Inhibin-α immunopositive ovarian granulosa cell tumor in a southern sea otter

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RESEARCH NOTE

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Ovarian granulosa cell tumors (GCTs) are uncommon neoplasms of humans (Jamieson and Fuller 2012) and other animals (Patnaik and Greenlee 1987; McCue et al. 2006; Heaps et al. 2017).GCTs are typically large, unilateral tumors with variable malignant potential, and are known to exert secondary systemic effects via hormone secretion (Ellenberger et al. 2007; Buijtels et al. 2010; Jamieson and Fuller 2012).

Few GCT cases have been described in wildlife, including marine mammals (Newman and Smith 2006), and among Mustelidae they have only been reported in domestic ferrets (*Mustela putorius furo*) (Patterson et al. 2003; Bakthavatchalu et al. 2016; Jekl and Hauptman 2017). Sea otters (*Enhydra lutris*), a mustelid marine mammal, are a federally listed threatened species in some regions of North America. These subspecies include southern sea otters (*Enhydra lutris nereis*) in California, and northern sea otters (*Enhydra lutris kenyoni*) in southwestern Alaska (Carter et al. 2022; Flannery et al. 2022). Although multiple cases of neoplasia have been diagnosed in sea otters (Newman and Smith 2006), no GCTs are documented. Here we report diagnosis of a putatively hormonally active ovarian GCT in a southern sea otter, as confirmed through gross necropsy, histopathology, and inhibin-α and vimentin immunohistochemistry (IHC). This is the first GCT reported in any mustelid other than domestic ferrets.

A free-ranging adult female southern sea otter was found dead in San Luis Obispo County, California, USA during September 2019. The carcass was frozen and transported to the California Department of Fish and Wildlife (CDFW) in Santa Cruz, California for necropsy. Sections of a left ovarian mass, right ovary, both uterine horns, cervix, and vagina were formalin-fixed, trimmed, and submitted to the University of California, Davis Veterinary Medical Teaching Hospital for paraffin embedding, sectioning, hematoxylin and eosin (H&E) staining, and IHC for inhibin-α and vimentin. We used inhibin (R1) mouse monoclonal antibody (Cell Marque 271M-16) and vimentin (Vim3B4) mouse monoclonal antibody (Dako M7020) for the IHC assays with positive and negative controls included. CDFW employees collected samples in accordance with U.S. Fish and Wildlife Service regulations implementing the U.S. Marine Mammal Protection Act at 50 CFR 18.22(a) and the U.S. Endangered Species Act at 50 CFR 17.21(c)(3). A veterinary pathologist (M. Miller) performed all microscopic evaluations.

At necropsy, the carcass was moderately decomposed with slight scavenging and moderate subcutaneous fat. Multifocal acute skin and soft tissue incisions and lacerations characteristic of white shark (*Carcharodon carcharias*) bite were present, along with diffuse moderate myocardial and hepatic pallor, possibly suggestive of severe blood loss. Severe gastric mucosal ulcers, intestinal melena, and mild enteric *Corynosoma enhydri* acanthocephalan infection were also present. A large (10.0 x 3.5 x 9.0 cm) fluctuant, light pink, encapsulated, expansile, multilobulated mass encompassed and obliterated the entire left ovary (**Fig. 1A**). On cut surface, the mass had a thin outer capsule and was composed of irregular lobules of moist, pale-yellow to light pink tissue that surrounded a central cavity filled with red-tinged to bloody gelatinous fluid and necrotic debris (**Fig. 1B**). The contralateral (right) ovary was abnormally small (1.4 x 1.0 x 0.3 cm) and smooth with no grossly apparent secondary or Graafian follicles, corpora lutea, corpora albicantia, or paraovarian cysts. The uterine endometrium was diffusely congested and hyperplastic with prominent linear mucosal ridges (**Fig. 1A**) and no visible placental bands. The vulva and cervix were diffusely enlarged, swollen, and edematous. There was no gross evidence of recent pregnancy or lactation.

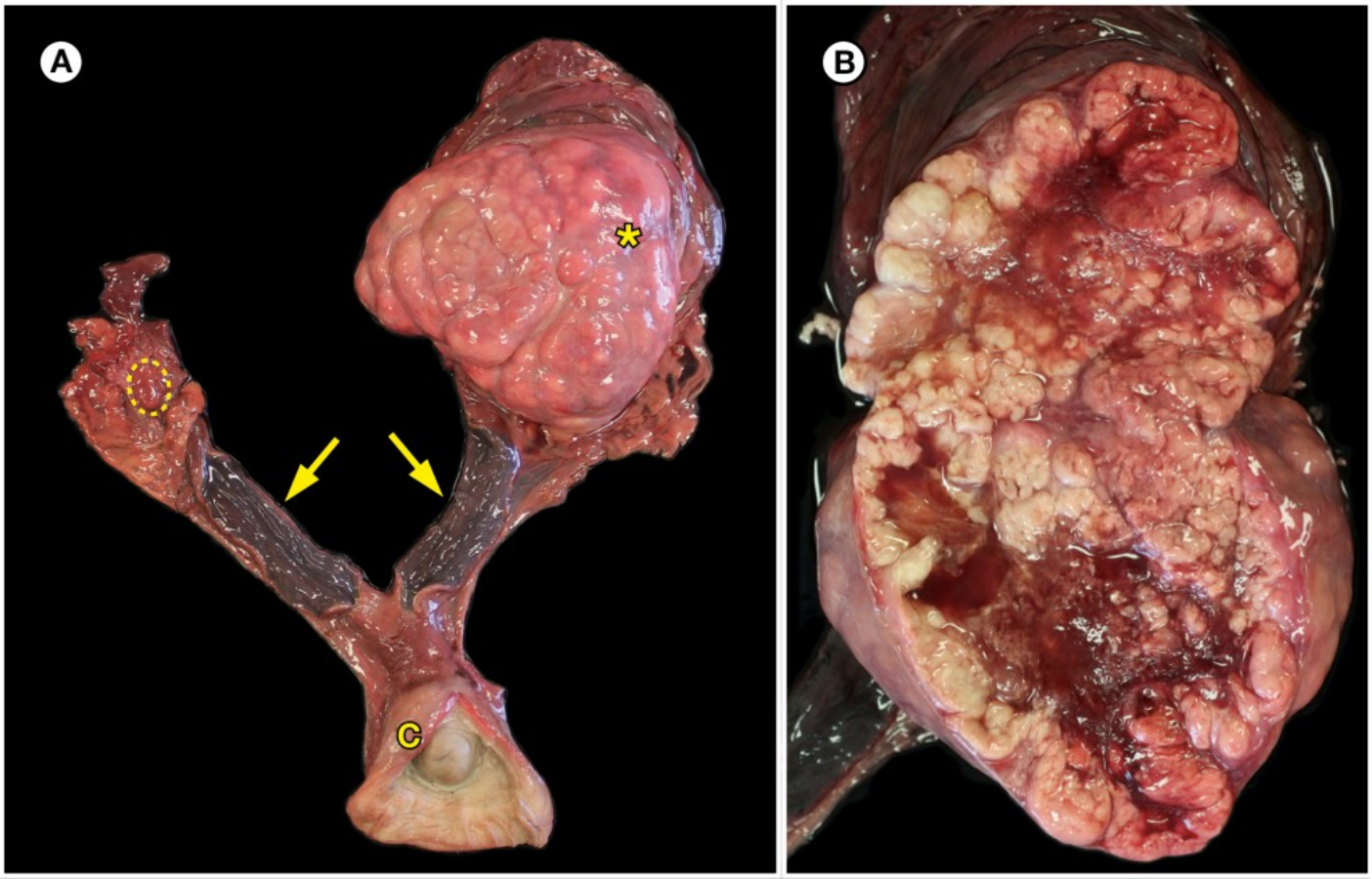

Figure 1. Ovarian granulosa cell tumor in a southern sea otter. (A) Reproductive tract from a moderately decomposed, frozen-thawed sea otter. The left ovary (top right) was effaced by a large unilateral, multilobulated mass surrounded by a thin outer capsule (asterisk). The contralateral ovary was small and atrophic (encircled by yellow dashed oval). The cervix (C) was moderately enlarged, hyperplastic, and edematous while the uterine endometrium was diffusely congested and moderately hyperplastic (arrows). (B) On cut surface, the soft, fluctuant mass was composed of lobules of pale yellow to light pink tissue surrounding a central core of red-tinged to bloody gelatinous fluid and necrotic debris.

Histologic examination of the right ovary confirmed the gross impression of no secondary or Graafian follicles, corpora lutea, corpora albicantia, or paraovarian cysts. Variably mineralized remnant oocytes and fragments of zona pellucida within scattered atretic follicles were surrounded by large polygonal cells with moderate eosinophilic vacuolated cytoplasm, and similar aggregates of polygonal cells were scattered throughout the ovarian stroma (Fig. 2).

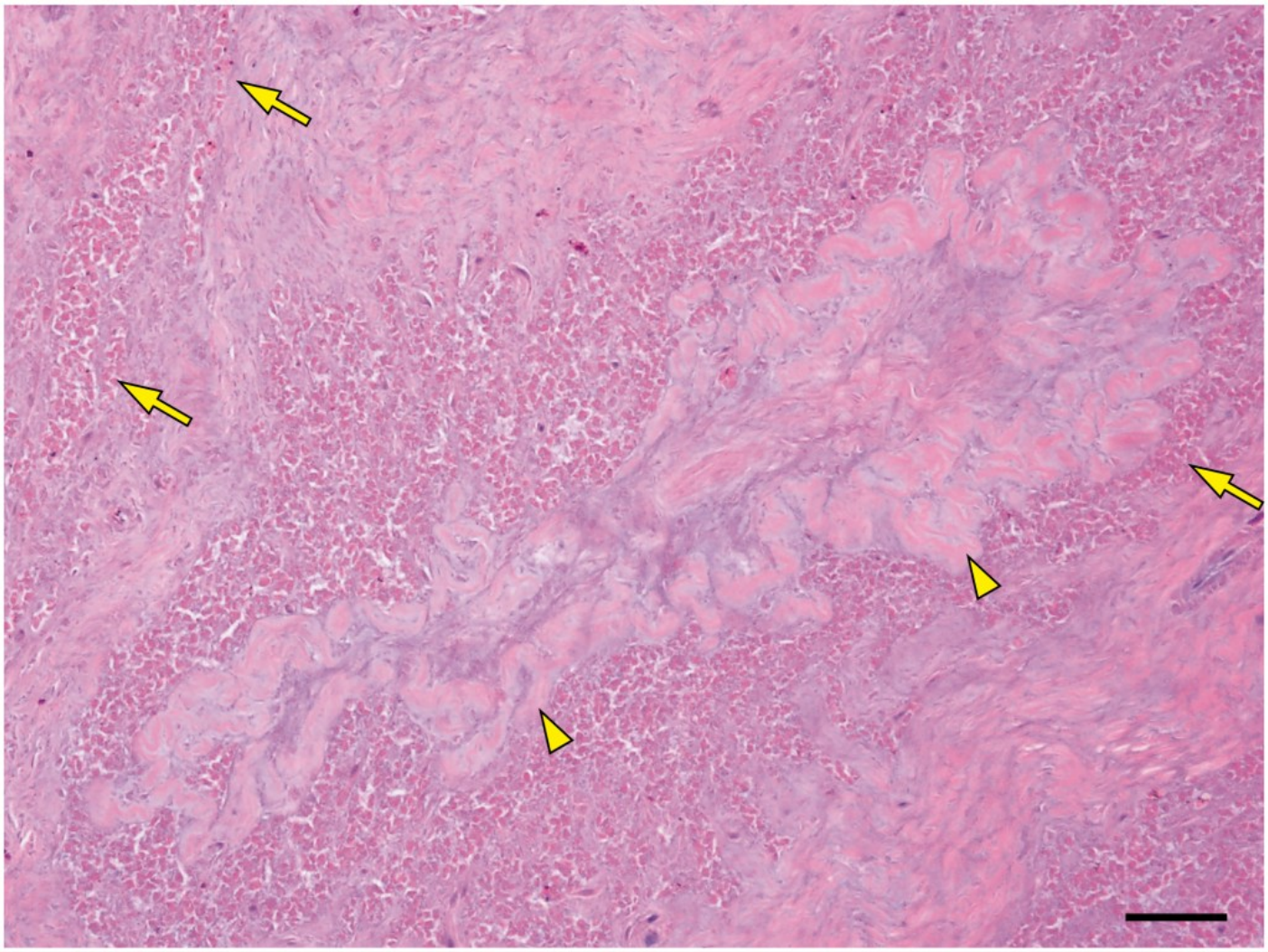

Figure 2. Atretic follicle surrounded by large polygonal cells in the right ovary of a southern sea otter with a large left ovarian granulosa cell tumor. Although the right ovary was small and smooth without grossly or microscopically apparent secondary or Graafian follicles, corpora lutea, corpora albicantia, or paraovarian cysts, several atretic follicles were visible on histopathology. These were characterized by a central elliptical region of deeply involuted connective tissue (arrowheads). These central scars were surrounded by aggregates of large polygonal cells with moderate vacuolated eosinophilic cytoplasm, and similar aggregates of these cells were scattered throughout the ovarian stroma (arrows). These resemble the large polyhedral cells described by Sinha et. al. (1966) that are only visible in sea otter ovaries during late proestrus and estrus and are presumed to have a secretory function (Bar = 80 µm).
Uterine histopathology confirmed the gross impression of endometrial hyperplasia, characterized by moderately thickened endometrium forming prominent luminal ridges. No endometrial cysts were observed grossly or microscopically. The endometrial surface was lined by tall columnar epithelial cells with basal nuclei, and endometrial glands were long, mildly coiled, and lined by similar columnar epithelial cells. The cervical and vaginal mucosa was lined by three or more layers of squamous epithelium, and luminal and mucosal bacteria and leukocytes were sparse.

Histologic examination of the left ovarian mass revealed complete effacement of normal ovarian architecture. Variable nests, tubules, and sheets of densely packed polyhedral cells were arranged in a fine to dense fibrovascular stroma with few intervening theca cells and multifocal areas of coagulation necrosis (**Fig. 3A**). In some areas, irregular nests of tumor cells were separated by thin bands of

fibrovascular tissue resulting in a lobular appearance (Fig. 3B). Several regions exhibited a microfollicular pattern with central circular spaces containing scant eosinophilic material surrounded by rosettes of neoplastic cells (Call-Exner bodies) (Fig. 3B). Neoplastic cells were also arranged as diffuse sheets of round to fusiform cells with deeply basophilic nuclei, scant eosinophilic cytoplasm, and indistinct cell borders. Multifocal coagulation necrosis and dystrophic mineralization were associated with intravascular fibrin thrombi. There was no gross or microscopic evidence of tumor metastasis.

Neoplastic cells from the left ovary were strongly but variably inhibin-α immunopositive (Fig. 3C) and vimentin immunonegative (Fig. 3D), while cells from the atrophic right ovary were inhibin-α and vimentin immunonegative. Capillary endothelium (Fig. 3D) and scant stromal leukocytes were vimentin immunopositive in both tissues.

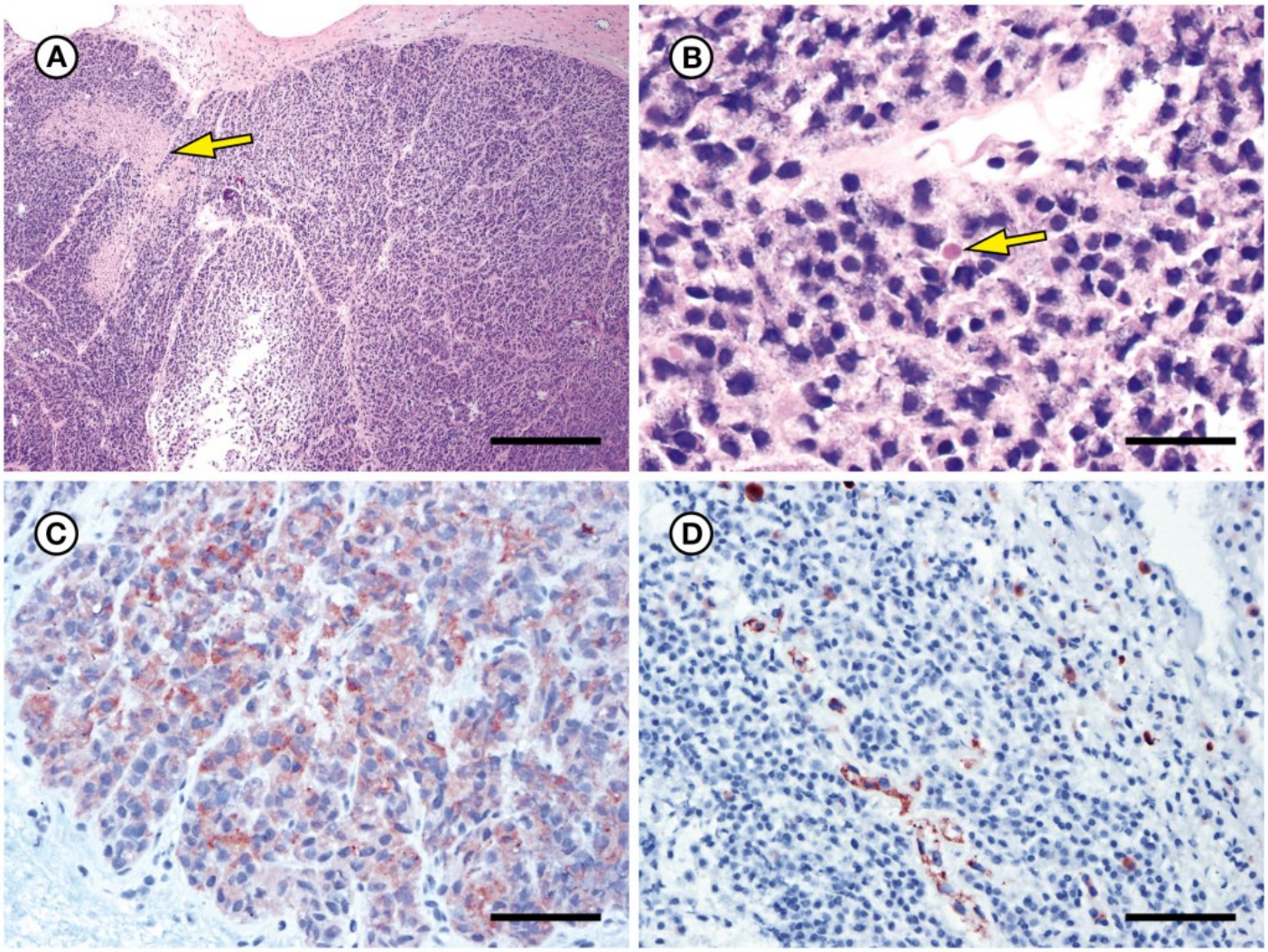

Figure 3. Histopathology and immunohistochemistry (IHC) of an ovarian granulosa cell tumor in a southern sea otter. (A) Histopathology revealed variable nests, tubules, and sheets of densely packed polyhedral cells in a fine to dense fibrovascular stroma with few intervening theca cells and multifocal areas of coagulation necrosis (arrow). (B) In some areas, the neoplastic cells clustered into a microfollicular or rosette pattern surrounding central deposits of eosinophilic material (Call-Exner bodies) (arrow). (C) The cytoplasm of neoplastic cells in the enlarged left ovary stained strongly but variably immunopositive for inhibin-α. (D) Vimentin IHC revealed immunopositive staining of capillary endothelium although immunonegative staining of neoplastic cells. [(A–B) Hematoxylin and eosin stain,

bar = 315 and 30 µm, respectively; (C–D) immunohistochemical stains of the left ovarian tumor for inhibin-α (C) and vimentin (D), bar = 30 and 60 µm, respectively].
Diagnosis of an ovarian GCT was made based on the following gross characteristics: a large, soft, unilateral, encapsulated and variably necrotic ovarian mass with a smooth lobulated surface, a densely cellular pale-yellow periphery, and marked internal cystic change. Histopathology and IHC also supported a diagnosis of ovarian GCT due to morphology and arrangement of the neoplastic cells, the presence of Call-Exner bodies, and confirmation of inhibin-α IHC staining. Gross and microscopic findings in other reproductive organs, summarized below, further support this diagnosis.

This is the first report of an ovarian GCT in sea otters. Reproductive tract neoplasms previously reported in this species include seminoma (Reimer and Lipscomb 1998), and uterine and cervical leiomyoma (Williams and Pulley 1981). In humans and domestic animals, GCTs are commonly large, unilateral, well-encapsulated masses that are often yellow on cross-section due to steroid production (Patnaik and Greenlee 1987; Schumer and Cannistra 2003; McCue et al. 2006; Ellenberger et al. 2007; Jamieson and Fuller 2012). They can be solid or cystic, smooth or multilobulated, with variable hemorrhage and necrosis (Patnaik and Greenlee 1987; Schumer and Cannistra 2003; McCue et al. 2006; Ellenberger et al. 2007; Jamieson and Fuller 2012). The gross characteristics of the sea otter tumor were generally consistent with GCTs in other species. The potential for tumor malignancy and metastasis varies among species (Patnaik and Greenlee 1987; Ellenberger et al. 2007; Jamieson and Fuller 2012; Heaps et al. 2017). The adult form in humans is of low-grade malignant potential (Jamieson and Fuller 2012), and GCTs are commonly benign in equines (Carter et al. 2022). Although there was no gross evidence of metastasis in this case, some features of malignancy were observed within the tumor including hemorrhage, intravascular fibrin thrombi, coagulation necrosis with central cavitation, and dystrophic mineralization.

The histologic appearance of GCTs also varies within and between species. Granulosa cells that comprise the tumor may exhibit microfollicular, macrofollicular, tubular, trabecular, insular, diffuse, or other patterns, and are often separated by fibrovascular stroma (Patnaik and Greenlee 1987; Schumer and Cannistra 2003; Ellenberger et al. 2007; Heaps et al. 2017; Matos et al. 2019). Call-Exner bodies (regions of fluid and cellular debris encircled by granulosa cells) are a characteristic feature of GCTs in many species (Patnaik and Greenlee 1987; Patterson et al. 2003; Schumer and Cannistra 2003; Heaps et al. 2017; Matos et al. 2019). In adult form human GCTs, neoplastic granulosa cells are typically small, pale, round to oval, and have grooved “coffee-bean” shaped nuclei (Schumer and Cannistra 2003; Jamieson and Fuller 2012), while neoplastic cells of the juvenile form typically exhibit hyperchromatic or irregular nuclei without nuclear grooves (Jamieson and Fuller 2012). In other species, tumor cell morphology varies, although round, polyhedral, or spindloid cells with eosinophilic cytoplasm, indistinct cell borders, and anisocytosis (Heaps et al. 2017) are often described (Patnaik and Greenlee 1987; Patterson et al. 2003). Nuclei may appear round to ovoid and basophilic, contain scattered chromatin, and exhibit anisokaryosis (Patnaik and Greenlee 1987; Patterson et al. 2003). Mitotic figures are typically few to rare (Patnaik and Greenlee 1987; Heaps et al. 2017; Matos et al. 2019). Hemorrhage and necrosis may be present (Patnaik and Greenlee 1987; Patterson et al. 2003). The histologic features of this sea otter tumor, most notably the presence of Call-Exner bodies and the observed patterns of tumor cell arrangement, fit within histologic descriptions of GCTs in other species. Because theca cells appeared to be sparse in this tumor, a diagnosis of granulosa cell tumor was deemed most appropriate.

GCTs can be hormonally active, impairing normal function of the hypothalamic-pituitary axis (Ellenberger et al. 2007; Riccardi et al. 2007; Buijtels et al. 2010; Jamieson and Fuller 2012).Tumor-mediated secretion

or expression of sex hormones (e.g., estrogens, testosterone, inhibin, and anti-Müllerian hormone) and intermediate filaments (e.g., cytokeratin, desmin, and vimentin) may be altered (Ellenberger et al. 2007; Riccardi et al. 2007; Buijtels et al. 2010; Jamieson and Fuller 2012). This can lead to systemic effects including menstrual or estrus abnormalities (Schumer and Cannistra 2003; Jamieson and Fuller 2012), behavioral changes such as nymphomania or aggression (McCue et al. 2006; Ellenberger et al. 2007), contralateral ovarian atrophy (Ellenberger et al. 2007), endometrial hyperplasia and cysts (Patnaik and Greenlee 1987; Jamieson and Fuller 2012; Jekl and Hauptman 2017), vulvar hypertrophy, genital edema (Jekl and Hauptman 2017), alopecia (Patterson et al. 2003; Jekl and Hauptman 2017), and other changes.

Although case workup was limited due to postmortem autolysis, multiple gross, histological, and immunohistochemical findings suggest that this sea otter GCT was hormonally active. These findings include gross detection of endometrial, cervical, and vulvar hypertrophy, inhibin-α immunopositive staining of the ovarian neoplastic cells, and microscopic confirmation of endometrial hypertrophy. Endometrial hypertrophy was characterized by a thickened endometrium that formed luminal folds, prominent endometrial glands, and presence of tall columnar epithelium with basal nuclei on the endometrial surface and within endometrial glands (Sinha et al. 1996).

Additionally, microscopic examination of the right ovary revealed atretic follicles surrounded by large polygonal cells with moderate eosinophilic vacuolated cytoplasm, and similar aggregates of polygonal cells were scattered throughout the ovarian stroma (Fig. 2). The appearance, pattern, and distribution of these large polygonal cells matches ovarian histopathology described for estrus sea otters by Sinha et. al. (1966). The authors of that paper concluded that these polygonal cells, which were only visible during late proestrus and estrus, have a secretory function. In this sea otter the presence of these specialized cells in a relatively atretic right ovary may be indicative of aberrant hormonal secretion by the left ovarian tumor, neoplasm-associated disruption of the hypothalamic-pituitary-ovarian axis, or both conditions concurrently. When viewed collectively, the observed vulvar and cervical enlargement, endometrial hypertrophy, endometrial gland hyperplasia, and the clusters of large polygonal cells surrounding atretic follicles and scattered throughout the ovarian stroma are characteristic of proestrus or estrus sea otters (Sinha et al. 1966). As described in the literature for GCT in other species, this sea otter neoplasm may have been secreting other hormones in addition to inhibins, such as androsterones or estrogens.

Also supporting a hypothesis of disruption of the hypothalamic-pituitary axis was the absence of gross or microscopic evidence that this animal had ever been pregnant; a finding that is extremely unusual in adult female southern sea otters. At gross necropsy, the mammary glandular tissue was not visible, the diameter of both uterine horns was relatively small and symmetrical for an adult female sea otter, and no placental scars were observed within either uterine horn. Additionally, histopathology confirmed the absence of secondary or Graafian follicles, corpora lutea, corpora albicantia, or paraovarian cysts in the intact right ovary, and there was no indication of prior pregnancy on uterine histopathology, as previously described.

As GCTs can be functional, IHC is a valuable diagnostic tool to assess tumor marker expression. Inhibins (glycoprotein hormones synthesized by granulosa cells) are common markers for immunodiagnosis of GCTs, along with glutathione-S transferase α, vimentin, and cytokeratin in some species (Schumer and Cannistra 2003; Ellenberger et al. 2007; Riccardi et al. 2007). The tumor cells in this case were inhibin-α immunopositive, supporting a diagnosis of GCT and suggesting that hypersecretion of inhibin-α could have been exerting negative feedback for pituitary follicle-stimulating hormone (FSH) release (Schumer

and Cannistra 2003; Jamieson and Fuller 2012). Tumor-mediated inhibin hypersecretion can reduce pituitary FSH release and thus downregulate function of the contralateral ovary, possibly explaining the unusually small size and smooth surface on the right ovary (Ellenberger et al. 2007). Although the tumor cells were immunonegative for vimentin, this does not rule out GCT diagnosis as vimentin expression is more variable in GCTs (Ellenberger et al. 2007; Riccardi et al. 2007). Other than endothelial cells and occasional round cells in the stroma, the atrophic right ovary was immunonegative for both inhibin-α and vimentin. Collectively these results suggest that sea otter endothelium can be labeled with mouse monoclonal antibodies to vimentin, and that neoplastic sea otter granulosa cells may not express vimentin, or that vimentin staining of the neoplastic cells may be more sensitive to freeze-thaw and decomposition artifact. Although we were unable to assess estrogen concentrations via serology, the observed vulvar and cervical enlargement, endometrial hyperplasia with enlargement of endometrial glands and epithelial hypertrophy, and proliferation of polygonal cells in the stroma of the right ovary are similar to tissue responses in other mammals with GCTs and suggest estrogen hypersecretion (Sinha et al. 1966; Patnaik and Greenlee 1987; Jekl and Hauptman 2017). While alopecia is documented in ferrets with GCTs (Patterson et al. 2003; Jekl and Hauptman 2017), alopecia was not observed in this sea otter.

The major limitations of this case were moderate postmortem autolysis with delayed tissue fixation and freeze-thaw artifact. These postmortem changes resulted in some unavoidable limitations on gross and histologic interpretation of lesions, including reduced ability to distinguish between apoptosis, necrosis, and autolysis. However, scattered areas of tissue necrosis within the neoplasm could be identified as well-defined areas of cellular pallor and loss of cytological detail in tissues immediately adjacent to intravascular fibrin thrombi. Freeze-thaw artifact also limited cytological detail via disruption of cell architecture. The effect of delayed fixation can also limit the ability to accurately quantify mitotic figures as part of tumor characterization (Cross et al. 1990), so estimates of mitoses and abnormal mitotic figures were not attempted for this case.

Additionally, autolysis, freeze-thaw artifact, and delayed fixation can positively or negatively affect antigen expression via IHC (Scudamore et al. 2011). Despite these limitations, we are confident that inhibin-α staining of the sea otter GCT was not an artifact for the following reasons: our inhibin IHC assay included both internal positive and negative controls which stained appropriately, as well as sections from both the right ovary and the left ovarian GCT. The right ovary was inhibin-negative, as would be expected. Although the strength of inhibin-α IHC staining in the left ovarian GCT was not uniform (relatively common in neoplastic tissue), the pattern and distribution of the staining was very specific; positive staining was concentrated within the neoplastic cells, the staining pattern was not random, and it was not associated with open spaces with no tissue, section edges, tears, non-neoplastic tissue, or other artifacts. As shown in Figure 3C, inhibin-α IHC staining was strongest within the cytoplasm of neoplastic cells. If the staining was artifactual, this highly specific staining pattern would not be expected. We suspect that if this tissue had not been negatively impacted by autolysis and freeze-thaw artifact, this highly specific staining pattern could have been even stronger and more uniform than we observed. Although we are very confident that the sea otter GCT was inhibin-α immunopositive, the results of IHC testing on autolyzed tissues must always be interpreted with caution.

Based on our results and comparison with ovarian tumors in other species, we conclude that this is the first case of an ovarian GCT reported in sea otters and in any mustelid species other than ferrets. While the proximate cause of death was likely shark bite, the tumor may have caused morbidity via systemic endocrine effects and this sea otter appeared to be non-reproductive. Risk factors for GCT development are poorly understood, although recent studies of human GCTs implicate cytogenic and genetic factors,

including a missense point mutation in the *FOXL2* gene (Jamieson and Fuller 2012). Clear inhibin-α immunostaining of this sea otter GCT confirms the value of attempting IHC for tumor characterization in wildlife and validating commercially available IHC assays for use in threatened species. Further IHC validation studies are planned for southern sea otters. This case contributes to our collective knowledge of neoplasms affecting southern sea otters (a federally listed threatened species), mustelids, and marine mammals.

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