain could be responsible for causing disease[109]. Because of the multiple factors apparently involved in outbreaks of bighorn sheep pneumonia, Post could not definitively list pasteurellosis as the primary cause of this disease, but he did suggest that, regardless of other contributing factors, *Pasteurella* be more closely considered as the ultimate cause of death for bighorns with pneumonia[109].

As the focus of bighorn sheep respiratory disease research has shifted from lungworm to *Pasteurella*, a great deal of information has been accumulated supporting the hypothesis that *Pasteurella* bacteria are the ultimate cause of fatal bighorn sheep pneumonia, and that these organisms may have been introduced to bighorn sheep by domestic species[87,139]:

1) *Pasteurella* bacteria are consistently isolated from the pneumonic lungs of bighorn sheep[23,48,50,59,98,133]
2) Contact between bighorn sheep and domestic species has been documented directly prior to outbreaks of fatal bronchopneumonia[51,59,116].
3) Experimental comingling of domestic sheep with bighorn sheep results in fatal bronchopneumonia in bighorn sheep while domestic sheep remain clinically normal[97].
4) Experimental infection of healthy bighorn sheep with *Pasteurella* bacteria from healthy domestic sheep results in fatal bronchopneumonia in bighorn sheep[52].
5) The differential pathogenicity of *Pasteurella* bacteria to domestic sheep and bighorn sheep has been explained based on differences in the leukotoxin gene of bacteria that are pathogenic to bighorn sheep but not domestic sheep[27].

Despite the above evidence, researchers still face the same issues that prevented Post from concluding that *Pasteurella* bacteria were the primary cause of bighorn sheep pneumonia; the presence of disease-causing bacterial strains in the upper respiratory tract of normal healthy bighorn sheep[5,15], and the variability of bacteria isolated. This problem may be due, in part, by inaccurate classification of *Pasteurella*-type bacteria. Throughout this literature review I have chosen to use "*Pasteurella* bacteria" to refer to the *Pasteurellaceae* family, a group of gram-negative bacterial organisms that has experienced multiple taxonomic revisions throughout history. This diverse group contains both commensal and pathogenic organisms, and the most relevant genera to this discussion are those which have been associated with bighorn sheep pneumonia, including species within the genera *Pasteurella, Mannheimia,* and *Bibersteinia*.

Classically, the *Pasteurella* bacteria have been classified based on growth characteristics

of the bacteria under various conditions, on serologic differentiation and, more recently, on biochemical properties[69]. All of these classification schemes are based on properties of the bacteria measured *ex vivo*, however, which may not correlate with pathogenicity of the bacteria *in vivo*. Additionally, genotype data generated on a large archive of bighorn sheep *Pasteurella* bacteria isolates suggest that our current classification schemes based on phenotypic properties of the bacteria may not correlate with genotypic properties. However, neither the specific genes responsible for the genetic variation seen in this study, nor the biological relevance of these genes, have been determined[89].

While much work is left to be done, one certain benefit of moving towards a genotypic classification scheme for *Pasteurella* bacteria is the ability to use culture-independent methods for bacterial classification. The challenges of collecting, transporting, and culturing bacteria from remote locations and long-deceased carcasses has likely contributed to the historic variation in quality and consistency of culture results. This has prevented relevant comparisons between samples and between outbreaks, which will ultimately be necessary to make conclusions regarding the biological relevance of various pathogens. Culture-independent methods may help to alleviate some of these complications[118].

Because researchers are most concerned with detecting the presence of pathogenic bacteria, one promising culture-independent diagnostic approach to targeting pathogenic versus nonpathogenic *Pasteurella* bacteria is to focus on what virulence factors are responsible for producing disease *in vivo*, regardless of species. Multiple virulence factors have been identified, but the virulence factor which appears to be most correlated with disease is leukotoxin [70]. Leukotoxin is a bacterial enzyme capable of lysing/rupturing leukocytes, including neutrophils, in the lung. This lysis of leukocytes is used by bacteria to eliminate bactericidal neutrophils,

preventing removal of the bacteria. As a side-effect, lysing the neutrophils also spills the digestive enzymes which are present in neutrophils into the surrounding tissue[13]. The lung tissue is degraded by these digestive enzymes, and tissue degradation may in fact be more harmful to the lungs than the bacteria themselves. With the advent of PCR, researchers can target the leukotoxin gene as an indicator of pathogenic bacteria in the lungs, instead of relying on the growth of the organisms in culture where results can be difficult to interpret[61].

Just as early investigators were unable to pinpoint a single cause for bighorn sheep pneumonia, researchers today continue to consider factors other than bacterial infection as possible primary causes for this disease. Besides lungworm, other infectious agents considered have included several viruses. The respiratory viruses with the greatest serological prevalence in bighorn sheep populations have consistently been parainfluenza-3 virus, and bovine respiratory syncytial virus[5,106,117,122,132,133]. Serological data suggest that, while viral infections may predispose bighorn sheep to pneumonia, infections with these agents may also be a common occurrence, causing mild or subclinical disease in herds of otherwise healthy bighorn sheep[87]. Non-infectious factors have also been considered as causes of bighorn sheep pneumonia including nutritional deficiencies and stress[87,102,133]. These studies indicate that, like lungworm infection and viral infections, non-infectious factors can be associated with outbreaks of bronchopneumonia and may contribute to the pathogenesis of this disease[87].

In recent literature, *Mycoplasma* species of bacteria have been suggested to be a primary cause of bighorn sheep pneumonia. The consideration of a role for *Mycoplasma* spp. in bighorn sheep respiratory disease is not a recent development. In 1970[142] Mycoplasma was first isolated from cases of bighorn sheep pneumonia. At that time, Mycoplasma was a known cause of pneumonia and other diseases in livestock, but had not been previously associated with

respiratory disease in bighorn sheep. This 1970 paper identified Mycoplasma in the pneumonic lungs of captive bighorn sheep during an outbreak, as well as from nasal swabs of live sheep. Attempts to culture *Pasteurella*-type organisms from the same samples were unsuccessful, but histopathological findings were suggestive of a typical bacterial pneumonia. The conclusion of the paper was that "it seems likely that when the [bighorn sheep respiratory disease] complex is better defined there will be multiple etiological agents (viruses, bacteria, parasites) – some of which will be found in clinically normal animals – that may interact with each other as well as environmental variables to result in overt disease. This report suggests that mycoplasmas should be considered one of these possible agents and further investigated"[142].

More recently, experimental studies have confirmed that infection with *Mycoplasma* spp. can predispose bighorn sheep to bronchopneumonia induced by infection with *Pasteurella* bacteria[28], and Mycoplasma species have been identified in pneumonic bighorn sheep lungs using culture-independent methods[14,141]. Although evidence is mounting that Mycoplasma may play a very important role in the pathogenesis of bighorn sheep pneumonia, the inability to produce pneumonia with Mycoplasma alone[14] suggests that Mycoplasma's important role is to predispose the lungs to invasion by bacterial pathogens such as *Pasteurella* bacteria.

It is interesting that even in the earliest investigations into various pathogens as factors in bighorn sheep respiratory disease, the same conclusion was reached: multiple factors are likely involved in the bighorn sheep respiratory disease complex. Features of the disease from a clinical, histopathological, and epidemiological standpoint all point to various factors predisposing bighorn sheep to bacterial pneumonia, which is the ultimate cause of death. Experimental transmission studies of any pathogen alone (except *Pasteurella* species) fail, suggesting that this family of bacteria is the etiologic agent of bighorn sheep bacterial

bronchopneumonia. However, it does seem that a predisposing factor is necessary for the proliferation of these pathogenic bacteria in the lungs, particularly in natural settings where *Pasteurella* bacteria are present in apparently healthy animals. While the study undertaken here focuses minimally on bacterial bronchopneumonia, it is possible that the disease described here (bighorn sheep sinus tumors) may be yet another contributing factor to colonization of the lungs by bacteria.

Initiation of Sinus Tumor Investigation

In 2009, the lower Poudre Canyon in Fort Collins, Colorado had suffered for at least 10 years with summer lamb mortality. Over a 10 year period of observation, ewes in the herd successfully lambed, but every year 100% of the lambs born succumbed to fatal bronchopneumonia at approximately 2-3 months of age. As the population age structure continued to shift towards old ewes and the overall population size slowly declined, it was decided to cull the small (7 elderly animals) herd to prevent transmission of disease to other nearby herds.

All seven animals were submitted for necropsies. Several showed evidence of mild, chronic bronchopneumonia, and many showed signs of resolved pneumonia (fibrous pleural adhesions). There were no signs of active pneumonia such as those associated with die-offs or lamb mortalities. A mild to moderate lungworm load was noted in all cases based on the presence of scattered lungworm nodules in the dorsal caudal lung lobes. While there was no evidence of active pneumonia to explain transmission of fatal bronchopneumonia to newborn lambs, we did find something that had never been previously described: All sheep examined had soft masses present within the paranasal sinuses. Chapter two discusses the features of these

masses in depth, but several features of the masses are reminiscent of oncogenic retroviruses of domestic sheep and goats, which will be discussed more thoroughly in this literature review.

The only previously described disease in bighorn sheep with somewhat similar features to the disease described here (sinus tumors of bighorn sheep) is chronic sinusitis of desert bighorn sheep. This disease was largely described by Bunch in the 1970's and 1980's, and is hypothesized to be caused by the migration of nasal bot (*Oestrus ovis*) larvae[2,19,107]. Bunch hypothesizes that bots migrate aberrantly from the nasal sinuses into the complex trabecular regions of the paranasal sinuses soon after infection as an L2 larvae, but are unable to migrate back out to the nasal sinus after maturing to a larger L3 larva[18]. These displaced larvae then incite a dramatic inflammatory response resulting in severe osteonecrosis and suppurative exudate within the sinuses. Fistulous tracts commonly form to the outside of the skull and infection leads to the demise of the animal[18]. While chronic sinusitis has some overlapping characteristics with bighorn sheep sinus tumors such as inflammation and reaction of the sinus lining, there are distinct differences. Chronic sinusitis lacks significant tissue growth, bighorn sheep sinus tumors lack a necrotizing process, and the consequences of chronic sinusitis are fatal versus nonfatal sinus tumors[18].

Based on a thorough review of the literature and personal communications with wildlife pathologists, veterinarians, and biologists from across western North America, it seems that this study is the first to describe and investigate the entity of sinus tumors in bighorn sheep. The most similar diseases published in the literature are the oncogenic retroviral diseases of domestic sheep and goats. This observation has led us to our hypothesis that sinus tumors of Rocky Mountain bighorn sheep are caused by an infectious agent, and we have specifically searched for the presence of oncogenic retroviruses in cases of bighorn sheep sinus tumors. The

characteristics of oncogenic retroviral diseases of domestic sheep and goats are explored below.

Oncogenic Retroviruses of Domestic Sheep and Goats

Basic Retrovirology

The retrovirus family (*Retroviridae*) is an interesting group of viruses for many reasons, one of which is the ability of these viruses to integrate viral genetic material into the genome of the infected host cell. This ability to alter host cell DNA creates two relatively unique opportunities for retroviruses. First, when viral genetic material is inserted into the host genome, it can alter the expression of normal host cell genes in that region. Second, if the virus manages to infect the germ cells of the host and integrate into the DNA of the host's sperm or egg, the genetic material of the virus can be permanently incorporated into the DNA of every cell of the host's progeny, termed endogenization. These two concepts will be specifically explored in regards to oncogenic retroviruses of domestic sheep and goats below, but are best understood given a brief background on basic retrovirology.

Retroviruses are an incredibly unique group of viruses, with an elaborate but fascinating replication strategy. Retroviruses contain a genome of two, positive-sense, single strands of RNA[22]. Retroviruses are the only diploid viruses we know of, and the reason for this diploidy is poorly understood[22]. The current model for retroviral replication indicates that only one molecule of RNA is needed to create the single strand of DNA which is integrated into the host genome, so having two molecules of RNA seems extraneous for replication[22]. One theory on the advantage of having two RNA molecules is that the second molecule is used to repair damage to the genome [22].

In addition to diploidy, retroviruses are also unique in how they use their positive sense RNA genome for replication. Most positive sense RNA viruses release their genome into the

cytoplasm of the infected host cell. This is because positive sense RNA, by definition, is in a form that can be recognized by the host cell as a message for translation. In other words, positive sense viral RNA can be recognized as messenger RNA by the host cell translation machinery and immediately translated by the cell into viral proteins which are then assembled, packaged, and released as new virions. But retroviruses don't use this approach. Instead, retroviruses have a much more complicated, but in many ways more effective strategy for replication, whereby the virus reverse transcribes its own RNA into DNA, and that DNA (termed cDNA) is then integrated into the host cell genome (and termed proviral DNA)[22]. Viral replication and assembly is then permanently directed by the host cell, with viral messages emanating from the host cell nucleus.

The RNA genomes of the retroviruses are around 7-13 kilobases (kb), on the smaller side of the range of viral genomes[22]. For reference, circoviruses, which encode for only 2 genes, are approximately 2 kb in length[96], while herpesviruses, encoding over around 100 genes, are around 100-200 kb long[96]. The genes encoded by retroviruses are fairly simple, with 4 mandatory genes encoded by all retroviruses: *gag*, *pro*, *pol*, and *env*[22]. The *gag* gene derives its name from **g**roup-specific **a**nti**g**en, one of the first genes identified to differentiate this family of viruses. The *gag* gene is responsible for production of the viral capsid, the protein shell which encases the viral genome[22].

The *pro* and *pol* "genes" are variable between retroviruses, and are ultimately extensions of the *gag* gene. The *pro* region encodes for proteases which cleave the *gag* and *pol* proteins to produce the final, active proteins which function in the mature virus[22]. The *pol* region encodes for the retroviral polymerase, an essential viral protein. The retroviral polymerase is the enzyme which drives reverse transcription of viral RNA into cDNA. The *pol* region also encodes for

integrase, the enzyme responsible for integration of the newly synthesized proviral DNA into the host cell genome[22].

Finally, the *env* gene encodes for the viral envelope, the surface and transmembrane proteins which are embedded in the lipid bilayer derived from the host cell and surround the viral capsid. The viral envelope functions in cell entry/infection by the virus[22]. While all retroviruses contain *gag*, *pro*, *pol*, and *env*, some retroviruses (complex retroviruses) also have additional coding regions that are variably-named depending on the virus, and in many cases have unknown functions[22]. In addition to these "coding" regions, or regions that are eventually translated into proteins, there are also non-coding regions of the retroviral genome which do not encode for proteins, but serve other functions that are necessary for replication of the virus. One of these noncoding regions is the long terminal repeat (LTR) region.

The LTR, along with an elaborate acrobatic feat of the viral reverse transcriptase enzyme, allows the virus to encode a signal for its own transcription. Within the integrated proviral DNA, there are two copies of the LTR, both of which are composed of the regions U3-R-U5. One LTR is located at the upstream end of the DNA, and the other at the downstream end (Figure1.2).

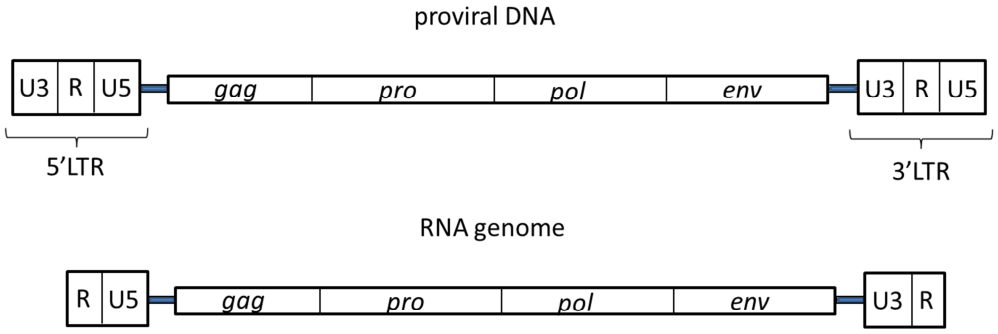

Figure 1.2: Retroviral structure, comparing proviral DNA and RNA genome.

The LTR at the upstream end functions as a promoter to direct the host cell machinery to begin transcription of proviral DNA into RNA. However, the transcriptional start site is at the U3-R junction, and therefore only the R-U5 regions are present at the 5' end of the resulting RNA molecule. The downstream LTR is important, in part, because it includes a copy of the LTR for restoring the upstream sequence. However, at the downstream end, the R region signals the termination of transcription, and only R-U3 regions are present in the resulting downstream 3' end of the viral RNA. During reverse transcription, both the missing 5' and 3' sequences are restored. This is accomplished because copies of the missing sequences are present at the opposite ends of the molecule and can serve templates for DNA synthesis. The final result is maintenance of the LTR, and therefore enhanced transcription of the area of the host genome containing the viral sequence[22]. This becomes important in regards to how retroviruses can enhance the expression of other nearby host genes and in some cases allow for oncogenesis (tumor formation).

Retroviral Oncogenesis

As previously mentioned, retroviral oncogenesis results from the ability of retroviruses to integrate into host cell DNA and alter gene expression. This is classically accomplished in one of two ways, separating most oncogenic retroviruses into two groups: acute-transforming viruses and non-acute transforming viruses[47,66]. The acute-transforming viruses are the more aggressive of the two groups. These viruses have an oncogene built into their genomes which, when expressed, enhances cell growth and replication[47,66]. Because the oncogene (part of the proviral DNA) is located downstream from an LTR promoting transcription, oncogene expression is enhanced and a tumor results regardless of where the virus integrates. Tumors can form very quickly, and often multiple integration events produce a tumor. Therefore, phenotypically,

acute-transforming retroviruses are characterized by rapid tumor formation and tumor foci are multifocal and polyclonal[47,66].

Interestingly, it is thought that retroviral oncogenes of acute-transforming viruses have been acquired through recombination events with host sequences, whereby normal growth-enhancing "proto-oncogenes" of the host cell are ultimately swapped into the viral genome. In most cases, this process requires swapping out some other portion of the viral genome, and because all of the retroviral genes are fairly critical for replication, the acquisition of an oncogene typically equates to loss of replication competence of the virus[47,66]. Therefore, despite rapid oncogenesis and aggressive tumors, the acute-transforming viruses typically require co-infection by a related virus to provide the missing proteins necessary for replication.

In contrast, the non-acute transforming viruses do not carry a viral oncogene. These viruses induce tumor formation when proviral DNA, including an upstream transcription-enhancing LTR, is inserted into the host genome in a region which encodes for normal growth and replication (in a region of a host "proto-oncogene")[66]. When this happens, the proviral LTR directs enhanced transcription of the proviral DNA, but also enhances transcription of nearby growth-promoting genes of the host. This is an imperfect process, and the integration of proviral DNA into the region of a proto-oncogene takes great numbers of infections, and therefore long incubation times to tumor formation[66]. The resulting tumors are typically focal and monoclonal, with the single successful integration of proviral DNA near a proto-oncogene resulting in genetically identical tumor cells, with identical integration sites by the provirus[66].

While the lack of an oncogene makes the non-acute transforming viruses less aggressive with longer incubation periods to tumor formation than acute-transforming viruses, they do have an advantage. The lack of an oncogene means no viral genes need to be swapped out in order to

swap in the oncogene. This allows for retention of critical viral genes, and therefore most non-acute transforming retroviruses are replication competent and do not require a viral co-infection to reproduce.

The two major groups of oncogenic retroviruses described here, acute transforming and nonacute transforming virus encompass most, but not all of the oncogenic retroviruses. These two major groups result in tumors by either acquisition of an oncogene from the host, or integration upstream from a cellular proto-oncogene. In addition to these mechanisms, there are also viruses which contain an oncogene that is part of the viral genome and has not been acquired from the host[10]. These include nonstructural genes that serve as oncogenes, as seen in viruses such as bovine leukemia virus, and structural genes that serve as oncogenes, as will be explained for viruses such as JSRV and ENTV[10].

Retroviral Endogenization

As described above, the integration of retroviral genetic material into the host genome provides a means for oncogenesis. Additionally it provides a means for endogenization. Throughout evolutionary history, many species including humans have become infected with retroviruses that have integrated into the host's genome via reverse transcription, and replicated using the host's nuclear machinery. As some of these viruses infected the germ cells of the host, some retroviruses could be passed vertically to offspring as a permanent part of the genome. Over time, mutations accumulated in the proviral sequence, and eventually these mutations became incompatible with replication. Today, only endogenous sequences of these now non-replication-competent viruses are left as a reminder of ancient infections. In fact, 5-8% of the human genome is composed of endogenous retroviral sequences[39]. Interestingly, some endogenous retroviruses maintain replication competence and may play a role in normal host

functions, particularly in the reproductive tract[45,103] or possibly in resistance to infection by exogenous (infectious, disease-causing) viruses[4,104,130]. However, most endogenous viruses have no known function.

As fascinating as endogenous retroviral sequences are, they provide a frustrating block to molecular diagnostics. For the group of exogenous retroviruses focused on in this study, there are at least 27 highly-homologous endogenous viral sequences identified in domestic sheep[3]. This means that, when using tumor tissue as a template (which contains DNA from the host genome), for every copy of an integrated exogenous virus that one attempts to amplify, there are a minimum of 27 similar endogenous sequences which may be amplified from the host genome instead. The similarity between exogenous viral sequences and endogenous sequences is incredibly high, and only a few small regions of the genome can be used to distinguish exogenous from endogenous sequences[6,7]. Therefore, discovery of a new, related virus is difficult by standard methods such as the use of degenerate PCR primers.

Additionally, sheep and goats that are naturally infected with oncogenic retroviruses do not mount a detectable immune response to these viruses[100,130]. This lack of an immune response is hypothesized to be due to similarities between coding regions of the endogenous and exogenous viral sequences[100]. The inability to use serological assays as diagnostic tools adds to the complications involved when investigating emerging retroviral diseases. Given these limitations, the accomplishments of researchers investigating now well-described oncogenic retroviruses are truly remarkable.

Jaagsiekte Sheep Retrovirus

Jaagsiekte sheep retrovirus (JSRV) is the causative agent of ovine pulmonary adenocarcinoma (OPA), an infectious disease of domestic sheep characterized by low-grade

neoplastic proliferations of bronchiolar and alveolar epithelial cells[33,105]. These tumors are found as multiple nodules within the lungs of affected sheep. Histologically, the nodules contain papillary projections of epithelial cells which can compress to form more solid structures[33]. Nuclear features of the tumor cells are relatively benign, with uniform nuclei containing infrequent mitotic figures. However, the extent of proliferation has led to the designation of adenocarcinoma (malignant) versus adenoma (benign)[33]. Typically, tumors are surrounded by a thin stroma, but occasionally fibrosis is a prominent feature. In some cases, tumors are infiltrated by aggregates of myxomatous tissue, presumably originating from mesodermal cells. These myxomatous structures are comprised of spindle-shaped cells embedded within a basophilic matrix, and are generally associated with neoplastic epithelial cells. Whether or not these myxomatous stromal cells are also neoplastic has not been determined[33,125].

An additional histologic feature present in many cases of OPA is polypoid growths arising from the bronchiolar epithelium[33]. These polyps are lined by normal epithelial cells and contain a central core of connective tissue. Often, polyps are accompanied by chronic lymphoplasmacytic inflammation, and hyperplasia of bronchiolar associated lymphoid tissue. It is uncertain whether polyps represent part of the neoplastic process, or if they are merely initiated by chronic inflammation[33].

The lung lesions of OPA cause significant respiratory distress and, in early descriptions of the disease, farmers in South Africa noted that affected sheep looked as if they had been chased or driven. This observation led to the naming of the disease as jaagsiekte, meaning "driving sickness" in Afrikaans[149]. Another feature of OPA is the production of abundant fluid by tumor cells in the lungs, which are secretory in nature. Clinically affected animals that are lifted by their hind end will exude abundant fluid (up to 300 mL) during the "wheelbarrow"

test[33]. This fluid contains infectious JSRV particles and has been used to induce disease in healthy sheep[82,149]. The first retroviral particles were observed in OPA tumors by electron microscopy in 1974[108], and the first report of transmission with OPA particles containing reverse transcriptase activity was published in 1976[82].

The oncogenesis of JSRV has been well-studied, in part because of similarities between ovine pulmonary adenocarcinoma and human lung cancer[92]. Initial investigations into whether JSRV was an acute or non-acute transforming retrovirus were not straightforward. JSRV exhibits phenotypic characteristics consistent with an acute-transforming virus; multifocal tumors, rapid induction of disease (in as little as 10 days experimentally[126]), and transformation of cells in culture[66]. However, investigations into the JSRV genome revealed none of the classically recognized oncogenes associated with acute-transforming retroviruses. This discrepancy was explained by experiments demonstrating that the envelope protein (Env) was necessary and sufficient to induce transformation of cells *in vitro* [80,146], demonstrating that JSRV does contain an oncogene, but that oncogene is part of the normal viral genome, the *env* gene. Exactly how Env contributes to oncogenesis is still under investigation.

As previously mentioned, the *env* gene encodes for proteins that are present within the envelope coating the mature virus. These proteins include a surface (SU) protein which binds to cellular receptors, and a transmembrane (TM) protein which spans the viral envelope and functions during fusion between the viral envelope and the host cell membrane[22]. The transmembrane protein has a tail which, when present in the host cell membrane, sticks into the host cytoplasm and can interact with host cell proteins. This cytoplasmic tail contains a YXXM motif, a motif that is well-recognized as an activator of the PI3/Akt pathway of cell growth and replication, commonly activated by neoplastic processes. This discovery theoretically provided

an explanation for how the *env* gene functions as an oncogene, and was supported by experiments examining PI3/Akt activity in JSRV and ENTV[75]. However, other experiments have suggested that while the PI3/Akt pathway is activated in transformed cells, it may not be due to an interaction with the YXXM motif[74], and it may not be the primary determinant of oncogenesis[152].

Additionally, multiple other pathways have been found to be activated by *env* and research is ongoing to elucidate the exact mechanism of oncogenesis[34,66,79]. Figure 1.3 outlines the multiple pathways thought to be involved in JSRV tumorigenesis, with question marks denoting unknown mechanisms for activation. Despite ongoing studies to further define JSRV oncogenesis, it remains constant that the *env* gene functions as oncogene, inducing transformation in JSRV-infected cells, consistent with an acute-transforming retrovirus.

Enzootic Nasal Tumor Virus

Enzootic nasal tumor virus (ENTV) is a close relative of JSRV, with high sequence homology. The amino acid sequences are 92-97% homologous for various regions of the genome[99,137]. Two species of ENTV are recognized, ENTV-1 which infects domestic sheep, and ENTV-2 which causes disease in domestic goats. Both viruses cause enzootic nasal tumors (ENT), also known as ovine nasal adenocarcinoma (ONA). Enzootic nasal tumors are characterized by neoplastic masses within the nasal cavity, typically originating from the ethmoid turbinates within the caudal portion of the nasal cavity. These masses are typically papillary in appearance, forming a cauliflower-like growth that obstructs the nasal passages and produces abundant mucus, the normal product of nasal epithelial cells.

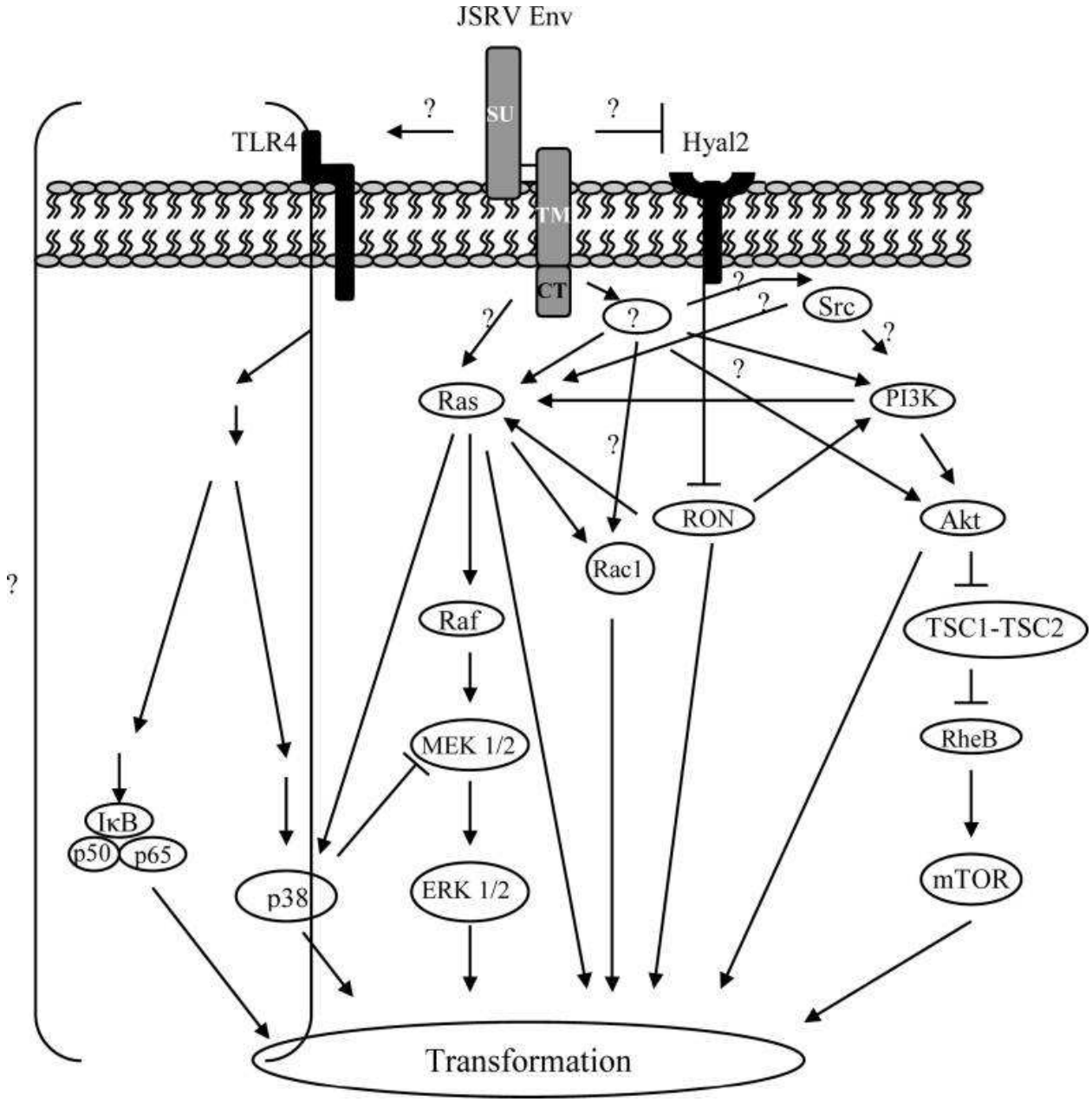

Figure 1.3: Signaling pathways involved in JSRV *env*-induced cell transformation (from Hofacre and Fan 2010)[66].

Like JSRV and lung tumors, virally-induced nasal tumors associated with ENTV are composed of papillary growths of epithelial cells that can consolidate to form solid masses. The epithelial cells of the tumors generally have a benign appearance and tumors very rarely

metastasize[33,112], but the severity of the disease has again led to a designation of adenocarcinoma versus adenoma. The most significant effects of this disease include respiratory distress, invasion of the tumors through the cribiform plate into the brain[43,114,148], and progressive loss of body condition with death due to bacterial infection and/or toxemia[33].

While ENTV nasal tumors are considered epithelial tumors, there is also a significant stromal component to many of the tumors[144]. In many cases, the submucosa of the affected ethmoid turbinates is markedly expanded by a hyperplastic and edematous stroma, within which neoplastic and hyperplastic epithelial cells are embedded. This mesenchymal population has not been described as a neoplastic population, and proviral DNA cannot be amplified from these regions of the tumor[144]. Similar to JSRV, polypoid masses are also often associated with ENTV tumors, particularly in nasal tumors of goats caused by ENTV-2[33].

Experimental transmission has not been as readily demonstrated for ENTV as it has been for JSRV. Early studies of ENTV transmission in the 1950's through the 1970's reported variable results. Some groups reported successful transmission and others were unable to reproduce disease using similar methods[33]. After viral particles were identified in nasal fluids of naturally infected goats with ENT, a transmission study using clarified and concentrated nasal fluids as an inoculum reproduced disease in 1 of 3 intranasally infected goat kids, and 2 of 5 goat kids infected by intrasinusal route[31]. The incubation period for this experiment was 12 to 16 months, much longer than the incubation periods reported in JSRV transmission studies (as short as 10 days). Other unpublished studies in domestic sheep have shown similar low rates of infection or have been unsuccessful[37,144]. The oncogenesis of ENTV appears to be very similar to that of JSRV based on experiments using plasmid constructs of the ENTV Env protein to induce transformation of rodent fibroblasts *in vitro*[1]. Further experiments using this model also

suggested that the signaling pathway for the *env* oncogenes of both JSRV and ENTV are similar[78].

Interestingly, experiments investigating the *env* gene have indicated that while the *env* gene is responsible for oncogenesis, it does not control the cellular tropism of these viruses[40,145]. Because JSRV appears to cause tumors strictly in the lungs and ENTV appears to cause tumors strictly in the nasal passages, it seems logical that this cellular tropism would be related to the *env* protein which interacts with a receptor on the host cell surface (Hyal 2)[86]. Instead, it seems that cell entry is not the limiting factor to cellular tropism, but rather it is the expression of the virus (after integration) that is limited by specific cellular conditions. This tissue-specific viral activation is suspected to occur by way of activation of the LTR[84]. Indeed, the LTR is a highly variable region between the viruses, and thus could explain highly variable cellular tropisms between JSRV and ENTV. Interestingly, a recent article has shown that, in mice, the LTRs of both JSRV and ENTV are activated in both the lung and the upper airway, arguing against the LTR as the sole determinant for tissue tropism[151].

While many questions still remain, a great deal of research has been accomplished investigating the molecular mechanisms behind JSRV infection and oncogenesis. Much of this work has been supported due to the similarities between JSRV and human lung cancer, with hopes that JSRV research may provide insights to mechanisms of oncogenesis in human cancer patients. Less work has been done to investigate the molecular mechanisms of ENTV and the likely myriad of differences between the two viruses. While the information provided here lends a background for mechanisms of retroviral oncogenesis, a virus related to ENTV and JSRV may have unique properties that cannot be explained based on our current understanding of sheep retroviruses.

CHAPTER TWO – DESCRIPTION OF A NEW DISEASE ENTITY[1]

Summary

Here we describe 10 cases of paranasal sinus masses in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). Among 21 bighorns from 11 herds in the state of Colorado, USA that were examined, 10/21 (47.6%) individuals from 4/11 (36%) of the sampled herds had masses arising from the paranasal sinuses. Affected animals included 9/17 (53%) of the females and 1/4 (25%) of the males, ranging in age from approximately 2 years to greater than 10 years. Defining gross features of these masses included unilateral or bilateral diffuse thickening of the respiratory lining of the maxillary and/or frontal sinuses, with abundant seromucinous exudate in the affected sinus cavities. Defining histological features of these masses included chronic inflammation, and proliferation of both mesenchymal and epithelial cells of the mucosa and submucosa. Epithelial changes included hyperplasia of mucosal epithelium, hyperplasia of submucosal glands and ducts, and neoplasia (adenocarcinoma). Mesenchymal changes included submucosal myxedema, submucosal fibroplasia/fibrosis, bone destruction, and neoplasia (myxomatous fibroma). Specific immunohistochemistry and polymerase chain reaction for Jaagsiekte sheep retrovirus and enzootic nasal tumor virus were performed with negative results.

Materials and Methods

Animals

In February, 2009 as part of ongoing respiratory disease investigation and management activities, a remnant (n=7) free-ranging band of female bighorn sheep from the Poudre River canyon in northern Colorado, USA was culled for population management purposes. Post-

[1] The work presented in this chapter was published in: Veterinary Pathology 2011 May; 48(3):706-12. Title: Paranasal Sinus Masses of Rocky Mountain Bighorn Sheep (*Ovis canadensis canadensis*). Authors: Karen A. Fox, Sarah K. Wootton, Sandra L. Quackenbush, Lisa L. Wolfe, Ivy K. LeVan, Michael W. Miller, and Terry R. Spraker.

mortem examinations were performed on each carcass. In addition to variable lesions of chronic bronchopneumonia, paranasal sinus masses were identified in all seven animals. Following the discovery of this novel lesion, all bighorn sheep submitted for necropsy to the Colorado Division of Wildlife were screened for the occurrence of similar masses. From February 2009 through September 2009, a total of 21 bighorn sheep one year of age or older were examined including 17 females and 4 males, ranging in age from approximately 2 years to greater than 10 years. For the majority of cases, the post-mortem interval was less than six hours, although some carcasses had been frozen prior to examination and post-mortem interval could not be determined.

Histopathology

For all carcass submissions, representative sections of all major organ systems present, including paranasal sinus masses, were fixed in 10% neutral buffered formalin. Selected sections were embedded in paraffin blocks, sectioned at 4-6 µm, and stained with hematoxylin and eosin for examination by light microscopy. Selected sections were additionally stained using the Periodic Acid Schiff (PAS) reaction, Alcian blue (pH 2.5) stain, and Masson's trichrome stain. Samples of all masses and exudates present within the paranasal sinuses were also collected aseptically and stored at -80° C for additional diagnostics.

Immunohistochemistry

To detect specific viral antigens of known sheep retroviruses, immunohistochemistry (IHC) was performed on paranasal sinus masses (n=7) using monoclonal antibody (mAb) with reactivity for the envelope protein of enzootic nasal tumor virus (ENTV) and Jaagsiekte sheep retrovirus (JSRV) as previously described [147]. Briefly, samples were deparaffinized and antigen retrieval was performed in a pressure cooker (heat to 120°C, hold for 3 minutes, allow to cool to 90°C, hold for 3 minutes) using Antigen Unmasking Solution (pH 6) (Vector Laboratories,

Burlingame, CA, USA). After cooling, endogenous peroxide was quenched with 3% hydrogen peroxide for 5 minutes. Slides were washed two times for 10 minutes each with phosphate buffered saline (PBS). The slides were incubated with a 1:50 dilution of anti-JSRV envelope mAb (from hybridoma cells) for 1 hour at room temperature. This anti-JSRV envelope antibody cross-reacts with the ENTV envelope[147]. Slides were washed and incubated with a 1:300 dilution of biotinylated horse-anti-mouse IgG (Vector Laboratories) for 30 minutes at room temperature. Slides were washed again and incubated with avidin:biotinylated enzyme complex (Vectastain Elite ABC kit, Vector Laboratories). 3,3'-diaminobenzidine tetrahydrochloride (DAB) with nickel chloride enhancement was used as a peroxidase substrate and the sections were counterstained with hematoxylin.

To further characterize proliferative and neoplastic cells, IHC for vimentin and pancytokeratin was performed using an indirect biotin-free system (ultraView Universal Alkaline Phosphatase Red Detection Kit, Ventana Medical Systems, Tuscon, AZ, USA) designed for use with an automated immunostainer (NexES immunohistochemical module, Ventana Medical Systems). Primary antibodies used were pan-keratin (Ventana Medical System) and vimentin (Ventana Medical Systems).

Polymerase Chain Reaction

For polymerase chain reaction (PCR), genomic DNA was extracted from sample (n=9), positive control (n=3), and negative control (n=1) tissue homogenates using phenol/chloroform extractions[119]. All tissues had been maintained at -80°C following removal at necropsy. Positive control genomic DNA for JSRV was extracted from lung tumor tissue of a 3 month old male domestic sheep (*Ovis aries*) with experimentally induced pulmonary adenocarcinoma. Positive control genomic DNA for ENTV-1 was extracted from nasal tumor tissue of a 4.5 year old

female domestic (Dorset breed) sheep with naturally occurring nasal adenocarcinoma. Positive control genomic DNA for ENTV-2 was obtained from an approximately 3 year old domestic goat (*Capra hircus)* with naturally occurring disease. Negative control genomic DNA used for both ENTV and JSRV PCR was obtained from lung tissue of a 3 year old female domestic (Suffolk breed) sheep lacking clinical signs of ENTV or JSRV. PCR was performed using specific primers for JSRV, ENTV-1, and ENTV-2.

PCR for ENTV-1 was performed using specific primers to amplify a 1400 base pair product from the *gag* gene: fw 5'-ATCCGTCCCTAGATTCGTC-3' and rv 5'-TGTTTAGACGGTGGAGGAAA-3'. Each 50 µL PCR reaction contained 45 µL Platinum® PCR Supermix (Invitrogen), 40 pmol of each primer, and 100 ng of genomic DNA. Thermocycling parameters included initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 68°C for 45 seconds. Final extension was at 68°C for 3 minutes. PCR for ENTV-2 was performed using specific primers to amplify a 180 base pair product from the U3 region[99]: fw 5'-GCAAAATGCCAGGACCTTGG-3' and rv 5'-GATCTTATCTGCTTATTTTCAG-3'. Each 25 µL PCR reaction contained 22 µL Platinum® PCR Supermix (Invitrogen), 20 pmol of each primer, and 500 ng of genomic DNA. Thermocycling conditions included initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for 30 seconds. Final extension was at 72°C for 3 minutes. PCR for JSRV was performed using specific primers to amplify a 300 base pair product from the *gag* gene: fw 5'-CCCCATCTCTGAAAATGCAC-3' and rv 5'-TGTTTAGACGGTGGAGGAAA-3'. Each 50 µL PCR reaction contained 45 µL Platinum® PCR Supermix (Invitrogen), 40 pmol of each primer, and 200 ng of genomic DNA.

Thermocycling conditions included initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 68°C for 45 seconds. Final extension was at 68°C for 3 minutes.

Results

Gross Pathology

Of the 21 carcasses examined, 10/21 animals had masses arising from the lining of the paranasal (maxillary and/or frontal) sinuses, including 9/17 females and 1/4 males from 4/11 sampled herds. In the index herd where all 7 individuals had paranasal sinus masses, all of the animals were greater than 10 years of age. Sample sizes for all other herds were very small ($n \leq 3$) and therefore prevalence for these herds was not calculated.

Grossly, masses ranged from moderate and diffuse thickening of the sinus lining to solid masses filling the sinus cavity. No discrete polypoid masses were identified, and in only one case, the mass extended to involve the palatine sinus and caudal nasal turbinates (case No. 10). Lesions were bilateral in 8/10 cases and unilateral in 2 cases. In all cases, the bone underlying the mass was either grossly invaded or had an irregular, pitted surface suggesting bone remodeling. The thickened sinus lining was easily separated from the underlying bone except in one case (case No. 8), for which the mass severely invaded the bone surrounding the cornual diverticulum of the frontal sinus, causing sloughing of the horn and protrusion of the mass from the top of the skull (Fig. 2.1). All masses were homogenous, white, shiny, soft to gelatinous, and frequently contained mucinous cysts. The affected sinus cavities were often filled with seromucinous to mucopurulent exudate. In at least two cases, similar mucinous material had been noted antemortem as nasal discharge.

Additional necropsy findings of the respiratory tract included moderate numbers of lungworm nodules in 9/10 animals, bronchopneumonia in 3/10 animals, fibrous pleural adhesions suggesting previous bronchopneumonia in 2/10 animals, and a tooth root abscess into the maxillary sinus of one animal. No *Oestrus ovis* larvae were seen in any of the carcasses examined. Cause of death in all animals was considered to be unrelated to the paranasal sinus masses, or other respiratory disease.

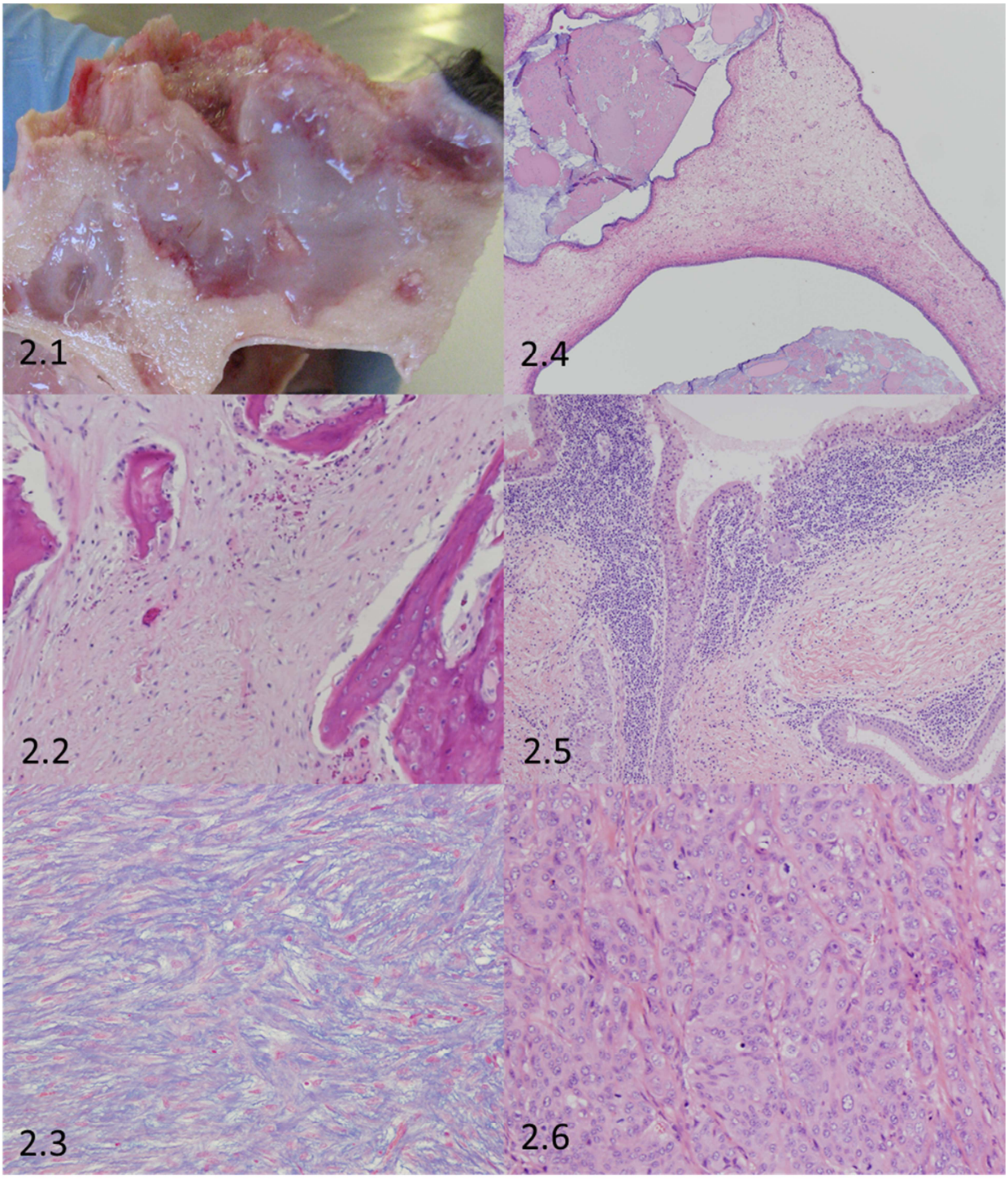

Fig. 2.1. Coronal section of right side of skull with frontal sinus mass; bighorn sheep, case No. 8. A homogenous, shiny, soft to gelatinous white mass fills the frontal sinus, with marked invasion of the surrounding bone. The mass has destroyed the bone of the cornual diverticulum, with sloughing of the horn and protrusion of the mass from the top of the skull.

Fig. 2.2. Histopathology of frontal sinus mass from Figure 2.1; bighorn sheep, case No. 8. The mass (myxomatous fibroma) is composed of dense intersecting bundles of plump spindle cells supported by scant myxomatous stroma. The neoplastic cells have effaced the underlying bone, with few remnant bone trabeculae present. Bone trabeculae are often surrounded by a rim of osteoblasts and fewer osteoclasts. Hematoxylin and eosin.

Fig. 2.3. Histopathology from the frontal sinus mass from Figure 2.1; bighorn sheep, case No. 8. In this area of loosely arranged fibroblasts, staining with Alcian blue demonstrates a background of acid mucopolysaccharides (blue). Alcian blue (pH 2.5).

Fig. 2.4. Histopathology of maxillary sinus mass; bighorn sheep, case No. 6. The mass is composed of a hyperplastic myxomatous stroma, with multifocal large cystic mucin-filled glands lined by well-differentiated epithelial cells. Hematoxylin and eosin.

Fig. 2.5. Histopathology of maxillary sinus mass; bighorn sheep, case No. 7. This section of the mass is composed of hyperplastic ductular and surface epithelial cells with multifocal areas of dysplasia. Associated with the hyperplastic surface epithelium is marked, dense plasmacytic inflammation. Hematoxylin and eosin.

Fig. 2.6. Histopathology of frontal sinus mass; bighorn sheep, case No. 10. The mass (adenocarcinoma) is composed of sheets and nests of neoplastic epithelial cells severely invading the submucosa, with moderate anisokaryosis, anisocytosis, and many mitotic figures. Hematoxylin and eosin.

Histopathology

All paranasal sinus masses examined had components of both epithelial and mesenchymal proliferation within the mucosa and submucosa of the sinus lining, and lesions varied from hyperplasia to neoplasia (Table 1). Epithelial and mesenchymal origin of cells was confirmed using immunohistochemistry (IHC) for pancytokeratin and vimentin respectively.

The majority of the masses examined were predominated by mesenchymal proliferation within the submucosa. These masses contained a population of well-differentiated spindle to stellate cells forming a well-vascularized, loose edematous or myxomatous stroma, to dense fibroplasia and fibrosis (Table 2.1).

Table 2.1. Histologic features for 10 cases of paranasal sinus masses in Rocky Mountain bighorn sheep. A "+" sign indicates presence of the listed histologic feature.

	Case Numbers									
Histologic features	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Lymphoplasmacytic Sinusitis	+	+	+	+	+	+	+	+	+	+
Stromal Myxedema	+	+	+	+	+	+	+	+	+	
Stromal Fibrosis/Fibroplasia	+	+	+	+	+	+	+	+	+	
Bone Invasion						+	+	+	+	
Myxomatous Fibroma								+		
Ductular Hyperplasia	+	+	+	+	+	+	+	+	+	+
Glandular Hyperplasia				+	+	+	+	+	+	+
Surface Epithelial Hyperplasia					+	+	+	+	+	+
Adenocarcinoma									+	+

In selected cases, the presence of a myxomatous matrix composed of acid mucopolysaccharides was confirmed by blue staining with Alcian blue, and a lack of magenta staining with the Periodic Acid Schiff (PAS) reaction. The presence of collagen fibers was demonstrated by blue staining with Masson's trichrome. In all cases, spindle cells were well differentiated despite frequent invasion of the underlying bone.

A single mass (case no. 8) was diagnosed as a myxomatous fibroma based on cellular features including massive proliferation of well-differentiated fibroblasts and collagen bundles (Fig. 2.2), and a variably loose myxomatous background (Fig. 2.3). Although the cells were well differentiated with minimal anisocytosis and anisokaryosis and rare mitotic figures, the diagnosis of neoplasia was made based on marked invasion and destruction of the surrounding bone in the absence of significant inflammation. The remnant bone spicules were often rimmed by osteoblasts and fewer osteoclasts (Fig. 2.2), suggesting either bone remodeling, or bone production by the tumor. Additional differentials considered for this mass included myxoma, myxosarcoma, ossifying fibroma, fibrous dysplasia, and periosteal fibrosarcoma.

Although typically less prominent than the mesenchymal proliferation, all masses were characterized by hyperplasia of epithelial components including the pseudostratified ciliated surface epithelium, submucosal serous and mucous glands, and submucosal simple cuboidal ductular epithelium (Table 2.1). Frequently, masses contained clusters of well-differentiated acini deep within the submucosa, as well as many large cystic structures containing abundant intraluminal (PAS-positive) mucin, lined by well-differentiated epithelial cells (Fig. 2.4). Hyperplastic epithelial cells occasionally demonstrated multifocal dysplasia but lacked prominent features of neoplasia (Fig. 2.5).

Two masses were diagnosed as adenocarcinoma based on the presence of poorly differentiated epithelial cells forming sheets and solid nests of cells deep within the submucosa, in addition to more differentiated tubuloacinar structures. One of these cases (case No. 10) additionally had frequent mitotic figures and moderate anisocytosis and anisokaryosis (Fig. 2.6).

Both hyperplastic and neoplastic masses frequently contained a significant population of inflammatory cells. Lymphoplasmacytic sinusitis was diagnosed in 10/10 cases, characterized by dense infiltrates of well-differentiated plasma cells and fewer lymphocytes, associated with proliferating epithelial cells (Fig. 2.5), and occasionally located within perivascular spaces. Additionally, cystic masses occasionally contained intraluminal suppurative exudate suggesting secondary bacterial infection. Aerobic culture of sinus exudate from one case (Case no. 10) yielded heavy growth of *Pasteurellaceae*. Similar inflammatory lesions were not present in the sinus linings of unaffected animals.

Histopathology of retropharyngeal lymph node was performed for all cases, with no evidence of neoplastic metastasis. Moderate to marked lymphoid hyperplasia, and increased numbers of plasma cells in lymph node sinuses, were noted in all cases.

Molecular diagnostics

Genomic DNA was extracted from 9/10 masses as well as positive and negative controls. None of the 9 samples, nor negative controls were positive by PCR using specific primers for JSRV and ENTV. IHC for envelope protein, with demonstrated reactivity for both JSRV and ENTV[147] was performed for 7/10 cases, and results were negative for all cases examined. The single case that was not evaluated by PCR was evaluated by IHC.

Discussion

Following the discovery of paranasal sinus masses in 7/7 Rocky Mountain bighorn sheep from a single herd in Colorado, we hypothesized that these masses represented a novel disease entity. To further investigate this hypothesis, we sampled additional animals to document the occurrence of this disease, to further characterize the disease, and to test for viral agents that are known to cause similar diseases in domestic sheep and goats.

Although the gross pathology and histopathology of these masses was variable, defining gross and histologic features were present in all cases. Defining gross features of all masses included diffuse thickening of the respiratory lining of the maxillary and/or frontal sinuses, and abundant seromucinous exudate in the affected sinus cavities. This gross lesion varied in severity, with the most severe cases characterized by masses filling the sinus cavity and markedly invadion and destruction of the underlying bone.

The defining histopathological feature of all masses was proliferation of both mesenchymal and epithelial cells, with neoplasia (mesenchymal or epithelial) at the most extreme end of a presumed continuum of changes.

These features are not unlike features of the recognized disease entity, enzootic nasal tumor (ENT), of domestic sheep and goats. Clinically, domestic sheep and goats affected by ENT have abundant seromucinous nasal discharge[32,38,43,85,95,114,148,150]. Grossly, masses originate from the ethmoid turbinates and expand to fill the nasal cavity, with frequent invasion of the surrounding paranasal sinuses associated with tumor expansion[32,43,85,148]. Tumors vary from soft, shiny, white gelatinous masses to firm, meaty, or granular, grey-red masses[38,43,85,148,150]. Histologically, these tumors are classified as adenomas[38,85,114,148], adenopapillomas[38,95,150], or low

grade adenocarcinomas[32,38,43,85,114,148,150]. Non-neoplastic, hyperplastic inflammatory polyps are occasionally found adjacent to neoplastic masses[32,38,114].

Inflammatory polyps are focal, raised, often pedunculated masses, with an edematous, often chronically inflamed fibrovascular core, lined by mucosal epithelium[44]. When associated with ENT, these hyperplastic masses have been proposed to be pre-neoplastic lesions, but this association has not been proven[114]. Histologically, polyps of affected domestic sheep and goats have a highly edematous stroma, and marked lymphoplasmacytic infiltrates[32,114]. The overlying epithelium is often hyperplastic[32,114].

Similarities between ENT and paranasal sinus masses in bighorn sheep include the presence of seromucinous nasal discharge clinically, the gross finding of a soft white mass in the sinus cavity, and classification of some masses as adenocarcinoma. Additionally, the inflammatory nasal polyps often associated with ENT share characteristics with the hyperplastic masses described here for bighorn sheep, although in bighorn sheep the mass is a diffuse thickening of the sinus lining, and not a discrete polypoid mass.

Prominent differences between ENT and the masses described here are location (paranasal in bighorn sheep and nasal in domestic sheep and goats) and malignancy (predominantly hyperplastic masses in bighorn sheep and predominantly neoplastic masses in domestic sheep and goats). Interestingly, in one bighorn sheep case the mass did extend to involve the nasal turbinates (Case no. 10), and this mass was classified as an adenocarcinoma.

Other prominent differences between the two entities include the papillary appearance and often grey-red color of ENT tumors that is not characteristic of bighorn sheep masses, and the prominent mesenchymal population present histologically in bighorn sheep masses but infrequently described for ENT.

Because the masses discovered in bighorn sheep were somewhat reminiscent of ENT, we considered enzootic nasal tumor virus (ENTV) or a closely related retrovirus as a possible etiology. Due to the close-relatedness of ENTV-1 (the causative agent of ENT in domestic sheep), ENTV-2 (the causative agent of ENT in domestic goats), and Jaagsiekte sheep retrovirus (JSRV), we screened samples for all 3 viruses. Our negative findings indicate that these specific retroviruses are unlikely to be involved in the pathogenesis of the lesion described here. Alternative hypotheses for the cause of these masses include infection by other viral agents, genetic predisposition, toxins, and chronic inflammation including chronic bacterial infections.

Chronic inflammation associated with the nasal bot, *Oestrus ovis*, has been suggested as a possible associated condition occurring with ENT[42,95] , although no association has been proven. *Oestrus ovis* infection is relatively common in bighorn sheep and thus may be a source of chronic inflammation in the sinus cavities. However, *Oestrus ovis* larvae or eosinophilic infiltrates were not found in the cases examined in this study. A syndrome of chronic sinusitis has been reported in desert bighorn sheep (*Ovis canadensis nelsoni*), and is attributed to aberrant migration of *Oestrus ovis* larvae[2,19,107]. These lesions are characterized by extensive bone destruction in the maxillary and frontal sinuses and are described predominantly as osteonecrosis and osteolysis[2,19,107]. However, some cases of chronic sinusitis with osteolysis have been diagnosed after decomposition of soft tissues[19], and therefore the diagnosis of either chronic sinusitis or paranasal masses should be made with caution for desiccated specimens.

While the cause of bighorn sheep paranasal sinus masses remains uncertain, the continuum of lesions among cases suggests a shared etiology. However, the examination of additional cases and more extensive diagnostics will be necessary to further define this disease and investigate possible infectious etiologies.

Regardless of etiology, the changes to the normal sinus respiratory mucosa, space occupying nature, abundant mucus production, and bone invasion of these masses may affect normal upper respiratory function, and are factors to consider when investigating bighorn sheep respiratory disease. Therefore, continued surveillance for paranasal sinus masses, in addition to continued attempts to identify an inciting cause, will be important for future management of affected herds.

CHAPTER THREE – FACTORS ASSOCIATED WITH BIGHORN SHEEP SINUS TUMORS

Summary

To determine possible factors associated with bighorn sheep sinus tumors, we explored the distribution of sinus tumors based on variables of age, sex, geographic distribution, morbidity, and co-infections with bacterial organisms. We used a series of Fisher's exact tests to investigate potential associations between these factors and the presence of sinus tumors. We found sinus tumors to be nonrandomly distributed geographically, with nearly all cases of sinus tumors restricted to three herds of bighorn sheep. This supports the hypothesis that sinus tumors are an infectious disease, maintained within certain infected populations. We also found sinus tumors to be associated with the presence of pneumonia-causing bacterial agents in sinus lining tissues, suggesting the potential for sinus tumors to allow increased proliferation of these potentially pathogenic organisms in the upper respiratory tract. No significant relationships were found between sinus tumors and age, sex, or morbidity, although trends in these categories may warrant further investigation.

Introduction

Bighorn sheep sinus tumors are a recently-described disease in Rocky Mountain bighorn sheep. While several characteristics of the disease resemble oncogenic retroviral diseases of domestic sheep and goats, the cause of bighorn sheep sinus tumors remains unknown.[53] We suspect that bighorn sheep sinus tumors are caused by an infectious agent, likely a viral agent, based on similarities to known virally-induced tumor diseases. Such a disease could have significant implications for bighorn sheep populations, and we were interested in further examining this disease at a population level. To do so, we created specific diagnostic criteria, based on gross pathology, to categorize lesions in bighorn sheep paranasal sinuses as tumor-

positive, tumor-suspect, and tumor-negative. We then retrospectively examined population variables to determine if tumor-positive and tumor-negative cases were randomly or nonrandomly distributed across the population for each variable. Variables we considered included geographic distribution, morbidity (based on cause of death and presence of concurrent respiratory disease), co-infections with bacterial organisms, age, and sex.

Methods

Cases

From February 2009 through July 2012 we examined a total of 136 bighorn sheep carcasses. Carcasses were obtained through Colorado Parks and Wildlife (free-ranging or captive animals found dead or euthanized and submitted for necropsy) and through taxidermists (after removing the skull cap/horns for taxidermy, the rest of the skull was donated for examination). Cases included full carcasses (n=62) and partial carcasses (n=74). Partial carcasses included taxidermy cases, and other cases for which at least the head, but not the entire carcass, was available for examination. Cases included male (n=68) and female (n=68), with ages ranging from 9 months to >10 years. Animals younger than 9 months were not included in the study based on the inability to assess the maxillary sinus cavity for lesions, as no molars have erupted from the maxillary sinus cavity and immature tooth material fills the sinus cavity. Six of the 136 carcasses were desert bighorn sheep (*Ovis canadensis nelsoni*), and the remaining 130 carcasses were Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*).

The most likely cause of death was recorded based on history and necropsy findings. Causes of death included hunter-killed (n=43), struck by vehicle (n=28), capture mortality (n=8), predation (n=5), fall from cliff (n=5), other trauma (n=5), cull due to ill thrift (n=5), cull due to disease in population (n=7), cull due to interaction with domestic animals (n=2), death from

respiratory disease (n=5), death from other disease (n=1), and unknown history (n=22). If lungs were present for examination, lesions of ongoing or previous pneumonia were recorded. Geographic location was recorded as the Colorado Parks and Wildlife bighorn sheep management unit (SMU) and data analysis unit (DAU).

Diagnostic Criteria

For each carcass, we grossly examined the paranasal sinus cavities, and categorized each carcass as tumor-negative, tumor-suspect, or tumor-positive based on the criteria provided in Table 3.1. Criteria were determined after all cases had been examined, due to the known continuum of gross lesions that characterize this disease[53]. With this continuum of lesions, it was difficult to determine cutoffs for negative, suspect, and positive criteria prior to seeing the entire spectrum of lesions. After all of the lesions had been examined and recorded, categorical criteria were determined with the intention of making the "suspect" category inclusive to cases which could be part of the bighorn sheep sinus tumor disease spectrum, but could also be due to other nonspecific diseases causing thickening of the sinus lining. The "positive" criteria were designed to be exclusive to cases of bighorn sheep sinus tumors. Each lesion examined fell exclusively into one of the three categories based on the criteria developed. Histologic criteria were not included in the categorical criteria because not all samples were suitable for histological examination. However, for those tissues examined histologically, the tumor-suspect cases demonstrated primarily features of hyperplasia, while the tumor-positive cases demonstrated hyperplastic, as well as dysplastic and/or neoplastic features. Examples of gross lesions of tumor-negative, tumor-suspect, and tumor-positive cases are illustrated in Figures 3.1 through 3.14.

Table 3.1: Gross criteria for bighorn sheep tumor-negative, tumor-suspect, and tumor-positive cases.

Category	Criteria for inclusion
Negative	No thickening of sinus lining, and No mucus in sinus cavities, and No cystic expansions of sinus lining
Suspect	Sinus lining thickened, but < 5 mm, and/or Mucus present in sinus cavities, and/or Cystic expansions of sinus lining
Positive	Sinus lining thickened to > 5 mm and Thickened tissue is edematous with a wet appearance and Invasion or remodeling of underlying bone

Gross Images, Bighorn Sheep Sinus Tumors

Figure 3.1: Bighorn sheep with snotty nose, reflecting mucus within the sinus cavities.

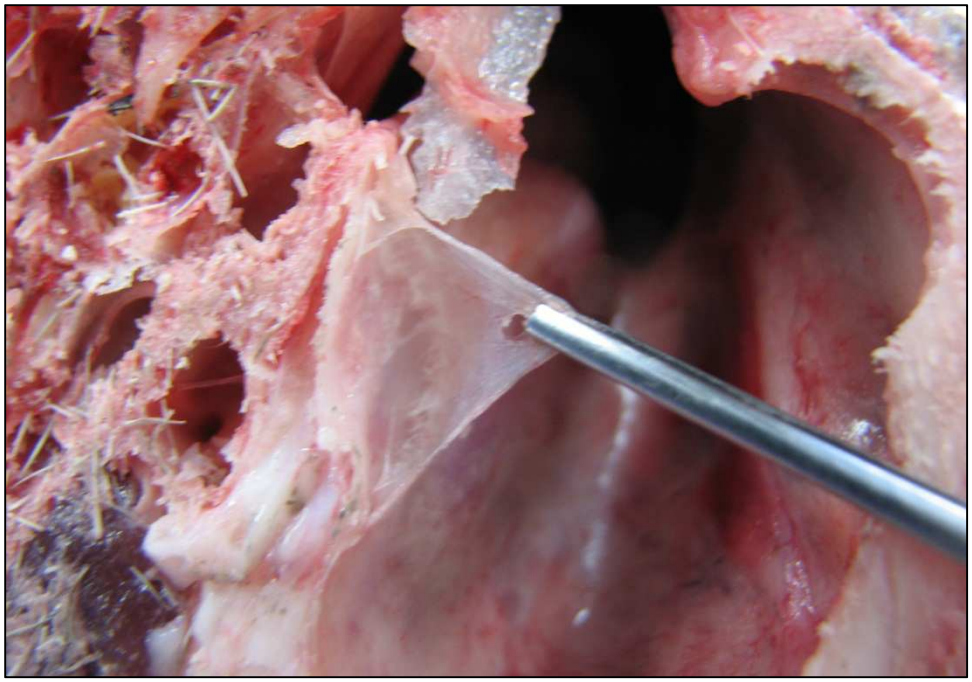

Figure 3.2: Tumor-negative case demonstrating normal, paper-thin sinus lining.

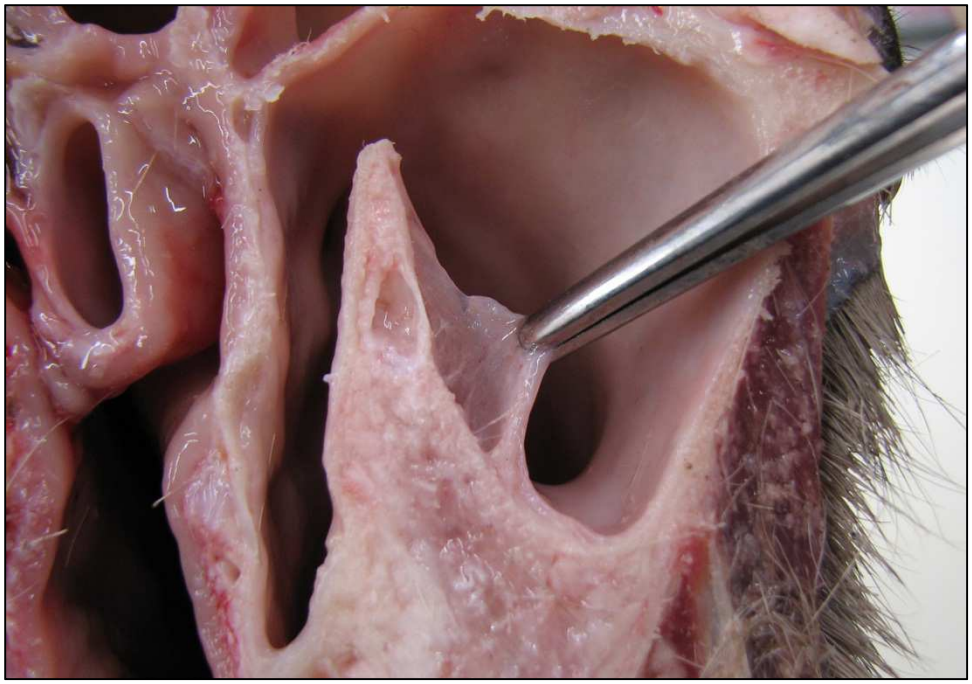

Figure 3.3: Tumor-suspect case with thickened sinus lining < 5 mm thick.

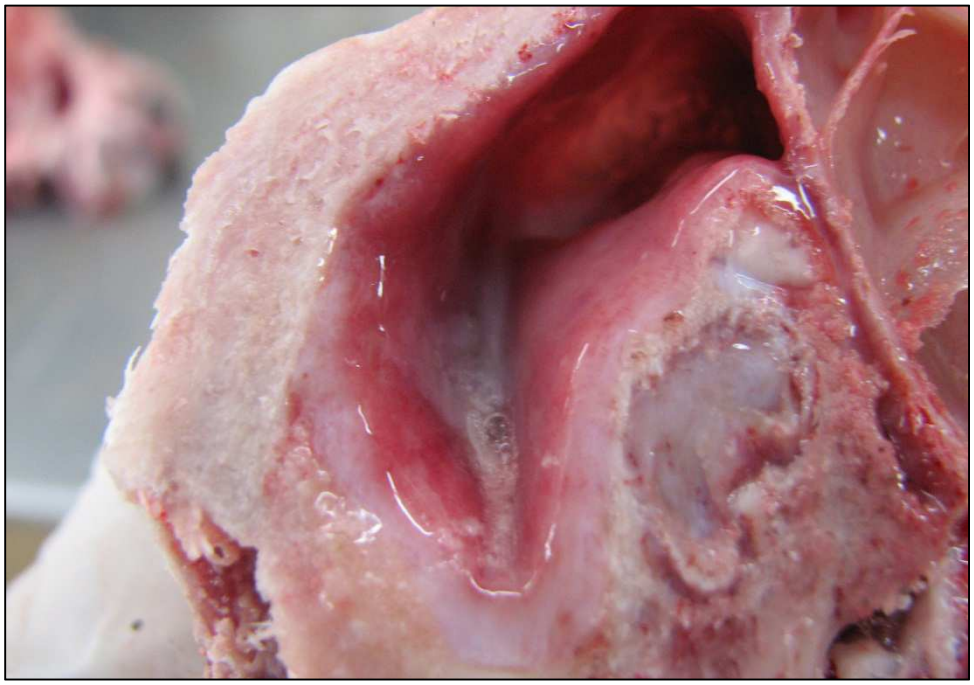

Figure 3.4: Tumor-positive case with thickened maxillary sinus lining > 5 mm thick. Also note wet/edematous appearance to the tissue and pool of mucus within the sinus cavity.

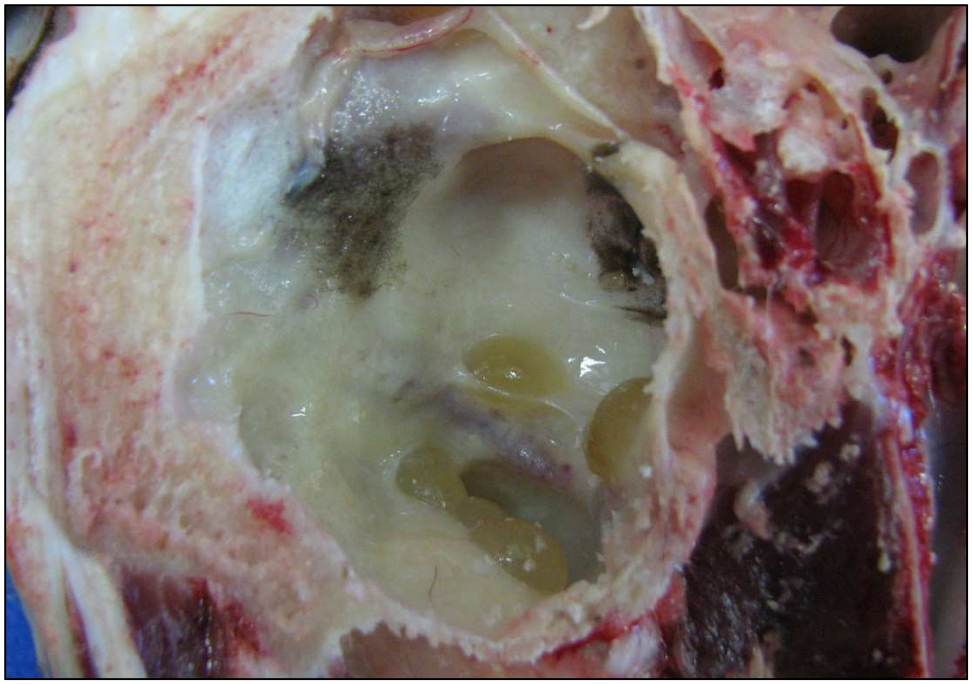

Figure 3.5: Tumor-suspect case with cystic expansions of the maxillary sinus lining.

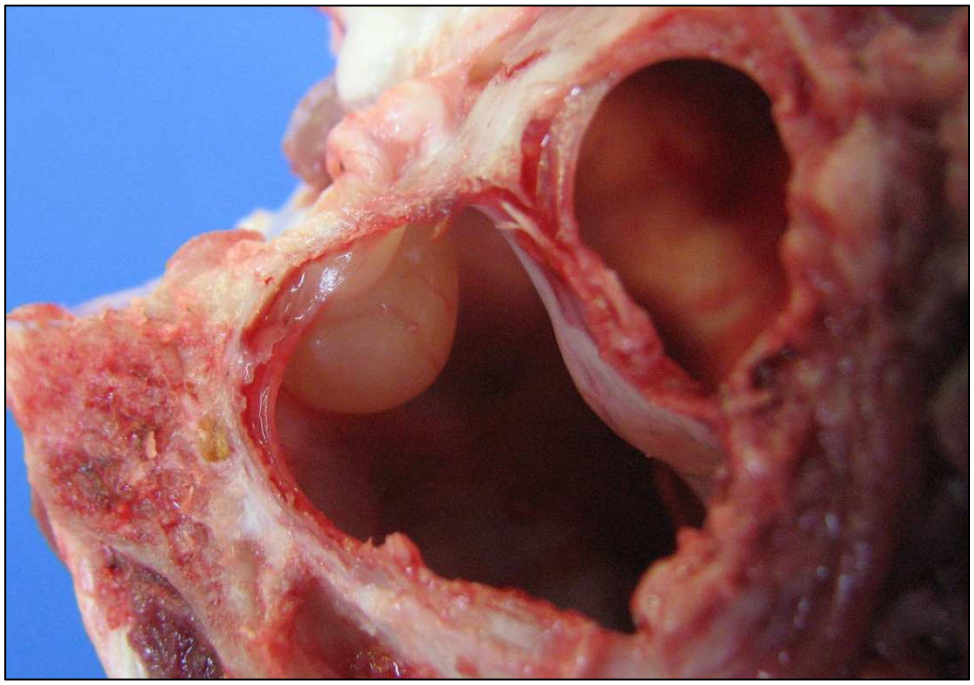

Figure 3.6: Tumor-positive case with cystic expansions of the maxillary sinus lining. This lesion was found in association with a sinus tumor (pictured in Figure 3.7).

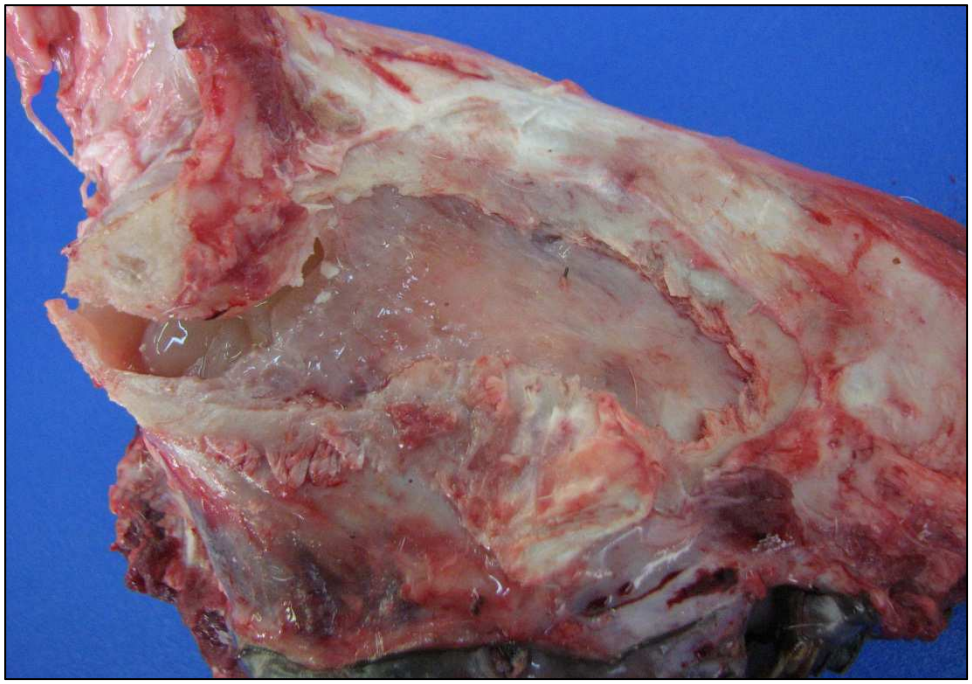

Figure 3.7: Tumor filling right maxillary sinus of bighorn sheep skull. Lateral view, with outer bone removed, exposing maxillary sinus which is filled with tumor material.

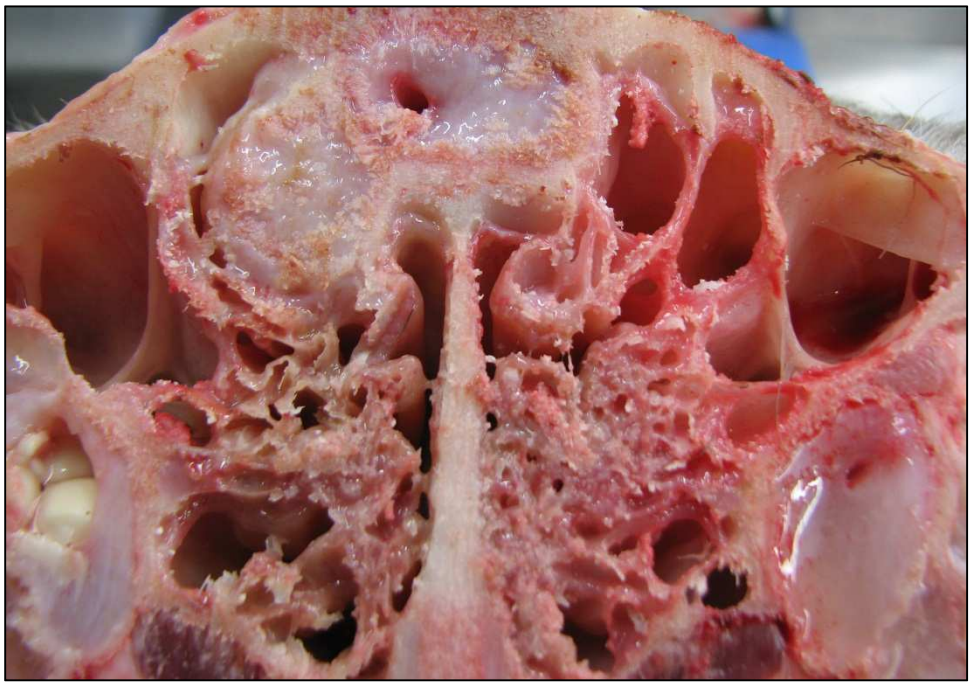

Figure 3.8: Sinus tumor filling maxillary sinuses bilaterally (note inspissated purulent exudate in maxillary sinus on left side of image), and partially filling frontal sinus, with destruction of the surrounding bone (near top of image).

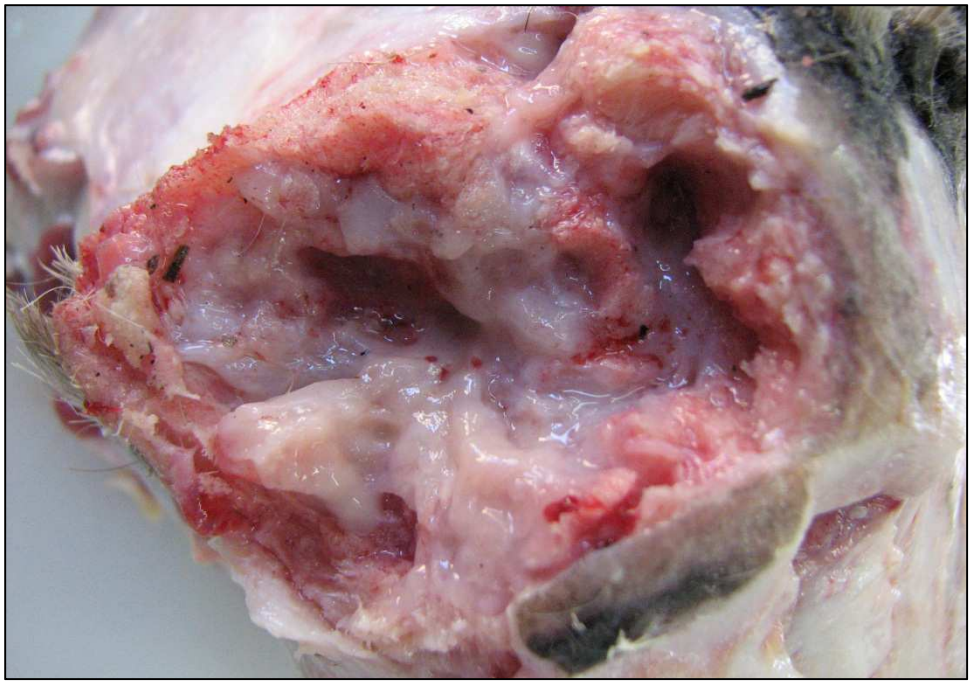

Figure 3.9: Bighorn sheep sinus tumor of frontal sinus. Invasion through skull has caused sloughing of the horn. View is through top of head at previous horn base.

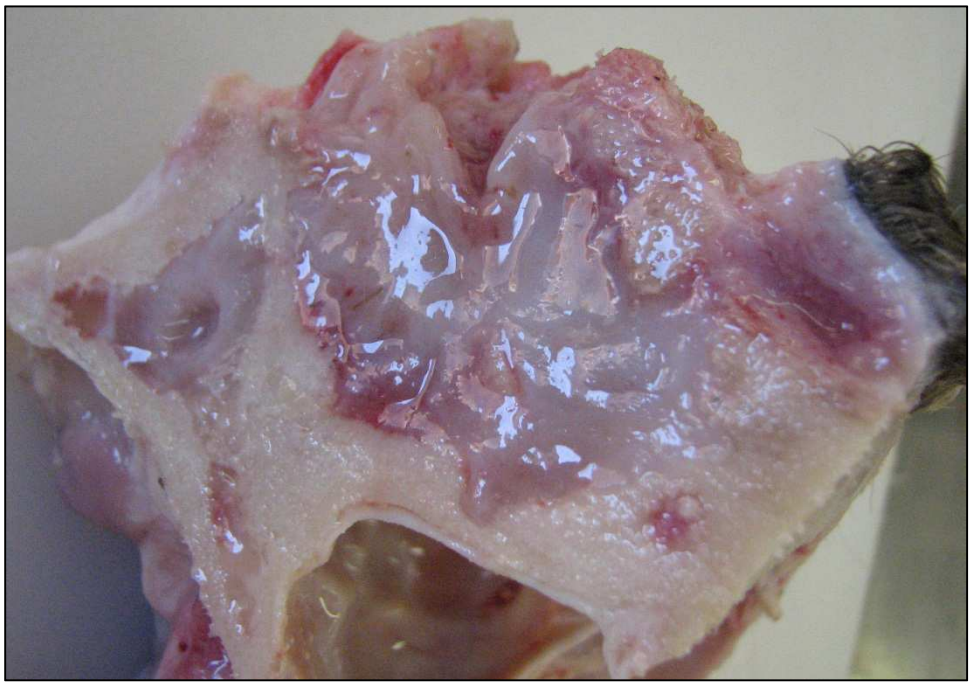

Figure 3.10: Cross section of skull from Figure 3.9, showing tumor filling the frontal sinus and destroying normal bone.

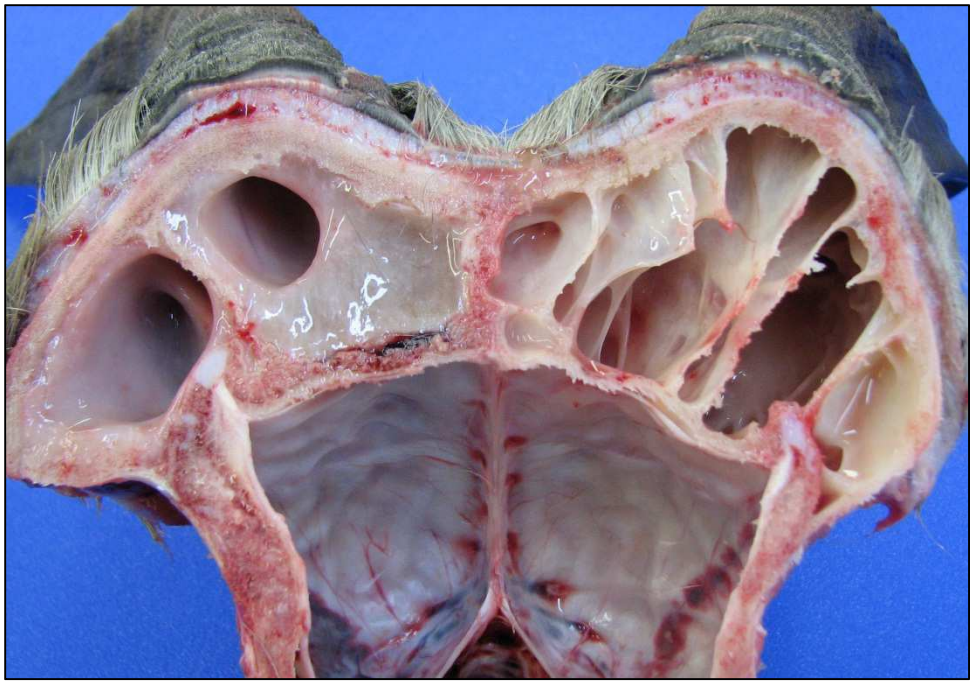

Figure 3.11: Unilateral tumor in frontal sinus (left side of image) of a yearling bighorn sheep ram.

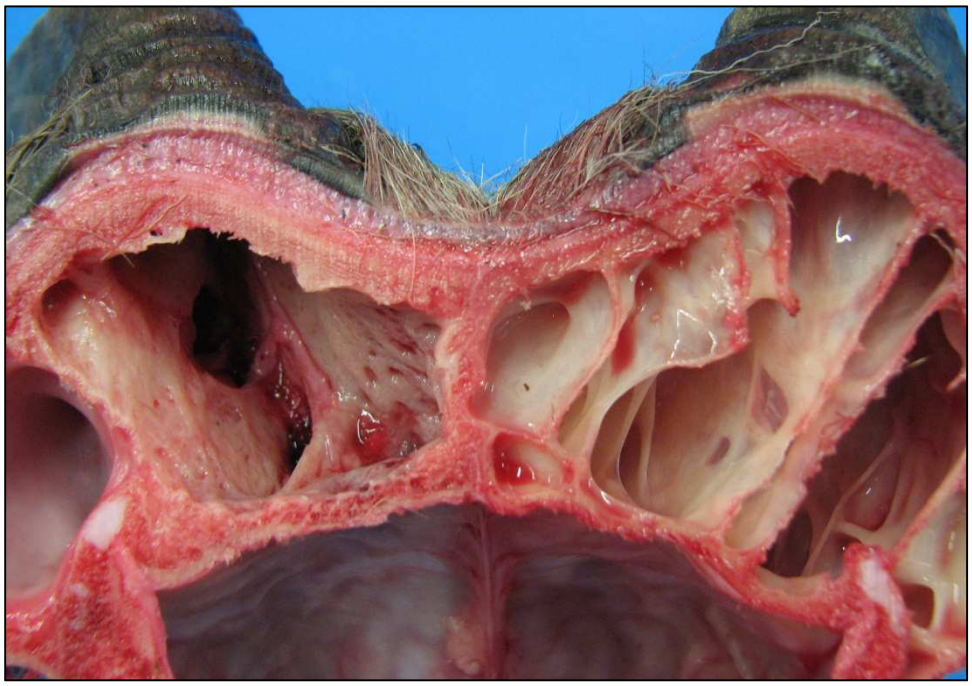

Figure 3.12: Skull from Figure 3.11 with tumor tissue removed, showing extensive remodeling of bone underlying the tumor.

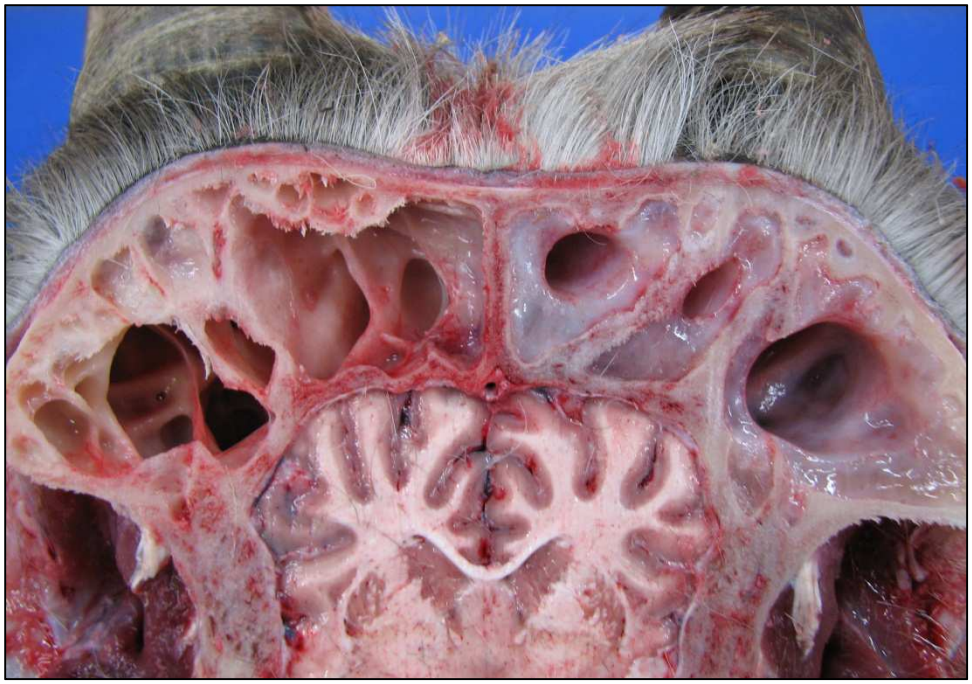

Figure 3.13: Unilateral sinus tumor filling frontal sinus (on right side of image).

Figure 3.14: Sinus tumor from Figure 3.13, extending into horn.

Analysis of factors associated with sinus tumors

To investigate bighorn sheep sinus tumors at a population level, we retrospectively conducted statistical analyses of various factors available for each carcass including geographic location, morbidity (determined by cause of death and concurrent respiratory disease), bacterial co-infections, age, and sex. To evaluate our dataset for possible associations between sinus tumors and these specific variables, we applied the following methods for each factor:

Geographic Location

Epidemiologically-relevant geographic location data were available for 127/136 carcasses. The remaining 9 carcasses were captive animals from the Colorado Parks and Wildlife Foothills Wildlife Research Facility (FWRF), and these carcasses were not included in the geographic location analysis based on captive animals originating from multiple free-ranging source herds in Colorado. For the 127 free-ranging animals, geographic location was recorded as the Colorado Parks and Wildlife sheep management unit (SMU) and Colorado Parks and Wildlife data analysis unit (DAU) in which each carcass was found. A DAU may be composed of multiple SMUs, and reflects epidemiologically-relevant groupings of herds that create larger populations, accounting for herd movements and interactions. To statistically examine whether or not geographic location was a factor in the occurrence of sinus tumors, our null hypothesis was that tumors are randomly distributed across SMUs and DAUs. To test this hypothesis, we performed a series of two-tailed Fisher's exact tests comparing the number of tumor-positive and tumor-negative bighorn sheep from each individual SMU or DAU to the number of tumor-positive and tumor-negative bighorn sheep from all other SMUs or DAUs combined. Tumor-suspect animals were not included in the analysis. A p-value <0.05 was considered statistically significant.

Morbidity

Bighorn sheep sinus tumors do not appear to be a fatal disease. However, we hypothesized that the presence of sinus tumors in the upper respiratory tracts of bighorn sheep might make sheep more susceptible to other respiratory diseases and causes of death (morbidity). We specifically hypothesized that disruption of the upper respiratory sinuses by tumors might decrease clearance of bacterial pathogens and lead to increased lesions of bronchopneumonia in the lungs.

A direct measurement of morbidity was not available from our dataset. Therefore, to investigate morbidity, we evaluated causes of death within our dataset to identify categories for which morbidity could have been a contributing factor. Suspected causes of death were available for 113/136 carcasses examined. Causes of death included hunter-killed (n=42), struck by vehicle (n=28), capture mortality (n=7), predation (n=5), fall from cliff (n=5), other trauma (n=5), cull due to ill thrift (n=4), cull due to disease in population (n=7), cull due to interaction with domestic animals (n=2), death from respiratory disease (n=6), and death from other disease (n=2). Of these causes of death, hunter-killed was the only category for which we considered morbidity to be an unlikely contributing factor. This is because bighorn sheep, like most hunted animals, are hunted primarily for trophy or consumption. It is therefore unlikely that hunters would purposely select for an unhealthy animal. In fact, hunters will potentially select for the healthiest of the animals in the population. For all other categories besides “hunter-killed”, we considered the causes of death to potentially be more likely for an animal which is otherwise compromised. To statistically examine whether or not sinus tumors are associated with morbidity, our null hypothesis was that tumors are randomly distributed across all causes of death, including hunter-killed. To test this hypothesis, we performed a two-tailed Fisher’s exact

tests comparing the number of tumor-positive and tumor-negative bighorn sheep that were hunter-killed to the number of tumor-positive and tumor-negative bighorn sheep from all other causes of death combined. Tumor-suspect animals were not included in the analysis. A p-value <0.05 was considered statistically significant.

In regards to morbidity, we specifically hypothesized that disruption of the upper respiratory sinuses by tumors might decrease clearance of bacterial pathogens and lead to increased lesions of bronchopneumonia in the lungs. Lungs were present for examination in 57/136 carcasses examined. For each of these carcasses, the lungs had been assessed for lesions of bronchopneumonia including consolidation of cranioventral lung lobes, pleuritis, or fibrous adhesions (suggesting previous, healed bronchopneumonia). To statistically examine whether or not sinus tumors are associated with pneumonia, our null hypothesis was that tumors are randomly distributed across all categories of lung lesions, including lesions of pneumonia and normal lungs. To test this hypothesis, we performed a two-tailed Fisher's exact test comparing the number of tumor-positive and tumor-negative bighorn sheep that also had evidence of pneumonia to the number of tumor-positive and tumor-negative bighorn sheep that had no significant lung pathology. Tumor-suspect animals were not included in the analysis. A p-value <0.05 was considered statistically significant.

Presence of Potentially Pathogenic Bacteria

To further investigate the hypothesis that bighorn sheep sinus tumors prevent optimal clearance of bacterial pathogens from the upper respiratory tract, we used PCR assays to assess sinus lining tissues for the two leading candidates of bacterial pathogens believed to be associated with fatal bronchopneumonia in bighorn sheep; *Pasteurellaceae* bacteria[59,87,109,139] and *Mycoplasma ovipneumoniae*[14,15,28,87]. These PCR results were compared between tumor-positive

and tumor-negative groups of animals.

Fresh tissue from the sinus lining was collected, and yielded amplifiable DNA as assessed by GAPDH PCR, from 97/136 carcasses. PCR assays for leukotoxin A (the main virulence factor of *Pasteurellaceae* bacteria[27,29,70]), and *Mycoplasma ovipneumonia* were performed for each sample. PCR primers and cycling conditions are given in Table 3.2. Primers for the leukotoxin A gene (*lktA*) were designed by aligning all *Pasteurellacaea lktA* gene sequences available through GenBank[94], and identifying conserved regions between all of the species. Conserved regions were then screened for primer candidates using Primer3 software[115]. *Mycoplasma ovipneumonia* primers (LMF and LMR) have been previously used to amplify this organism from postmortem bighorn sheep lung tissue[15]. Positive and negative PCR results for each assay were compared between tumor-negative and tumor-positive sheep groups. Tumor-suspect animals were not included in the analysis. A p-value <0.05 was considered significant.

Table 3.2: PCR primers and reaction conditions for bacterial PCR assays.

Primers	Expected product	Reaction Details	Cycling Conditions
Leukotoxin A Lkt900fw: 5'-GCCCGTTATCTTGCGAATTT-3' Lkt900rv: 5'-TACCACCAAATAAGCGGTCA-3'	945 bp	25 uL reaction -22.5 uL Platinum PCR SuperMix (Invitrogen) -100 ng genomic DNA -0.4uM each primer	1 cycle 95C x 10 min 35 cycles 95C x 30 sec 60C x 30 sec 72C x 60 sec 1 cycle 72C x 3 min
Mycoplasma ovipneumoniae LMF: 5'-TGAACGGAATATGTTAGCTT-3' LMR: 5'-GACTTCATCCTGCACTCTGT-3'	419 bp	25 uL reaction -22.5 uL Platinum PCR SuperMix (Invitrogen) -100 ng genomic DNA -0.4uM each primer	1 cycle 94C x 5 min 35 cycles 94C x 30 sec 55C x 30 sec 72C x 30 sec 1 cycle 72C x 7 min

Age

Age data were available for 106/136 carcasses examined. Age was determined by examination of horn growth rings, based on disproportionate growth of horns throughout the season as nutrition quality varies from spring to winter. This aging system is likely accurate to about 10 years of age, and therefore animals were not aged beyond 10+ years. Animals younger than 9 months were not included in the study based on the inability to accurately assess the paranasal maxillary sinus cavity due to unerupted tooth material.

Because the 10+ year age category included multiple age groups, we could not evaluate each year individually. Therefore, we grouped ages into categories of 0-3 years, 4-6 years, 7-9 years, and 10+ years. To statistically examine whether or not age was a factor related to bighorn sheep sinus tumors, our null hypothesis was that tumors are randomly distributed across all age classes. To test this hypothesis, we performed a series of two-tailed Fisher's exact tests comparing the number of tumor-positive and tumor-negative bighorn sheep from each age class to the number of tumor-positive and tumor-negative bighorn sheep from all other age classes combined. Tumor-suspect animals were not included in the analysis. A p-value <0.05 was considered statistically significant.

After conducting our analyses for various factors, we identified a possible confounding factor for our age analysis – a single SMU likely contributing to over-representation of the 10+ age group in the tumor-positive category, with tumor-positive status likely related to geographic distribution and not age of the animals. We controlled for this factor and repeated the analysis.

Sex

Sex data were available for all 136 carcasses examined, with 68 males and 68 females examined. To statistically examine whether or not sex was a factor associated with bighorn

sheep sinus tumors, our null hypothesis was that tumors are randomly distributed across both sex categories. To test this hypothesis, we performed a two-tailed Fisher's exact test comparing the number of tumor-positive and tumor-negative male bighorn sheep to the number of tumor-positive and tumor-negative female bighorn sheep. Tumor-suspect animals were not included in the analysis. A p-value <0.05 was considered statistically significant.

After conducting all of our analyses for various factors, we then identified two possible confounding factors for our sex analysis. First, as described for age, we identified a single SMU that was likely contributing to over-representation of the 10+ age group in the tumor-positive category, with tumor-positive status likely related to geographic distribution and not age of the animals. Second we identified likely over-representation of males in the tumor-negative category, with tumor-negative status likely related to being hunter-killed and not sex of the animals. We controlled for these factors and repeated the analysis.

Results

Geographic Location

To analyze geographic location as a possible factor related to the occurrence of bighorn sheep sinus tumors, we performed a series of Fisher's exact tests analyzing the variables of tumor category and geographic location. Our null hypothesis was that bighorn sheep sinus tumors are randomly distributed across all sheep management units (SMUs) and data analysis units (DAUs). For most SMUs, p-value was not significant, possibly reflecting small sample sizes. A significant p-value was found for only two free-ranging SMUs. One of these units contained no positive cases, consistent with a nonrandom distribution of negative cases in this unit ($p=0.0360$). The other unit contained our 7 index cases of bighorn sheep sinus tumors and a total of 8 positive cases, consistent with a nonrandom distribution of positive cases in this unit

(p<0.0001). When the analysis was performed based on DAUs, we found a significant p-value for four DAUs. One of these DAUs contained the SMU which showed a nonrandom distribution of negative cases (p=0.0360). The other three DAUs showed a nonrandom distribution of positive cases within each of these three units (p-value<0.0001, p-value=0.0269, and p-value=0.0403). Taken together, these three DAUs contained all but one of the SMUs with tumor-positive animals (Figure 3.15). This finding is highly suggestive that bighorn sheep sinus tumors are nonrandomly distributed geographically, with location in three specific DAUs being a significant factor associated with having sinus tumors.

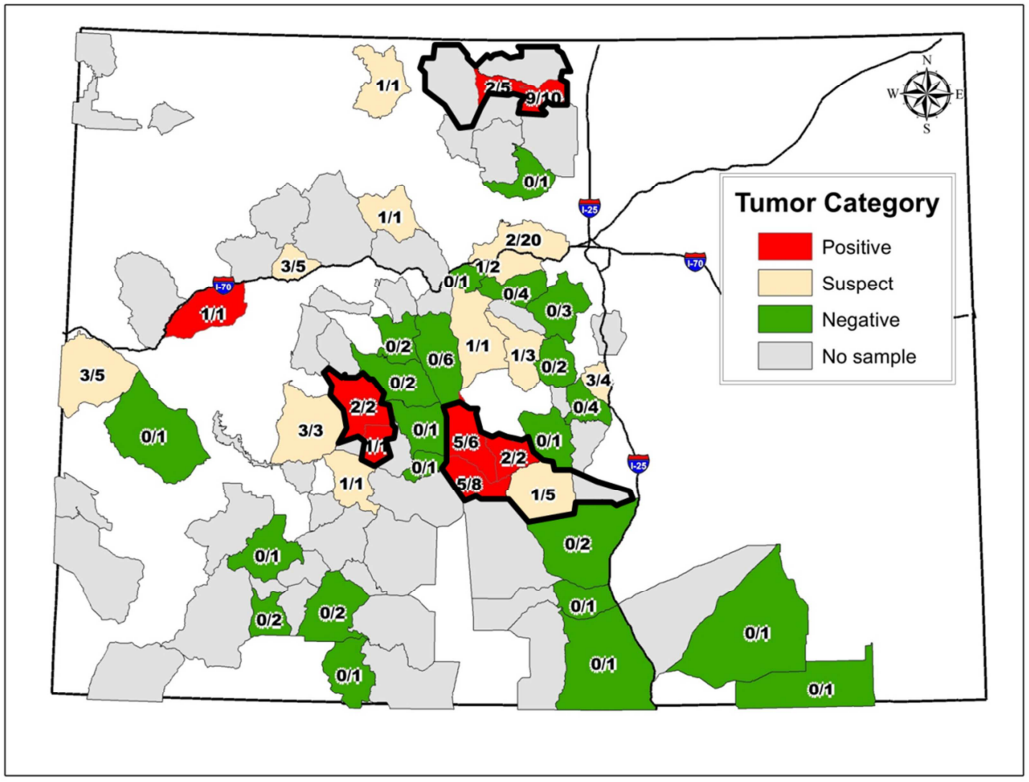

Figure 3.15. Map of Colorado Parks and Wildlife sheep management units (SMUs), illustrating the distribution of bighorn sheep sinus tumor cases. Number labels represent the number of positive and suspect cases combined, over the total number of cases examined for each SMU. Bold outlines represent data analysis units (DAUs) for which we determined a nonrandom distribution of tumor-positive cases.

Morbidity

To analyze morbidity as a possible factor related to the occurrence of bighorn sheep sinus tumors, we performed a Fisher's exact test analyzing the variables of tumor category and cause of death. Our null hypothesis was that bighorn sheep sinus tumors are randomly distributed across all causes of death, including hunter-killed and non-hunter-killed animals. We found 0/33 (0%) cases of sinus tumors in hunter-killed bighorn sheep versus 18/53 (34%) cases of sinus tumors in non-hunter-killed bighorn sheep (Figure 3.16). These data demonstrate a non-random distribution of tumor-negative cases among hunter-killed animals ($p<.0001$). Given our presumptions about morbidity and cause of death, these data support the hypothesis that sinus tumors may be associated with morbidity in bighorn sheep populations.

Additionally, in regards to morbidity, we specifically hypothesized that disruption of the upper respiratory sinuses by tumors might decrease clearance of bacterial pathogens and lead to increased lesions of bronchopneumonia in the lungs. To analyze pneumonia as a possible factor related to the occurrence of bighorn sheep sinus tumors, we performed a Fisher's exact test analyzing the variables of tumor category and pneumonia lesions. Our null hypothesis was that bighorn sheep sinus tumors are randomly distributed across all categories of lung lesions including animals with pneumonia lesions and lacking significant lung lesions. We found 9/15 (60%) of animals with pneumonia lesions to have sinus tumors, and 7/26 (27%) of animals lacking significant lung lesions to have sinus tumors ($p=.0506$) (Figure 3.16). While this p-value minimally exceeds the significance cutoff of $p<0.05$, there does appear to be a trend towards a nonrandom distribution of sinus tumors in animals with lesions of pneumonia. Taken together, our data regarding morbidity support the hypothesis that bighorn sheep sinus tumors can cause morbidity in a population, with a trend towards concurrence of pneumonia and sinus tumors.

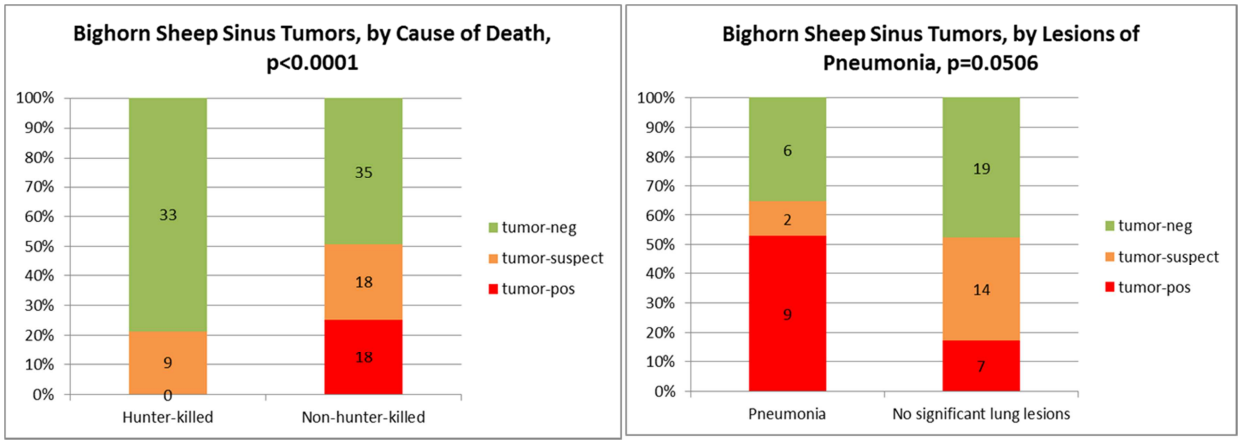

Figure 3.16: Evaluation of morbidity in bighorn sheep with sinus tumors. P-values were calculated using a two-tailed Fisher's exact test. The tumor-suspect category was omitted from the analysis but is included in the figure for reference.

Presence of Potentially Pathogenic Bacteria

To further evaluate the hypothesis that bighorn sheep sinus tumors prevent normal clearance of potentially pathogenic bacterial organisms from the upper respiratory tract, we compared PCR results for two assays detecting previously documented bacteria associated with fatal bronchopneumonia in bighorn sheep; *Mycoplasma ovipneumoniae*, and *Pasteurellaceae* bacteria carrying the *lktA* gene. We compared the results of these assays between tumor-positive and tumor-negative animals and found positive PCR results for *M ovipneumoniae* in 1/50 (2%) tumor-negative and 5/14 (36%) of tumor-positive animals (p=0.0014). Similarly, we found positive PCR results for *lktA* in 3/50 (6%) tumor-negative and 5/14 (36%) tumor-positive animals (p=0.0097) (Figure 3.17). These data suggest a nonrandom distribution of positive PCR results for bacterial agents among tumor-positive animals, supporting the hypothesis that bighorn sheep sinus tumors can interfere with the normal clearance of potentially pathogenic bacterial organisms from the upper respiratory tract.

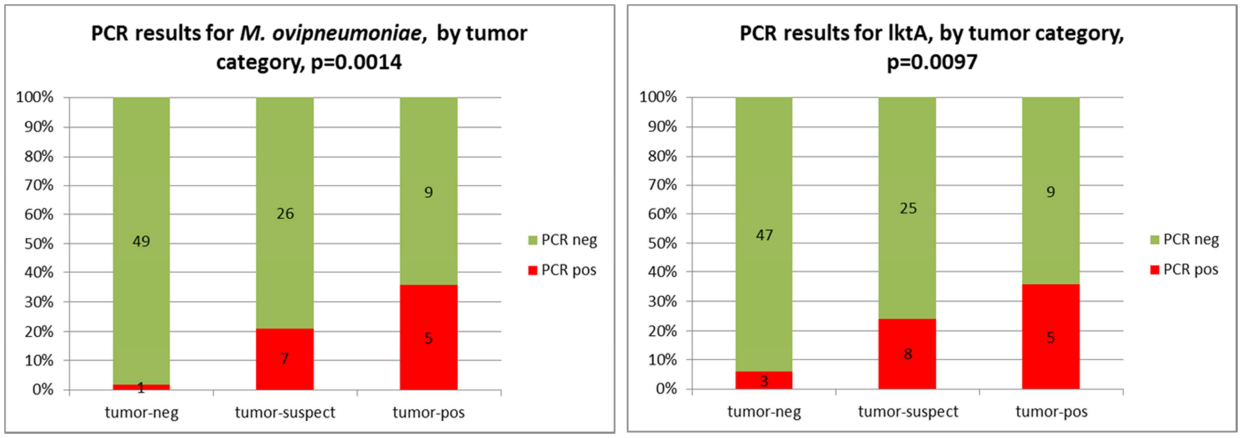

Figure 3.17: Evaluation of potentially pathogenic bacterial organisms within the sinus tissues of bighorn sheep sinus tumor-positive and tumor-negative animals. P-values were calculated using a two-tailed Fisher's exact test. The tumor-suspect category was omitted from the analysis but is included in the figure for reference.

Age

To analyze age as a possible factor related to the occurrence of bighorn sheep sinus tumors, we performed a series of Fisher's exact tests analyzing the variables of tumor category and age class. Our null hypothesis was that bighorn sheep sinus tumors are randomly distributed across all age classes. We initially found a nonrandom distribution of tumor-positive cases for the 10+ age group. However, after completing all of our analyses, we identified one factor which may have confounded these results. The only SMUs found to have a nonrandom distribution of tumor-positive cases when compared to all other SMUs contained our 7 index cases of bighorn sheep sinus tumors. All seven of these cases were females in the 10+ age class. Based on our analysis of geographic location, we concluded that tumor-positive status of these index cases was likely based on location. Because this group of index cases accounted for 7/10 (70%) of the positive cases in the 10+ age class, these 7 cases were omitted from the analysis and the analysis was repeated. No significant difference was then found between the 10+ age class and all other age classes (p=0.0604). Results are shown in Figure 3.18.

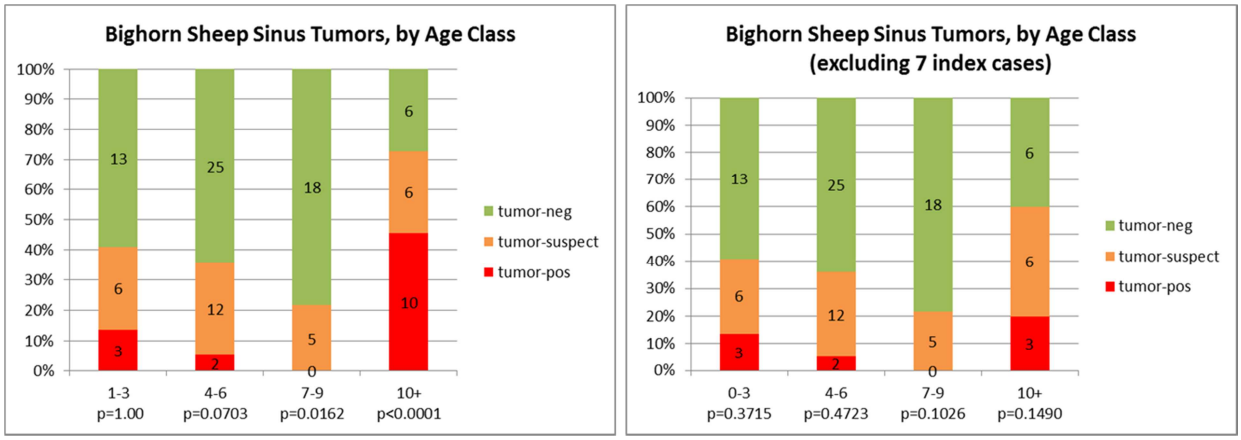

Figure 3.18: Bighorn sheep sinus tumors, by age class. P-values were calculated using a two-tailed Fisher's exact test. The tumor-suspect category was omitted from the analysis but is included in the figure for reference.

Sex

To analyze sex as a possible factor related to the occurrence of bighorn sheep sinus tumors, we performed a Fisher's exact test analyzing the variables of tumor category and sex. Our null hypothesis was that bighorn sheep sinus tumors are randomly distributed between both sex categories. Our initial findings suggested a nonrandom distribution of tumor-positive and tumor-negative cases, with males more likely to be tumor-negative, and females more likely to be tumor-positive (p=0.0006) (Figure 3.18). However, after completing all of our analyses, we identified two factors that might have confounded our analysis of sex. First, the only SMUs found to have a nonrandom distribution of tumor-positive cases when compared to all other SMUs contained the herd with our 7 index cases of bighorn sheep sinus tumors. All seven of these cases were females in the 10+ age class. Based on our analysis of geographic location, we concluded that tumor-positive status of these index cases was likely based on location. Because this group of index cases accounted for 7/16 (44%) of the positive female cases, these 7 cases were omitted from the analysis.

Second, we concluded in our morbidity analysis that hunter-killed animals had a

nonrandom distribution of tumor-negative cases. All hunter-killed animals were male, accounting for 33/49 (67%) of tumor-negative male cases. We therefore considered hunter-killed status to be a confounding factor for the sex analysis. We re-analyzed our data, omitting all hunter-killed animals and the 7 index cases of bighorn sheep sinus tumors in aged female animals. Our revised results showed no significant difference between male and female animals, based on tumor status (p=0.7353) (Figure 3.19). These results suggest that tumor-positive and tumor-negative cases are randomly distributed among male and female sex categories. This result implies that sex was not likely to be a confounding factor in our analyses of hunter-killed animals and geographic distribution.

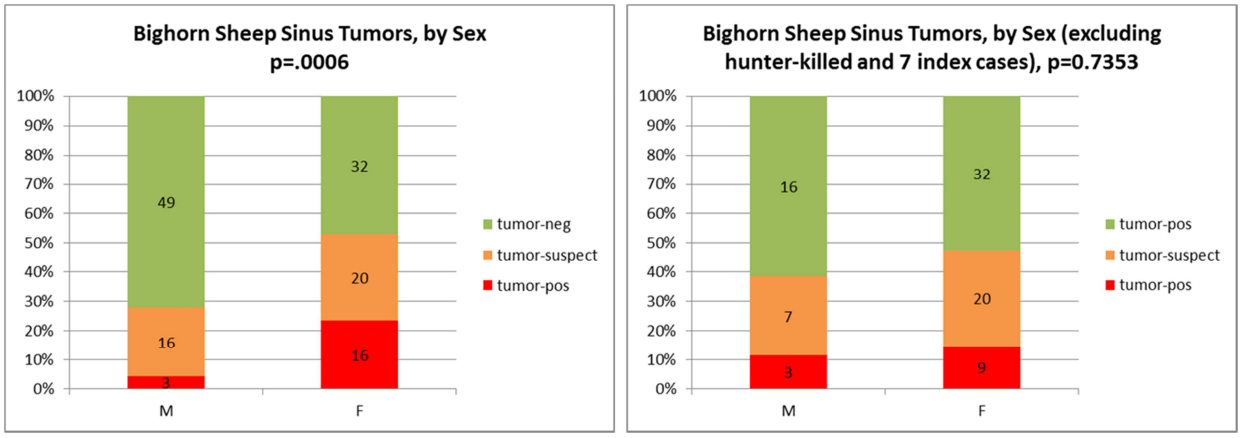

Figure 3.19: Bighorn sheep sinus tumors, by sex category. P-values were calculated using a two-tailed Fisher's exact test. The tumor-suspect category was omitted from the analysis but is included in the figure for reference.

Discussion

To assess bighorn sheep sinus tumors at a population level, we created diagnostic criteria, based on gross pathology, to categorize cases into tumor-positive, tumor-suspect, and tumor-negative groups. We then evaluated the distribution of tumor-positive and tumor-negative groups across multiple population variables. These factors included geographic location, morbidity, co-infections with potentially pathogenic bacteria, age, and sex. Of these factors, we

found a nonrandom distribution of positive cases when considering geographic location, morbidity, and co-infections with potentially pathogenic bacteria. We found a random distribution of positive and negative cases when considering age and sex.

Our analysis of geographic location demonstrated a nonrandom distribution of cases across the state of Colorado. We chose to analyze our data based on sheep management units (SMUs) and data analysis units (DAUs). We conducted an analysis based on SMUs, which are designed for ease of management purposes. While these units often contain fairly distinct herds of sheep, they do not necessarily reflect the migratory patterns and interactions of multiple herds. For this reason we also conducted our analysis based on DAUs, which are designed to account for larger, connected populations of sheep herds. Based on these analyses, we found only a single SMU to have a nonrandom distribution of tumor-positive cases, but three DAUs to have a nonrandom distribution of tumor-positive cases. In fact, all tumor-positive bighorn sheep cases, except one, were clustered within three DAUs (Figure 3.15), with each DAU showing a significantly increased chance of containing a positive animal as compared to all other DAUs combined. This finding supports our hypothesis that bighorn sheep sinus tumors are an infectious agent, with maintenance of an infectious agent within specific geographic regions.

Our analysis of morbidity suggests that hunter-killed animals, assumed to be predominantly healthy animals, show a nonrandom distribution of tumor-negative cases, while animals with evidence of pneumonia show a trend towards a nonrandom distribution of tumor-positive cases. We hypothesized that animals with sinus tumors may be more susceptible to pneumonia due to decreased clearance of potentially pathogenic bacterial organisms from the upper respiratory tract. To further examine this possibility, we used PCR assays to screen sinus lining tissue from animals with and without tumors for potentially pathogenic bacteria including

leukotoxin A-carrying *Pasteurellaceae*, and *Mycoplasma ovipneumoniae*. For both agents, there was a nonrandom distribution of positive PCR results for tumor-positive sheep versus tumor-negative sheep. While these data may suggest that bighorn sheep sinus tumors allow for decreased clearance of bacterial pathogens and predispose to pneumonia, we cannot conclude cause and effect from our analysis. It is possible that sheep with bacterial pneumonia are more likely to accumulate pathogenic bacteria in the sinus cavities, causing chronic inflammation that can progress to neoplasia. This possibility can be further examined by controlled experimental transmission studies.

Our initial analyses of sex and age appeared to show a nonrandom distribution of tumor-positive cases in female animals over the age of 10 years, and a nonrandom distribution of tumor-negative cases in males. However, we felt that these data might be skewed due to confounding factors. We previously determined that hunter-killed animals showed a nonrandom distribution of tumor-negative cases, presumably based on categorization as non-morbid animals. We also previously determined that a single SMU showed a nonrandom distribution of tumor-positive cases, presumably based on geographic location in an area with high tumor prevalence. This SMU contained all female animals over the age of 10 years and we felt that this fact could have skewed our age and sex analyses. By omitting hunter-killed animals from our sex analysis, and these 7 index cases from our age and sex analyses, we found a nonrandom distribution of tumor-positive and tumor-negative cases based on age and sex.

Taken as a whole, our assessment of bighorn sheep sinus tumors on a population level suggest that bighorn sheep sinus tumors are highly associated with specific geographic locations, and are likely associated with morbidity, particularly in regards to bacterial bronchopneumonia. Implications of these findings for management include prevention of interactions between

animals from tumor-positive and tumor-negative populations, and heightened surveillance of tumor-positive populations for bacterial bronchopneumonia. Interpretations of these data may evolve as additional information is obtained regarding the cause of bighorn sheep sinus tumors and what role this disease may play in the larger picture of bighorn sheep respiratory disease.

CHAPTER FOUR – EXPERIMENTAL TRANSMISSION

Summary

Bighorn sheep upper respiratory sinus tumors are a recently-described disease affecting Rocky Mountain bighorn sheep. While an infectious etiology is suspected for the tumors, a specific etiologic agent has not yet been identified. To test the hypothesis that bighorn sheep sinus tumors are caused by an infectious agent, we inoculated four bighorn sheep lambs and four domestic sheep lambs intranasally with a cell-free filtrate prepared from tissues and exudates associated with a naturally-occurring bighorn sheep sinus tumor. Within 18 months post-inoculation, three of the four inoculated domestic sheep and one of the four inoculated bighorn sheep developed a tumor at the site of inoculation, with features similar to naturally-occurring bighorn sheep sinus tumors. These findings support the hypothesis that bighorn sheep sinus tumors are caused by an infectious agent. Histologically, the experimentally-induced tumors were composed of stellate to spindle cells embedded within a myxomatous matrix (myxoma), with marked bone proliferation that was highly reminiscent of fetal bone production by intramembranous ossification. Stellate to spindle cells stained positively with vimentin, S100, and alpha smooth muscle actin. A periosteal origin for these tumors is suspected.

Introduction

Bighorn sheep sinus tumors occur primarily within the maxillary and frontal paranasal sinuses of Rocky Mountain bighorn sheep, and range from a thickening of the sinus lining to solid, gelatinous masses filling the sinus cavities[53]. Associated with the masses, it is also common to see mucinous exudate within the sinus cavities, and cystic expansions of the sinus lining (Chapter 3). Histologically, bighorn sheep sinus tumors contain proliferative epithelial and stromal cells, with both populations ranging from hyperplasia to neoplasia[53]. However, in

the vast majority of cases, the stromal population is predominant, and is composed of spindle cells embedded within a mucinous matrix. These fibromyxomatous masses often invade and destroy the underlying bone, although minimal histochemical staining has been performed to characterize the invasive spindle cell population. Additionally, chronic lymphoplasmacytic inflammation is commonly associated with bighorn sheep sinus tumors[53], but the role of inflammation as a primary or secondary process has not been evaluated.

Due to some similarities between bighorn sheep sinus tumors and oncogenic retroviruses of domestic sheep and goats, these tumors were originally hypothesized to be caused by enzootic nasal tumor virus (ENTV), Jaagsiekte sheep retrovirus (JSRV), or a closely-related oncogenic retrovirus. However, initial screens of naturally-occurring bighorn sheep sinus tumors were negative for these viruses by PCR and IHC[53].

Multiple characteristics of bighorn sheep sinus tumors are suggestive of an infectious cause, including geographically clustered cases suggesting local maintenance of an infectious agent (Chapter 3), and a lack of predilection for aged animals that would be expected with a non-infectious tumor (Chapter 3). Although a specific etiology has not been identified for bighorn sheep sinus tumors, we suspect that this disease is caused by an infectious agent. To test this hypothesis, we experimentally inoculated bighorn sheep and domestic sheep lambs with a cell-free filtrate derived from homogenates of a naturally-occurring case of a bighorn sheep sinus tumor. We monitored these inoculated animals for signs of tumor development over a period of 18 months using radiographic methods. Any tumors that developed were examined histologically and characterized by histochemical staining to further define this disease.

Materials and Methods

Animals and Facilities

A total of 10 animals were used for this study, including five domestic sheep (*Ovis aries*) and five Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). The domestic sheep were a mixed breed of Dorset, East Friesian, and Lacaune breeds, and were acquired from a sheep dairy herd in Bushnell, Nebraska (Irish Cream Dairy). The bighorn sheep were acquired from the Colorado Parks and Wildlife, Foothills Wildlife Research facility (FWRF) in Fort Collins, Colorado, with breeding animals descended from multiple wild herds within the state of Colorado. All experimental animals were housed at the FWRF throughout the study.

Lambs were allowed to suckle colostrum from their dams at birth, but were separated from dams within 48 hours of birth. The domestic and bighorn sheep were housed at opposite ends of the facility throughout the experiment, and for each species lambs were separated into groups of treatment (n=4) and control (n=1) animals. Treatment animals were housed together, but treatment and control pens were separated by at least six feet and double fencing. Biosecurity measures were taken to avoid transfer of pathogens between pens by staff.

Preparation of Inoculum

Tumor material and associated mucinous exudates were collected post-mortem from an adult female bighorn sheep with a naturally-occurring sinus tumor, within two hours of death. The tumor was present within the maxillary sinus and frontal sinus, with extension into the cornual sinus, causing a deformity of the horn. After collection of the tumor tissue and associated exudates, this material was immediately transferred on ice to the laboratory, where it was homogenized in sterile phosphate-buffered saline using a dounce homogenizer. The resulting homogenate was clarified by centrifugation at 8,000 g for 30 minutes, and the

supernatant was passed through a 0.45 micron filter, creating a cell-free filtrate. The filtrate was divided into 1.75 mL aliquots, and frozen at -80°C until inoculation (within 8 weeks of preparation).

Inoculation of Animals

At approximately 48 hours of age, all treatment lambs were inoculated intranasally and unilaterally with 1.75 mL of filtrate prepared as described above and thawed on ice. Domestic sheep were inoculated in the right nasal cavity and bighorn sheep in the left nasal cavity. The inoculum was administered using a rigid plastic nasal vaccine applicator, inserted through the nostril to approximately the level of the medial canthus. This level of the nasal cavity corresponds to the rostral aspect of the ethmoid turbinates. The inoculum was administered into the nasal cavity while the lamb's head was positioned parallel to the ground to avoid swallowing of the inoculum. The lambs were calm and breathing normally during inoculation. The inoculum was heard bubbling within the nasal cavity with the animals' breaths. An identical procedure was used to administer sterile saline intranasally and unilaterally to each of the negative control lambs.

Monitoring of Animals

All animals were clinically assessed at least every two weeks for any signs of respiratory distress, discomfort, or nasal exudate. Every two months for the first eight months, all animals were assessed radiographically using standard radiographic films. At nine months post-inoculation, all animals were assessed by computed tomography (CT), which was repeated at the anticipated termination of the study, 18 months post-inoculation. For three bighorn sheep, the study was extended past 18 months, and for these individuals, CT was repeated at the termination of the study 22.5 months post- inoculation. Every month, nasal swabs were

collected and stored in RNAlater (Qiagen, Inc. Valencia, CA, USA) at -80°C.

Post-mortem Examinations and Histopathology

At the termination of the study for each animal, sheep were euthanized and necropsied immediately following final screening by CT. At necropsy, two sets of tissues were collected from each animal, including samples of the frontal and maxillary sinus lining, ethmoid turbinates, and nasal scrolls, including any masses or other abnormal tissues. One set of tissues was collected in 10% neutral buffered formalin, and the other was frozen at -80°C for molecular diagnostics. Selected fixed tissues were embedded in paraffin blocks, sectioned at five microns, and stained with hematoxylin and eosin.

Histochemical and Immunohistochemical Stains

For tumors identified in the upper respiratory sinuses, additional staining was performed including the histochemical stains Alcian blue (pH 2.5), and periodic acid-Schiff (PAS), as well as the immunohistochemical stains vimentin (Leica Biosystems, PA0033), S100 (Leica Biosystems, PA0900), alpha smooth muscle actin (Leica Biosystems, PA0943), and osteocalcin (Thermo Scientific Pierce Antibodies, MA1-20786). Immunohistochemical staining was performed using a Leica BOND-MAX automated IHC stainer (Leica Biosystems).

Results

Clinical Assessment

Mild nasal exudates were rarely noted in lambs throughout the study, and occurrence was transient. The degree and character of exudate did not differ between treatment and control animals, and these findings were attributed to transient rhinitis within both treatment and control groups. No other significant clinical findings were noted, including no evidence of discomfort and no respiratory distress.

Radiology and Computed Tomography

Domestic Sheep

At two months post-inoculation (mpi), no significant changes were seen in any of the domestic sheep lambs radiographically. At four mpi, a slight opacity was noted on the inoculated side of the nasal cavity in two of four treatment domestic sheep (DS 3, DS 4, data not shown). In both cases, slight progression of the lesion was noted by radiology at six and eight mpi. Due to the low resolution of the lesions by standard radiographs, computed tomography was then conducted at nine mpi to further assess the lesions. Computed tomography confirmed soft tissue opacities in the nasal cavity of two domestic sheep (DS 3, DS 4) on the side of inoculation, at approximately the level of inoculation at the rostral ethmoid turbinates (Figure 4.1). The masses measured 1.6 cm x 1.9 cm (DS 3), and 1.4 cm x 1.1 cm (DS 4). Both masses appeared to be arising from the soft tissues lining the nasal turbinates, and both were characterized by a rim of relatively radiodense tissue surrounding a core of more radiolucent tissue, consistent with a core of low-density solid tissue or fluid (Figure 4.1). Additionally, radiodense stippling was noted within the lesions, consistent with calcification or ossification in these areas. No other lesions were noted in any other treatment or control domestic sheep at nine mpi.

At 18 mpi, nine of the ten animals were re-scanned with computed tomography. One of the treatment domestic sheep with a mass noted at nine mpi (DS 3) was not re-scanned at 18 mpi because the animal died in the interim, for reasons unrelated to the study. At necropsy, the mass appeared similarly-sized to its proportions at the nine mpi scan (Figure 4.1). The other domestic sheep with a mass noted at nine mpi (DS 4), was re-scanned at 18 mpi, and CT demonstrated progression from the nine mpi scan (from 1.4 cm x 1.1 cm to 4.7 cm x 1.2 cm). An additional

treatment domestic sheep that did not have a tumor at the nine mpi scan (DS 5), had a mass lesion within the nasal cavity, on the inoculated side, at approximately the level of inoculation near the rostral ethmoid turbinates at 18 mpi (Figure 4.1). This mass lesion measured 3.2 cm x 1.5 cm at the 18 mpi scan. As with the lesions for DS 3 and DS 4, the mass lesion seen for DS 5 also had significant radiodense stippling, suggesting significant ossification or mineralization (Figure 4.1).

In addition to three of four inoculated domestic sheep lambs (DS 3, DS 4, and DS 5) showing tumors at the site of inoculation by 18 mpi, additional lesions were also seen in other regions of the upper respiratory tract. One of the four inoculated domestic sheep (DS 2) did not develop a tumor at the site of inoculation, but at 18 mpi this animal did have a small (2.0 cm x 0.75 cm) mass on the uninoculated side of the nasal cavity within the nasal scrolls. Interestingly, this lesion on the uninoculated side was much further rostral in the nasal cavity as compared to the lesions in the other domestic sheep. This lesion is hypothesized to be the result of animal-to-animal transmission within the treated domestic sheep. An additional, smaller mass (1.9 cm x 1.0 cm, not shown in images) was also seen in DS 5, rostral to the inoculation site at 18 mpi. No lesions were noted in the control domestic sheep at 18 mpi.

Bighorn Sheep

For the bighorn sheep, no masses were noted in any of the animals by nine mpi by standard radiographs or computed tomography. At 18 mpi, CT scan demonstrated a soft tissue mass within one of the four inoculated bighorn sheep (BHS 5), on the side of inoculation, at approximately the level of inoculation, within the rostral ethmoid turbinates (Figure 4.1). This mass was approximately 0.5 cm x 0.6 cm in size, with a rim of relatively radiodense tissue and a core of more radiolucent tissue, similar to findings in the domestic sheep. No other bighorn

sheep showed lesions at this time. Two of the treatment bighorn sheep (BHS 2, BHS 3) were euthanized and tissues collected at 18 mpi, while the control bighorn sheep (BHS 1) and remaining treatment bighorn sheep (BHS 4 and BHS 5) were given an extended incubation period to assess progression of the lesion in BHS 5 and to allow additional time for lesion formation in BHS 4. At 22.5 mpi, the remaining bighorn sheep were scanned by CT. No additional lesions were noted, and no significant progression of the mass in BHS 5 was noted. The study was terminated, and the remaining animals were euthanized and necropsied.

Mucus-filled cysts of the Maxillary Sinus Lining

One additional finding in both the bighorn sheep and domestic sheep was the presence of mucus-filled cystic expansions of the maxillary sinus lining. One inoculated bighorn sheep (BHS 4) and one inoculated domestic sheep (DS 2) showed multifocal cystic structures arising from the sinus lining of the maxillary sinus (data not shown). In one case, the cysts were contralateral to the side of inoculation, and in the other case, the cysts were ipsilateral to the side of inoculation. These cysts have previously been observed in bighorn sheep sinuses, often associated with sinus tumors, but not exclusively associated with sinus tumors (Chapter 3).

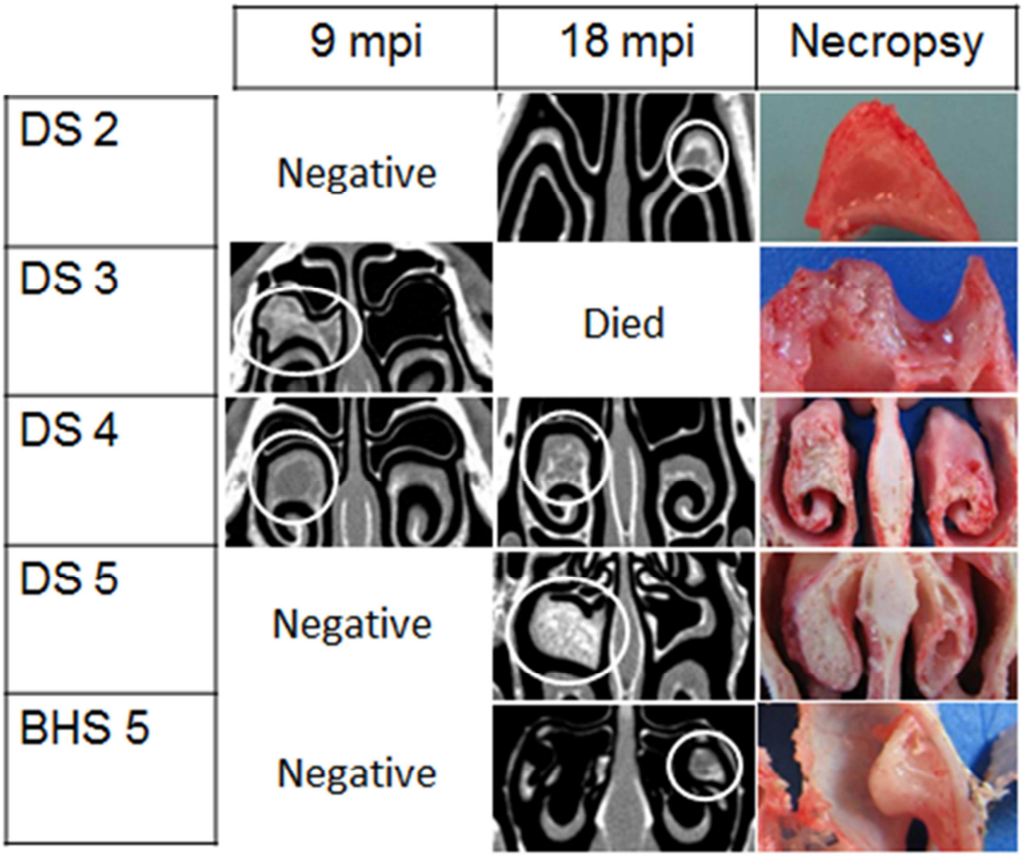

Figure 4.1: Summary of findings for CT scans and necropsy exams of experimentally-transmitted sinus tumors in domestic sheep and bighorn sheep. Most tumors developed only on the side of inoculation (right side for domestic sheep, left side for bighorn sheep, with images representing the animals facing towards the reader). For CT images of tumors, note the rim of radiodense tissue surrounding a core of more radiolucent tissue (most clearly demonstrated for DS 4, nine mpi scan) and the radiodense stippling of all tumors at the 18 mpi scans and necropsy images, consistent with mineralization or ossification of the tumors. All animals not represented by images here did not develop sinus tumors. For DS 5, an additional smaller mass was also seen on the side of inoculation further rostral to the image shown here.

Gross Pathology

Post-mortem examinations of the nasal and paranasal sinuses demonstrated lesions consistent with the CT radiographical interpretation of soft tissue masses with multifocal mineralization or ossification. The masses were variably ossified/mineralized, but were otherwise soft, with a gelatinous consistency, and a white to translucent color. Also consistent with the CT findings, on gross examination two of the animals (DS 2, BHS 4) examined had

cystic, mucus-filled structures, arising from the lining of the maxillary sinus. The cystic structures could be peeled away from the underlying bone, although the exposed bone showed remodeling beneath the cyst, with apparent bone resorption in this area. In both the treatment and control animals, scant mucus was present lining the surfaces of nasal turbinates and not significantly associated with tumors or cysts.

Histopathology

Histologically, the experimentally-induced tumors in this study had characteristics similar to those observed in naturally-occurring bighorn sheep sinus tumors. Tumors were predominated by stellate to spindle cells embedded within a mucinous matrix (Figure 4.2) that was positive for Alcian blue (pH 2.5) (Figure 4.3) and negative for PAS, consistent with acid mucopolysaccharides found within other myxomatous tumors. As expected, this stromal component demonstrated invasion and remodeling of the surrounding bone. Cells were relatively benign in appearance, but the experimentally-induced tumor cells demonstrated a more stellate phenotype than is typically seen in naturally-occurring cases, and these stellate cells had occasional mitotic figures (Figure 4.4). Epithelial involvement was limited, with adenomatous hyperplasia of submucosal glands occurring adjacent to one tumor (DS 3), and associated with cystic expansions of the maxillary sinus lining in one case (Figure 4.5, DS 2). Interestingly, experimentally-induced tumors lacked the hyperplasia of surface epithelium that is often seen with naturally-occurring cases. Also unlike naturally-occurring bighorn sheep sinus tumors, inflammation was not a prominent finding in the experimentally-induced tumors. No lymphoplasmacytic inflammation was seen, and only mild neutrophilic inflammation was seen, associated with submucosal gland hyperplasia.

One prominent finding in the experimentally-induced tumors, seen occasionally in

naturally-occurring cases but to a lesser degree, was marked production of bone. Frequent islands of bone and osteoid were embedded within the tumors (Figure 4.2), consistent with the radiodense stippling seen on CT scans. Interestingly, these islands of osteoid and bone demonstrated organization that was highly reminiscent of fetal bone formation by intramembranous ossification, with features suggesting orderly progression through phases of bone production and maturation. These features included proliferation of stellate cells reminiscent of primitive mesenchyme (Figure 4.4), and nests of suspected osteoprogenitor cells, appearing to arise from the periosteum of adjacent bone spicules. Nests of osteoprogenitor cells appear to demonstrate differentiation to osteoblastic cells, with or without small central lakes of osteoid (Figure 4.6), and larger islands composed of woven bone (Figure 4.7) and mature bone (Figure 4.8).

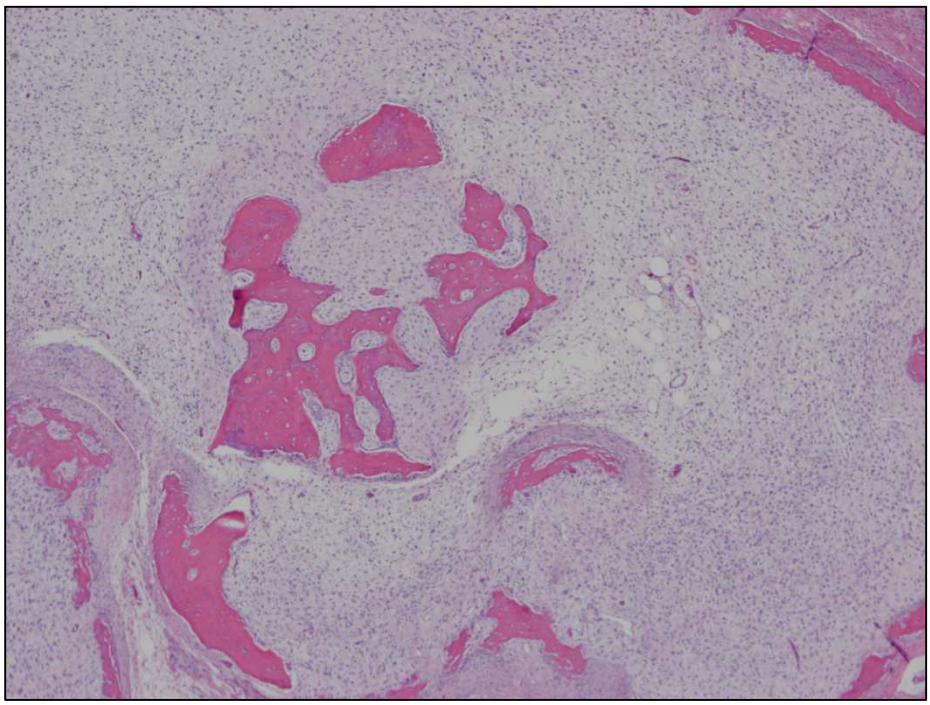

Figure 4.2: Experimentally-induced tumor predominated by stellate to spindle cells embedded within a myxomatous matrix, with prominent bone production. DS 4, H&E, 4x objective.

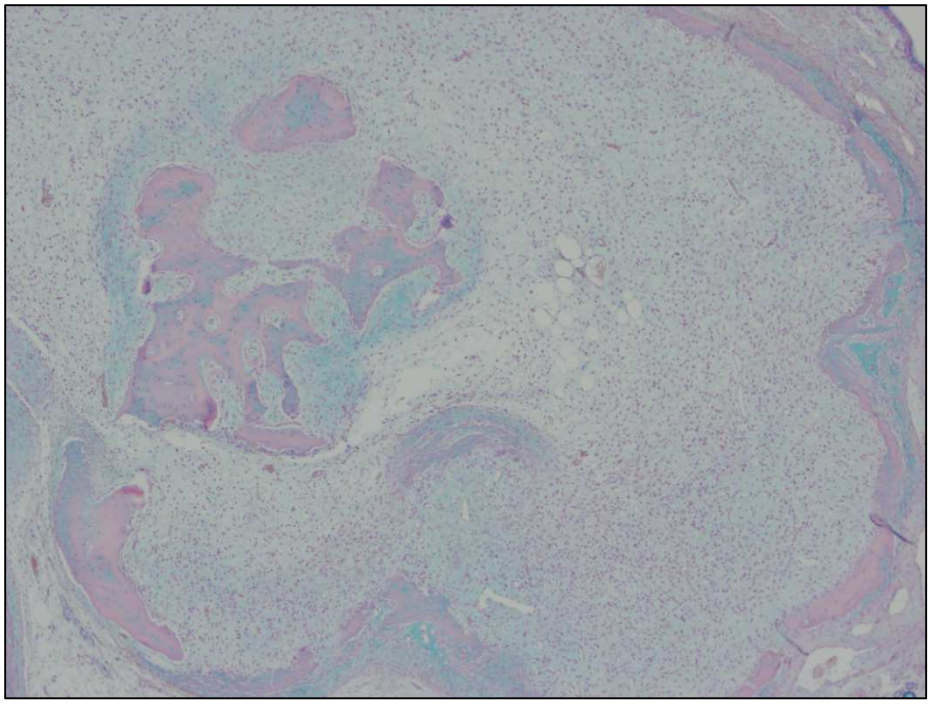

Figure 4.3: Staining of myxomatous background light blue, consistent with acid mucopolysaccharides that characterize myxomas. DS 4, Alcian blue (pH 2.5), 4x objective.

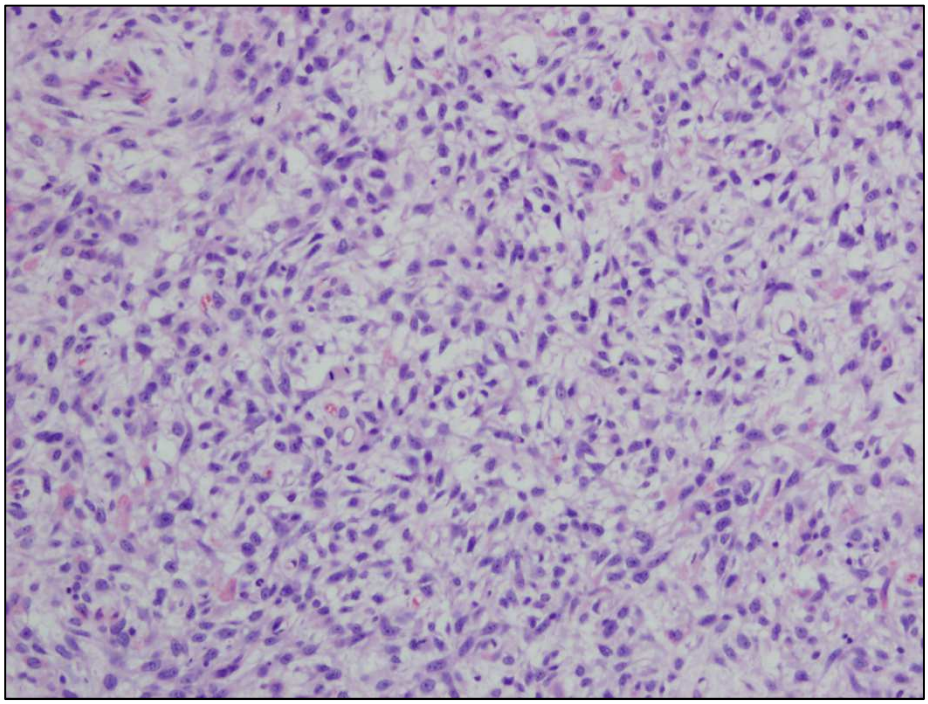

Figure 4.4: Experimentally-induced tumor composed of stellate to spindle cells embedded within a myxomatous matrix. DS 4, H&E, 20x objective.

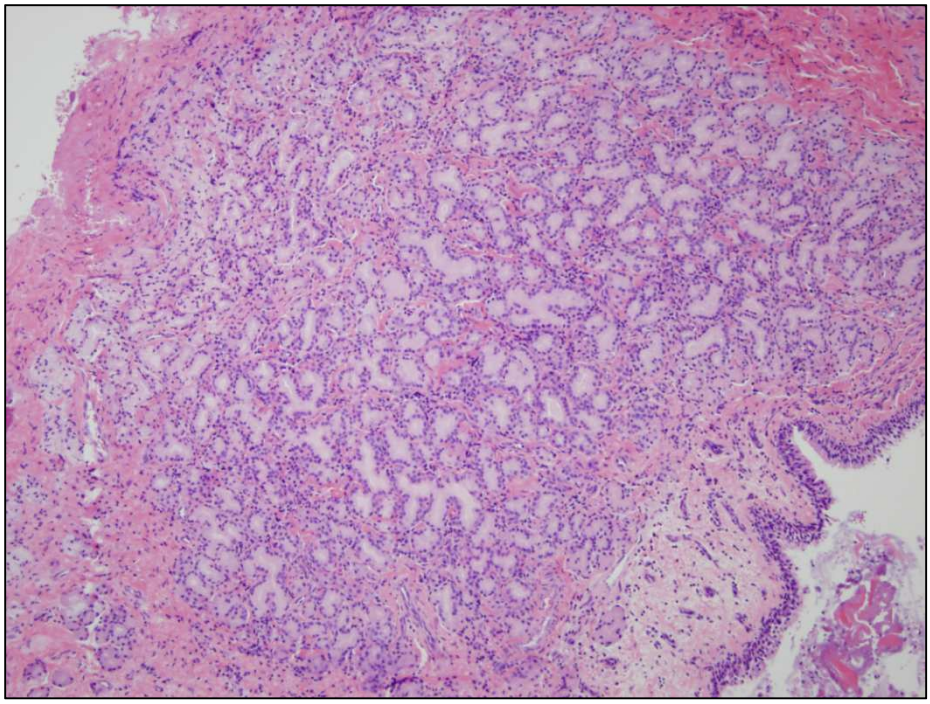

Figure 4.5: Submucosal gland hyperplasia associated with cystic expansions of the maxillary sinus lining. DS 2, H&E, 10x objective.

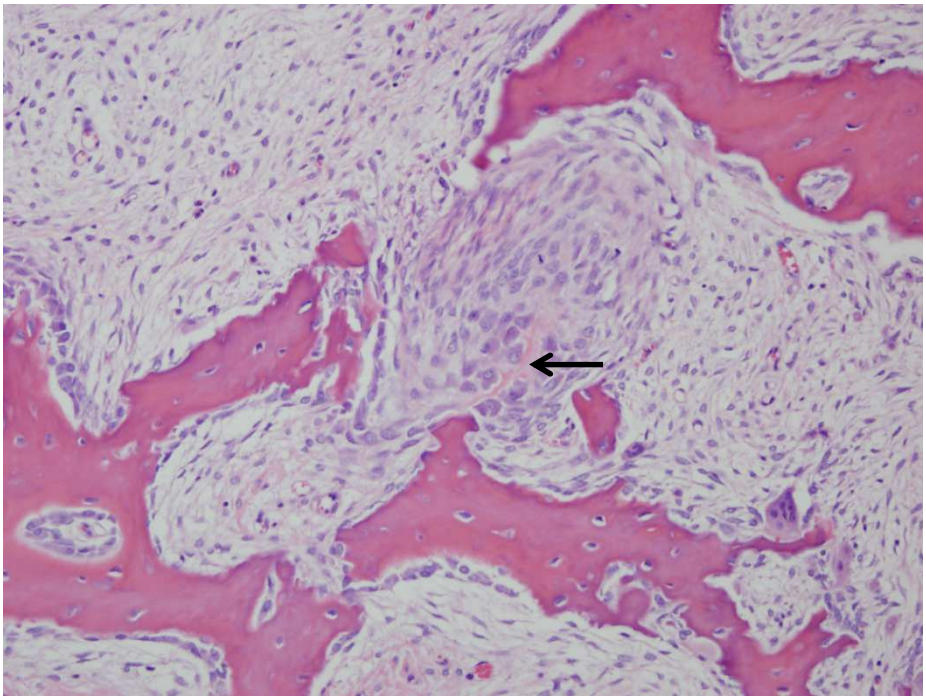

Figure 4.6: Experimentally-induced tumor showing features reminiscent of fetal bone formation by intramembranous ossification, including osteoid formation (arrow). DS 5, HE, 20x objective.

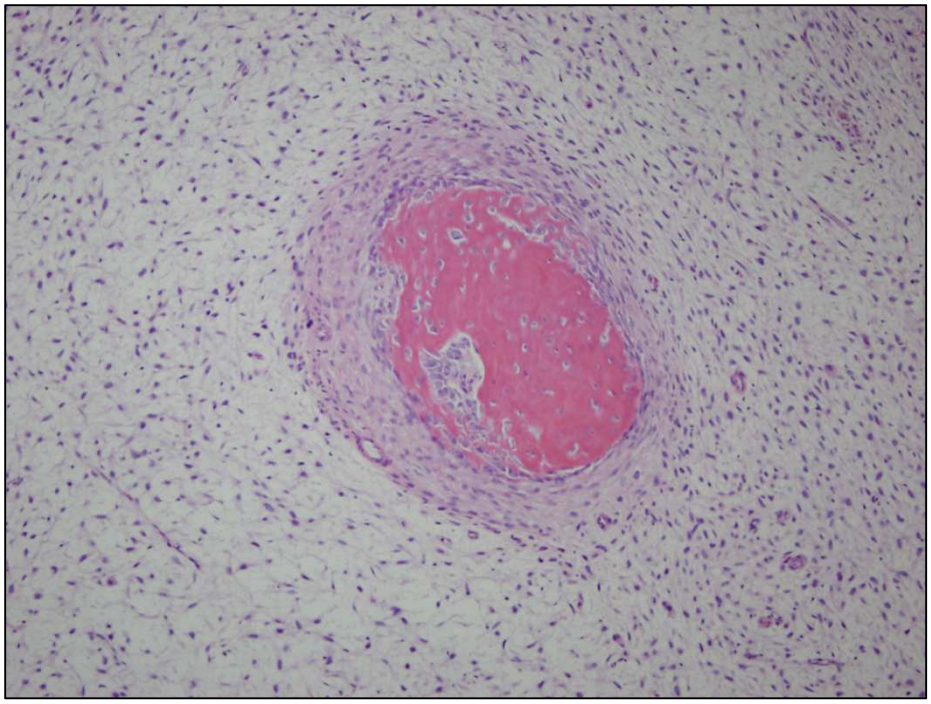

Figure 4.7: Experimentally-induced tumor showing features reminiscent of fetal bone formation by intramembranous ossification, including formation of woven bone. DS 5, HE, 10x objective.

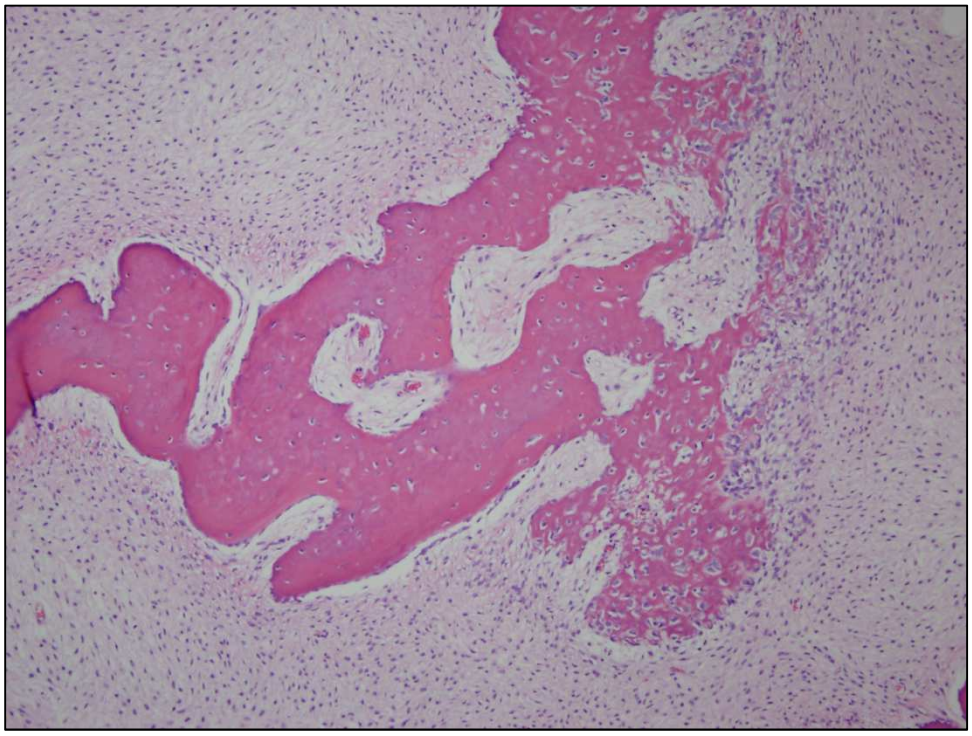

Figure 4.8: Experimentally-induced tumor in a domestic sheep showing features reminiscent of fetal bone formation by intramembranous ossification. Note the orderly progression from woven bone (to right of image) to mature bone (to left of image). DS 5, H&E, 10x objective.

Immunohistochemistry

To further characterize the population of stromal cells which predominated in the experimentally-induced tumors, we applied a panel of IHC markers including vimentin, alpha smooth muscle actin (SMA), S100, and osteocalcin. The stellate to spindle stromal cells were strongly positive for vimentin, SMA, and S100 (Figures 4.9, 4.10 and 4.11). This staining pattern can be consistent with a diagnosis of myxoma, as myxomas are consistently positive for vimentin, with variable staining reported for S100 and SMA. Specifically regarding sheep, there are only two case reports of myxomas that include characterization by IHC, and both of these are case reports of pulmonary myxomas. In both cases, staining of stellate to spindle tumor cells with vimentin was positive[68,101]. In one case staining of the tumor cells with S100 and SMA was negative[68], while in the other case staining with S100 was positive and staining with SMA was not performed[101]. In the human literature, odontogenic and cardiac myxomas have been extensively studied, with variable IHC positivity for S100 and SMA[76,77]. Both of these myxoma subtypes in humans are hypothesized to originate from pluripotent mesenchyme[60,134], perhaps explaining the lack of a definitive immunohistochemical profile. Based on these previous reports, the findings in this study of tumor cell positivity for vimentin, SMA, and S100 are not inconsistent with other cases of myxomas.

Additionally, in the cases of experimentally-transmitted tumors examined here, positive staining with osteocalcin occurred only within the cells lining islands of osteoid and bone (Figure 4.12) and not the osteoprogenitor-like cells which appear intermediate in phenotype between the osteoblasts and the stellate to spindle cells of the surrounding mesenchyme. This finding suggests multifocal, gradual differentiation to osteoblastic cells, consistent with intramembranous ossification originating within the multipotent cells of the periosteum.

Because the experimentally-induced tumors demonstrate orderly and heterogeneous proliferation, consistent with normal periosteal proliferation (during fetal life), these findings argue against an origin from a clonally expanding, virus-infected, transformed neoplastic cell. Rather, these tumors may represent a normal response by the periosteum to abnormal levels of growth factors, perhaps provided by an infected cell.

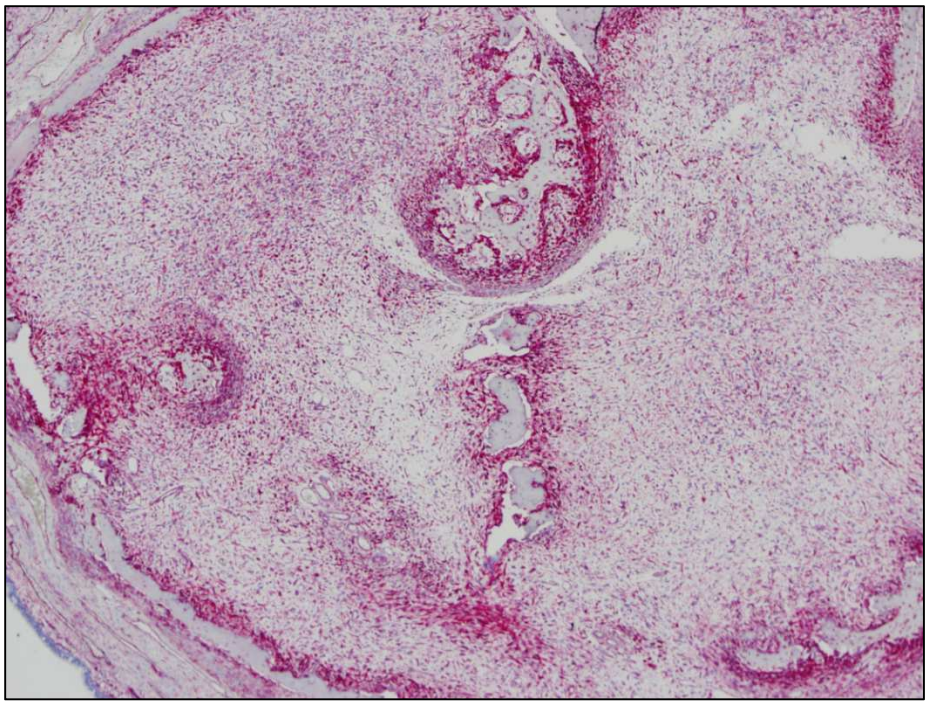

Figure 4.9: Experimentally-induced tumor with demonstration of mesenchymal origin of tumor cells by positive staining with vimentin. DS 4, vimentin IHC, 4x objective.

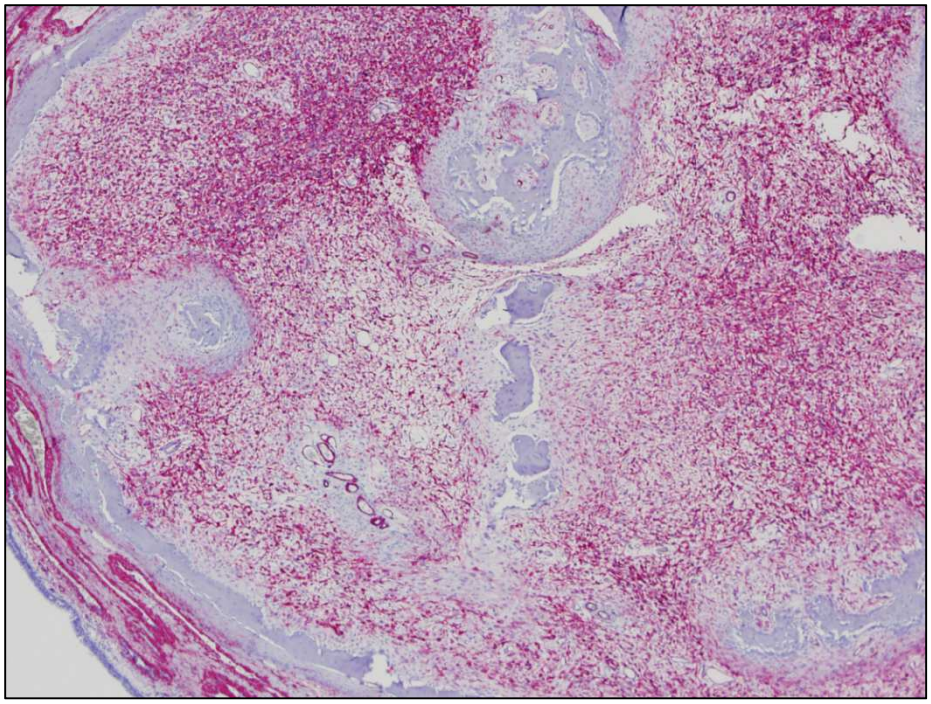

Figure 4.10: Experimentally-induced tumor showing positive staining with alpha smooth muscle actin (SMA). SMA is a variable IHC marker of myxomas. DS 4, SMA IHC, 4x objective.

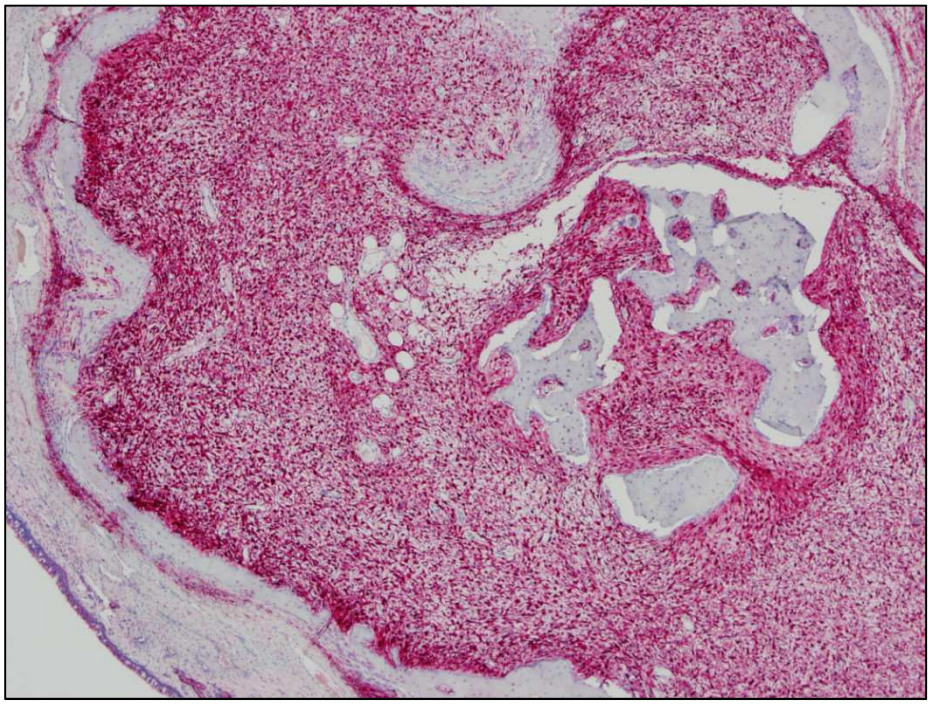

Figure 4.11: Experimentally-induced tumor with demonstration of S100 positive staining. S100 is a variable IHC marker of myxomas. DS 4, S100 IHC, 4x objective.

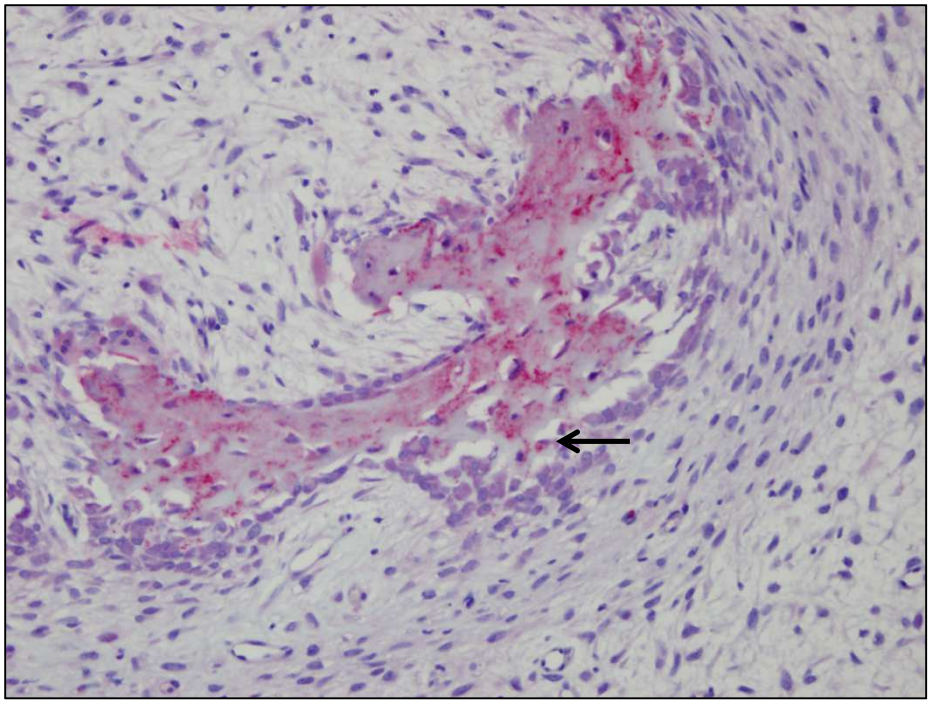

Figure 4.12: Bone production by an experimentally-induced tumor demonstrating positive staining for differentiated osteoblasts (arrow) and bone. DS 4, osteocalcin IHC, 20x objective.

Discussion

Recently, we described a syndrome of sinus tumors in Rocky Mountain bighorn sheep[53] with multiple features suggestive of an infectious etiology. These features include occurrence within wild populations at high numbers with no apparent age predilection (Chapter 3), and distinct geographical clustering of cases (Chapter 3). However, despite indications that bighorn sheep sinus tumors are an infectious disease, initial screens for likely infectious agents were negative.

To investigate the hypothesis that these tumors are caused by an infectious agent, we inoculated bighorn sheep and domestic sheep lambs with a cell-free filtrate originating from the tissues and exudates of a naturally-occurring bighorn sheep sinus tumor. The results of this experiment demonstrate transmission of the tumors to both domestic sheep and bighorn sheep. While sample sizes in this experiment were small and no statistical analysis was performed, the

occurrence of tumors was highly suggestive of transmission due to the administered inoculum. Only inoculated animals developed tumors, the tumors which developed all had characteristics consistent with bighorn sheep sinus tumors, and in most cases the tumors formed specifically at the site of inoculation.

For the domestic sheep, all four of the inoculated animals developed tumors. Three of those four inoculated animals developed tumors specifically at the site of inoculation. While an additional, smaller tumor formed in one case at another location within the nasal cavity, the tumor at the site of inoculation was the first and largest tumor seen. In one of the four inoculated domestic sheep no tumor formed at the site of inoculation, but a small tumor did form at a more rostral location in the nasal cavity late in the study. This tumor, and possibly the smaller tumor in the previously-mentioned sheep, may represent animal-to-animal transmission between the treatment animals which were housed together throughout the study.

For the bighorn sheep, only one of the four inoculated animals developed a tumor. This tumor was similar to those that formed in the domestic sheep in that it occurred at the site of inoculation, and had characteristics consistent with naturally-occurring bighorn sheep sinus tumors. The lack of tumors in the other three inoculated bighorn sheep seems inconsistent with the more dramatic results observed in the domestic sheep. Because the sample sizes are small for this study, is it possible that there is no significant difference between the two species' responses to administration of the inoculum. Alternatively, domestic sheep may be more susceptible to the disease, possibly suggesting some degree of host adaptation by the etiologic agent to bighorn sheep that is lacking in domestic sheep.

The mucus-filled cystic expansions of the maxillary sinus lining seen in one bighorn sheep and one domestic sheep in this experiment have been seen associated with naturally-

occurring bighorn sheep sinus tumors, but it is uncertain whether or not these are part of the same disease process. The locations of the cysts were not consistent with the locations at which the sheep were inoculated, but cysts did develop only in inoculated animals. Discovery of the specific etiology for this disease will help to further define whether these cystic structures are nonspecific findings or are related to tumor development.

Because of the consistently-observed intense chronic inflammation seen in naturally-occurring cases of bighorn sheep sinus tumors, and because of the well-described bacterial pneumonias of bighorn sheep, we considered bacterial infection as a potential cause for these tumors via initiation of chronic inflammation which may progress to neoplasia. In this study, bacterial agents were eliminated from the inoculum by filtration through a 0.45 micron filter, and the resulting tumors lacked significant inflammation. The successful transmission of tumors using an inoculum that excluded bacteria, and the lack of inflammation in the experimentally-induced tumors, suggest that bighorn sheep sinus tumors are not caused by bacterial infections leading to chronic inflammation. Rather, we suspect that bacterial infections seen in naturally-occurring cases of bighorn sheep sinus tumors are probably a secondary process, due to decreased clearance of agents by the altered sinus lining. This may have implications for sinus tumors as predisposing agents to bacterial infections in the upper, and possibly lower respiratory tracts.

In addition to testing whether or not bighorn sheep sinus tumors are infectious, this study also provided an opportunity to further characterize the histologic and immunohistochemical features of these masses. The experimentally-induced tumors were composed of stellate to spindle cells embedded within a myxomatous matrix but with relatively benign features, consistent with myxomas. Bone production by the tumors was prominent, but orderly and highly

reminiscent of fetal bone formation by intramembranous ossification, suggesting a periosteal origin for the cells. Immunohistochemical staining results supported the diagnosis of myxoma and confirmed multifocal osteoblastic differentiation in the tumors. Taken together, these findings suggest that the tumors examined here may result from inappropriate stimulation of the periosteum by growth factors promoting orderly proliferation of primitive mesenchyme, rather than clonal expansion by a virus-infected, transformed neoplastic cell. The epithelial component of the naturally-occurring tumors may be a combination of a response to growth factors, hyperplasia in response to secondary bacterial infections or, possibly, these cells may be targeted by the infectious agent.

This experiment has demonstrated transmission of bighorn sheep sinus tumors to both domestic and bighorn sheep via intranasal inoculation of a cell-free filtrate originating from a naturally-occurring bighorn sheep sinus tumor. The location of the tumors at the site of inoculation, the lack of tumors in control animals, and the reproduction of features consistent with naturally-occurring cases all indicate successful transmission of the disease, and therefore an infectious etiology for the tumors. Further characterization of the tumors by histopathology and immunohistochemical staining suggests origin from periosteum, with orderly proliferation of primitive mesenchyme and bone production. Future studies to identify a specific infectious etiology for this disease, and to identify the host cell targeted for infection, will allow further investigation into the pathogenesis of this lesion.

CHAPTER FIVE – INVESTIGATION OF A SPECIFIC ETIOLOGY

Introduction

Bighorn sheep sinus tumors are a recently-described disease in Rocky Mountain bighorn sheep[53]. Tumors are typically located within the paranasal maxillary and frontal sinuses, and are characterized by stromal proliferation of spindle cells[53], likely arising from the multipotent periosteum lining the bone of the sinus cavities (Chapter 4). Epithelial proliferation is also common, but not as prominent as the stromal population which invades and destroys underlying bone. Multiple features of these tumors are suggestive of an infectious etiology, including a nonrandom distribution of cases geographically (Chapter 3), and experimental reproduction of the disease by intranasal inoculation of a cell-free filtrate into the nasal cavities of domestic sheep and bighorn sheep lambs (Chapter 4).

Bighorn sheep sinus tumors are reminiscent of oncogenic retroviral diseases of domestic sheep and goats including Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus (ENTV). In domestic sheep JSRV causes multifocal pulmonary adenocarcinomas[33,105], while ENTV causes nasal adenocarcinomas within the ethmoid turbinates of domestic sheep (ENTV-1)[35,43] and domestic goats (ENTV-2)[24,31]. Despite similarities, initial screens of bighorn sheep sinus tumors by PCR and IHC for JSRV, ENTV-1, and ENTV-2 were negative[53]. Based on a suspected viral etiology for bighorn sheep sinus tumors, we conducted a series of experiments to investigate the specific etiology of this disease.

Cell Culture

Histologically, bighorn sheep sinus tumors do not contain viral inclusions or other hallmarks of viral infection. However, we were interested to determine if signs of viral infection (cytopathic effects) may be seen if the cells were grown in tissue culture. Additionally, growing

infected cells in culture may ultimately provide a method for virus growth and production for experimental studies, as well as an *in vitro* platform for researching the virus without requiring the use of live animals. To further examine the cellular features of bighorn sheep sinus tumors, and to possibly provide material for future experiments, three bighorn sheep sinus tumors were propagated in cell culture during the course of this project.

Methods

Approximately 1 gram of bighorn sheep sinus tumor tissue was collected at necropsy within 2 hours of death, placed in a 50 mL conical tube with PBS, and transported to the laboratory on ice. The material was then washed three times with sterile PBS, with a centrifugation step following each wash. Four washes were then performed using a solution of PBS with 5% penicillin/streptomycin antibiotic added. Tissues were then only handled within a laminar flow hood and sterile instruments.

The tissue was split among at least 4 tissue culture flasks with DMEM media supplemented with 10% fetal bovine serum, 1% L-arginine, 1% penicillin/streptomycin antibiotics, and 0.1% amphotericin antifungal agent. After 24 hours, cells had begun to adhere to the bottom of each flask, and the large pieces of tissue were removed. The adhered cells were washed with sterile PBS and new media was added. Cells were subsequently washed and new media added every 3-5 days. If cells were split to begin new passages, the cells were trypsinized until approximately 75% of the cells were detached, and those cells were transferred to a new flask, or frozen in liquid nitrogen for archival.

Results

All of the tissues grown in cell culture proliferated well for approximately three passages, after which cell proliferation stopped or slowed dramatically. During the first passage, there was

a mix of epithelial (Figure 5.1) and fibroblastic (Figure 5.2) cells, although as the cultures matured, the epithelial cells were quickly outgrown by the fibroblasts. Cytopathic effects were observed in all tumor cell cultures and included multinucleated cells (Figure 5.3) and cytoplasmic vacuolization (Figure 5.4). These cytopathic effects can be associated with viral infection but are not suggestive of a specific virus.

Indicators of neoplasia included foci of cells robustly proliferating despite contact with surrounding cells (lack of contact inhibition). While this effect was seen in both epithelial (Figure 5.5) and fibroblastic (Figure 5.6) cells, the fibroblastic population was more dramatically affected. In one tumor cell culture, fibroblasts repeatedly formed balls of cells that would pull up off of the culture flask surface (Figure 5.7). When these cells were mildly trypsinized, agitated to separate the cells, and re-plated, the cells would form a monolayer and then repeat the process of balling up and pulling off of the culture flask surface. The predominance of fibroblast proliferation versus epithelial cell proliferation recapitulates the histologic features of the naturally occurring tumors, which are predominated by invasive, proliferating fibroblastic cells.

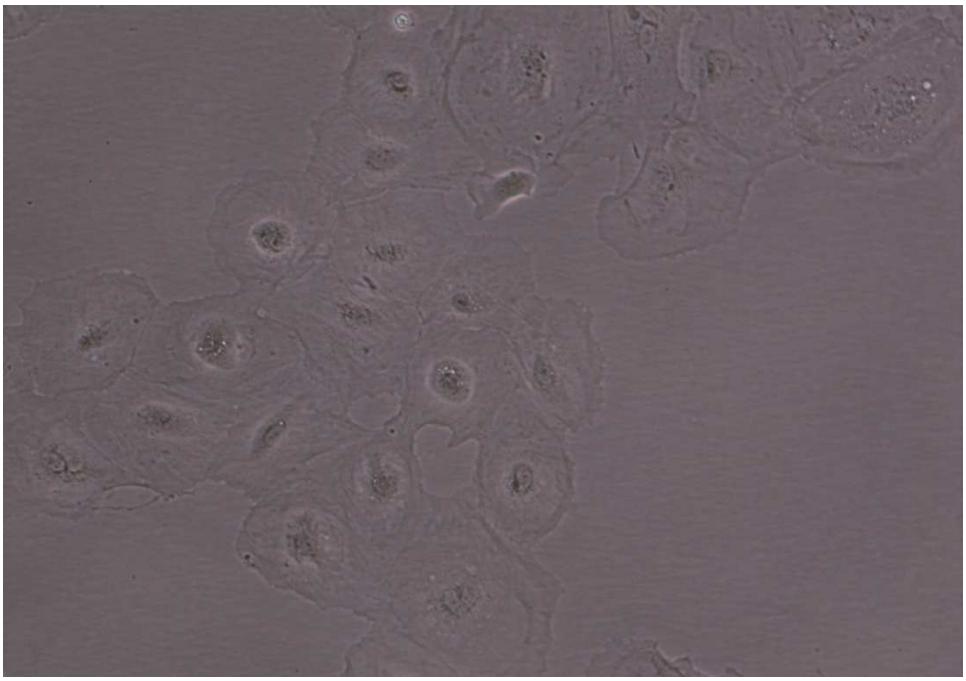

Figure 5.1: Epithelial cells in tissue culture, bighorn sheep sinus tumor.

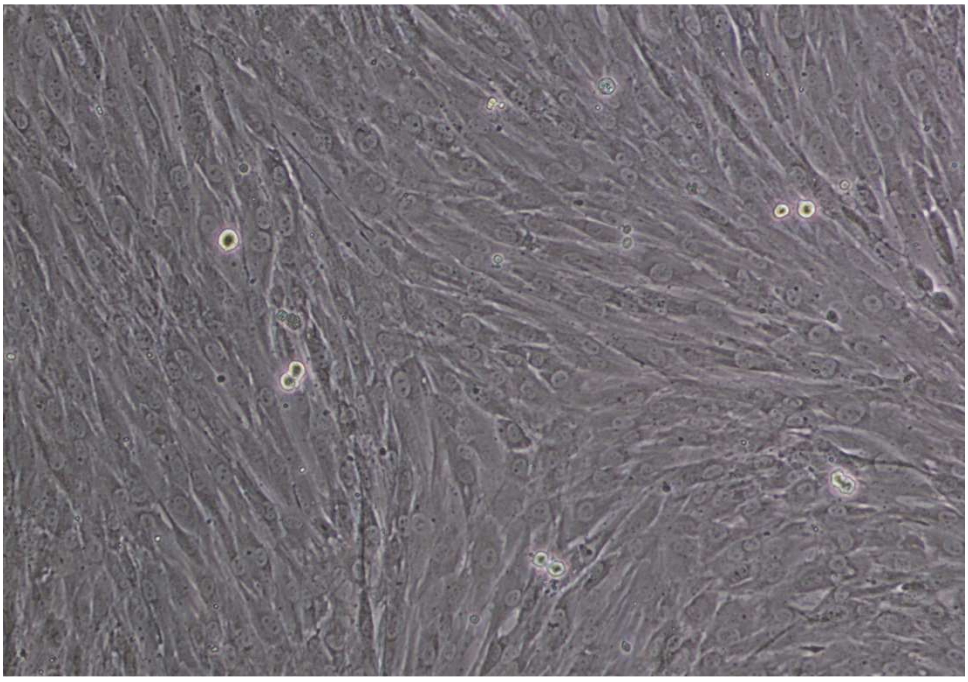

Figure 5.2: Fibroblasts in tissue culture, bighorn sheep sinus tumor.

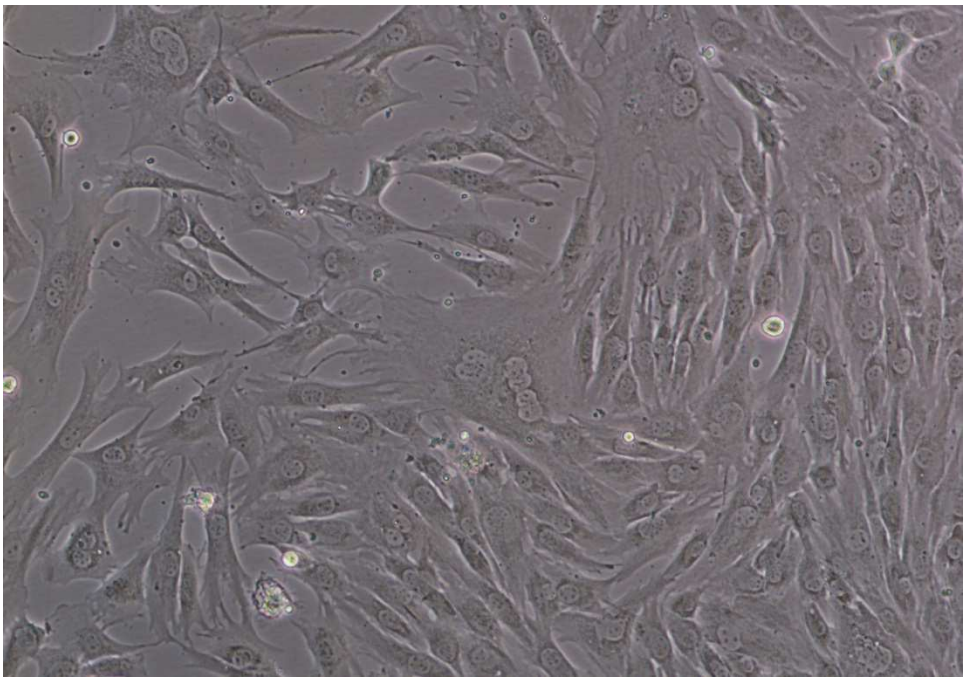

Figure 5.3: Multinucleated cell in tissue culture, bighorn sheep sinus tumor.

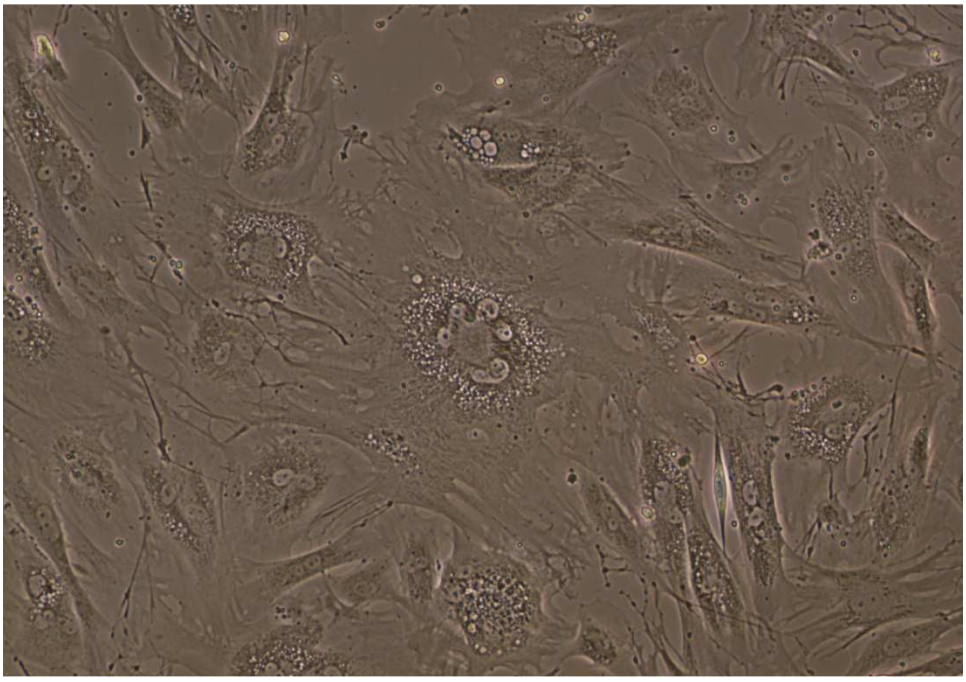

Figure 5.4: Cytoplasmic vacuolation in tissue culture, bighorn sheep sinus tumor.

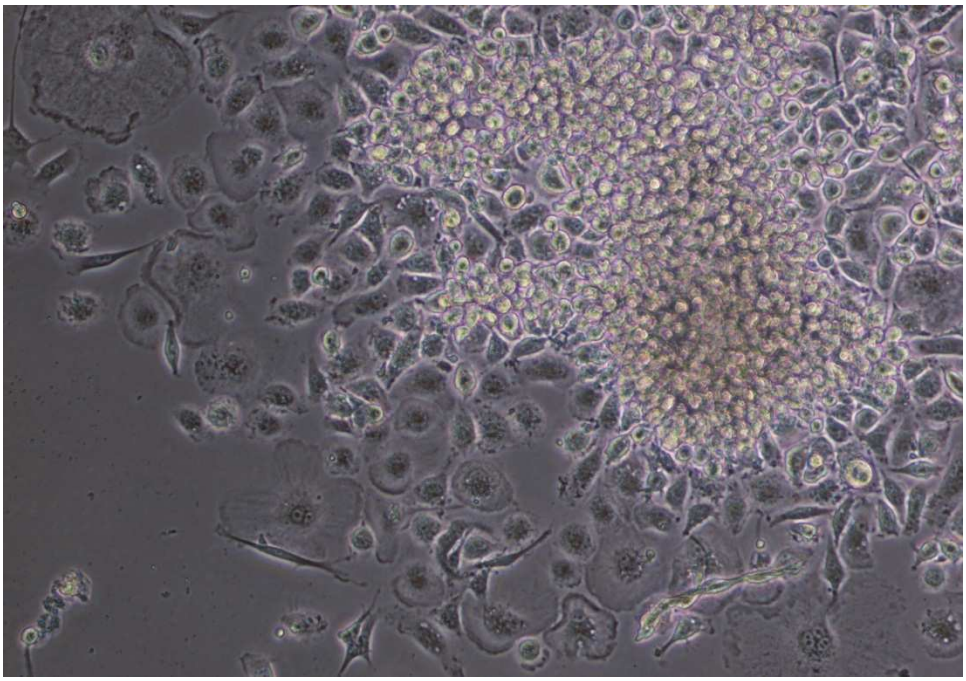

Figure 5.5: Focus of robust epithelial cell proliferation in tissue culture, demonstrating lack of contact inhibition which is a characteristic of neoplasia, bighorn sheep sinus tumor.

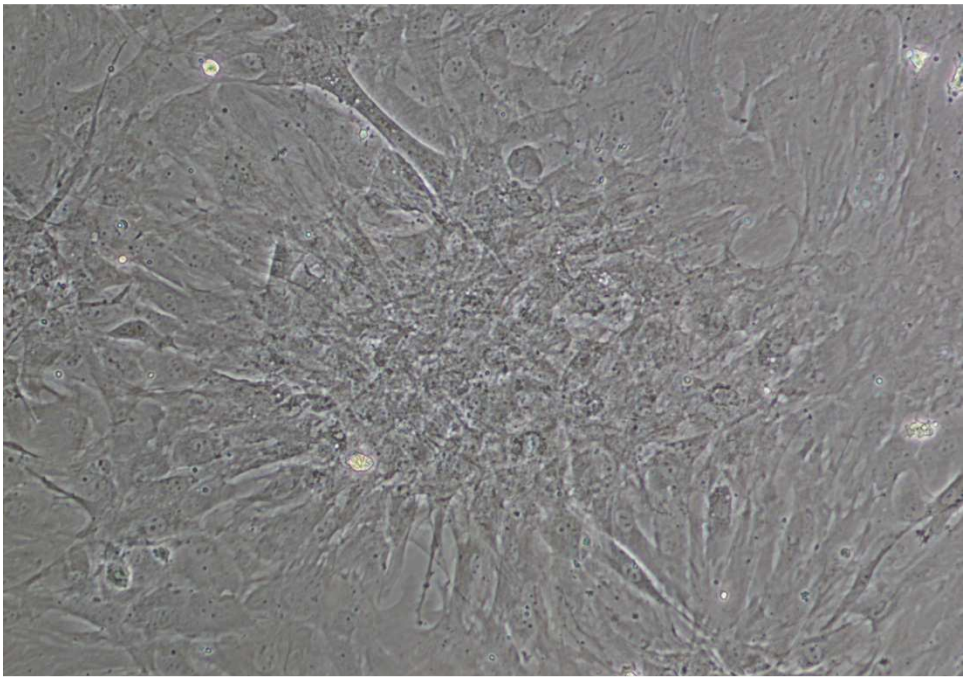

Figure 5.6: Focus of robust fibroblastic proliferation in tissue culture, demonstrating lack of contact inhibition which is characteristic of neoplasia, bighorn sheep sinus tumor.

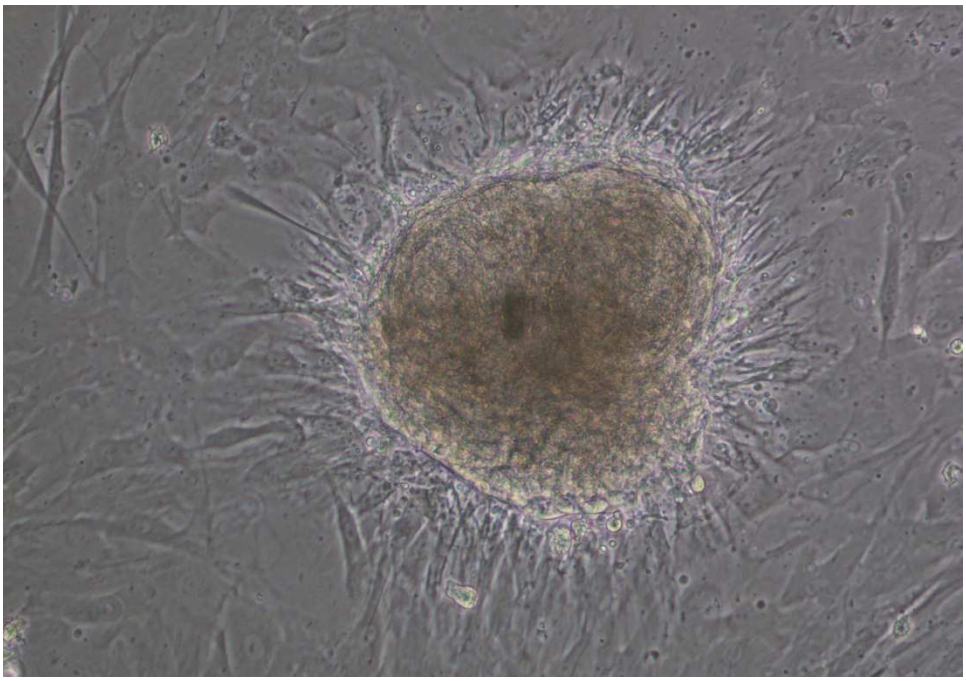

Figure 5.7: Progression of fibroblastic focus of proliferation in tissue culture, as shown in Figure 5.6. The fibroblastic cells have grown on top of each other in multiple layers, which have then balled up and pulled off of the surface of the flask, bighorn sheep sinus tumor.

Conclusions

We successfully propagated three naturally-occurring bighorn sheep sinus tumors in tissue culture, and observed cytopathic effects (multinucleated cells, vacuolization) consistent with viral infection. We also observed a lack of contact inhibition and the formation of foci with uncontrolled proliferation, consistent with neoplasia. Both epithelial and fibroblastic cells were present in culture, and both populations demonstrated cytopathic effects and neoplastic features. Of the two populations, fibroblastic cells demonstrated more dramatic proliferation. This is consistent with histologic features seen in naturally-occurring tumors, where stromal cells predominate and account for much of the invasive behavior of the tumors.

Electron Microscopy

Nasal secretions

Given our early observations of copious mucus within the nasal and paranasal sinuses of bighorn sheep with sinus tumors, and the apparent transmissibility of this disease, we suspected that these mucinous secretions might be harboring large numbers of infectious virus particles, and that these viral particles might be visible by negative contrast electron microscopy (EM). We prepared nasal secretions from three bighorn sheep affected by sinus tumors, and one animal categorized as tumor-suspect that had abundant mucinous nasal discharge, but no tumor at necropsy.

Methods

Approximately 5 mL of mucus for each sample was clarified by centrifugation at 8,000 rpm for 30 min at 4°C. The supernatant was then concentrated at 100,000 g for 2 hrs at 4°C. The resulting pellet was resuspended in 250 µL of TNES (Tris, Na, EDTA, SDS) buffer, and centrifuged again at 100,000g for 2 hrs at 4°C over a 60%/20% sucrose gradient. The sucrose interface was collected, including 1 mL on either side of the interface. The collected material was centrifuged again at 100,000 g for 2 hrs at 4°C, and the supernatant poured off of the pellet. The pellet was saved in the small amount of TNES/sucrose at the bottom of the tube, the tube covered in parafilm, and transported on ice to the EM facility (Wyoming State Veterinary Laboratory, Laramie, WY) where pellets were diluted, stained, and loaded onto grids for EM evaluation.

Results / Conclusions

The three samples of nasal exudates from animals with tumors all demonstrated variably-sized and shaped blebs ranging from 70 nm to 100 nm in diameter. The particles were smudged,

with no distinct zones of electron density. Extracellular viral particles from ENTV are described as being "round in shape, 90-110 nm in diameter with an electron-dense zone surrounded by a clear zone and a membrane with numerous spikes (6-10 nm)"[35]. No particles matching this description were seen in the samples of nasal exudates from bighorn sheep with sinus tumors. No particles at all were seen in the sample from the bighorn sheep with nasal exudates, but lacking a sinus tumor. We concluded that we were unable to detect viral particles by negative contrast EM within these samples.

Cell culture

Based on observations of cytopathic effects in bighorn sheep sinus tumors propagated *in vitro*, we attempted EM using tissue culture material from a naturally-occurring bighorn sheep sinus tumor. This material grew well in culture, producing expansive nests of epithelial cells early on (Figure 5.5) and robustly proliferating fibroblasts in later passages. The proliferative fibroblasts showed robust growth past the point of expected contact inhibition, forming balls of cells growing on top of each other, which often peeled off the bottom of the flask (Figure 5.6). These balls of cells could be quickly lifted from the culture with minimal trypsinization, agitated by pipetting to disrupt the aggregate, re-plated, and the same process of growth and balling up was noted for two additional passages. The vast nests of epithelial cells contained perinuclear dark aggregates that we could not further identify (Figure 5.8). To further assess the perinuclear material, we prepared the sample for thin-section EM.

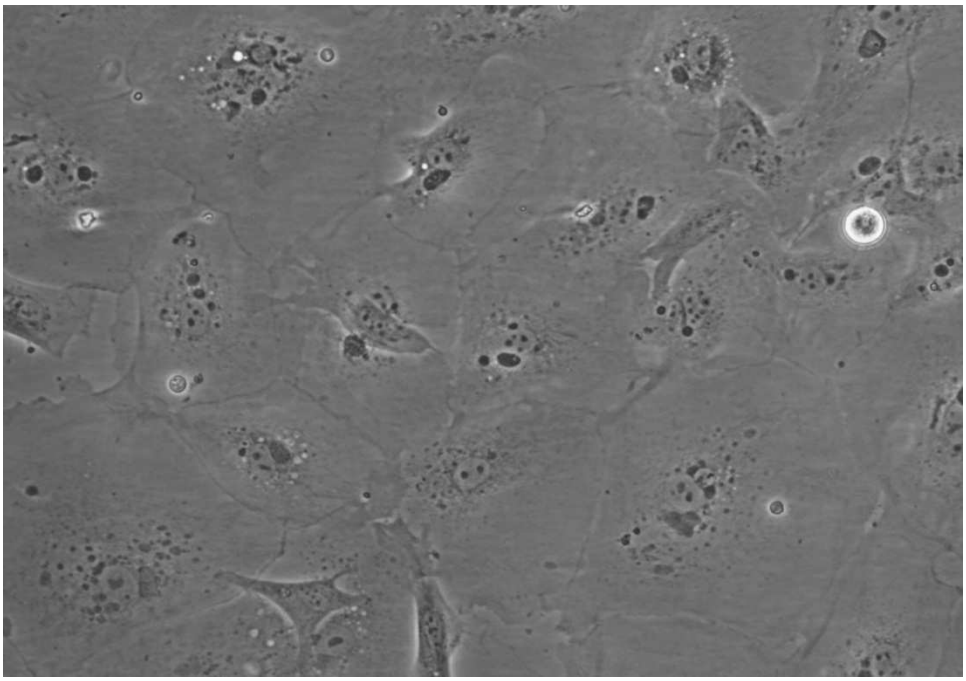

Figure 5.8: Perinuclear aggregates seen in epithelial cells in tissue culture and further examined by thin section electron microscopy. Bighorn sheep sinus tumor.

Methods

We fixed the contents of the tissue flask containing abnormal epithelial cells (Figure 5.8) by washing the cells and adding 4% gluteraldehyde for two hours. We then used a sterile tissue culture scraper to selectively scrape only the nests of epithelial cells, releasing these fixed cells from the bottom of the flask. We collected the fixative/cells, and centrifuged the material at 200 g for 5 minutes, to form a pellet of cells. These cells were embedded in amber, and examined by thin section EM (D. N. Rao Veeramachaneni, Colorado State University, Animal Reproduction and Biotechnology Laboratory).

Results/Conclusions

Three cells were examined by thin-section EM. All of the cells examined contained intracytoplasmic structures that were most consistent with degenerative organelles, but could not be further identified. Other features of the cells noted by Dr. Veeramachaneni included nucleolar

fusion to the nuclear membrane, which he recognized as a possible feature of neoplasia. No viral particles were seen. We concluded that the perinuclear structures observed in cell culture were likely degenerate organelles, and that we were unable to detect viral particles in bighorn sheep sinus tumor cells propagated in tissue culture.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was the predominant method we used to attempt to identify the causative agent of bighorn sheep sinus tumors. Given our hypothesis that this disease is similar to the oncogenic retroviruses of domestic sheep and goats (JSRV and ENTV), we first tested specifically for those agents with negative results[53]. Because we suspected a viral etiology for bighorn sheep sinus tumors, we then used degenerate PCR primers designed to amplify well-conserved regions of the viral genome to screen naturally-occurring tumors for the presence of oncogenic retroviruses and herpesviruses. When applicable, sequences amplified using degenerate PCR primers were used to create PCR primers specific to the sequences amplified from bighorn sheep samples. These specific primers were then used to screen tumor-positive, tumor-suspect, and tumor-negative bighorn sheep sinus tissue samples to determine if there was an association between the identified virus and the occurrence of tumors. Multiple primer sets were employed during the course of this investigation. Those with results reported here are included in Table 5.1, including the expected product size, reaction details, and cycling conditions.

Table 5.1: Primer sequences and cycling conditions for PCR reactions used to investigate possible infectious etiologies for bighorn sheep sinus tumors.

Primers	Expected product	Reaction Details	Cycling Conditions
Herpesvirus *pol* gene DFA(fw): 5'-gayttygcnagyytntaycc-3' ILK(fw): 5'-tcctggacaagcagcarnysgcnmtnaa-3' KG1(rv): 5'-gtcttgctcaccagntcnacnccytt-3' TGV(fw): 5'-tgtaactcggtgtayggnttyacnggngt-3' IYG(rv): 5'-cacagagtccgtrtcnccrtadat-3'	2nd round: ~220 bp	Round 1: 50 µL reaction -45 µL Platinum PCR SuperMix (Invitrogen) -1000 ng genomic DNA -0.2 µM each primer (DFA, ILK, KG1) Round 2: 50 µL reaction -45 µL Platinum PCR SuperMix (Invitrogen) -3 µL product (Rd 1) -0.2 µM each primer (TGV, IYG)	Round 1 and Round 2: 1 cycle 94°C x 3 min 60°C x 2 min 72°C x 1 min 45 cycles 94°C x 30 sec 46°C x 1 min 72°C x 30 sec 1 cycle 72°C x 7 min
Bighorn sheep rhadinovirus 5'BHS(fw): 5'-ccatgcttaaaaattgcagagact-3' 3'BHS(rv): 5'-cacgcaaactagcgtgttctt-3'	~150 bp	25 µL reaction: 22.5 µL Platinum PCR SuperMix -0.8µM each primer -100 ng genomic DNA	1 cycle 94°C x 3 min 39 cycles 94°C x 30 sec 56°C x 30 sec 72°C x 30 sec 1 cycle 72°C x 7 min
Retrovirus *pol* gene LPQG(fw): 5'-tggaaagtgytrccmcargg-3' YMDD(rv): 5'-ctiagiakrtcrtccatrta-3'	~130 bp	50 µL reaction: -45 µL Platinum PCR SuperMix -0.2µM each primer -1000 ng genomic DNA	10 cycles: 94°C x 1 min 37°C x 2 min 72°C x 3 min 30 cycles: 94°C x 30 sec 55°C x 1 min 72°C x 1 min
Retrovirus tRNA binding site and *gag* gene K12 tRNA (fw): 5'-canbtggcgcccaacgtggggc-3' GAG-D (rv):	~1140 bp	25 µL reaction: -22.5 µL Platinum PCR SuperMix -0.4 µM each primer -200 ng genomic DNA	1 cycle: 92°C x 2 min 15 cycles: 92°C x 10 sec 61°C x 30 sec 68°C x 45 sec

5’-cawtkttcaaaaaaytcagatttcca-3’			20 cycles: Increase extension by 40 sec every 4 cycles 1 cycle: 68°C x 7 min
JSRV *gag* gene JSRV Scr(fw): 5’-ccccatctctgaaaatgcac-3’ JSRV Scr (rv): 5’-tgtttagacggtggaggaaa-3’	~320 bp	25 µL reaction: -22.5 µM Platinum PCR SuperMix -0.8 µM each primer -100 ng genomic DNA	1 cycle: 95°C x 2 min 40 cycles: 95°C x 30 sec 56°C x 30 sec 68°C x 45 sec 1 cycle: 68°C x 3 min
ENTV-1 *gag* gene FragB(fw): 5’-atccgtccctacattcgtc-3’ FragB(rv): 5’-ccttgaacatctgttttggacc-3’	~1400 bp	25 µL reaction: -22.5 µL Platinum PCR SuperMix -0.8 µM each primer -100 ng genomic DNA	1 cycle: 95°C x 2 min 40 cycles: 95°C x 30 sec 56°C x 30 sec 68°C x 2 min 1 cycle: 68°C x 2 min
ENTV-2 LTR, U3 region (hemi-nested) PCI(fw): 5’-gcaaaatgccaggaccttgg-3’ PCII(rv): 5’-gatcttatctgcttattttcag-3’ PCIII(fw): 5’-ccctcaggaagtcttaaaag-3’	1st Round: ~180 bp 2nd Round: ~75 bp	25 µL reaction: -22.5 µL Platinum PCR SuperMix -0.8 µM each primer -100 ng genomic DNA	1st Round (PCI, PCII): 1 cycle: 95°C x 10 min 35 cycles: 95°C x 30 sec 55°C x 30 sec 72°C x 1 min 1 cycle: 72°C x 3 min 2nd Round: (PCII, PCIII) Decrease annealing temp to 53°C
ENTV-2 LTR (BHS specific) kfLTR(fw): 5’-gccaccctcaggaagtctta-3’	~100 bp	25 µL reaction: -22.5 µL Platinum PCR SuperMix	1 cycle: 95°C x 10 min 40 cycles:

kfLTR(rv): 5'-caatcaccggatccttatgt-3'		-0.8 µM each primer -100 ng genomic DNA	95°C x 30 sec 53°C x 30 sec 72°C x 1 min 1 cycle: 72°C x 3 min

Herpesvirus degenerate PCR primers

The oncogenic potential of herpesviruses is well-recognized, with tumors caused by herpesviruses found in a variety of species including birds, amphibians, primates, and humans[26,71]. Oncogenic herpesviruses are typically within the subfamily *Gammaherpesvirinae,* and these viruses can cause neoplasia by viral transformation of individual cells, leading to clonal expansion and neoplasia, and/or by paracrine signals causing proliferation of normal, non-transformed cells[26]. To investigate herpesviruses as a potential cause for bighorn sheep sinus tumors, we used degenerate PCR primers against a well-conserved region of the DNA polymerase gene to amplify a novel gammaherpesvirus from samples of bighorn sheep sinus tumors. We then screened samples of normal and abnormal bighorn sheep sinus lining tissues to determine whether or not this novel virus is associated with bighorn sheep sinus tumors.

Methods

Bighorn sheep sinus lining tissues were collected as described in Chapter 3. Genomic DNA was extracted by phenol/chloroform extractions as previously described[53], and screened for amplifiable DNA by GAPDH PCR. A subset of samples from tumor-positive tissues was screened for the presence of herpesviruses by PCR using degenerate primers designed to amplify a well-conserved region of the DNA polymerase gene[136]. The primer sequences and cycling conditions are listed in Table 5.1. Resulting products were analyzed by gel electrophoresis on a 1.5% agarose gel, and bands of the expected size (~220 bp) were extracted from the gel. This extracted DNA was purified, cloned, and sequenced. Cloning was performed by ligation with a

pGEM-T Easy cloning vector (Promega) and transformation into One Shot TOP10 chemically competent E. coli (Invitrogen). Sequencing was performed using a 3130xL Genetic Analyzer (Applied Biosystems) at Colorado State University Proteomics and Metabolomics Facitily (Fort Collins, CO). Based on the resulting sequence, we created PCR primers internal to the degenerate primer sequences, specific to our samples.

Results/Conclusions

We successfully amplified an approximately 220 base pair fragment of DNA from the polymerase gene of a gammaherpesvirus. This sequence was similar, but not identical, to a previously published rhadinovirus described in mouflon sheep[73]. The bighorn sheep virus contained two consistent base pair substitutions from the mouflon sequence (Figure 5.9).

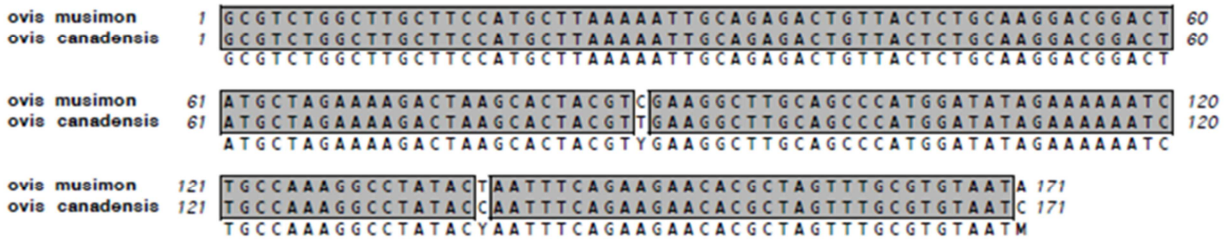

Figure 5.9: Alignment of a portion of the *pol* gene from two ruminant rhadinoviruses. The top line (O*vis musimon*) is a previously published sequence amplified from the peripheral blood of a mouflon sheep. The bottom line (*Ovis canadensis*) is the sequence of a novel rhadinovirus, amplified from sinus tissue of a Rocky Mountain bighorn sheep sinus tumor.

Additional sequence information could not be obtained from the bighorn sheep virus using degenerate primers exterior to the 220 base pair sequence. In addition to the sequence shown in Figure 5.9 from a mouflon sheep, similar sequences have also previously been amplified from the peripheral blood of animals representing numerous ruminant species[73]. None of the animal of various species in this study displayed illness, suggesting a nonpathogenic role for the viruses[73]. However, to investigate whether or not this bighorn sheep rhadinovirus could be the

cause of bighorn sheep sinus tumors, we created PCR primers specific to the sequence amplified from bighorn sheep tissues, and screened all archived samples (including tumor-positive, tumor-negative, and tumor-suspect tissues) using these specific primers.

Herpesvirus specific PCR primers

Methods

Specific PCR primers were designed to amplify a 150 bp fragment of the bighorn sheep rhadinovirus identified by degenerate primer PCR as described above. We used these primers to screen 97 bighorn sheep tissue samples for the presence of viral DNA. Tissue samples originated from bighorn sheep sinus lining that was categorized as tumor-positive, tumor-suspect or tumor-negative by criteria previously described (Chapter 3). DNA was extracted from tissues by phenol-chloroform extraction, and amplifiable DNA was demonstrated by GAPDH PCR. Primer sequences and cycling conditions are listed in Table 5.1.

Results/Conclusions

We screened 97 bighorn sheep sinus lining tissue samples by PCR using primers specific for bighorn sheep rhadinovirus. We found 50/97 (52%) of samples to be positive for the bighorn sheep rhadinovirus. Based on tumor category, we found 7/14 (50%) of tumor-positive tissues, 18/33 (55%) of tumor-suspect tissues, and 25/50 (50%) of tumor-negative tissues to be positive for the bighorn sheep rhadinovirus. There was no statistical difference in PCR results between tumor-positive and tumor-negative groups ($p=1.0$, Figure 5.10) based on a Fisher's exact test (GraphPad QuickCalcs, graphpad.com).

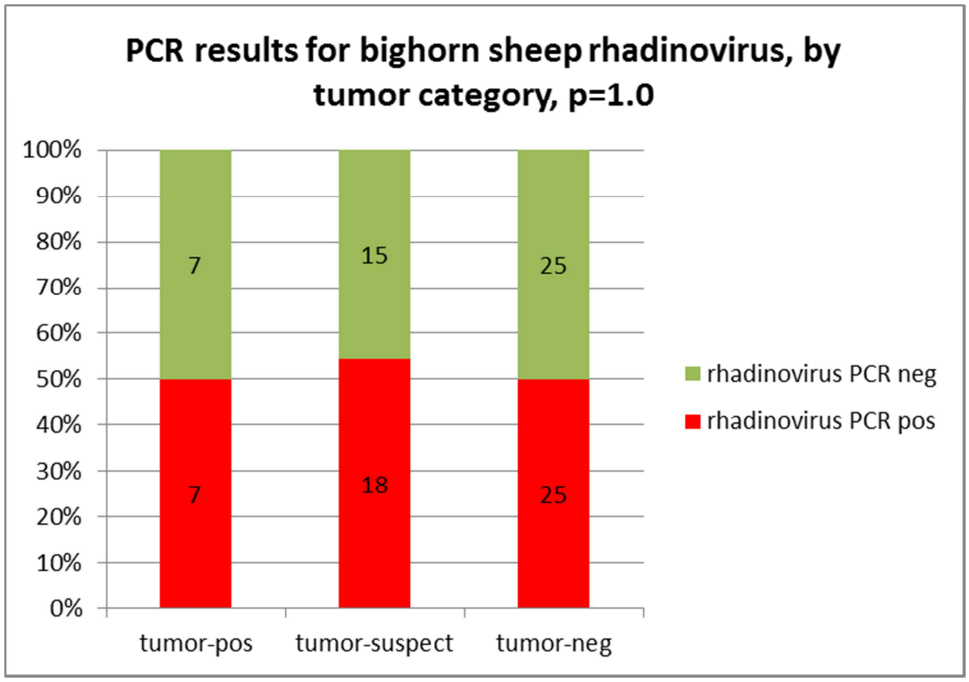

Figure 5.10: Results of PCR assay for bighorn sheep rhadinovirus, based on tumor category. No significant difference was found for PCR results between tumor-positive and tumor-negative groups. Tumor-suspect data are shown for reference, but was not included in the statistical analysis.

Conclusions, Herpesvirus PCR

To screen bighorn sheep sinus tumors for the presence of a herpesvirus, we employed established degenerate PCR primers to amplify a portion of the herpesvirus DNA polymerase gene. This proved to be an effective strategy, and we successfully amplified DNA from a novel gammaherpesvirus (rhadinovirus) from our samples. We found this virus to be present in 52% of bighorn sheep sinus lining tissue samples, with no apparent association between presence of virus and presence of tumors (Figure 5.10). While the role of this virus in bighorn sheep is unknown, we found no evidence to suggest a role for the bighorn sheep rhadinovirus in the development of bighorn sheep sinus tumors. A similar rhadinovirus was amplified from domestic sheep during the initial search for an infectious agent causing enzootic nasal tumors[37] and this virus was also determined to be an incidental finding. Additionally, similar

rhadinoviruses have been found widespread in ruminant populations in the absence of disease[73]. While these viruses are an interesting finding and could play a role in future research, the bighorn sheep rhadinovirus described here does not appear to be the cause of bighorn sheep sinus tumors.

Retrovirus degenerate PCR primers

The oncogenic potential of retroviruses is well known, and retrovirally-induced tumors have been described in species ranging from fish[111] to humans[55]. Oncogenic retroviruses of domestic sheep and goats (JSRV, ENTV-1, and ENTV-2) cause neoplastic diseases in the upper and lower respiratory tracts of these animals by expression of the viral oncogene, *env* [80,146], causing neoplastic transformation and clonal expansion of infected cells. Given some similarities of bighorn sheep sinus tumors to previously-described oncogenic retroviruses of domestic sheep and goats, we focused much of our efforts on attempting to amplify exogenous retrovirus proviral DNA from bighorn sheep sinus tumor samples.

Methods

To screen bighorn sheep sinus tumor samples for the presence of retroviral integrated proviral DNA, we employed degenerate PCR primers, designed to amplify a well-conserved region of the retroviral RNA polymerase gene, as well as specific primers for JSRV, ENTV-1, and ENTV-2. Any products amplified of the expected size were cloned and sequenced as described above. If a resulting sequence was found likely to represent an exogenous virus, we screened tumor-positive, tumor-suspect, and tumor-negative bighorn sheep sinus lining tissue samples for the presence of this proviral DNA to determine if the virus was associated with bighorn sheep sinus tumors.

Bighorn sheep sinus lining tissues were collected and genomic DNA was extracted by

phenol-chloroform extractions previously described[53]. A subset of tumor-positive samples were screened for the presence of retroviruses by PCR using degenerate primers (LPQG (fw) and YMDD (rv)) designed to amplify a well-conserved region of the RNA polymerase gene[41]. Primer sequences and cycling conditions are listed in Table 5.1. Because genomic DNA extracted from sinus tumors was expected to contain endogenous retroviral sequences, we also extracted RNA to use as a starting template, expecting that most endogenous viruses are not replication-competent and therefore do not produce viral RNA. We extracted RNA from tissues and fluids associated with naturally-occurring bighorn sheep sinus tumors using RNA-bee (Tel-Test Inc.) according to the manufacturer's instructions. The extracted RNA was then DNAse treated and cDNA was synthesized using reverse transcription and the reverse primer YMDD. The resulting cDNA was then used as a template for conventional PCR using the degenerate retrovirus primers as described above. Control reactions were conducted using cDNA synthesized in the absence of reverse transcriptase and/or DNAse treatment.

A second set of retrovirus degenerate PCR primers were employed that were designed to selectively amplify exogenous sequences from genomic DNA. The forward primer (K12 tRNA) was designed within the retrovirus tRNA binding site, and the reverse primer (gag-D) was designed within a variable portion of the *gag* gene[21]. Primer sequences and cycling conditions can be found in Table 5.1.

For all PCR products resulting from retrovirus degenerate primer PCR, products were visualized by gel electrophoresis, and any products of the expected size were extracted from the gel, cloned, and sequenced as described above. When applicable, sequences were aligned with known exogenous and endogenous retroviral sequences and phylogenetically analyzed with MacVector software (utilizing the ClustalW program) to create a basic guide tree.

Results

Using PCR with genomic DNA from sinus tumor as a starting template, and degenerate retrovirus primers for the *pol* gene (LPQG, YMDD), we amplified a product of the expected length, approximately 130 base pairs. The product was gel-extracted, cloned, and two colonies were selected for sequencing. Both sequences grouped with endogenous retroviral sequences (Figure 5.11, 061909B and 060909C), not unexpectedly considering the starting template of genomic DNA.

To avoid amplifying endogenous sequences, we then attempted reverse transcriptase PCR methods using RNA (from tumor material and exudates) as a starting template. Using this approach, we again successfully amplified products of the expected length. These products were cloned and sequenced, with an additional 19 sequences analyzed. Again, all sequences aligned with endogenous sequences (Figure 5.11). The reason for this result may have been the presence of contaminating DNA (despite DNAse treatment and the lack of a product in our DNAse treated control with no reverse transcriptase), or the presence of replication-competent endogenous viruses.

Because our approach using degenerate PCR primers targeting the *pol* gene (LPQG, YMDD) identified only endogenous-like sequences, we attempted another previously published PCR approach using degenerate retrovirus PCR primers (K-12 tRNA (fw) and gag-D (rv)) designed to amplify only exogenous retroviral sequences from genomic DNA[21]. Using this approach, we successfully amplified a product of the expected length from genomic DNA extracted from bighorn sheep sinus tumor samples. Five products were cloned and sequenced, with sequences having high similarity to endogenous JSRV sequences (data not shown).

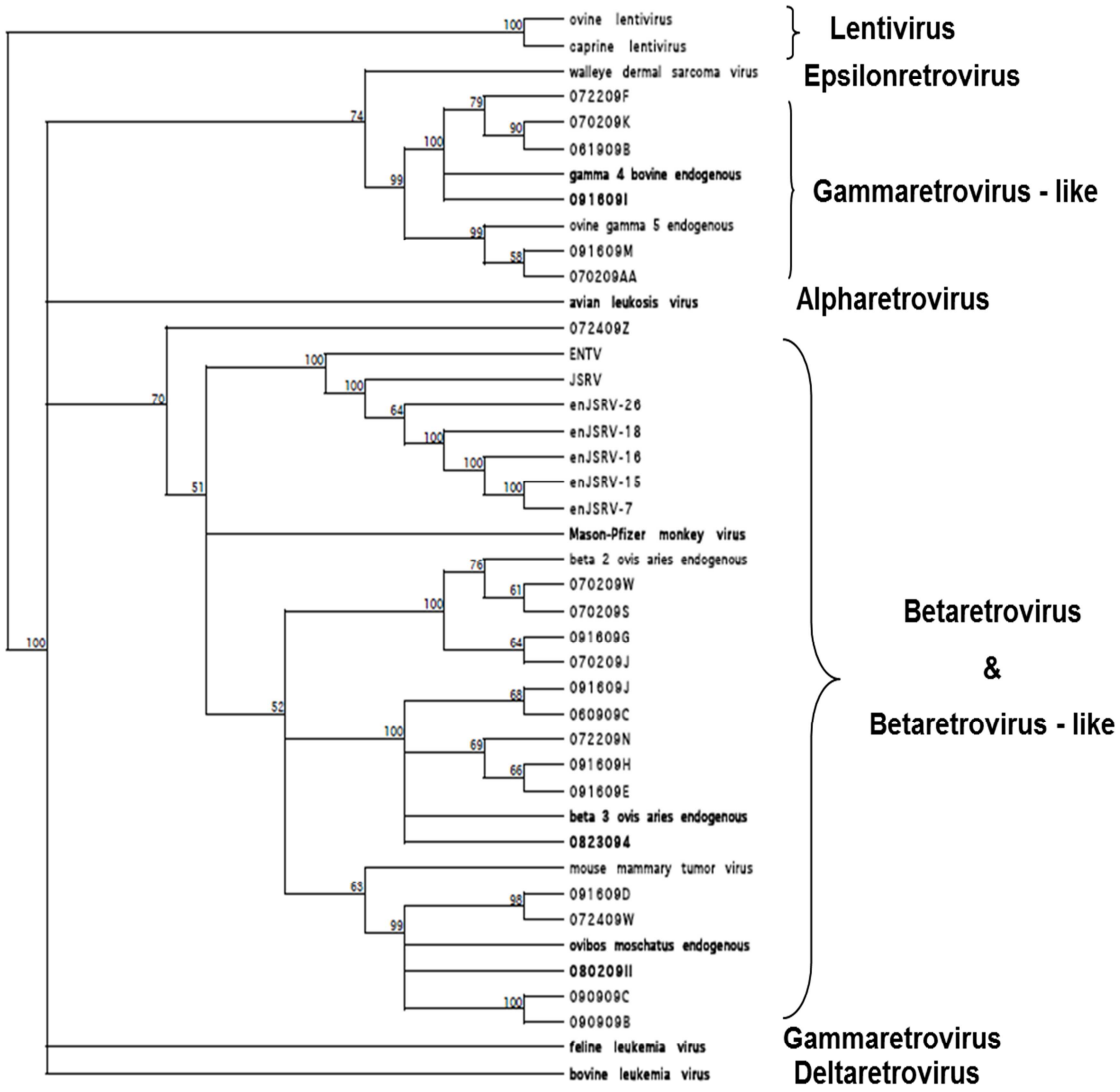

Figure 5.11: Phylogenetic analysis of 130 bp fragments of the retroviral *pol* gene, amplified using degenerate primers LPQG (fw) and YMDD (rv). Previously published endogenous and exogenous retroviral sequences were retrieved from GenBank and are included in the tree. Novel sequences amplified in this study are included as 7-8 character alphanumeric coded entries in the tree.

Conclusions

Because we have been highly suspicious of an oncogenic retrovirus as the cause of bighorn sheep sinus tumors, we attempted to amplify retroviral proviral DNA from bighorn sheep sinus tumor tissues and exudates. We used established degenerate retroviral primers to amplify conserved regions of the genome using both DNA and RNA as starting templates. We

hypothesized that if virus-infected cells were present in high numbers then we would readily amplify that sequence, even in the presence of endogenous sequences. As the results reflect, we did not readily amplify an exogenous virus. It is likely that contaminating DNA from endogenous sequences, or RNA from replication-competent endogenous viruses, interfered with our ability to demonstrate an exogenous virus using these methods. Alternatively, the causative agent of bighorn sheep sinus tumors is not a retrovirus. In either case, the methods we attempted did not appear to be an effective strategy for finding the cause of this disease.

Retrovirus specific PCR primers

Methods

Because bighorn sheep sinus tumors are reminiscent of oncogenic retroviruses of domestic sheep and goats (JSRV, ENTV-1, and ENTV-2), we performed PCR assays to screen bighorn sheep sinus tissue samples for the presence of proviral DNA from each specific virus. Tissues screened included tumors, as well as tumor-negative and tumor-suspect tissues. Genomic DNA was extracted with phenol-chloroform as described above. Due to possible contamination issues with ENTV-2 amplicon, final ENTV-2 PCR was performed using freshly extracted genomic DNA and new reagents. For these subsequent extractions, tissues were homogenized in sterile PBS using a Mini-Beadbeater-1 (BioSpec) system, with tubes containing Lysing Matrix A (MP Biomedical). DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions.

PCR reactions were performed using primers specific for regions of the exogenous retroviral sequences that varied from endogenous sequences, and also varied between JSRV, ENTV-1, and ENTV-2. Because of the high homology between these three viruses, as well as endogenous viruses, specific primers are difficult to design, and the location of adequate primers

is limited to a few small regions including a variable region of the *gag* gene and portions of the LTR. The JSRV specific primers (JSRV Scr (fw and rv)) were designed to amplify an approximately 320 bp fragment located within a variable region of the *gag* gene. The ENTV-1 primers (Frag B (fw and rv)) were designed to amplify an approximately 1500 bp fragment located within a variable region of the *gag* gene. The ENTV-2 primers (PCI (fw), PCII (rv), and PCIII (fw)) were designed to amplify either a 180 bp fragment (PCI and PCII), or an approximately 75 base pair fragment of the U3 region of the LTR, by hemi-nested approach (PCI and PCII followed by PCII and PCIII). Primer sequences and cycling conditions can be found in Table 5.1.

Results

A total of 97 bighorn sheep sinus lining tissues were screened for the presence of JSRV, ENTV-1 and ENTV-2 by PCR specific to each virus. All samples were negative for JSRV and ENTV-1. However, we did obtain some positive results for ENTV-2 by various PCR methods. While these results do not definitely identify ENTV-2 as the causative agent of bighorn sheep sinus tumors, there is indication of an association between the virus and the disease. The process by which these results were obtained and interpreted is outlined below.

Early in this investigation, a subset of bighorn sheep sinus tumors were screened for the presence of ENTV-2 provirus[53] using previously published primers designed to amplify a fragment of the U3 region of the LTR[99], which is variable between endogenous/exogenous sequences as well as between the exogenous sequences for JSRV, ENTV-1, and ENTV-2. The primer sequences and cycling conditions can be found in Table 5.1. The published protocol for these ENTV-2 specific primers included a hemi-nested approach, with PCI (fw) and PCII (rv) used in the first round, and PCIII (fw, internal to PCI and PCII) and PCII (rv) used in the second

round. However, as described in the original publication of these primers, only the first round was required to produce a PCR product of the expected size when applied to tumor material from domestic goats[99]. For this reason, our initial screen of bighorn sheep sinus tumors only included the first round of PCR, yielding negative results[53]. Later in the investigation, we screened additional samples for ENTV-2 using the complete, hemi-nested approach with some inconsistent positive results. Based on these results, we screened all previously extracted bighorn sheep sinus lining tissues using the hemi-nested PCR approach. Our results were inconsistent, however, and we struggled with possible contamination of the PCR reaction. For this reason we did not statistically analyze the results of the hemi-nested PCR approach, but we did take the results as evidence that ENTV-2, or a related virus, may be present in some of the bighorn sheep sinus tumor samples.

To further investigate ENTV-2 as a possible etiology for bighorn sheep sinus tumors, we attempted to acquire more sequence data using ENTV-2-specific PCR primers, and to then design new PCR primers, specific to the products amplified from bighorn sheep. Because the hemi-nested PCR product was only 75 base pairs in length, designing internal primers based on this sequence was not possible. To acquire more sequence information, we attempted to optimize the PCR conditions to yield the larger, 180 base pair product, expected from the first-round reaction. We were unable to visualize any bands of the expected size in the first round, but optimization of the hemi-nested protocol allowed visualization of a very faint second, larger band in the 2nd round of PCR in addition to the expected second-round 75 bp product.

The larger band, presumed to represent low amplification of the expected first round product, was amplified by band-stab[16] approach. While visualizing the gel by UV-trans-illumination, the band of interest was stabbed in the gel multiple times with a P20 pipette tip.

The tip was then submerged in a PCR tube containing the reagents for the first-round PCR reaction, the reaction mix was agitated, the pipette tip was removed, and the first round PCR protocol was repeated using this gel-stabbed material as a starting template. PCR conditions were slightly altered, by lowering the number of amplification cycles from 35 to 25. The resulting product was an intense, single band at approximately 180 base pairs of length. The PCR product was purified, and sent for direct sequencing which yielded 134 base pairs of sequence internal to PCI and PCII, identical to the published ENTV-2 sequence. We used this sequence information to develop primers internal to PCI and PCII. Primers were designed using Primer 3 software[115], and the expected product was 106 bp in length. Primer sequences and cycling conditions are listed in Table 5.1. These new primers (kfLTR (fw and rv)) were used to screen a subset of samples, and we successfully amplified a product of the expected length (approximately 100 bp) from several bighorn sheep tissue samples.

Based on previous contamination issues, we wanted to be sure that the bighorn sheep tissue samples were not contaminated with ENTV-2 amplicon. Therefore, all bighorn sheep tissue samples were re-extracted using the QIAamp DNA Mini kit (Qiagen) as described above. One sample was unavailable for re-extraction. The resulting 96 newly-extracted genomic DNA samples were screened for the presence of ENTV-2 using the kfLTR bighorn sheep specific primers. The PCR results were consistent based on duplicate runs of randomly-selected samples, and there was no evidence of contamination by PCR amplicon based on no-template-control reactions in each PCR run.

The results of the ENTV-2 PCR demonstrated 21/96 (22%) of the samples tested were positive for ENTV-2. Of these, 2/14 (14%) of tumor-positive, 7/49 (14%) of tumor-negative, and 12/33 (36%) of tumor-suspect tissues were positive for ENTV-2 (Figure 5.12). While there

was so significant difference between the tumor-positive and tumor-negative groups, there was a significant difference between the tumor-suspect and tumor-negative categories (p=.0300) using a Fisher's exact test (GraphPad QuickCalcs, graphpad.com). This may suggest an association between the virus and early cases of bighorn sheep sinus tumors. However, the tumor-suspect category was created specifically because this group could also include other disease processes besides early tumor formation. To further evaluate the significance of the ENTV-2 positive results in tumor-suspect cases, we analyzed these results based on geographic location.

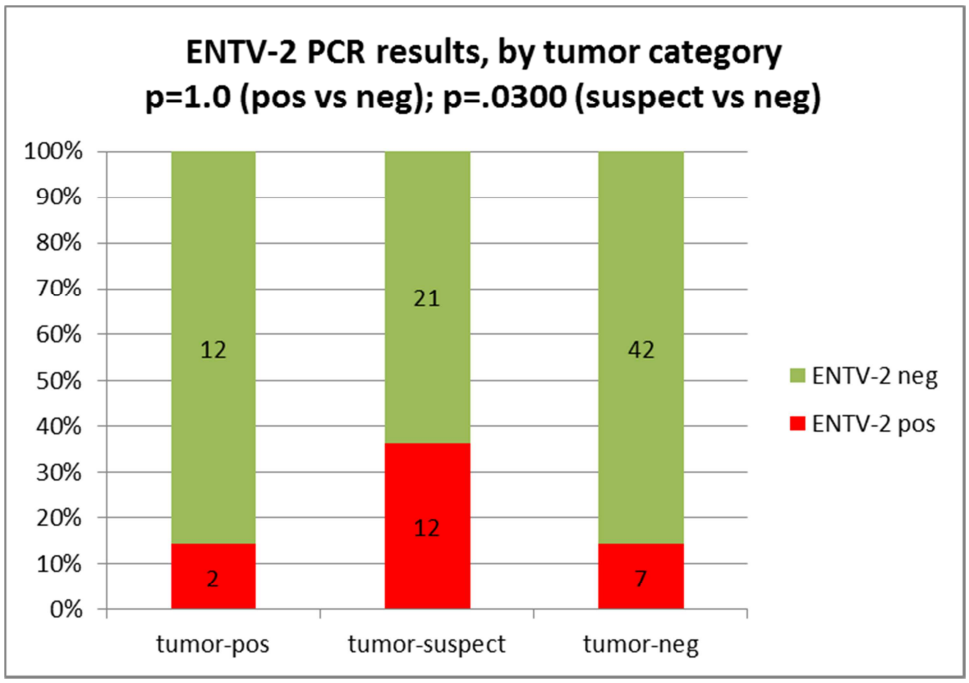

Figure 5.12: ENTV-2 PCR results, by tumor category. No significant difference was found between tumor-positive and tumor negative samples, but there was a significantly higher percentage of tumor-suspect cases that were PCR-positive for ENTV-2 when compared to tumor-negative cases.

Previously, we determined that bighorn sheep sinus tumors are non-randomly distributed geographically in free-ranging bighorn sheep herds, with tumor-positive cases clustered into a few specific populations of sheep (Chapter 3). We hypothesized that if ENTV-2 was associated with a disease process other than tumors, geographic location would not make a difference when

considering the ENTV-2 PCR result. We therefore re-analyzed our ENTV-2 PCR results for the tumor-suspect category based on location within a tumor-positive or non-tumor-positive herd. Only free-ranging populations were included in the analysis. We found that in tumor-positive herds, 7/11 (64%) of tumor-suspect cases were positive for ENTV-2, while in non-tumor-positive herds, only 3/18 (17%) of tumor-suspect cases were positive for ENTV-2 by PCR (p=.0143). This significant difference between tumor-positive and non-tumor-positive herds suggests that the association between tumor suspect cases and ENTV-2 positive PCR results is likely associated with early cases of bighorn sheep sinus tumors.

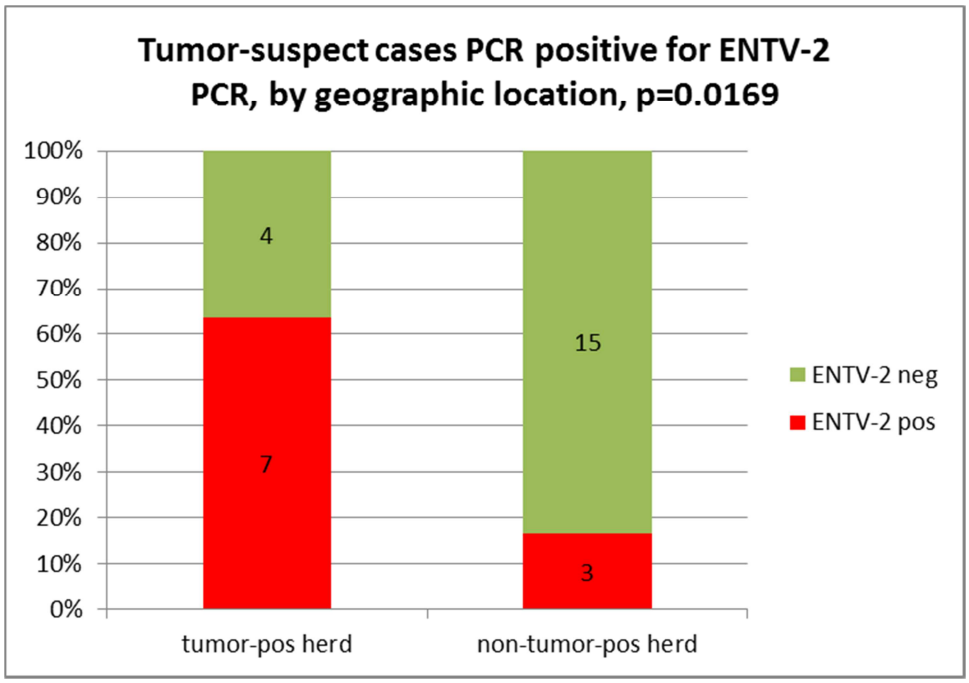

Figure 5.13: Evaluation of tumor-suspect cases from tumor-positive and non-tumor-positive herds, based on PCR results for ENTV-2. When evaluated by herd tumor status, we found a significantly increased percentage of ENTV-2-positive, tumor-suspect cases in herds with tumors, than in herds lacking tumors.

Conclusions

We evaluated bighorn sheep sinus tissues that were tumor-positive, tumor-suspect, and tumor-negative for the presence of specific oncogenic retroviruses of domestic sheep and goats

(JSRV, ENTV-1, and ENTV-2) using previously-published specific PCR primers. Results for JSRV and ENTV-1 were consistently negative, but we did amplify ENTV-2 from some tissues. Based on this finding, we obtained additional sequence specific to the bighorn sheep samples, created specific PCR primers based on this sequence, and screened 96 bighorn sheep sinus tissue samples for the presence of this ENTV-2-like virus. We found an association between the virus and tumor-suspect (early tumor) cases.

The discrepancy in ENTV-2 PCR results between tumor-suspect and tumor-positive cases may be due to the proportion of epithelial cells versus stromal cells in these tissues. Previous experiments have indicated that the stromal portion of bighorn sheep sinus tumors likely represents proliferation of the periosteum in response to growth factors, versus clonal expansion by an infected, transformed cell (Chapter 4). Based on our knowledge of ENTV-1 and ENTV-2 it is likely that only epithelial cells, and not stromal cells, are infected by the virus[144]. Because bighorn sheep sinus tumors appear to result from proliferation of an un-infected population of cells, we hypothesize that as tumors grow, infected (epithelial) cells comprise a smaller and smaller proportion of the tumor, making these infected cells less and less likely to be represented in a tumor tissue sample. These insights help to explain our inability to amplify integrated provirus from well-developed tumors versus early tumor cases.

Immunohistochemistry

Immunohistochemistry was performed by Dr. Sarah Wootton on the initial 10 bighorn sheep sinus tumors identified, as described in Chapter 2. The antigen used for this preliminary IHC was against the envelope protein of ENTV-1, and demonstrated cross reactivity with JSRV. No positive staining was noted in the naturally-occurring tumors that were examined. Immunohistochemistry was repeated following the successful transmission of tumors to domestic

and bighorn sheep (Chapter 4). The experimentally-induced tumors were evaluated by IHC, again using an antigen directed against the envelope protein of ENTV and JSRV.

Methods

Immunohistochemistry was performed as previously described using a monoclonal antibody (mAb) with reactivity for the envelope protein of JSRV, with demonstrated cross-reaction for ENTV[53,147]. Briefly, samples were deparaffinized and antigen retrieval was performed in a pressure cooker (heat to 120°C, hold for 3 minutes, allow to cool to 90°C, hold for 3 minutes) using Antigen Unmasking Solution (pH 6) (Vector Laboratories, Burlingame, CA, USA). After cooling, endogenous peroxide was quenched with 3% hydrogen peroxide for 5 minutes. Slides were washed two times for 10 minutes each with phosphate buffered saline (PBS). The slides were incubated with a 1:50 dilution of anti-JSRV envelope mAb (from hybridoma cells) for 1 hour at room temperature. Slides were washed and incubated with a 1:300 dilution of biotinylated horse-anti-mouse IgG (Vector Laboratories) for 30 minutes at room temperature. Slides were washed again and incubated with avidin:biotinylated enzyme complex (Vectastain Elite ABC kit, Vector Laboratories). 3,3'-diaminobenzidine tetrahydrochloride (DAB) with nickel chloride enhancement was used as a peroxidase substrate and the sections were counterstained with hematoxylin.

Results

IHC staining was evaluated microscopically, and positive staining was identified as punctate, granular, dark-brown staining of the cytoplasm or similar staining of cell-product such as mucus. This staining was significantly different from the lighter-brown, less aggregated staining present throughout the slides, characteristic of background staining.

Positive staining was only identified in scattered surface epithelial cells lining the sinus

tissues (Figure 5.14). Positive-staining epithelial cells often were mucus-producing cells, and the mucus within these cells stained intensely positive for the *env* protein (Figure 5.15). A lack of staining of adjacent mucus-producing cells ruled out the possibility of nonspecific staining of mucin (Figure 5.15). Positive staining was not seen in the stromal portions of the tumors (Figure 5.16), or other stromal components of the tissues. No staining was seen in submucosal glands, including regions of submucosal gland hyperplasia. Tissues evaluated included nasal turbinates, maxillary sinus lining, and/or tumor material from inoculated and un-inoculated animals. Slides were read blindly, and categorized as IHC positive or negative without knowledge of tumor status. Positive staining was noted in at least one tissue sample from all animals that developed tumors, as well as a sample from a single bighorn sheep that did not develop a tumor, but did develop a mucus-filled cyst in the maxillary sinus lining. Positive staining was not identified in tissues from negative control animals, or from infected animals that failed to develop lesions.

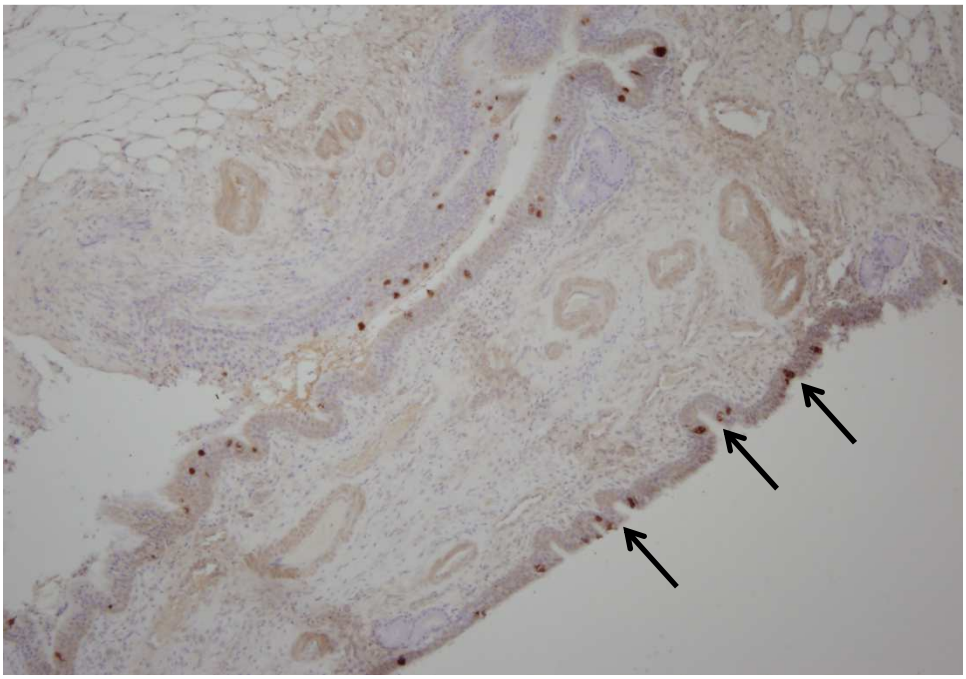

Figure 5.14: IHC for ENTV, sinus lining adjacent to experimentally-induced tumor. Note the patchy staining of surface epithelial cells (arrows). BHS 5.

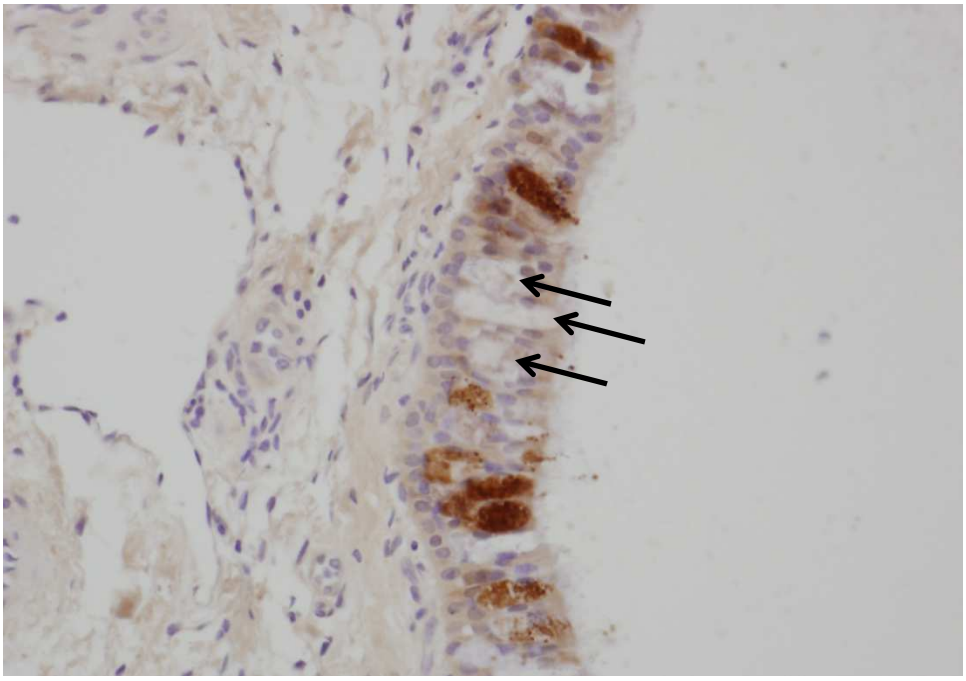

Figure 5.15: IHC for ENTV, nasal turbinates adjacent to experimentally-induced tumor (presumed to represent animal-to-animal transmission). Note the staining of scattered mucus-producing cells. The lack of staining in adjacent cells (arrows) rules out nonspecific staining of mucin. DS 2.

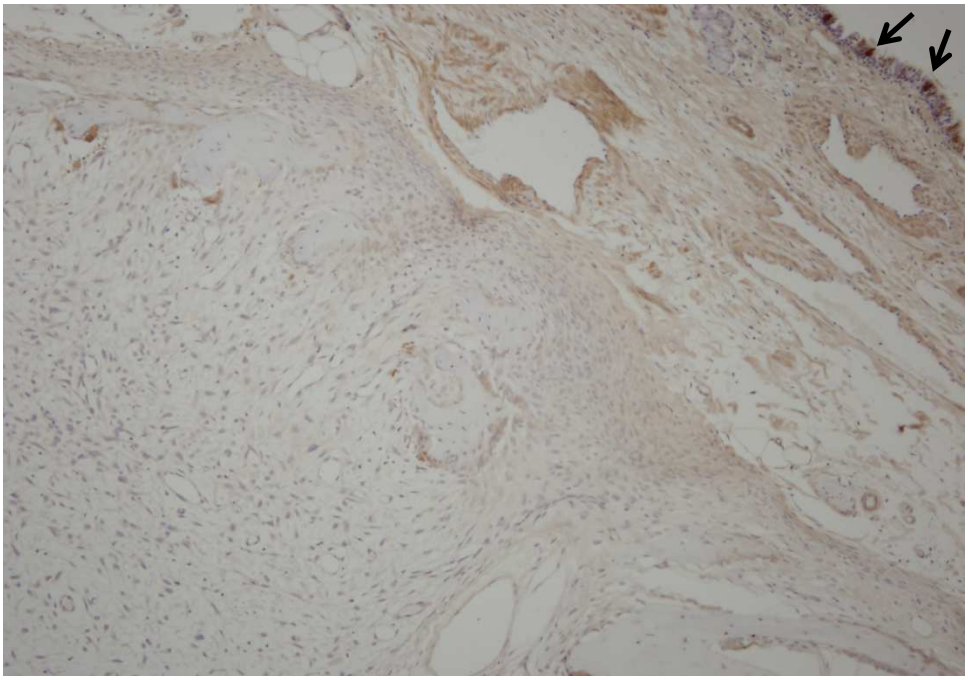

Figure 5.16: IHC for ENTV, experimentally induced tumor. Note the lack of staining in the stromal tumor (towards bottom left), but scattered positive staining in the overlying surface epithelial cells (arrows). DS 4.

Conclusions

Experimentally-induced sinus tumors of bighorn sheep and domestic sheep (Chapter 4) were stained for the envelope protein of JSRV and ENTV using a monoclonal antibody against this protein. While the tumors themselves, predominated by stromal cells, were negative for the envelope protein, scattered positive staining was demonstrated in mucus-producing surface epithelial cells scattered throughout the tissues of the sinus cavities. Specifically, positive staining was seen in the cytoplasm and the mucus product of these cells. Positive staining was seen only in tissues from animals which developed lesions in the sinus cavities. No positive staining was noted in tissues from inoculated animals that failed to develop lesions, or from negative control animals. These findings support the hypothesis that bighorn sheep sinus tumors are caused by ENTV-2 or a similar virus. Additionally, these findings support the hypothesis that bighorn sheep sinus tumors are predominated by uninfected stromal cells, complicating PCR diagnostics targeting integrated proviral DNA in the tumors.

CHAPTER SIX – CONCLUSIONS

Rocky Mountain bighorn sheep populations have struggled for nearly a century with fatal respiratory disease. In the course of investigating this disease in bighorn sheep in Colorado, USA, we discovered a high incidence of previously undescribed sinus tumors in the upper respiratory tracts of these animals. Tumors were characterized by epithelial and stromal proliferation, although the stromal component was often predominant, and responsible for invasion and destruction of the underlying bone. Associated with these tumors, it was common to find abundant mucinous exudate, presumably originating from hyperplastic epithelial cells. Chronic inflammation was also a common and prominent histologic finding, with inflammatory cells concentrated near the epithelial surface.

On a population level, we found that tumors were non-randomly distributed geographically, with all tumor-positive animals clustered within a few specific populations of sheep, suggesting that bighorn sheep sinus tumors are an infectious disease. Further supporting this hypothesis, we did not find an association between age and tumor formation that would be expected for a non-infectious tumor. Additionally, we found that bighorn sheep with sinus tumors were more likely to be infected with potentially pathogenic bacterial organisms in the upper respiratory tract than bighorn sheep lacking sinus tumors. Although the cause and effect relationship between bacterial infections and sinus tumors cannot be determined from our data, it is possible that sinus tumors may predispose bighorn sheep to upper respiratory, and perhaps lower respiratory, tract infections by potentially pathogenic bacteria. This hypothesis is further supported by our finding of a trend towards the increased occurrence of pneumonia lesions in bighorn sheep with sinus tumors as compared to bighorn sheep lacking sinus tumors. We suspect that bighorn sheep sinus tumors are an infectious disease that, in addition to tumor

formation, predisposes bighorn sheep to bacterial infections of the respiratory tract through decreased clearance of pathogens by the altered sinus lining.

In addition to our findings at the population level suggesting that bighorn sheep sinus tumors are an infectious disease, some features of these tumors are also reminiscent of oncogenic retroviral diseases of domestic sheep and goats (JSRV, ENTV-1, and ENTV-2), further suggesting a possible infectious, and perhaps retroviral, etiology for the disease in bighorn sheep. To test our hypothesis that bighorn sheep sinus tumors are caused by an infectious agent, we inoculated bighorn sheep and domestic sheep lambs intranasally with a cell-free filtrate originating from a naturally-occurring bighorn sheep sinus tumor. We successfully transmitted this disease to both bighorn sheep and domestic sheep species, and concluded that bighorn sheep sinus tumors are an infectious disease.

The experimental transmission study also allowed us to analyze experimentally-induced tumors without many of the confounding factors seen in naturally occurring cases, helping us to understand a bit more about the pathogenesis of these lesions. Naturally-occurring cases frequently contained abundant chronic inflammation, raising the question of whether or not any or all of the lesions observed were a result of chronic inflammation and progression to neoplasia. Experimentally-induced tumors developed in the absence of significant secondary bacterial infections. Bacteria were excluded from the inoculum by filtration, and the tumors which developed lacked significant inflammation. From these findings we concluded that experimentally-induced tumors were not caused by chronic inflammation. This helps us to interpret the findings from naturally-occurring cases where we observed an association between sinus tumors and bacterial infections but could not determine cause and effect. The results of the experimental transmission study rule out bacterial infections as the driving force for tumor

formation, supporting our hypothesis that bacterial infections are secondary to sinus tumor formation.

The experimental transmission study also allowed us to examine the histologic features of early tumors, which can provide information about processes occurring early in tumor formation, and the pathogenesis driving formation of the lesions. Detailed histologic and immunohistochemical analysis of the experimentally-induced tumors demonstrated that the predominant, stromal portion of the tumor was most consistent with a myxoma, likely originating from the periosteum. Additionally, features of the tumors were highly reminiscent of fetal bone formation by intramembranous ossification, a normal function of the periosteum. This suggests that the stromal portion of bighorn sheep sinus tumors may result from stimulation of the periosteum to proliferate in an orderly fashion, and not by uncontrolled clonal expansion of a transformed/infected cell.

The findings of the experimental transmission study help to interpret our PCR results from naturally-occurring cases of bighorn sheep sinus tumors. We screened tissues from bighorn sheep tumor-positive, tumor-suspect, and tumor-negative cases by PCR for specific retroviruses of domestic sheep and goats. Our results indicated an association between tumor-suspect cases and ENTV-2, an oncogenic retrovirus of domestic goats that causes nasal adenocarcinoma in this species. Interestingly, while we were often able to detect proviral DNA from ENTV-2 in tumor-suspect cases, tumor-positive cases were typically negative for ENTV-2. Our findings regarding stromal cell proliferation in experimentally-induced tumors become relevant when considering these PCR results. Based on our knowledge of retroviral oncogenesis in domestic sheep and goats, ENTV-1 and ENTV-2 target epithelial cells for infection. While stromal proliferation may be associated with tumors, stromal cells are typically negative for integrated proviral DNA.

Based on this information, we hypothesize that bighorn sheep sinus tumors are predominated by proliferative stromal cells of the periosteum that are uninfected cells, but are responding to growth factors released by infected (epithelial) cells. We suspect that, as tumors grow, the stromal population progressively outnumbers the epithelial population, and therefore early cases which contain a lower proportion of stromal cells have a greater chance of yielding infected epithelial cells when sampled. This would explain our PCR results which suggest the ability to detect proviral DNA in early tumor cases, but not later in the disease process when the tumor is predominated by uninfected stromal cells.

Our IHC data support this hypothesis and help us to further understand our PCR results. IHC using an antibody against the envelope protein of JSRV and ENTV demonstrated positive staining in tissues from animals with experimentally-induced tumors. Positive staining was only associated with surface epithelial cells, including the intracellular mucus being produced by these cells. Positive staining was often very patchy, with large areas of negatively staining cells interspersed with clusters of strongly positive cells. These IHC findings support the hypothesis that the stromal population of bighorn sheep sinus tumors is not infected by virus, and that rather epithelial cells are the infected population.

The patchy, intensely-positive staining of surface epithelial cells also helps us to understand the discrepancy between our inability to detect infected cells, and the ease of transmission of this disease. If infected cells are patchy, it would be expected to see rare PCR positive cases, especially as stromal cells become more and more predominant and epithelial cells are less likely to be sampled. However, if the epithelial cells that are infected are highly productive, it helps to explain why the disease is so infectious, despite our inability to consistently detect provirus.

Taking all of these findings together, it seems likely that bighorn sheep sinus tumors are caused by ENTV-2. Our working hypothesis is that ENTV-2 infects mucus-producing surface epithelial cells in a patchy manner, but that infected cells produce high amounts of virus. We suspect that these infected cells also produce growth factors to stimulate stromal proliferation of nearby cells such as the multipotent periosteum. Because these uninfected proliferative stromal cells predominate in the tumors, detection of ENTV-2 proviral DNA in tumors is unlikely to be an effective strategy for disease diagnosis and surveillance. However, based on our successful transmission of the disease, and our hypothesis that infected cells produce high quantities of virus along with mucus, we suspect that detection of viral RNA from nasal secretions may be a more effective strategy, which can also be applied antemortem to populations of bighorn sheep.

Future directions for this project include:

1. Development of an assay for detection of ENTV-2 viral RNA.
2. Application of the RNA assay to wild populations of bighorn sheep for disease surveillance.
3. Detection of viral RNA in the inoculum used for experimental transmission, and from swabs or tissues collected from animals with experimentally-induced tumors to fulfill Koch's postulates and more definitively identify ENTV-2 as the cause of bighorn sheep sinus tumors.
4. Cloning and sequencing the entire virus to determine any differences between published sequences of ENTV-2 and this virus in bighorn sheep.
5. Further investigation into the role of growth factors in driving stromal proliferation of bighorn sheep sinus tumors, and investigation into why infected epithelial cells do not undergo transformation to neoplasia.

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