UNIVERSITÀ DEGLI STUDI DI MILANO

Department of Veterinary Medicine

Role of the Transcription Factor SOX9 in the tumorigenesis of some domestic animal neoplasms

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PhD course of Veterinary Hygiene and Animal Pathology (Coordinator Prof. Giuseppe Sironi)
XXVIII cycle | Academic Year 2014/2015

To Claudia and Giovanni,
who gave me life.
To Samuele and Davide,
who fulfill my life.

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Structure of the work—The *first section* of this thesis gives a brief overview of the literature about the transcription factor Sox9, focusing the attention on its main functions during developmental processes and in acquired diseases. The *second section*, after a short introduction on Sox9 in Veterinary Medicine, is concerned with the assessment of Sox9 immunohistochemical staining in normal tissues of various animal species, in order to validate this marker in domestic animals. The *other sections* present the findings of the research, with six original research studies concerning Sox9 immunohistochemical expression in several groups of neoplasms. To better explain Sox9 role in nervous tissue development and in its neoplastic lesions they are treated together with Sox10 in the last work. To conclude, an appendix with other publications/communications realized during the 3-academic-year–period (end of 2012, beginning 2016).

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The SOX family

Sox family transcription factors is one of the earliest class of master regulatory genes coordinating the main phases of embryonic development (Chew and Gallo, 2009). They regulate and organize precise activation and silencing of gene expression during cell, tissue differentiation and organogenesis (Pritchett et al., 2011). They are involved in adult tissue homeostasis and regeneration, as cellular turnover or wound healing, they are able to reprogram differentiated cells into somatic or pluripotent stem cells, or they can even maintain stem cell state (Sarkar and Hochedlinger, 2013). Sox transcription factors are expressed in multiple types of stem cells and progenitor cells types, but Sox factors can be expressed at subsequent stages of differentiation within a cell lineage or in complementary patterns within a developing or adult tissue (Sarkar and Hochedlinger, 2013).

Sox family identification is related to the identification of a gene responsible for testis determination in mammals (sex determining region Y –SRY-) (Sinclair et al., 1990). Sry transcription factors conserve a characteristic 79 amino acid DNA binding domain known as High Mobility Group (HMG) box (Wegner, 1999). Proteins showing at least 50% amino acid similarity with the HMG domain of Sry are grouped into Sox (SRY box-containing) family (Gubbay et al., 1990). Outside the HMG domain, SOX sequences are quite variable suggesting recent shared ancestry (Bowles et al., 2000). To date, about 20 SOX proteins and their genes have been identified in vertebrates, while 8 in Drosophila (Kamachi et al., 2000; Chew and Gallo, 2009). According to Sox HMG similarities Wright et al., (1993) classified the known murine Sox genes into 6 groups (A-F). Further studies allowed to confirm Wright first classification and to introduce 4 more Sox protein groups (G-J) (Table I) (Bowles et al., 2000). Sox members within a group share an HMG domain with more than 80% identity and strong homologies in amino acid sequence and structural organization even outside the HMG domain (Wegner, 2010; Sarkar and Hochedlinger, 2013; Kamachi and Kondoh, 2013). The amino acid sequence identity of the HMG domain decreases to ~60% between distant groups (Kamachi et al., 2000). Thus, members of the same group show similar biochemical properties and overlapping functions, while Sox proteins from different groups have distinct functional properties despite recognizing the same domain (Wegner, 2010; Sarkar and Hochedlinger, 2013). Each Sox protein is able to play different roles in different tissues depending on both cellular and target gene context. Frequently, members of the same Sox group co-regulating the same target are expressed in the same developing tissues with small differences in spatio-temporal patterns. This redundancy between the group members with equivalent functions safeguards the developmental processes against genetic variations. Anyway, modifications induced by individual members of a Sox group to a developmental process are not always equivalent, with regard to the timing of expression, expression levels and the activity of a protein (Kamachi and Kondoh, 2013). This Sox functional versatility is further achieved through different molecular mechanisms as interaction with specific cofactors, homo- or heterodimerization among Sox proteins, post-translational modifications (Kamaki et al., 2000; Chew and Gallo 2009; Sarkar and Hochedlinger, 2013; Kamachi and Kondoh, 2013). Sox genes moreover are often controlled by other sox proteins or subjected to auto-regulation. Their function, indeed, is known to be dose-dependent (Kamachi and Kondoh, 2013). In this way, each protein is expressed in many different cellular contexts and a specific cell type can co-express many Sox factors (Wegner, 1999; Chew and Gallo, 2009). Sox genes has emerged as potent modulator factors in developmental processes, tissue homeostasis and stem cells maintenance and their role have been investigating in several diseases, cancerogenesis included (Pritchett at al., 2011).

Sox9

An introduction

Sox9 is part of the SoxE group, together with Sox8 and Sox10. Their protein organization show a total length of 300-500 amino acids with HMG domain located close to

the N-terminus and an activation domain in the C-terminal region (Kamachi and Kondoh, 2013). Sox9 mutations were identified as responsible for campomelic dysplasia (Foster et al., 1994). Campomelic dysplasia (CD) is a rare, often fatal congenital skeletal malformation syndrome related to an autosomal dominant condition caused by hap-

Table I. Sox genes with their main target organs and functions.

Sox Family	Sox Member	Foetus	Adult	Functions
Sox A	SRY	Gonad	Testis Hypothalamus Midbrain	Testis determination. Expression/role in SCs undefined.
Sox B1	Sox1	NPCs Lens	NPCs	Specification and maintenance of undifferentiated SCs. Knockout mice show microphthalmia, cataracts.
	Sox2	NPCs Trachea Lung Tongue Esophagus Stomach Anus Cervix Inner ear Lens Teeth Skin Bone Ovary Testis	NPCs Trachea Lung Tongue Esophagus Stomach Anus Cervix Inner ear Lens Teeth Skin Bone Ovary Testis	Development regulator.
	Sox3	NPCs Lens	Spermatogonia	Specification and maintenance of undifferentiated SCs. Genetic deletion leads to loss of undifferentiated spermatogonia. May act redundantly with Sox1 and Sox2.
Sox B2	Sox14	NPCs	N/A	Transcriptional repressor. Specification of a subset of ventral interneurons in the spinal cord and neuronal subtypes in the brain.
	Sox21	Hair bulge Developing CNS	Hair bulge	N/A
Sox C	Sox4	Developing neurons Early embryonic cells	N/A	Organ hypoplasia in case of deletion Roles in cardiac outflow tract development and B-cell development revealed by phenotype of knockout mice.
	Sox11	Developing neurons Early embryonic cells^	Kidney^	Organ hypoplasia in case of deletion.^
	Sox12	^	^	^

loinsufficiency of Sox9 (Kwok et al., 1995). This syndrome is characterized, in various extent, by short stature, bowing of the long bones, hypoplasia of the scapula, abnormal pelvic bones, talipes equinovarus, narrow iliac wings, small thorax, 11 pairs of ribs. Other anomalies include low ears, long philtrum, micrognathia, depressed nasal bridge, car-

Table legend. NPCs = neural progenitor cells, SC = stem cell, ^ = expression/role in stem cells largely undefined, N/A = not assessed yet (data from: Bowles et al., 2000; Sarkar and Hockedlinger, 2013; Kamachi and Kondoh, 2013).

Sox Family	Sox Member	Foetus	Adult	Functions
Sox D	Sox5 Sox6	Chondrocytes^	Chondrocytes Oligodendrocytes Neocortex neurons^	In case of defects: chondrogenesis defects, precocious oligodendrocyte differentiation, alopecia, aberrant histogenesis of the neocortex. May act redundantly and both interact with Sox9.
	Sox13	Arteries Thymus	^	N/A
	Sox23	Ovary Brain		Homodimerization.
Sox E	Sox 8	Neural crest SCs Branchial arches Limb Heart Dorsal root ganglia Testes	Muscle satellite cells	N/A
	Sox9	Hair follicle SCs Chondrocytes Gonad Lung Retinal progenitor cells NPSCs Neural crest SCs Oligodendrocytes progenitors Glial progenitors Pancreatic progenitors	Hair follicle SCs Oligodendrocytes NPSCs Exocrine pancreatic duct cells Liver duct cells Intestinal SCs Mammary SCs Chondrocytes	Specification and maintenance of undifferentiated SCs. Genetic deletion leads to alopecia, failure of neural SCs and mammary gland SCs maintenance, loss of SCs differentiation potential to Muller glial lineage, loss of pancreatic progenitors, chondrogenesis and otic placode invagination defects.
	Sox10	Neural crest SCs (melanocytes and Schwann cells) Oligodendrocytes progenitors	N/A Oligodendrocytes	Maintenance of stem cells.
Sox F	Sox7	^	Endoderm	Gut defects in case of deletion
	Sox17	Hematopoietic SCs Extraembryonic endoderm SCs in preimplantation embryo	N/A Vascular endothelium Endoderm Spermatogenesis	Gut defects in case of deletion Spermatogenesis
	Sox18	Blood vessel	Dermal papilla of hair follicle Lymphatic endothelium Vascular endothelium	Blood vessel and hair follicle development
Sox G	Sox15	N/A	Satellite cells	Inhibitor of myoblast differentiation
	Sox16	N/A	N/A	N/A
	Sox20	Fetal testes	N/A	N/A
Sox H	Sox30	Male germ cells	N/A	N/A
Sox I	Sox31	Late blastula, gastrula, and neural tissues	N/A	Neural induction
Sox J	SoxJ	N/A	N/A	N/A

diac and renal defects, absence of the olfactory bulbs, dilatation of cerebral ventricles (Kwok et al., 1995). Male to female sex reversal (XY-female) is often observed in CD patients, with genital morphological anomalies ranging from minor conditions such as hypospadia and cleft scrotum to female genitalia with streak-like gonadal rudiments. This variety in phenotypes demonstrates that Sox9 plays a key role not only in cartilage and skeletal development but even in sex determination (Kent et al., 1996).

Sox9, of primary importance for cartilage differentiation (Akiyama and Lefebvre, 2011), is moreover involved in a wide variety of embryonic developmental processes and in differentiation of several tissues and organs, including testis, pancreas, intestine, brain, kidney, lung, heart valves, bile ducts, hair follicles and derivatives of neural crest (Akiyama et al., 2004; Vidal et al., 2005; Sakai et al., 2006; Seymour et al., 2007; Davis and Zur Nieden, 2008; Akiyama, 2008; Barrionuevo and Scherer, 2010; Antoniou et al., 2010; Pritchett et al., 2011; Zhu et al., 2012). Sox9 is required at the right time, place and amount during development: that is why inappropriate or ectopic expression can result in disease (Pritchett at al., 2011). In recent years, indeed, the role of Sox9 in several disease states, as fibrosis or sclerosis and even cancer, has emerged (Pritchett et al., 2011). Sox9 expression has been reported in several adult organs such as intestine, pancreas, testis and skin (Nowak et al., 2008); many of its functions have been investigated and explained during these years, but further studies are needed to better understand its role in postnatal tissues homeostasis.

Sox9 in tissues

Cartilage

Sox9 plays an essential role in determining chondrocyte fate and differentiation during embryonic and postnatal development of bone tissue through a process known as endochondral ossification (Akiyama and Lefebvre, 2011). To activate genes encoding for cartilage-specific extracellular matrix (ECM) components, such as Collagens and Aggrecan, Sox9 works together with Sox5 and Sox6 (the Sox-trio) (Pritchett et al., 2011).

Sox9 has been demonstrated to be of primary importance in the early phases of cartilage differentiation of mesenchymal stem cells; its expression is detected in all chondroprogenitors and differentiated chondrocytes until the cells in the growth plate develop into hypertrophic chondrocytes (Akiyama, 2008). As reported above, mutations in this gene can lead to a severe skeletal malformation syndrome (Bi et al., 2001; Akiyama and Lefebvre, 2011). The effects of Sox9 gene mutations have been studied in Sox9+/- mutant mice too. Sox9+/- mice die shortly after birth with skeletal alterations, such as bowing of long bones and hypoplasia of various skeletal elements, that strongly resemble the CD ones. Embryo skeletal analysis revealed abnormalities in cartilage primordia, impaired development of pre-cartilaginous mesenchyme, that was delayed and smaller in size, and premature skeletal mineralization (Bi et al., 2001). Many signalling pathways regulate Sox9 expression during chondrogenesis and bone repair either directly or indirectly; inappropriate Sox9 pathways activation with excessive or ectopic ECM deposition can lead to fibrosis and related disorders (Pritchett et al., 2011).

Pancreas

Examinations of CD cases revealed abnormal pancreatic morphology, with epithelial cells less densely packed and islet less clearly formed with variable expression of hormone and beta-cell markers (Piper et al., 2002).

Several members of Sox family, Sox9 included, have been investigated for their role in mice pancreas development, especially in endoderm specification, beta cell proliferation, insulin secretion, endocrine cell differentiation and pancreatic stem cells maintenance (McDonald et al., 2012). Inactivation of Sox9 in mouse pancreas leads to significant depletion of pancreatic progenitor cells, resulting in pancreatic hypoplasia (McDonald et al., 2012). Only Sox9 expression has been detected in the developing and in the adult human pancreas (Piper et al., 2002). Sox9 is important for the maintenance of early pancreatic progenitors; moreover it determines their endocrine fate, because formation of endocrine progenitors is sensitive to Sox9 gene dosage. On the other hand, formation of exocrine progenitors is insensitive to reduced Sox9 gene dosage (Seymur et al., 2007).

In the first embryo developmental stages, Sox9 is detectable in the precursor cell population that will give rise to intestinal epithelium, hepatic ductal cells and exocrine/endocrine pancreatic cells. Later, Sox9 detection becomes restricted to the pancreatic ducts. Sox9 embryonic pancreatic progenitors lose their capacity for endocrine differentiation shortly after birth, at time of Langerhans islet formation (Furuyama et al., 2011). In adult, pancreatic exocrine and endocrine functions are mediated separately by independent functional units, represented respectively by pancreatic acinus and islet of Langerhans. The functional units of intestine, liver and ex-

ocrine pancreas receive a continuous supply of cells from a common Sox9-positive progenitor zone; the islet of Langerhans, on the other hand, lose their histological connection to the ductal tree and do not receive cells from this Sox9-positive precursors (Furuyama et al., 2011). In adult, indeed, Sox9 is expressed in intestinal crypts, pancreatic ducts and bile ducts. In pancreas, it is present in duct cells, centroacinar cells included, but not in exocrine acinar cells or in endocrine islet cells (Furuyama et al., 2011).

In adult man, pancreatic progenitor pool seems to be limited to a subset of ductal and centroacinar cells, hypothesized to be the pancreatic stem cell compartment (Belo et al., 2013).

Liver and gastrointestinal tract

The upper digestive tract includes organs as the liver, pancreas, and duodenum. They derive from the primitive foregut endoderm of the embryo, and share Sox9 expression in their progenitor populations (Jo et al., 2014). Because of this, there is a contiguous Sox9 expression in the liver, pancreas and intestine; this progenitor zone involves hepatic and pancreatic ductal tree and intestinal crypt, and is anatomically connected through the duodenal papilla (Furuyama et al., 2011).

With regard to liver, hepatoblasts are the precursors of both embryonic hepatocytes and bile duct cells. Sox9 is absent in hepatocytes, the cells that secrete bile, but instead is detected in cholangiocytes and mucin-producing cells of bile ducts (Carpino et al., 2011; Jo et al., 2014).

Sox9 starts to be detected in the extrahepatic biliary tract at embryonic day 13.5 and retained at the adult stages. During mid-to-late embryogenesis Sox9 expression begins to be detected in the intrahepatic bile duct cells, when the structure of the ductal tree starts to develop in preparation for the adult function of the liver as metabolic organ. In early embryonic stages, indeed, the liver is a hematopoietic organ. As the intrahepatic ductal tree develops, Sox9 negative hepatoblasts switch to Sox9 positive embryonic hepatocyte progenitors. At the late embryonic stages intrahepatic and extrahepatic bile duct will connect (Furuyama et al., 2011).

Evidence suggests that Sox9 determines the timing of bile duct morphogenesis: the biliary tube is entirely composed of Sox9 positive cholangiocytes, and liver inactivation of Sox9 results in delayed duct maturation (Jo et al., 2014). Further studies showed involvement of Sox9 positive cells in liver regeneration by mean of lineage analysis and hepatic injury experiments: it seems that cells located at the junction of the duct structure and the functioning units of the liver (canal of Hering) are the Sox9 expressing cells representing the progenitor cells of the liver involved in tissue homeostasis maintenance (Furuyama et al., 2011). A recent surprising data is that Sox9 positive cells in the liver can be reprogrammed into insulin-secreting duct cells, implying that developmentally related cells can be modified to be used in a potential therapy for diabetes (Jo et al., 2014).

During duodenal development, Sox9 is expressed in most early epithelial progenitors and becomes gradually restricted to the nuclei of crypt cells, stem cells in the lower crypts, as well mature Paneth cells, enteroendocrine cells and in a subset of Transit-amplifying cells that orchestrate stem cell activity and tissue regeneration (Mori-Akiyama et al., 2007; Belo, 2013; Hsu et al., 2014). Sox9 indeed is required for the differentiation of Paneth cells in the intestinal epithelium. In the absence of Sox9, Paneth cells were not formed, crypts were enlarged due to a marked increase in cell proliferation throughout the crypts, and Paneth cells were replaced by proliferating epithelial cells (Mori-Akiyama et al., 2007). Sox9 expression in intestinal crypt cells is regulated by the Wnt/β-catenin signaling pathway, which has been identified as one of the key pathways in the initiation and development of colorectal cancer and in gastrulation and morphogenesis and maintenance of crypt stem cell self-renewal (Sun et al., 2012). Intestinal Sox9-expressing progenitors indeed are capable to retain multipotency from the early developmental stages throughout the lifespan (Furuyama et al., 2011). In human embryonic tissues at 7.5 weeks gestational age, mesodermal Sox9 expression is detected in the posterior region of the stomach at the pyloric area (Kimura et al., 2011). Sox9 indeed specifies the pyloric sphincter epithelium through mesenchymal to epithelial transition signals (Moniot, 2004). However, Sox9 expression in human adult stomach has not yet been clarified (Kimura et al., 2011). Inactivation of the Sox9 gene in the intestinal epithelium alters the morphology of the colon epithelium with hyperplastic appearance and local crypt dysplasia confirming a regulating role for Sox9 in cell proliferation (Kimura et al., 2011). Sox9 *in vivo*, indeed, suppresses proliferation in mouse intestinal epithelium, while inactivation of Sox9 results in increased proliferation (Jo et al., 2014).

Lung

Lung originates from cells of the foregut of early embryo and subsequently differentiates into multiple lineages of lung cell types (Zhu et al., 2012).

The morphogenesis of the lung, as well as the injury repair of the adult lung, is tightly controlled by a network of

signaling pathways with key transcriptional factors, and Sox9 is one of them (Zhu et al., 2012). Sox9 regulates lung epithelial development during lung branching (Chang et al., 2013; Jo et al., 2014). Up to now several studies have attempted to point out its role in lung branching program, but with conflicting results, maybe due to the different genetic line of the mice employed (Jo et al., 2014). In a study, Sox9 inactivation resulted in substantial no alterations in lung structure, postnatal survival and recovery after oxygen injury (Perl et al., 2005). On the other hand, other investigations revealed aberrant epithelial movements, cytoscheletal disorganization and defects in extracellular matrix (Chang et al., 2013; Rockich et al., 2013). Moreover, transgenic mice lacking Sox9 expression, showed a tracheal collapse because cartilage rings were missing; mutant mice were unable to breathe and died at birth, demonstrating the importance of Sox9 in tracheal development too (Turcatel et al., 2013). Sox9 indeed is highly expressed throughout lung morphogenesis as a downstream gene of Sonic Hedgehog (Shh) (Zhu et al., 2012); Shh is a glycoprotein expressed in the notochord and floor plate that upregulates Sox9 to generate chondrogenic precursors. In this way, Shh signalling controls the patterning and formation of tracheal cartilage by means of spatiotemporal regulation of Sox9 (Park et al., 2010; Jo et al., 2014).

Sox genes seem to have an oncogenic potential in lung cancer; overexpression of Sox9 seems to promote lung adenocarcinoma cell proliferation (Zhu et al., 2012).

Heart

Epithelial-to-mesenchymal transition (EMT) is a process occurring repeatedly throughout embryogenesis (Pritchett et al., 2011) and is involved in the formation of endocardial cushions, primordia of valves and septa too (Akiyama et al., 2004). In CD patients ventricular septal defects and Fallot tetralogy have been signalled (Montero et al., 2002; Sanchez-Castro et al., 2013). Sox9 and SoxE genes play an important role in cardiac septa formation and valvulogenesis; indeed they seem to be responsible for connective tissue differentiation. Sox9 in particular, together with other Sox proteins, regulates aggrecan and type II Collagen genes expression: these are extracellular matrix components of the cushion tissue mesenchyme and developing valve (Montero et al., 2002). Previous work on chick embryos showed that Sox9 is expressed in mesenchymal tissue of the out flow tract and endocardial cushions of the heart chick embryos between days 3.5 and 4.5 of incubation. In the following stages its expression in these structures increases and reaches the atrioventricular sulcus. By day 7 of incubation, transcripts are concentrated in the anlage of the valve leaflets and in the membranous portion of the interventricular septum. From day 8, expression is restricted to the connective portion of the atrioventricular and arterial valve leaflets. Sox8 and Sox10 are detectable in subendothelial mesenchyme, while Sox9 is present in the whole core of the developing valves (Montero et al., 2002).

Testicle

Since the discovery of Sox9 mutations in CD, concurrently with studies concerning skeletal development, Sox9 has been investigating for its role in sex determination.

In mammals, the presence of Y chromosome led the bipotential gonad to differentiate in testicle (Barrionuevo and Scherer 2010). Sertoli cells development is the first sign of testis differentiation and represents a critical event in male sex determination (Jakob and Lovell-Badge, 2011). Experimental studies reducing or repressing Sox9 function, showed that Sox9 plays a key role in Sertoli cells differentiation during testis development. Two-thirds of patients affected by CD exhibit varying degrees of XY male-to-female sex-reversal, whereas Sox9 duplication is related to human patients XX female-to-male sex-reversal (Pritchett et al., 2011). In XY Sox9-/- mice, Sertoli cells do not develop, and thus no testis cord; whereas conditional deletion of Sox9 in the gonad can result in XY male-to-female sex reversal (Jakob and Lovell-Badge, 2011). During foetal development Sox9 is found in both male and female gonads; gradually its expression becomes upregulated in male gonad and decreases till silencing in the female one (Kent et al., 1996; Morais da Silva et al., 1996). In developing gonad, SRY stimulates Sox9 expression for Sertoli cells differentiation in a restricted time window: in mouse between 10.75 and 12.5 days after coitus. It was demonstrated that SRY has to act by this critical time or Sox9 levels stay low and ovary development follows (Hiramatsu et al., 2009). SRY activates Sox9 working in combination with Steroidogenic factor-1 (SF1) via the Testis-Specific Enhancer of Sox9 (TES) (Sekido and Lovell-Badge, 2008). TES has been recently identified as a regulatory sequence responsible for specific Sox9 testis expression; within TES, a more definite region, TESCO, highly preserved between mouse, rat, dog and man was found (Jacob e Lovell-Badge, 2011). SF1 and SRY together upregulate Sox9 and then, with SF1 help, Sox9 also binds to the enhancer to help maintaining its own expression after that of SRY has ceased (Sekido and Lovell-Badge, 2008). Sox9 then activates other genes involved in male sex determination (Kamaki and Kondoh, 2013): it

up regulates, i.e., the production of anti-muellerian hormone (Mackay, 2000; Josso et al., 2001), secreted during the embryonic development by Sertoli cells and promoting the regression of the Muellerian ducts (Josso et al., 1993).

To maintain Sox9 expression at high levels in Sertoli cells, two positive feedback loops have been identified. According to recent studies, it seems that fibroblast growth factor 9 (FGF9) and FGF receptor 2 (FGFR2) may play a part in Sox9 expression maintenance, as both mutant mice FGF9-/- and FGFR2-/- show a XY male-to-female sex reversal related to the absence of Sox9 signal. It has been shown, however, that Sox9 itself is required for FGF9 expression, generating a positive feedback loop (Jakob and Lovell-Badge, 2011). Another positive feedback loop is represented by a cell-intrinsic mechanism involving Sox9 itself together with SF1 (Jakob and Lovell-Badge, 2011). Moreover Sox9 promotes Prostaglandin D2 (PGD2) synthesis and Sox8 activation, another SoxE gene, that seems to have a reinforcing role for Sox9 and is involved in the maintenance of testicular functions at later stage (Jakob and Lovell-Badge, 2011; Kamachi and Kondoh, 2013).

Prostate

Mammalian prostate originates from urogenital sinus, an embryonal structure composed by an epithelial layer surrounded by mesenchymal tissue. In mouse, prostate morphogenesis begins at embryonic day 17.5, when epithelial cells grow into mesenchyme to form buds (Thomsen et al., 2008). Sox9 plays an important role in the early stages of prostate development and it is found in cell nuclei of the epithelial buds (Thomsen et al., 2008). Prostate development is androgen dependant with the initial androgenic effects occurring through mesenchymal-dependent FGF signalling (Huang et al., 2012), similarly to testis development. Deletion of FGFR2 and Sox9 in mice show comparable prostate defects, suggesting that these genes and their products are both necessary for early prostate development (Thomsen et al., 2008). A prostate specific deletion of Sox9 showed a lack in ventral prostate development and an abnormal anterior prostate differentiation (Thomsen et al., 2008). Sox9 is needed not only for ventral lobe development, where it regulates cellular proliferation and differentiation, but even for adult prostate regeneration (Huang et al., 2012).

Skin and hair follicle

Sox9 role in hair follicle (HF) development has been described first by Vidal et al., (2005), that demonstrated Sox9 expression in the developing hair placode at embryonal day (E) 14.5 and at E 12.5 in whisker pads of mice embryos by *in situ* hybridization analysis . Sox9 was detected in the thickening epithelial tissue but was absent in the underlying mesenchyme. At E18.5 its expression is restricted to the presumptive outer root sheet (ORS) layer. Sox9 expression was present at all stages of hair cycling but, at telogen stage positive Sox9 cells are detected at the base of the hair-follicle epithelium, presumably representing the bulge compartment (Vidal et al., 2005). The bulge is a part of the ORS and represents a highly specialized hair follicle niche that preserves the proliferative potential of quiescent adult stem cell (Nowak et al., 2008).

To evaluate the role of Sox9 in hair formation, transgenic mice with a skin-specific inactivation of Sox9 were employed [Y10:Cre/Sox9(fl/fl)]. Sox9 knock-out mice showed severe hair abnormalities, appeared hairless in the caudal part of the body, defects in hair development and hair follicle stem cells disappearance (Vidal et al., 2005). Despite the nude appearance of Sox9 knockout mice, careful observation demonstrated the presence of small, atrophic hair (Vidal et al., 2005). Histological alterations are detectable from day 8 after birth with increased cellularity in the dermis, and a decreased number of bulb matrix cells. Absence of CD34 positive cells and reduction of cytokeratin 15 (K15) cells, both stem cells markers, are indicative of bulge absence. These results indicated a role for Sox9 in hair follicle maintenance, especially for the formation of the bulge and for stem cells maintenance (Vidal et al., 2005). Sox9, indeed, besides to be essential for outer root sheath differentiation and the formation of hair stem cell compartment itself (Vidal et al., 2005), it is expressed in epithelial stem cells in mouse and human adult hair follicle (Nowak et al., 2008).

Further studies allowed to understand that Sox9 is not expressed throughout the early placode, but rather taking over in the later stages and exclusively in a small subset of supra-basal cells that do not correspond to the basal layer expressing Lhx2, another transcription factor involved in bulge formation. These two distinct populations of placode cells may have distinct roles during HF morphogenesis; Lhx2 participate in early events and disappears by about 7 days after birth (P7), while Sox9 takes over in the later stages and gives rise to a long-lived stem cell population with several essential functions, one of which is the completion of hair follicle morphogenesis (Nowak et al., 2008, Christiano, 2008). The initial matrix cells are Sox9 negative: that is why in K14- Cre;Sox9flox/flox mice employed for this study the early stages of hair follicle morphogenesis are preserved; and this explains also why they undergo arrested development when Sox9-positive matrix cells should replace the Lhx2-positive cells at P7 (Nowak et al.,

2008, Christiano, 2008). Notably, this is the same time point at which Vidal et al., (2005) reported the initial signs of hair follicle degeneration in their mice, beginning with a depletion of matrix cells in Y10:Cre;Sox9flox/flox mutant animals.

In Nowak et al. (2008) study , authors demonstrated that hair follicle Sox9 positive cells and their progeny do not contribute to interfollicular epidermis either during its development or its subsequent homeostasis. However these cell are robustly recruited in case of a wound response. In Sox9 cKO mice indeed, interfollicular epidermis regeneration is highly impaired (Nowak et al., 2008; Christiano, 2008).

In subsequent studies, Sox9 expression in the skin has been detected in the sebaceous gland, sweat gland, outer root sheath of the hair follicles (Vidal et al., 2008; Shi et al., 2013) and in the bulge (Vidal et al., 2008). Differently from what observed in mouse, in human Sox9 is expressed in keratinocytes basal layer too. Overexpression of Sox9 promotes human keratinocyte proliferation and inhibits keratinocyte differentiation (Shi et al., 2013).

To conclude, it has emerged that the hair follicle represents an important stem cell reservoir in the skin, both for epithelial stem cell (Vidal et al., 2008; Shi et al., 2013) and for mesenchymal stem cell (Mercati et al., 2009), to maintain tissue homeostasis and contribute to wound healing processes (Nowak et al., 2008). Sox9 is expressed in epithelial stem cells in adult hair follicle, that give rise to all epithelial cells of the hair follicle, the sebaceous gland, and the interfollicular epidermis (Christiano, 2008).

Mammary gland

The transcription factor Sox9 plays an important role in mammary gland development. Most of the studies have been performed in mice and human beings (Guo et al., 2012a; Malhotra et al., 2014; Ye et al., 2015; Fazilaty et al., 2015; Pomp et al., 2015). Conditional Sox9 deletion results in defective mammary gland development with substantial delay in ductal elongation and branching (Malhotra et al., 2014). A study performed on Sox9 cKO mice revealed that Sox9 deletion resulted in defects that were particularly severe 3 weeks postnatal and were followed by gradual recovery, with essential no defects beyond 8 weeks of age. Recovery from this transient branching block imposed by Sox9 deletion seems to be related to the expansion of mammary epithelial cells in which Sox9 deletion did not occur. Moreover, in the same study, fate mapping of Sox9 deleted cells demonstrated that Sox9 is essential for luminal, but not myoepithelial, lineage commitment and proliferation (Malhotra et al., 2014).

Mammary gland represents a useful model to study epithelial staminal cells, as it contains a subpopulation of multipotent mammary stem cells (MaSCs) in the adult mammary gland, with a remarkable regeneration capacity (Guo et al., 2012a; Malhotra et al., 2014). It has been demonstrated that Sox9 and Slug serve as a master regulators of the gland-reconstituting activity of normal mammary stem cells (Ye et al., 2015).

In healthy mammary gland, indeed, Sox9 and Slug, through epithelial to mesenchymal transition (EMT) mechanisms, cooperate reprograming differentiated luminal cells of the mammary gland into MaSCs (Guo et al., 2012a; Ye et al., 2015; Pomp et al., 2015), while Sox9 alone converted these cells into luminal progenitors (Guo et al., 2012a; Malhotra et al., 2014). Mature mammary luminal cells expressing both Sox9 and Slug were able to activate in adult MaSCs an endogenous autoregulatory network and are capable of generating an entire mammary ductal tree when transplanted into a mammary fat pad (Guo et al., 2012a; Sarkar and Hochedlinger, 2013). In recent years identification and characterization of molecular controls that regulate MaSCs and progenitor cells homeostasis are critical to our understanding of normal mammary gland development and its pathology, neoplastic lesions included. Up to now there are few studies concerning this issue, and most of them are about Sox9 role in human brest carcinoma (Guo et al., 2012a; Ye et al., 2015; Pomp et al., 2015).

Sox9 in acquired diseases

Fibrosis and related disorders

As Sox9 plays multiple roles in embryogenesis and in control of proliferation and cell differentiation, it seems logical that its inappropriate or ectopic expression can result in disease (Pritchett et al., 2011). Sox9 implication in fibrosis-related disorders and even in neoplastic cells growth have been investigated (Akiyama, 2008). Ectopic Sox9 expression indeed mediates extracellular matrix (ECM) deposition (Piper-Hanley et al., 2008): fibrosis, sclerosis and related disorders can affect virtually all tissues and organs in the body and characterize several chronic diseases. They are characterized by excessive, inappropriate ECM deposition, resulting in the destruction of tissue architecture and function (Pritchett et al., 2011).

Type1 collagen is the major collagen subtype deposited

in organ fibrosis and its levels increase with Sox9 level growth (Piper-Hanley et al., 2008). Transforming growth factor β (TGF-β) and fibroblast growth factor 2 (FGF2) seems to augment ECM deposition, increasing Sox9 expression (Piper-Hanley et al., 2008), even if a recent study suggests that is Hedgehog signaling, not TGF-β, that lies upstream of Sox9 (Jo et al., 2014). Anyway, both TGF-β and FGF2, together with other transcription factors as Slug, promote epithelial-to-mesenchymal transition (EMT), converting normal and neoplastic epithelial cells into derivatives with a more mesenchymal phenotype (Piper-Hanley et al., 2008; Riemenschnitter et al., 2013). Through EMT indeed, cells lose epithelial markers as Cytokeratins and E-Cadherin, and gain mesenchymal markers as Vimentin, α-Sm Actin (Pritchett et al., 2011). In development, this process induces Sox9 expression during neural crest delamination for (i.e) the differentiation of chondrocytes and astrocytes (Jo et al., 2014). EMT is also a forerunner of organ fibrosis when the resultant mesenchymal cells express abundant type1 collagen as part of the fibrotic matrix (Piper-Hanley et al., 2008). Sox9 results overexpressed in glomerulosclerosis, vascular calcification, hepatic, and cardiac fibrosis (Piper-Hanley et al., 2008; Pritchett et al., 2011). In particular, in kidney with glomerulosclerosis, high expressions of Osteopontin and other TGF-β genes were observed, suggesting that Sox9 activity is similar in both glomerulosclerosis and liver fibrosis (Jo et al., 2014).

Tumors

Sox9, as emerged from the previous section, together with other transcription factors as Slug, is involved in EMT, an early developmental phenomenon that results in the acquisition of an invasive, mesenchymal phenotype by epithelial cells (Riemenschnitter et al., 2013). In the context of neoplasia, passage through an EMT results in the acquisition of cell-biological traits associated with high grade malignancy, including motility, invasiveness and increased resistance to apoptosis (Guo et al., 2012a,b).

With respect to neoplasms, Sox9 has been identified as a pro-oncogenetic factor in a wide range of human tumors; where it is overexpressed promotes cellular proliferation, bypasses senescence and it is able to immortalize primary stem cells (Matheu et al., 2012). On the other hand, as for some human and mice melanomas, Sox9 is likely to operate as tumor suppressor (Passeron et al., 2009; Matheu et al., 2012). These data present opposing roles for Sox9 in tumors, either inducing or potentially inhibiting cell proliferation. The differences between these studies could be attributed, in part, to the differences in cell lines and levels of Sox9 (Jo et al., 2014). Anyway in recent studies Sox9 has found to be overexpressed in most of human neoplastic cell lines, with the exception of lymphomas and kidney cancers (Matheu et al., 2012). There are strong analogies between normal tissue stem cells and tumor-initiating cells (TICs): for this reason some researchers proposed a common activation program (Ye et al., 2015). TICs seem to be responsible for both initiating the bulk of tumors and increasing cell migratory and invasive properties, a prerequisite for tumor metastasis (Luanpitpong et al., 2015; Ye et al., 2015). This is confirmed by the observation that the presence of TICs in primary tumors is strongly correlated with an increased incidence of metastasis and poor survival of patients (Luanpitpong et al., 2015).

Sox9 role in human cancer is context dependent and related to the transcription factors involved (Matheu et al., 2012). Slug (also known as Snai1) and Snail (or Snai2) are members of the Snail family of the EMT-inducing transcription factors. These transcription factors induce distinct EMT programs (Ye et al., 2015).

Slug and Sox9 determine the mammary stem cell (MaSC) state, and work as regulators of the gland-reconstituting activity of normal MaSCs (Guo et al., 2012a; Ye et al., 2015).

On the other hand, the induction of differentiated luminal epithelial cells into cancer cells exhibiting basal features is tightly associated with EMT activation with Snail participation. Shutdown of Snail, indeed, could selectively eliminate breast TICs (Ye et al., 2015).

This argument is still debated; in literature different and often contrasting results are reported, as this issue is still evolving. Forced expression of Slug in collaboration with Sox9 in several solid tumors was found to promote the formation of tumor-initiating cells (TICs) formation, but it is not required for EMT activation in lung cancer cells (Luanpitpong et al., 2015; Ye et al., 2015).

Previous works correlated high levels of Slug to poor prognosis in brest or lung cancer neoplasms (Guo et al., 2012a; Luanpitpong et al., 2015) but the prognostic power of Slug expression may be due in large part to its strong association with basal differentiation, which is, on its own, a well-known feature of aggressive breast cancers (Ye et al., 2015).

Knockdown of Slug and Sox9 repressed TICs, in agreement with the previous report implicating their role in breast tumor initiation, and inhibited experimental metastasis, which is likely due to their effect on TICs and not on the EMT process, as EMT remained activated after Slug knockdown (Luanpitpong et al., 2015).

Sox9 positive cancer cells are often detected in tissues

where Sox9 plays critical roles in their development and within the stem cell compartments of most of the corresponding normal tissues (Matheu et al., 2012), such as lung (Zhu et al., 2012), gastrointestinal tract (Lu et al., 2008; Kimura et al., 2011; Matheu et al., 2012; Sun et al., 2012) prostate (Wang et al.,2007; Huang et al., 2012), mammary gland (Chakravarty et al., 2011a) or hair follicle (Shi et al., 2013).

The high levels of Sox9 detected in some neoplasms might reflect the origin of the tumors from the expansion of Sox9-positive stem/progenitor cells. Activated stem cells indeed are required for injury repair, and chronic injury increases the risk of forming cancer (Pritchett at al., 2011). Together with a transforming agent, activated oncogene, or event such as chronic injury, higher levels of Sox9 significantly increase the rate of developing tumors (Matheu et al., 2012).

On the other hand, Sox9 seems to be expressed even in tissues where Sox9 is not known to be involved during normal physiology (Matheu et al., 2012).

Sox9 detection potentially carries prognostic value in a range of tumors including neurofibromatoma, medulloblastoma, colon cancer and prostate cancer. Although the identification, validation and application of biomarkers is complex, it has been suggested that immunostaining for Sox9 in biopsy samples and pathology resections could aid diagnosis and prognostication for patients (Pritchett at al., 2011). Sox9 expression in human breast cancer or in lung adenocarcinoma, i.e., is associated with tumor progression and malignancy (Chaktravarty et al., 2011a; Zhu et al., 2012).

In lung cancer Sox genes seem to have an oncogenic potential; overexpression of Sox9 indeed seems to promote lung adenocarcinoma cell proliferation (Zhu et al., 2012), TICs and metastases in a mouse model (Luanpitpong et al., 2015). In lung metastatic tumor cells, increased Slug expression stabilizes Sox9, inhibiting Sox9 ubiquitination and proteosomal degradation. Sox9 stabilization promotes the expansion of TICs and subsequent cancer metastasis (Luanpitpong et al., 2015).

Sox9 seems to be required for prostate carcinogenesis in animal models (Huang et al., 2012), but it has been found even in primary prostate cancer *in vivo*, at a higher frequency in recurrent prostate cancer and in prostate cancer cellular lines too (Wang et al., 2007). It is possible that Sox9 transactivates the androgen receptor, as some prostate cancers are androgen-dependent. On the other hand, one study showed that Sox9 suppresses growth and tumorigenesis in the prostate tumor cell line M12 (Jo et al., 2014).

In pancreas Sox9 is detected in all types of pancreatic lesions/neoplasms of ductal origin, but rarely in other pancreas neoplasms, representing a useful marker for the epithelial cells of pancreatic ductal system, including centroacinar cells, and for the ductal lineage of pancreatic neoplasms (Shroff et al., 2014). Sox9 moreover accelerates the formation of precursor lesions of pancreatic ductal adenocarcinoma (Jo et al., 2014). It was moreover reported that the Sox9 expression was decreased in pancreatic intraepithelial neoplasia and increased in pancreatic ductal adenocarcinoma (Matsuhima et al., 2015). As explained earlier, Sox9 as a biomarker may be helpful in immunophenotype detection, to better asses the tissutal origin of the neoplasm, as in case of hair follicle, neural crest or cartilagineous neoplasms (Wehrly et al., 2003; Vidal et al., 2008; Carbonelle-Puscian et al., 2011). Sox9 indeed has been employed to distinguish mesenchymal chondrosarcoma from other small blue round cell tumors, whose therapy and prognosis differ considerably (Wehrly et al., 2003). Sox9 is detected in benign cartilagineous neoplasms as chondromyxoid fibroma and chondroblastoma and may be useful for differential diagnosis of these tumors (Dancer et al., 2010) A recent study demonstrated that downregulation of Sox9 could inhibit migration and invasion of chondrosarcoma cells (Li et al., 2015).

With regard to liver, SRY and in particular Sox9 is expressed in hepatocellular carcinoma and the nuclear expression of SRY is associated with cancer progression and poor patient survival (Xue et al., 2015). In cases of intrahepatic cholangiocarcinoma a two-stage patterns of Sox9 expression has been observed: the first one with Sox9 expression decreasing, related to the early stage of the carcinogenesis, and the second with Sox9 increase due to biliary infiltration and poorer prognosis, promoting cell migration and invasion (Matsushima et al., 2015). This expression pattern has been observed for pancreatic ductal adenocarcinoma and gastric carcinoma too (Sun et al., 2012; Matsushima et al., 2015). Sox9 upregulation in carcinogenesis of the digestive system have been detected in esophageal squamous cell carcinoma (Hong et al., 2015), gastric cancer (Sun et al., 2012) and colorectal cancer (Shen et al., 2015).

Regarding colon carcinoma, Sox9 induces the expression of S-100P, a protein related to proliferation, tumorigenesis regulation, invasion and cancer cell motility, leading to increased cell invasiveness and metastasis as well as activation of EMT (Shen et al., 2015). As previously explained, the Wnt signaling pathway, together with Sox9, is crucial for crypts and Paneth cells maintenance: Wnt activating mutations are often found in colorectal cancers; the same happens for members of other developmental

programs as Notch or bone morphogenetic proteins, transcription factors involved with Sox9 in developmental processes of various tissues (Matheu et al. 2012). Sox9 is overexpressed in colorectal cancers; given the role of Sox9 in stem cells in the intestinal epithelium, the high levels of Sox9 might reflect the origin of the tumors from transformation of such cells. Alternatively, the high levels of Sox9 could reflect a loss of repression, or genetic alterations and/or other types of deregulation (Matheu et al. 2012). In addition Sox9 regulates B lymphoma Mo-MLV insertion region 1 (BMI1) expression, a well-established oncogene; their interactions are likely to be critical events in colorectal tumor development and progression. Sox9 and BMI1, cooperate in EMT, leading to cancer metastasis that occurs in the most advanced colorectal cancers (Matheu et al. 2012).

Skin and hair follicle neoplasms, mammary gland neoplasms, nervous tissue development and neoplasms are discussed in the following sections.

SOX9 in veterinary medicine

In veterinary literature there is few data concerning the role of Sox9, and most of them concern its role in gonadal development. Sox9 sequence in dog shows high homology to other Sox9 genes, since it is highly conserved among vertebrates. Similarly, Sox9 expression is implicated in canine testis determination. The pattern and timing of canine gonadal Sox9 expression is similar to those described for humans, pigs, goats, and mice (Meyers-Wallen, 2003) and it has been previously described. Sox9 has been investigated in cases of XX sex reversal syndrome, the most common form of intersexuality occurring in dogs, where testicular tissue develops in the absence of the key gene, SRY (Groppetti et al., 2012). This abnormality of gonadal sex determination is observed in human too, where at about 80% of XX sex reversed patients show a SRY translocation. The remaining 20% do not have SRY or any other genes normally located on the Y chromosome (Meyers-Wallen, 2006). As for human XX reverse syndrome, even in dog has been recently demonstrated the role of Sox9 duplication in the development of this anomaly (Rossi et al., 2014; Xiao 2013).

Other researches focused on stem cells demonstrated that canine mesenchymal stem cells, treated with suitable induction media, differentiate in chondrocytes exhibiting Sox9 transcription factor (Csaki et al., 2007). Sox9 positive stem cells have been identified in canine hair follicle bulge too (Kobayashi et al., 2010). From this study has emerged that canine staminal cells localization in the hair follicle matches the human one. In anagen canine primary hair follicles, indeed, multipotent cells were predominantly observed in the outermost layer of the outer root sheet (ORS) around a little bud that provides the insertion point of the erector pili muscle. In the same study, canine anagen hair follicles were microdissected into four fragments: P1 (bulb), P2 (suprabulbar), P3 (isthmus including bulge), and P4 (infundibulum). Genes crucial for the development and maintenance of mouse bulge stem cells like Sox9, Lhx2, Tcf3, and Nfatc1 were tested by means of real-time PCR; all of these genes were over-represented in P3 from dogs and human. Sox9 especially was the most evident. Thus, canine bulge cells share some crucial gene expression profiles with mouse and human bulge stem cells (Kobayashi et al., 2010).

SOX9 in normal tissues of various animal species

The results of a preliminary evaluation of Sox9 antibody used in the research studies of this thesis are shown below. The evaluation has been performed on tissues and organs of various animal species.

Anti-Sox9 antibody employed is a rabbit polyclonal serum (Sigma-Aldrich, St. Louis, MO, USA) obtained using a 117 amino-acids peptide corresponding to the N-terminal of human Sox9. This peptide sequence has been compared with the one of various domestic animals and it resulted highly conserved (table 1).

To assess the immunohistochemical staining for this marker serial diluition tests have been performed on several organs of different animal species (table 2).

Internal positive controls were represented by skin and hair follicle adnexa. Sox9 immunolabeling was scored as negative (-), weak (+), moderate (++), intense (+++). NP= not present. Sox9 expression was detected in many domes-

Table 1. Sox9 sequence identities and similarities in various animal species.

Specie	Identities (%)	Positive similarities (%)
Cat *Felis catus*	97	98
Dog *Canis familiaris*	96	98
Goat *Capra hircus*	89	95
Horse *Equus caballus*	98	99
Swine *Sus scrofa*	94	94
Cow *Bos taurus*	not reported	not reported
Rabbit *Oryctolagus cunicoli*	not reported	not reported

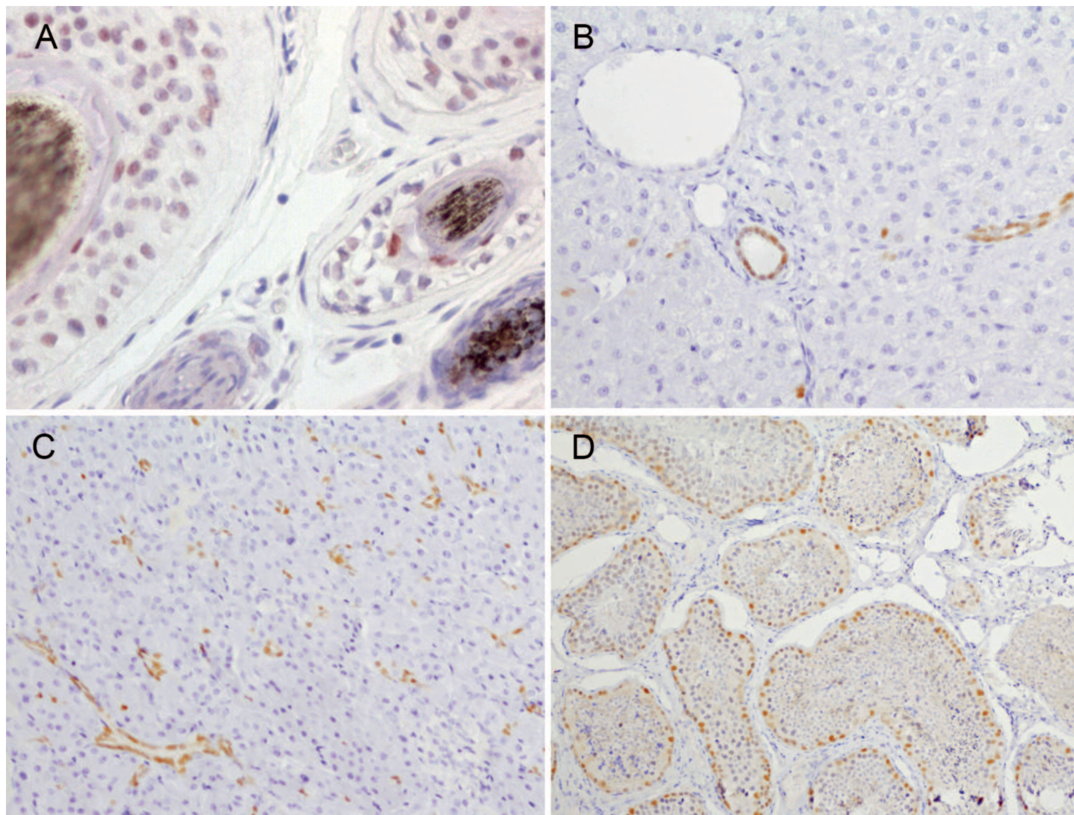

Figure 1. Sox9 immunohistochemical results on canine hair follicle (A) (obj. 40 x), bovine liver (B) (obj. 20 x), goat pancreas (C) (obj. 20 x) and testis (D) (obj. 10 x). Haematoxylin counterstain.

Table 2. Sox9 immunohistochemical results. Table legend:- = negative immunolabeling; + = weak, ++ = moderate, +++= intense immunolabeling. NP= tissue not present.

Tissue	Cat	Cow	Dog	Goat	Horse	Rabbit	Swine
CNS	++	++	++	++	++	+	+
Forestomach	NP	+	NP	++	NP	NP	NP
Heart	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-
Large Intestine	+	++	+	++	+	+	+
Liver	++	+++	++	+++	+++	++	+
Lung	+	+	+	++	+++	+	+
Lymph node	-	-	-	-	-	-	-
Mammary gland	++	++	++	++	++	NP	NP
Pancreas	+++	+++	+++	++	+++	++	+++
Salivary gland	+++	++	+	++	++	+	-
Skeletal muscle	-	-	-	-	-	-	-
Skin	+++	+++	+++	+++	+++	+++	+++
Small Intestine	++	++	++	+++	+++	++	+++
Spleen	-	+	-	+	-	-	+
Stomach	+	+	+	+	+	-	+
Testicle	++	+++	+++	+++	+++	NP	NP
Thyroid	-	-	-	-	-	-	-

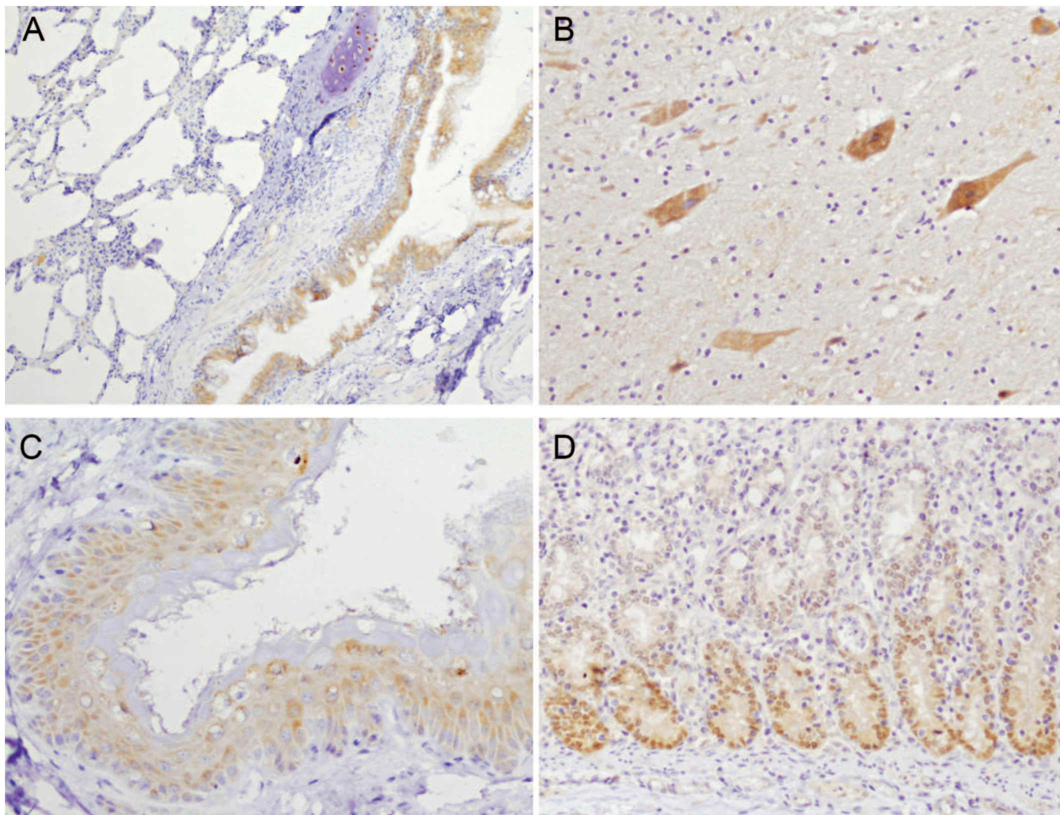

Figure 2. Sox9 immunohistochemical results on equine lung (A) (obj. 10 x), bovine central nervous system (B) (obj. 20 x), goat forestomach (C) (obj. 20 x) and bovine small intestine (D) (obj. 20 x). Haematoxylin counterstain.

tic animal tissues, but especially in the hair follicle and in other skin adnexa (Fig. 1A), in bile ducts of the liver (Fig. 1B), in pancreas ductal component (Fig. 1C), in basal cells of the seminiferous ducts of the testicle (Sertoli cells) (Fig. 1D) and in basal glandular cells of the small intestine (Fig. 2D). In lung, strong immunolabeling was detected in scattered cells in bronchial cartilage and diffusely in bronchial epithelium (Fig. 2A). In the spleen dendritic cells sometimes resulted mildly positive, as well as glandular cells in the stomach. Sox9 positivity in the central nervous system was detected in neuronal cytoplasm and in scattered glial cells (Fig. 2B). Forestomach of ruminants showed mild to moderate positivity in basal layers (Fig. 2C)

SOX9 in the skin and hair follicle

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Sox9 in canine epidermal skin tumors

Introduction

Sox9 in human is highly expressed in several skin diseases as psoriasis or acne (Shi et al., 2013), and generally in adnexal-related neoplasms (Vidal et al., 2008; Krahl and Sellheyher, 2010a,b; Shi et al., 2013), basal cell carcinoma (Vidal et al., 2008), squamous cell carcinoma (Shi et al., 2013) and metastatic melanoma (Rao et al., 2010). The role of Sox9 in skin tumorigenesis, however, needs to be clarified yet (Shi et al., 2013).

Vidal et al., (2008) investigated the role of Sox9 in case of human basal cell carcinoma (BCC); its expression was observed in all subtypes of BCC tested and in all adnexal skin tumors analyzed, suggesting a role for Sox9 to the pathogenesis of these tumors. According to the authors, the heterogeneous staining obtained may be related to these tumors are composed of cells from various origins and/or it might reflect the different status of the tumor cells.

Further studies on human skin found that Sox9 expression in during cutaneous embryogenesis and post-natal period strongly resembles the one observed in BCC and in pilomatrixoma in their spatial distribution within the various follicular subcompartments. Basaloid cells of pilomatricoma indeed are Sox-negative as the evolving hair matrix of the embryonic hair follicle, while BCC cells resulted positive, as the primordial outer root sheath (Krahl and Sellheyer, 2010a). BCC is still viewed by many dermatologists as originating from the interfollicular epidermis but there is an increasing body of evidence that links this neoplasm to the hair follicle and classifies the tumor as the most primitive follicular neoplasm. This study strongly underline the existing link between the embryology of the skin and the histogenesis of adnexal tumors: an appropriate immunophenotyping may allow a different classification of adnexal neoplasms (Krahl and Sellheyer, 2010b).

Among various specific human bulge markers that are similarly overexpressed in canine bulge cells, Sox9 is one of the most represented. Canine bulge stem cells, indeed, strongly resemble distribution and biochemical features of the human ones (Kobayashi et al., 2010). Some canine follicular tumors show an expression of hair follicle stem cell markers and it could be suggested that these positive cells, having some features of stem cells, may play a role in tumor development (Brachelente et al., 2013). The aim of this study was to investigate the immunopositivity for Sox9 in different canine epidermal and hair follicle neoplasms; differences in Sox9 expression would help to correlate tumor phenotype with molecular characteristics thus allowing to better define tumor development, contribute to its diagnosis and clinical management.

Materials and Methods

Case selection

Different types of canine epithelial tumors were retrieved from the files of the Department of Veterinary Medicine of Milan. Samples of squamous papilloma (n=5), squamous cell carcinoma (n=6), infundibular keratinizing acanthoma (n=4), inferior tricholemmoma (n=5), isthmic tricholemmoma (n=2), trichoblastoma (n=12), trichoepithelioma (n=7), malignant trichoepithelioma (n=3), pilomatricoma (n=2), subungual keratoacanthoma (n=1), subungual squamous cell carcinoma (n=4) were selected. Normal skin tissue surrounding the tumor was also included in the study.

The diagnosis of each tumor was revised according to the criteria of the World Health Organization classification.

Immunohistochemical staining

Four-micrometer thick sections from routinely formalin-fixed and paraffin-wax embedded samples were prepared on poly-L-lysine-coated glass slides for immunohistochemistry. The sections were de-paraffinized in xylene and rehydrated through graded alcohols. Endogenous peroxidase was blocked using 3% hydrogen peroxide in distilled water for 5 min, then, antigen retrieval for formalin-fixed samples was accomplished by microwave irradiation in cit-

rate buffer, pH 6.0, for 10 min. Normal goat serum for 30 min was employed to block sections. Sections were then incubated for 18 h at 4°C with anti-Sox9 rabbit polyclonal antibody (HPA001758; Sigma-Aldrich, St. Louis, MO, USA) diluted 1:200. The antibody recognizes a Sox9 specific peptide of 117 amino acids with 96% identity with Canis familiaris. The sections were incubated for 30 min with 1:200 goat anti-rabbit biotin conjugate antibody (Vector Laboratories, Burlingame, CA, USA; BA 1000) and subsequently stained for 30 min with streptavidin-biotin peroxidase kit (Vectastain Elite ABC, Vector Laboratories, PK-6100). Positive staining was visualized with 3.3-diaminobenzidine-4 HCl (Vectastain, Vector Laboratories, SK-4100) and nuclei were counterstained with Mayer's hematoxylin. Sox9 reactivity of follicular outer root sheath present in normal cutaneous tissue of tumor

Table 1. Immunohistochemical results

Neoplasm	Case no.	Positive cells	Signal intensity	Global Score
Squamous Papilloma	#1	1	1	2
	#2	3	2	5
	#3	1	2	3
	#4	2	2	4
	#5	3	3	6
Squamous Cell Carcinoma	#1	1	1	2
	#2	3	2	5
	#3	3	3	6
	#4	1	1	2
	#5	2	2	4
	#6	2	1	3
Infundibular Keratinizing Acanthoma	#1	1	1	2
	#2	2	2	4
	#3	1	1	2
	#4	4	3	7
Subungual Keratoacanthoma	#1	0	-	0
Subungual Squamous Cell Carcinoma	#1	2	2	4
	#2	1	2	3
	#3	1	1	2
	#4	1	2	3
Inferior Tricholemmoma	#1	3	3	6
	#2	2	2	4
	#3	2	1	3
	#4	2	3	6
	#5	4	3	7
Isthmic Tricholemmoma	#1	3	3	6
	#2	3	3	6
Trichoblastoma	#1	4	3	7
	#2	4	3	7
	#3	4	3	7
	#4	4	2	6
	#5	4	3	7
	#6	4	3	7
	#7	3	3	6
	#8	4	3	7
	#9	3	3	6
	#10	4	3	7
	#11	4	1	5
	#12	2	2	4
Trichoepithelioma	#1	2	2	4
	#2	4	3	7
	#3	3	2	5
	#4	3	3	6
	#5	2	3	5
	#6	1	2	3
	#7	3	2	5
Malignant Trichoepithelioma	#1	3	2	5
	#2	3	2	5
	#3	2	3	5
Pilomatricoma	#1	3	2	5
	#2	2	3	5

sample was used as internal positive control. Negative control sections were produced by omission of the primary antibody.

Evaluation of immunohistochemical data

The immunohistochemically stained tissue slides were examined using standard light microscopy. The staining results were independently scored by the authors as follows: Sox9 protein expression was assessed by categorizing immunoreaction of each tumor cell type into four groups according to the proportion of positive cells: 0, no positive cells; 1, from 1% to 25% positive cells; 2, from 25% to 50%; 3, from 50% to 75%; 4, from 75% to 100%. The intensity of labeling was graded as: W, weak positive staining; M, moderate positive staining; I, intense positive staining, and scored as follows: W=1, M=2, I=3. A global score was conferred adding together the positive cells percentage score and the intensity of immunolabeling score.

Results

Sox9 immunohistochemical results are reported in table 1.

In every section a portion of healthy skin next to the neoplasms was always included, as Sox9 internal positive tissue control (Fig. 1A). Immunohistochemical examination resulted always positive. In normal skin, Sox9 positive reaction was restricted to the bulge region and outer root sheath of the hair follicles and to germinative cells of sebaceous glands. Well differentiated sebocytes, sweat glands and epidermal cells were mostly negative. Only in a few cases Sox9 positive epithelial cells were present in basal and spinous layers of hyperplastic, reactive epidermis near and above the neoplastic tissue.

Squamous papilloma

In 4 of 5 squamous papillomas the number of Sox9 positive cells varied from 1-25% (1 case) to 25-50% (1 case), or 50-75% (2 cases) showing moderate to intense immunolabeling, staining especially basal and suprabasal cells. Only in 1 case, the percentage of Sox9 positive cells ranged from 1% to 25% and appeared weakly positive.

Squamous cell carcinoma

Sox9 was positive in all 6 squamous cell carcinomas (Fig. 1B), with a variable number of positive cells, extending from 1-25% (2 cases) to 25-50% (2 cases) or 50-75% (2 cases). The intensity of staining being intense in 1 case,

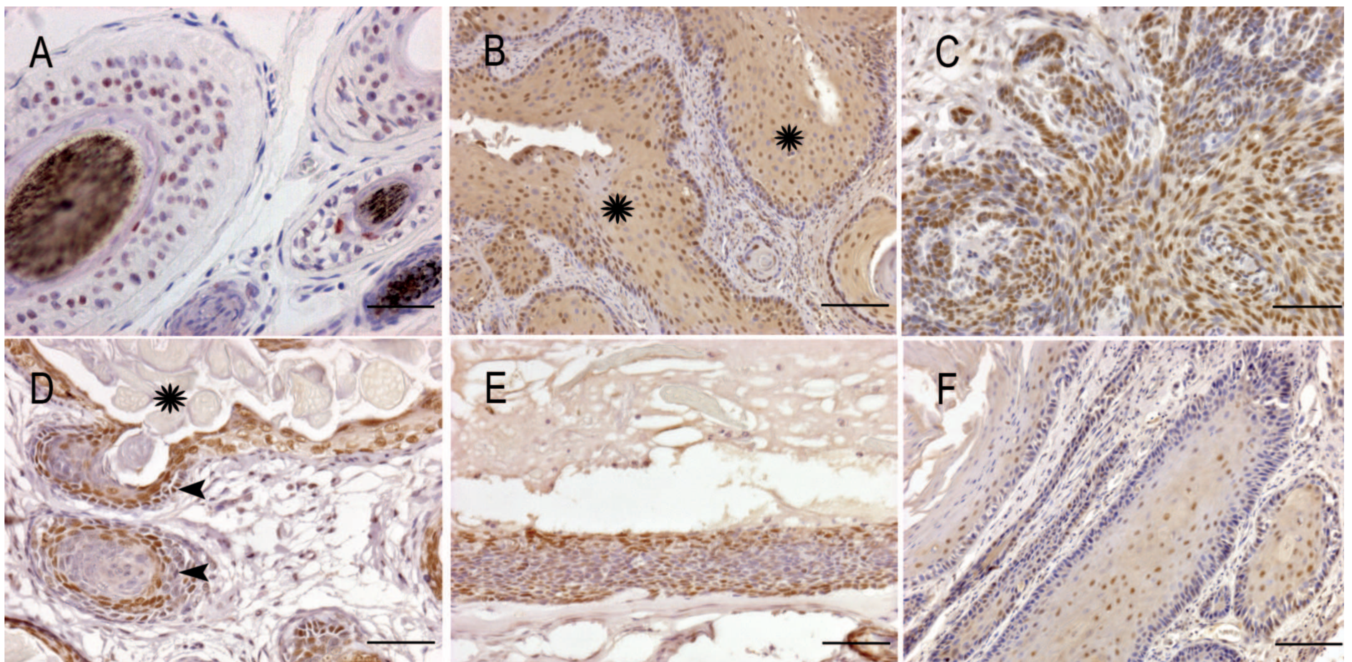

Figure 1. Sox9 immunohistochemistry. A: Hair follicle positive control: transverse sections with various positive nuclei. B: Squamous cell carcinoma. Intense, diffuse, irregular nuclear positivity in neoplastic cells with basal and spinous differentiation (asterisks). Positive cells are also detected in reactive fibrous stroma. C: Trichoblastoma. Cords and spirals of neoplastic epithelial cells almost entirely positive for Sox9. D: Trichoepithelioma. Abundant keratin (asterisk) surrounded by basal and suprabasal epithelial cells intensely positive (arrows). E: Pilomatricoma. Wall of a neoplastic cyst with positive cells mostly in superficial layers. F: Subungual squamous cell carcinoma with scatterd positive spinous cells (A, C, D Bar= 75 μm; B, F, E Bar = 150 μm).

moderate in 2, weak in 3. Immunolabeling involved especially basal cell layer and spinous cells, even if in 3 cases its distribution was very irregular.

Infundibular keratinizing acanthoma

Sox9 showed a variable positivity: in all 4 cases, ranging from a rare and weak staining (2 cases) to a diffuse and intense immunolabeling (1 case).

Inferior tricholemmoma

Sox9 showed a variable positivity in all 5 tricholemmoma cases. The number of positive cells usually ranged from 25% to 50% (3 cases), with intensity of staining from weak to intense. Two tumors were diffusely (50-100% of positive cells) and strongly positive.

Isthmic tricholemmoma

In both isthmic tricholemmomas, positive cells varied from 50% to 75% of neoplastic cells. An intense immunolabeling was detected and involved mainly peripheral basal cells.

Trichoblastoma

All 12 trichoblastoma (Fig. 1C) were Sox9 positive; 9 tumors were diffusely positive (75-100% of neoplastic cells in 7 cases, 50-75% in 2 cases) with an intense staining, whereas in 1 case Sox9 showed diffuse (75-100% of cells) but weak, immunolabeling. In 2 cases there was a variable percentage of positive cells (from 25% to 100%) with a moderate intensity of staining.

Trichoepithelioma

Four of 7 trichoepitheliomas (Fig. 1D) showed a very wide percentage of positive cells, ranging from 1-25% to 75%; Sox9 immunolabeling was moderate. In 3 cases, 25-50% to 75-100% of the tumor cells showed an intense staining. Sox9 was detected mainly in peripheral basal cells and in suprabasal cells with spinous differentiation. Only in 1 case a rare and weak immunolabeling was observed.

Malignant trichoepithelioma

All 3 tumors expressed Sox9. The percentage of positive cells varied from 25-50% (1 case) to 75-100% (2 cases). The intensity was usually moderate (2 cases) or intense (1 case). Peripheral basal cells and suprabasal cells were mostly positive.

Pilomatricoma

The 2 tumors expressed from 25% to 75% of positive cells, with a moderate to intense Sox9 positivity (Fig. 1E).

Subungual keratoacanthoma

Sox9 was negative in neoplastic keratoacanthoma cells. Internal control, represented by normal skin hair follicles, was mildly positive.

Subungual squamous cell carcinoma

The percentage of positive cells in subungual squamous cell carcinomas (Figure 1 F) ranged from 1-25% (3 cases) to 25-50% (1 case) of positive cells. The intensity of immunolabeling varied from weak (1 case) to moderate (3 cases), especially in spinous and basal layer. Immunolabeling decreased or disappeared in most differentiated cells.

Discussion

Genes involved in developmental processes or tissue homeostasis maintenance are often overexpressed in tumorigenesis (Vidal et al., 2008; Matheu et al., 2012). Sox9 is implicated in a wide range of human neoplasms, especially in organs where it plays a role in their developmental stages and in stem cells maintenance (Vidal et al., 2008; Matheu et al., 2012), but it has been found even in other neoplasms, as fibrosarcomas, where Sox9 is not known to be required during normal physiology (Matheu et al., 2012).

As regards skin, Sox9 is present in the outer root sheet of human hair follicle and sebaceous gland, especially in basal layers; Sox9 has been also detected in basal layer of inter-follicular epidermis too (Shi et al., 2013). In canine normal skin, Sox9 positive cell distribution was similar to that reported in man (Vidal et al., 2008; Krahl and Sellheyer, 2010b). In a few cases scattered Sox9 positive epithelial cells were detected also in upper hyperplastic epidermis above the neoplastic tissue; similar findings have been described in man by Vidal et al. (2008) and Shi et al. (2013).

This gene, indeed, is required for the specification of early bulge cells and its progeny; moreover it is responsible of maintenance of stem cells characteristics in the adult bulge niche, whose action is necessary in a wound environment (Nowak et al., 2008).

As previously described by some Authors (Nowak et al., 2008; Krahl and Sellheyer, 2010b), various cutaneous neoplasms mirror some of Sox9 embryological expression patterns, sometimes even reflecting its spatial distribution within the various follicular subcompartments (Krahl and Sellheyer, 2010a,b).

Sox9 was detected in all the adnexal tumors tested, with the exception of subungual keratoacanthoma, maybe due

to its origin from the nailbed epithelium (Goldschmidt et al., 1998). Subungual squamous cell carcinomas resulted moderately positive. As for subungual keratoacanthoma, the origin from a highly differentiated epithelium could explain the reduced Sox9 expression as well as the loss of other stem cell markers (Brachelente et al., 2013). Sox9 expression in epidermal keratinocytes indeed, is prominently detected in undifferentiated rather than in differentiated keratinocytes (Shi et al., 2013).

Sox9 was expressed at the highest levels in neoplasms strictly related to embryonal hair follicle, as trichoblastomas. Both in human and veterinary medicine trichoblastomas are considered to be neoplasms deriving from hair follicle stem cells (Hurt et al., 2006); our findings therefore support this hypothesis and confirm the results of other studies performed in dogs employing different stem cell markers (Pascucci et al., 2006; Mercati et al., 2008; Brachelente et al., 2013). The intensity of immunolabeling and number of Sox9 positive cells may be related to the cycling and bulge-harboring portion of the follicle, respectively represented by the hair stem and the isthmus (Krahl and Sellheyer, 2010b).

Sox9 immunolabeling was observed in high percentage of cells also in tricholemmomas and trichoepitheliomas especially in peripheral basal cells and in less differentiated suprabasal cells. Regarding trichoepithelioma, the heterogeneity in Sox9 staining may reflect the origin of this neoplasm from the three different parts of the hair follicle (Goldschmidt et al., 1998; Brachelente et al., 2013). Malignant trichoepitheliomas, in lesser extent, show the same immunolabeling variability.

Tricholemmomas, both inferior and isthmic type, showed a variable positivity especially in basal and suprabasal layers, but no relevant differences in immunohistochemical reactivity were observed. There is no general agreement about the origin of these neoplasms; they are supposed to derive respectively from the inferior segment of the outer root sheet or from the isthmus segment of the outer root sheet; experimental data are rather controversial (Vidal et al., 2008; Brachelente et al., 2013). Squamous cell carcinomas, both epidermal and subungual type, and squamous papillomas expressed Sox9 with various degrees of positivity. Squamous cell carcinoma was irregularly Sox9 positive in accordance with previous studies where different markers for stem cells were considered (Sellheyer, 2011; Bongiovanni et al., 2011; Shi et al., 2013).

Sox9 expression patterns in pilomatricomas resemble those of the developing and the post-natal hair follicle (Krahl and Sellheyer 2010a,b). In the current study, according with the previous work by Krahl and Sellheyer, (2010b) basaloid cells/bulb germ cells were Sox9 negative, while a Sox9 positivity was observed in more differentiated cells. These neoplasms, therefore, have to be considered as trichoepitheliomas with a prominent matrical component.

In recent years many efforts have been made to find a logical classification of adnexal tumors according to a new molecular-morphological characterization, investigating neoplasm stem cell biology (Sellheyer, 2011). Regarding canine tumors, the association between hair follicle and various stem cell markers, as CD34, CK15 and Nestin, have been investigated (Pascucci et al., 2006; Mercati et al., 2008; Brachelente et al., 2013), each with its own characteristics feature and target. Sox9 may be proposed as another reliable stem cell marker for dermopathologists to better assess the role of stem cells in canine epidermal and follicular tumors.

SOX9 in male gonad

The present work has been recently published as:
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Immunohistochemical expression of Sox9 protein in immature, mature, and neoplastic canine Sertoli cells

Introduction

Sox9 is involved in testicular development being a key testis-determining gene for specifying the Sertoli cell (SC) lineage (Harley et al., 2003; Sharpe et al., 2003; Barrionuevo et al., 2010). Sox9 transcripts, initially expressed in the genital ridge of both sexes, becomes upregulated in SCs, whereas it is downregulated in the ovaries (Morais da Silva et al., 1996; de Santa Barbara et al., 2000). The inactivation of Sox9 before the sex determination stage results in XY sex reversal (Chaboissier et al., 2004; Barrionuevo et al., 2006). Two thirds of XY patients with a mutation in Sox9 show sex reversal indicating an essential role of this gene in the testis determination (Foster et al., 1994). In addition, recent studies reported that Sox9 upregulates the production of anti-Muellerian hormone (Mackay, 2000; Josso et al., 2001), secreted during the embryonic development by SCs and promoting the regression of the Muellerian ducts (Josso et al., 1993). This latter finding supports the crucial role of Sox9 in male sex determination and testicular development. In scientific literature, few papers investigating the gene expression and protein localization of Sox9 in normal testis have been published, and most of them are focused on embryonic and fetal gonads, both in human and laboratory animals (Pelliniemi and Frojdman, 2001; Carmona et al., 2009). In human undifferentiated gonads, Sox9 protein was initially demonstrated by immunofluorescence in the cytoplasm of somatic cells and then become restricted to the nuclei of SCs when sex cord formation begins (de Santa Barbara et al., 2000) and during all the normal testicular development, as demonstrated by immunohistochemistry and *in situ* hybridization as well (Hanley et al., 2000).

Besides studies about the embryologic and fetal development of the testis, few studies explore Sox9 protein expression in adult and/or in pathologic testes (Lan et al., 2013). In mice, the immunohistochemical pattern of Sox9 expression during testis formation and adulthood parallels humans (de Santa Barbara et al., 2000; Kobayashi et al., 2005; Nel-Themaat et al., 2011). On the other hand, in rats, Sox9 protein expression seems to be dependent on the age and stage of spermatogenesis: prominently expressed in gonadal blastemal of embryonic testis, Sox9 gradually declines in postnatal gonads but strongly reoccurs in pubertal and adult testis (Frojdman et al., 2000; Pelliniemi and Frojdman, 2001).

Regarding other animal species, data are relatively scarce and mainly focused on embryonal gonads (Carmona et al., 2009; Elzaiat et al., 2014). Concerning canine species, a polymerase chain reaction–based study investigated the expression of Sox9, both in female and male embryos from 27 to 37 days of gestation. Sox9 was found to be expressed in the urogenital ridge, in both sexes up to Day 30 of gestation, and then it becomes markedly elevated only in male gonads with little or no expression in other components of the urogenital ridge. Authors concluded that these findings similarly to those reported for humans and other domestic animals were consistent with the role of Sox9 in testis determination (Meyers-Wallen, 2003).

Nevertheless, besides its critical role in testicular determination and different timing of expression among species, the physiological relevance of Sox9 in adult testes and its possible role in the neoplastic counterpart deserve further investigation.

As a gene related to cellular proliferation and differentiation, Sox9 protein induction and its implication in the growth of neoplastic cells and extracellular matrix deposition has been recently considered (Akiyama, 2008) and Sox9's role in epithelial invasion, migration, and proliferation in various cancers and its prognostic potential has been reported (Wang et al., 2008; Chakravarty et al., 2011a). Moreover, Sox9 deregulation and/or mutation have been found in several human cancers (Bruun et al., 2014).

Recently, studies on the immunophenotype of developing, adult, and neoplastic testicular cells have been performed in dogs to prove their potential role as a relevant model for human testicular cancer (Banco et al., 2010).

Because spontaneous testicular cancers are quite common in dogs and Sox9 protein expression in normal and neoplastic SCs has never been examined in this species, the present study aimed to investigate the expression of Sox9 protein in canine SCs during testicular maturation and neoplastic transformation.

Materials and methods

Samples

No animals were killed for this study, and all selected samples were originally submitted for diagnostic purposes to departmental routine biopsy or necropsy service. For this retrospective study, paraffin blocks of formalin-fixed normal pairs of testes from three canine fetuses (50 days of gestation), four newborn dogs (from 0 to 20 days of age), and four prepubertal puppies (56, 90, 120, and 180 days of age) and from five adult/aged dogs (2, 4, 6, 10 and 13 year old) were selected from departmental archives. During sample selection, particular attention was posed to consider well-preserved testes: not macerated fetal testes, adult normal testes from routine spaying, immature testes from well-conserved puppies, submitted for necropsy after sudden traumatic death. Regarding neoplastic testes, from the same diagnostic archive, 31 SC tumors and five Leydig cell tumors (LCTs) were selected. Sertoli cell tumors (SCTs) were from dogs ranging in age from 4 to 15 years. One of these tumors was malignant, with metastases to the lumboaortic lymph nodes 2 years after surgical castration; 10 of 31 SCTs developed within cryptorchid testes. LCTs were from dogs ranging in age 9 to 13 years and all developed in eutopic testes.

Histology

For each sample, 5-μm-thick sections were obtained and stained with hematoxylin and eosin. As in previous studies, the histologic variants of canine SCTs were recorded as “typical” and “lipid rich” (Banco et al., 2010). The growth pattern of the SCTs was also recorded and defined as“intratubular” or “diffuse.” Tumors showing both intratubular and diffuse neoplastic growth were defined as “intratubular/diffuse.”

The histologic variants of LCTs were classified as “solid-diffuse” and “cystic-vascular” as reported in the “World Health Organization International Histological Classification of Tumors of Domestic Animals” (Kennedy et al., 1998). For all the neoplastic testes, the mitotic index (MI) was assessed by counting the mitotic figures in 10 high-power fields (400 x), and the mean value for each sample was then recorded.

Immunohistochemistry

The immunohistochemical staining of all samples was performed using the avidin–biotin–peroxidase complex procedure (Hsu et al., 1981) with a commercial immunoperoxidase kit (Vectastain Standard Elite; Vector Laboratories, Inc., Burlingame, CA, USA). Sections were dewaxed, treated with hydrogen peroxide 0.5% in methanol for 20 min, and rehydrated. Antigen retrieval was carried out by microwave in citrate buffer, pH 6.0 (10 min, 650 W). After pretreatment, the sections were incubated for 30 min in normal goat serum (diluted 1:60). As a primary antibody, a rabbit anti-human polyclonal antibody directed against Sox9 (Sigma–Aldrich Corporation, St. Louis, Missouri, USA) was applied and diluted 1:150 in Tris buffer. According to the manufacturer’s instructions, the antibody was raised against a human Sox9 synthetic peptide sharing 96% amino acid homology with Canis familiaris Sox9. Sections were incubated overnight in a humid chamber at 4°C.

After incubation with the secondary biotinylated anti-rabbit immunoglobulin (diluted 1:200; Vector Laboratories, Inc.) for 30 min, the avidin–biotin–peroxidase complex method (Vector Laboratories, Inc.) was performed. The peroxidase reaction was developed by 3-amino 9-ethyl carbazole (Vector Laboratories, Inc.), and sections were counterstained with Mayer’s hematoxylin. Negative controls were obtained replacing the primary antibody with normal goat serum.

Assessment of the immunohistochemical labeling

The percentage of positive cells was semiquantitatively assessed and scored as follows: - = 0; + = 20% or less; ++ = 21% to 50%; +++ = 51% to 80%; ++++ = 81% to 100%.

Results

Histology

Both in fetuses and newborns, the seminiferous tubules were small, with no recognizable lumen, and lined by undifferentiated SCs and rare early germ cells.

In the testes from prepubertal dogs, spermatogonia were recognizable in all samples together with few spermatocytes in the oldest one (180 day old). In this latter dog, a small central lumen was also evident. In the testes from adult dogs, complete seminal line, including spermatozoa, was evident into the seminiferous tubules.

Regarding SCTs, on the basis of their pattern of growth, 17 of 31 tumors were classified as “intratubular” and nine of 31 as “intratubular/diffuse.” Five cases were

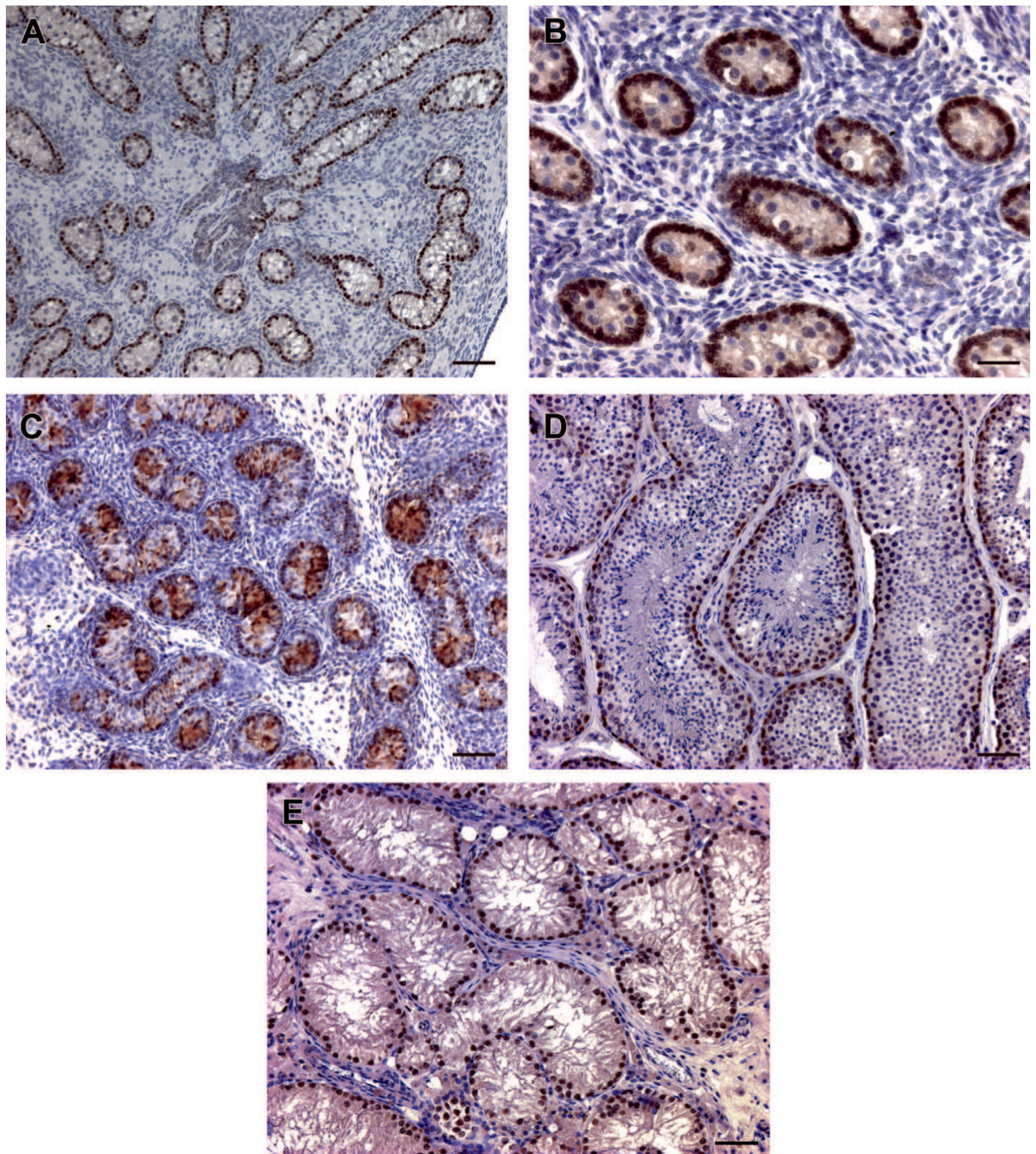

Figure 1. Sox9 protein immunohistochemical expression in non-neoplastic Sertoli cells. (A) Canine fetal testis (Sox9 immunolabeling). Strong nuclear staining detectable in all Sertoli cells lining the seminiferous tubules. Negative *rete testis* epithelial cells are evident at the center of the image. Magnification 100. Scale bar = 70 μm. (B) Canine neonatal testis (Sox9 immunolabeling). Strong nuclear staining detectable in all Sertoli cells. Magnification 200. Scale bar = 35 μm. (C) Canine prepubertal testis (Sox9 immunolabeling). Sertoli cells in seminiferous tubules from 90-day-old puppy were characterized by both nuclear and cytoplasmic staining. Magnification 100. Scale bar = 70 μm. (D) Canine adult testis (Sox9 immunolabeling). Strong nuclear staining detectable in all mature Sertoli cells. Magnification 100. Scale bar = 70 μm. (E) Canine adult testis. Atrophic testicular parenchyma (Sox9 immunolabeling). Atrophic tubules, generally at the periphery of the Sertoli cell tumors, were lined by Sertoli cells characterized by a strong nuclear labeling. Magnification 100. Scale bar = 70 μm.

characterized by a “diffuse” pattern of growth; 18 of the 31 tumors examined (12 intratubular, three “intratubular/diffuse,” and three “diffuse,” respectively) were classified as typical SCT. These tumors comprised neoplastic tubules, irregular in shape and diameter, and surrounded by thick basal membranes. Neoplastic tubules were separated by a variable amount of dense fibrous stroma and lined by one to two layers of tall and slender cells, oriented perpendicularly to the basal membrane in a palisading arrangement. Neoplastic cells were characterized by indistinct cell borders, oval to spindle basal nuclei with stippled chromatin, and scant to moderate, faintly eosinophilic cytoplasm.

In 13 of 31 SCTs (five intratubular, six “intratubular/diffuse,” and two “diffuse”, respectively), neoplastic SCs contained large intracytoplasmic, round, smooth contoured vacuoles displacing the nucleus at the periphery, giving the cell a signetring appearance. These cases, according with a previous report in the canine species (Banco et al., 2010), were classified as “lipid-rich” SCTs.

Three cases out 31 SCTs, two intratubular typical, and

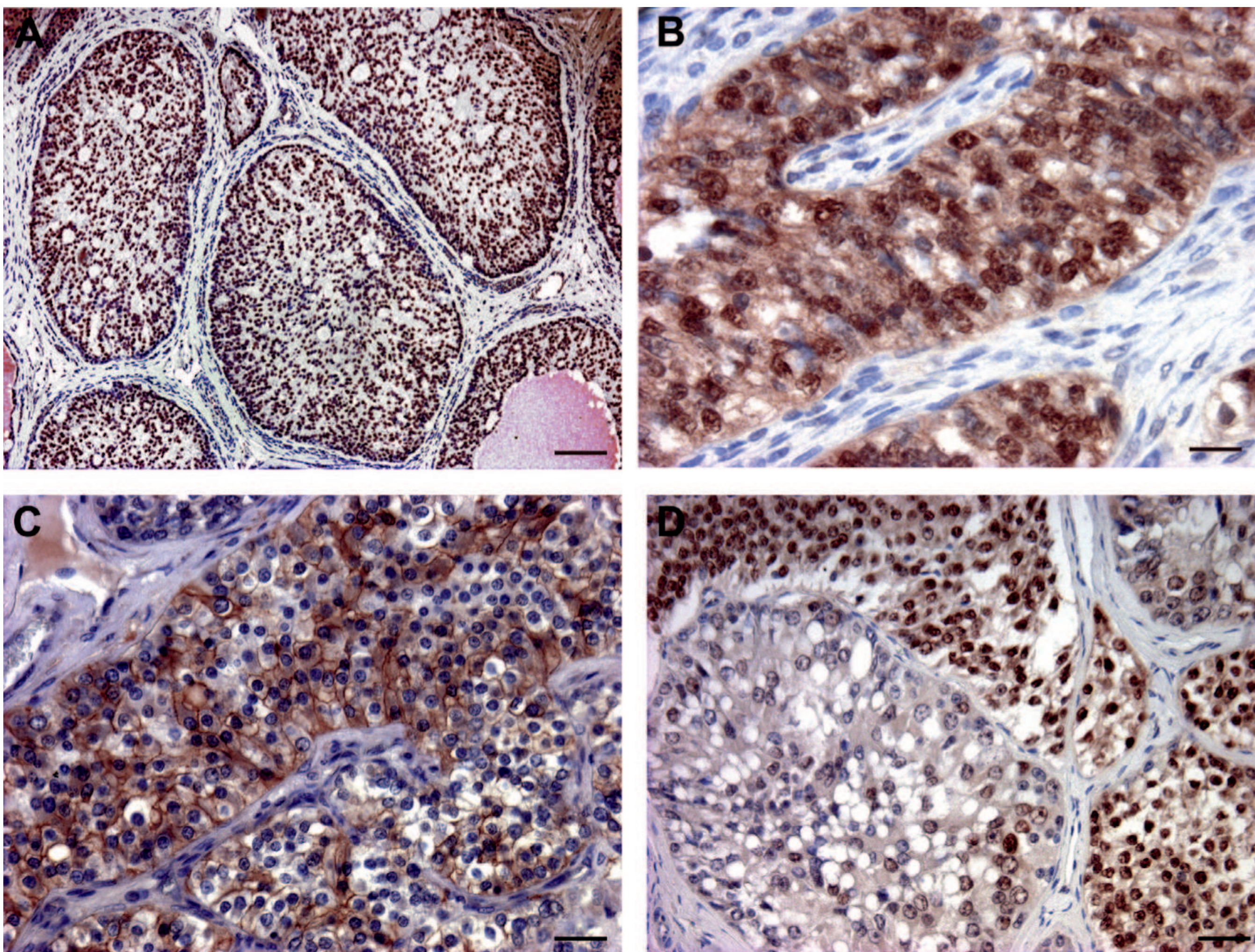

Fig. 2. Sox9 protein immunohistochemical expression in neoplastic Sertoli cells. (A) Canine intratubular typical Sertoli cell tumor (Sox9 immunolabeling). Strong nuclear staining in neoplastic SCs. Magnification 50. Scale bar = 130 μm. (B) Canine intratubular typical Sertoli cell tumor (Sox9 immunolabeling). Strong nuclear and cytoplasmic staining in neoplastic SCs. Magnification 200. Scale bar = 35 μm. (C) Canine intratubular/diffuse Sertoli cell tumor (Sox9 immunolabeling). Neoplastic tubules filled with Sertoli cells showing a strong membrane immunostaining. Magnification 400. Scale bar = 20 μm. (D) Canine intratubular, lipid-rich Sertoli cell tumor (Sox9 immunolabeling). Heterogeneous pattern of expression of Sox9. Neoplastic tubules at the periphery of the image are lined by neoplastic Sertoli cells characterized by diffuse nuclear labeling, whereas the centrally located tubule is characterized by lesser number of positive Sertoli cells. Strongly immunostained cells are closely opposed to weakly positive or completely negative ones. Magnification 200. Scale bar = 35 μm.

one intratubular lipid rich were additionally characterized by rare Call–Exner bodies, represented by neoplastic SCs arranged in rosettes surrounding microcavities filled with hyaline eosinophilic amorphous material.

In nine of 31 SCTs (four typical and five lipid rich), scattered areas of intratubular necrosis, cystic cavities, and hemorrhages were present. In three of these nine cases, neoplastic SCs were characterized by severe anisokaryosis and anisocytosis, infiltration of the albuginea, and large areas of necrosis. One case was associated with lumboaortic lymph node metastasis and, thus, was malignant.

In most SCTs (24 of 31), MI was low, ranging from 0 to 0.5. The remaining seven cases had high MI (0.7–2.4).

Concerning LCTs, three cases, composed by sheets and cords of polygonal cells separated by scant amount of fibrovascular stroma, were classified as "solid-diffuse" LCTs. One case, characterized by interconnecting cords of neoplastic Leydig cells (LCs), surrounding and delineating large lacunae filled with erythrocytes, was classified as "cystic vascular" LCT. Finally, in one case, both growth patterns were present and it was, therefore, classified as "solid-diffuse"/"cystic vascular" LCT. Neoplastic cells were polyhedral to cuboidal, with low N/C ratio, finely granular eosinophilic cytoplasm, variably distinct cell borders, small and round eccentric nucleus, and an indistinct nucleolus. In two cases, large clusters of cells had numerous vacuoles within the cytoplasm, compressing the nucleus to the periphery (signet ring cells). In three cases, focal hemorrhages were observed. In all the LCTs, MI was less than one.

Immunohistochemistry

In fetal and neonatal testes, a strong positive and exclusively nuclear immunolabeling for Sox9 protein was detected in all SCs (++++) (Fig. 1A; 1B).

In the testes of the four prepubertal puppies, the immunohistochemical staining was variable. In the youngest and oldest dogs, belonging to this category 56 and 180 days old, intense nuclear expression of Sox9 was detected in an high percentage of SCs (+++ and ++++, respectively), similarly to fetal, neonatal, and adult testes. Conversely, the SCs of the 90-day-old puppy exhibited both strictly nuclear or nuclear and cytoplasmic expression of Sox9 (Fig. 1C). In this case, independently from the localization of the immunohistochemical signal, the percentage of labeled SCs was assessed as +++. In the 120-day-old puppy, all SCs were negative.

In adult testes, all SCs exhibited a strong nuclear immunolabeling (++++) for Sox9 (Fig. 1D). In all testes examined, LCs and germ cells were constantly negative for Sox9.

Regarding the testicular tumors examined, LCTs were always negative for Sox9 protein, whereas in 31 of 31 SC tumors, Sox9 was constantly expressed. In 28 of 31, the percentage of stained SCs was high, ranging from +++/++++. In only three cases, the percentage of positive cells was low, less than 50% (++). The signal varied from nuclear (Fig. 2A), nuclear and cytoplasmic (Fig. 2B), and exclusively cytoplasmic in 18 of 31, 11 of 31, and two of 31 SCTs, respectively.

Nuclear labeling was observed in 12 typical and six lipid-rich SCTs; nuclear and cytoplasmic labeling was evident in six typical and in five lipid-rich SCTs, whereas a strictly cytoplasmic signal was recorded in two lipid-rich SCTs. Interestingly, in one lipid-rich SCT, exhibiting both nuclear and cytoplasmic signal, a strong positive staining of the membrane of neoplastic cells was observed (Fig. 2C).

Although all the SCTs were positively labeled for Sox9, they were characterized by an heterogeneous pattern of expression, particularly evident in the lipid-rich cases: within a single tubule, most cells could be immunolabeled, or the positive immunostaining could be restricted to a small number of cells. Moreover, strongly immunostained tubules could be adjacent to weakly positive or completely negative ones (Fig. 2D).

Within the atrophic testicular parenchyma surrounding both SCTs and LCTs, Sox9 immunolabeling was confirmed to be strictly limited to SC nuclei (Fig. 1E).

Sox9 immunohistochemical results are summarized in tables 1 and 2.

Discussion

In mammals, numerous studies focusing on the genetic regulation of mammalian gonads reported the pivotal role of Sox9 gene in male gonadal development. Highly conserved among vertebrates, Sox9 gene expression was already demonstrated by polymerase chain reaction in testes of canine fetuses from 27 to 37 days of gestation (Meyers-Wallen, 2003).

On the other hand, scarce information about immunohistochemical Sox9 protein expression and localization in postnatal testes is present in literature, and data about its expression in testicular tumors are lacking. The present study investigated the immunohistochemical expression of Sox9 in normal canine testes, from fetal to adult age, and in tumors derived from SCs and LCs.

In the dog, Sox9 immunolabeling was confirmed to be limited to the nucleus of SCs in fetal, neonatal, and normal

adult testes, as already described in human species and laboratory animals (Morais da Silva et al., 1996; de Santa Barbara et al., 2000; Frojdman et al., 2000; Hanley et al., 2000; Nel-Themaat et al., 2011; Lan et al., 2013). Interestingly, in the testes from the four prepubertal dogs, the immunohistochemical expression of Sox9 protein was highly variable. In the youngest and oldest dogs (56 and 180 days old), Sox9 was restricted to the nucleus of SCs, as observed in fetal and neonatal testes, whereas in the 120-day-old pup no expression of Sox9 was observed. In the 90-day-old dog, Sox9 expression reappeared and was detectable both in the nucleus and in the cytoplasm. Similar variations in Sox9 protein expression have been reported in rat gonads. In rats, nuclear Sox9 expression was described in SCs of fetal testes and then the immunoreaction gradually declined, becoming faintly positive to negative in postnatal rats. From the onset of puberty until 15-day-old rats, Sox9 protein expression returned and increased (Frojdman et al., 2000; Pelliniemi et al., 2001). These authors suggested that the presence and amount of Sox9 protein could be dependent on the age and the spermatogenetic stage within the seminiferous tubules. Although the present study only examined four prepubertal dogs and, in particular only one of 120 days, the results seem to parallel what is observed in rats, suggesting a similar biologic role. However, these results deserve to be verified on a larger number of samples.

In all testes examined, seminal and LCs were always negative, consistently with results obtained in other mammals (de Santa Barbara et al., 2000; Lan et al., 2013). In addition, Sox9 immunohistochemical signal was absent in all LCTs, whereas SCTs were constantly positive. These results confirmed that in canine testis Sox9 protein could be considered a reliable marker for normal and neoplastic SC and could be used to discriminate between LCTs and SCTs, in particular the lipid-rich variant. In fact, LCTs and SCTs share morphologic features although having different clinical outcomes: LCT is a benign tumor, whereas malignant and metastasizing SCTs have been described (MacLachlan and Kennedy, 2002).

In the present study, all the SCTs were positively labeled. A strong immunohistochemical signal, varying from 51% to 100% of neoplastic cells was observed, confirming that Sox9 protein remains a specific marker of SCs, also in the neoplastic counterpart. In most SCTs (18 of 31), Sox9 labeling was exclusively nuclear, as observed in normal testes, whereas in 11 of 31 cases the signal was nuclear and cytoplasmic and in two of 31 exclusively cytoplasmic.

The cytoplasmic expression of Sox9 protein observed both in neoplastic samples and in one prepubertal pair of testes is an interesting finding that deserves some consid-

Table 1. Expression of Sox9 in fetal, neonatal, prepubertal and adult testes.

No.	Breed	Age	SOX9 immunolabeling	
			% labeled cells	Signal localization
1	Mixed breed	Fetus (50d gestation)	++++	N
2	Mixed breed	Fetus (50d gestation)	++++	N
3	Mixed breed	Fetus (50d gestation)	++++	N
4	West Highland White terrier	Stillborn	++++	N
5	Boxer	Stillborn	++++	N
6	Mexican hairless dog	3d	++++	N
7	Mexican hairless dog	20d	++++	N
8	Rough collie	56d	+++	N
9	Mixed breed	90d	+++	N/C
10	English bulldog	120d	-	N
11	American staffordshire	180d	++++	-
12	Golden retriver	2y	++++	N
13	German shepard	4y	++++	N
14	Mixed breed	6y	++++	N
15	Dachshund	10y	++++	N
16	Mixed breed	13y	++++	N

- = 0; +++ = 51%–80%; ++++ = 81%–100%.
Abbreviations: C, cytoplasmic; N, nuclear; NC, nuclear and cytoplasmic.

eration. Cytoplasmic Sox9 protein expression has been described in the early stage of testicular development. In both murine and human species, during early gestation, Sox9 gene is upregulated in the male. Simultaneously, Sox9 protein, which appears cytoplasmic in the undifferentiated gonads, becomes nuclear in pre-SCs (Morais da Silva et al., 1996; de Santa Barbara et al., 2000). On the basis of these observations, we hypothesize that the cytoplasmic expression of Sox9 protein in prepubertal and neoplastic SCs could be related to functional immaturity or alternatively

Table 2. Expression of SOX9 in neoplastic testes.

No.	Breed	Age	Growth	Variant	Necrosis and hemorrhages	MI	SOX9 immunolabelling	
							Percentage of labeled cells	Signal localization
1	German Shepherd	4	D	T	X	2,4	+++	N/C
2	German Shorthaired Pointer	4	D	LR	X	1,1	++++	N/C
3	Boxer	5	D	T	X	1,6	+++	N/C
4	Chihuahua	6	I	T		0,1	++++	N
5	Mixed breed	6	I/D	T	X	1	++	N/C
6	Rough Collie	7	I	T		0,1	++++	N
7	Cocker spaniel	7	I	LR		0,1	++++	N
8	Alaskan malamute	7	I	T		0,3	++++	N
9	Mixed breed	7	I	T		0,3	++++	N
10	Mixed breed	8	I	LR		0	++++	N
11	Dalmatian	8	D	T		1,2	+++	N/C
12	German Shorthaired Pointer	8	I	LR		0,2	++++	N
13	Mixed breed	9	I	T		0	++++	N
14	Belgian Shepherd	9	I	T		0	++++	N
15	German Shepherd	10	I	T		0,1	+++	N
16	Labrador Retriever	10	I/D	LR		0,1	+++	C
17	Doberman Pinscher	10	I/D	LR	X	0,8	+++	N/C
18	Springer Spaniel	10	I	T		0,2	+++	N
19	Bergamasco Shepherd	11	I	T		0,3	+++	N/C
20	Mixed breed	11	D	LR	X	0,1	++++	C
21	German shepherd	12	I/D	T		0,3	++++	N
22	Beagle	13	I	T		0,1	++++	N
23	Mixed breed	14	I	LR	X	0,3	+++	N/C
24	Yorkshire Terrier	14	I/D	LR	X	0,2	+++	N/C
25	Mixed breed	15	I/D	LR		0,3	++++	N
26	Mixed breed	NR	I	T		0,2	+++	N/C
27	Shetland Sheepdog	7	I	LR		0,5	+++	N
28	Boxer	5	I/D	LR		0,4	++	N
29	German Shepherd	4	I/D	T		0,4	+++	N
30	Airedale Terrier	5	I/D	LR		0,5	++	N/C Membrane
31	German Shepherd	NR	I	T	X	0,7	++++	N
32	Beagle	9	SD			0	-	-
33	American Staffordshire Terrier	10	CV	L	X	0	-	-
34	Dalmatian	11	SD			0	-	-
35	Mixed breed	12	SD/CV	L	X	0	-	-
36	Yorkshire Terrier	13	SD		X	0	-	-

- = 0; ++ = 21%–50%; +++ = 51%–80%; ++++ = 81%–100%.
Abbreviations: C, cytoplasmic; CV, cystic vascular; D, diffuse; I, intratubular; I/D, intratubular/diffuse; LR, lipid rich; MI, mitotic index; N, nuclear; NR, not reported; SD, solid diffuse; SD/CV, solid-diffuse/cystic vascular; T, Typical.

could represent an undifferentiated, not fully developed SC transient phenotype, similarly to what observed during the testicular development. The dynamic subcellular redistribution of Sox9 protein in the gonads at the time of sexual differentiation has been described both in humans and mice (Morais da Silva et al., 1996; De santa Barbara et al., 2000) demonstrating that Sox9 is able to shuttle between the nucleus and the cytoplasm, hypothesizing the existence of a nuclear export signal triggering male-specific sexual differentiation (Gasca et al., 2002). In human medicine, no data regarding testicular neoplasm and Sox9 protein expression are present, but this marker has been used on other neoplastic tissues (mammary gland and ovaries) revealing interesting results in a subset of cases, such as cytoplasmic and/or membranous labeling (Zhao et al., 2007; Bratthauer and Vinh, 2009; Bruun et al., 2014).

Sox9 is a transcription factor; thus, the nuclear distribution of this protein is an expected finding, compared with cytoplasmic and membranous staining. According to Chakravarty et al. (2011a), cytoplasmic Sox9 in breast cancer is associated with invasive and metastatic breast carcinomas and it is related to increased cell proliferation. Similarly, in two thirds of ductal breast lesions, Sox9 protein was located in the cytoplasm and not in the nucleus (Bratthauer and Vinh, 2009). Moreover, cytoplasmic and membranous Sox9 staining has been found in "borderline" ovarian tumors and not in well differentiated ones (Zhao et al., 2007). Therefore, the results of our study could support the hypothesis that the cytoplasmic expression of Sox9 in neoplastic SCs reflects a less differentiated phenotype. This point could be relevant and should be explored in future studies.

Moreover, although all SCTs were Sox9 positive, a variable number of negative neoplastic cells were evident in all the SCTs examined. In addition, in this study, one prepubertal pair of testes was characterized by the lack of Sox9 expression, similarly to a minority of neoplastic SCs not labeled in the SCTs. Regarding normal canine prepubertal SCs, we hypothesize that the negative Sox9 labeling parallels the results observed in rat gonads (Frojdman et al., 2000), suggesting that Sox9 expression, before adulthood, is unstable and physiologically reduced. In the same way, negative neoplastic SCs could reflect a more immature or dedifferentiated immunophenotype.

The results obtained from this first study investigating Sox9 protein expression in normal and neoplastic canine testes suggest that this protein may play a role in SC proliferation and neoplastic transformation. However, further investigations on a larger number of samples are required to confirm these hypotheses.

On the other hand, the consistent expression of Sox9 in the SCTs, together with the consistent negative results obtained in LCTs, show that Sox9 is a reliable marker for confirmatory diagnosis of these tumors in dogs.

SOX9 in mammary gland neoplasms

In recent years identification and characterization of molecular controls that regulate mammary stem and progenitor cell homeostasis are critical to our understanding of normal mammary gland development and its pathology, neoplastic lesions included. Up to now there are few studies concerning this issue, and a consistent of them are about Sox9 role in human brest carcinoma.

Recent evidence suggests that human breast cancer stem cells are controlled by key regulators similar to those operating in normal mammary stem cells (Guo et al., 2012). Stem cells can be classified in two main groups: embryonic stem cells, with an unlimited self-renewing capacity, and adult stem cells or somatic stem cells, with a restricted lineage potential. Somatic stem cells are present in almost every tissue of the body and are capable of multilineage or uni-lineage differentiation only, as they are responsible for physiologic tissue turnover (Davis and zur Nieden, 2008).

Afonja et al. (2002) first, found Sox9 expression in breast cancer cell lines. In mammary neoplastic tissue, instead, Sox9 expression was found in 4 cases of matrix-producing carcinoma of the breast, an extremely rare and specialized variant of metaplastic carcinoma of the breast. Immunopositivity for Sox9 was more diffuse in the nuclei of metaplastic cancer cells than in carcinomatous cells. Sox9 protein in matrix-producing carcinoma of the breast was moreover associated with differentiation of neoplastic epithelial cells to chondrocytic cells (Kusafuka et al., 2008). Another study showed a correlation between Sox9 and breast tumor cell proliferation, demonstrating the prognostic significance of this marker in case of invasive ductal carcinomas and metastatic breast cancer. Patients with higher Sox9 expression indeed had significantly shorter overall survival. Sox9 expression in neoplastic cells was detected in the cytoplasm, instead of nucleus; this cytoplasmic accumulation, observed in about 30% of invasive ductal carcinomas and lymph node metastases, was significantly correlated with enhanced proliferation in breast tumors (Chakravarty et al., 2011a, 2011b); Sox9 cytoplasmic sequestration seems to be implicated in tumor progression (Chakravarty et al., 2011b). In case of invasive breast cancer, especially in triple negative phenotype (estrogen receptor, progesterone receptor and HER2/Neu negative breast cancer), Slug, Sox9 and Sox10 were overexpressed in the nuclea or in the cytoplasm of neoplastic cells and associated with poor overall survival (Riemenschnitter et al., 2013).

High Sox9 expression in basal-like breast cancer correlates with poor prognosis and activation of Wnt/β-catenin pathway; Sox9 silencing reduced cell proliferation and invasion (Malhotra et al., 2014). Wnt family is composed by a number of highly-conserved proteins involved in many developmental processes such as cell embryonal differentiation and migration, cell proliferation and regeneration in adult tissues, governing somatic stem cells function (Davis and zur Nieden, 2008). Moreover it has emerged that genes encoding for Wnt pathway components are often altered in human cancer and disease (Davis

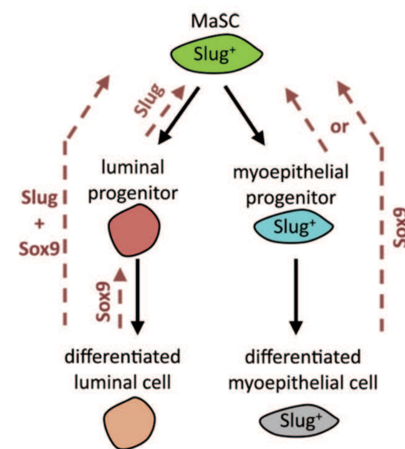

Figure 1 (*from Guo et al., 2012*). **Sox9 and Slug effects on different mammary epithelial lineages. Red dashed lines indicate the morph of a differentiated cells into stem (MaSCs) or progenitor cells through the induction of the transcriptor factor indicated. Sox9 together with Slug was sufficient in reprograming mature luminal mammary epithelial cells into MaSCs while Sox9 expression by itself converted these cells into luminal progenitors (Malhotra et al., 2014). It is not clear whether Sox9 by itself changes myoepithelial progenitors or myoepithelial differentiated cells into MaSCs and/or expands a preexisting MaSCs population (Guo et al., 2012).**

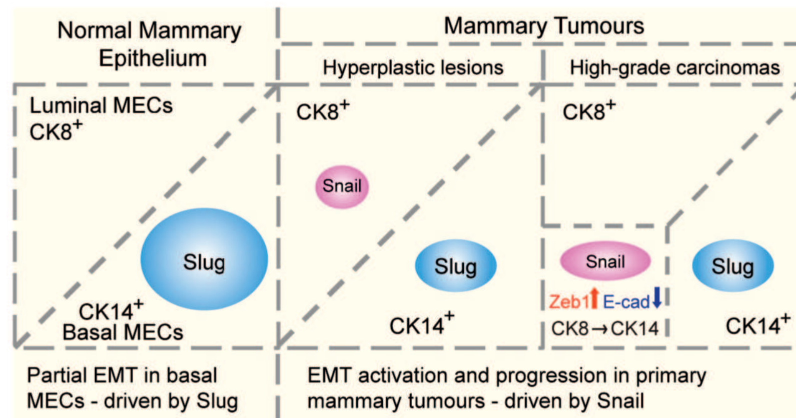

Figure 2 (*from Ye et al., 2015*). Snail activation is associated with invasiveness in mammary tumor cells. Expression patterns of Snail and Slug in normal mammary epithelium and in different stages of mammary tumor development. Markers CK8 and CK14 are expressed respectively in luminal mammary epithelial cells (MECs) and in basal cells MECs. As tumor progresses, cells acquire basal CK14 expression and lose luminal CK8 expression (Ye et al., 2015).

and zur Nieden, 2008). Sox9 overexpression in cell lines increased Wnt signaling transgenic overexpression and, *in vivo* in murine mammary glands increased secondary ductal branching (Malhotra et al., 2014). Sox9 acts in cooperation with Wnt in various stem cell systems (Blache et al., 2004), and it seems that Wnt/β-Catenin pathway plays a key role in MaSCs maintenance too. Thus, Sox9 is an important component of MaSCs maintenance by Wnt/β-Catenin and potentially other pathways (Malhotra et al., 2014).

With concern to other transcription factors families, forced expression of Slug and Sox9 in breast cancer cells was demonstrated to promote the tumorigenesis and metastasis; it induce, indeed, the entrance of cells in a TIC (tumor-initiating cells) state, that is responsible for metastatic dissemination and clinical relapse in several cancers, with subsequent poor patient outcomes (Guo et al., 2012; Ye et al., 2015; Fazilaty et al., 2015). Guo et al. (2012) investigated the correlation between EMT and staminal property acquisition from normal differentiated mammary cells and neoplastic cells through the expression of the transcription factors Sox9 and Slug. In healthy mammary gland, Sox9 and Slug function as master regulators of the gland-reconstituting activity of normal mammary stem cell; they cooperate inducing differentiated luminal cells of the mammary gland into mammary stem cells. Differentiated mammary luminal cells expressing both Sox9 and Slug were able to activate an endogenous autoregulatory network in somatic stem cells and reconstitute an entire mammary gland (Guo et al., 2012) (Fig. 1). In the same study coexpression of Slug and Sox9 was demonstrated to promote the tumorigenesis and metastasis of human breast cancer cells and are associated with poor patient outcomes (Guo et al., 2012).

The last study on this issue (Ye et al., 2015) gained new insights in mammary gland neoplasms development and in Guo et al. (2012) findings. Researchers found that Snail, member with Slug of the EMT-inducing family of transcription factors, was absent in normal mammary epithelial cells but become activated during breast cancer progression (Fig. 2). Slug and Snail induce distinct EMT programs; both Slug and Snail, in collaboration with Sox9, can efficiently induce a TIC phenotype in breast cancer cells, but Snail subpopulation showed more than two orders of magnitude higher proportions of TICs than the other subpopulations. Moreover Snail cells were more able to seed pulmonary metastases than Slug population. In a panel of human breast cancer cell lines, Snail knockdown suppressed tumor initiation in most of them, while Slug knockdown failed to do so (Ye et al. 2015). On the other hand, Slug knockdown significantly affected normal MaSCs in their gland reconstituting activity. It was moreover demonstrated that Snail TICs did not arise from basal MaSCs-like (expressing high Slug levels), but they came from a different population of cells. According to data obtained, the authors suggest that normal stem cells and TICs could arise from different cellular compartments and activate different signaling pathways. Moreover the high Slug expression previously associated to poor prognosis in breast cancer patients, may be thus related to Slug MaSCs basal differentiation, that on its own, represents a well-known feature of aggressive cancers (Ye et al., 2015).

This last investigation has thrown up many new questions regarding Snail, Slug and Sox9 role in cancer development. More research is needed to better understand the mechanisms of stem cells regeneration and maintenance in order to find maybe new strategies for treating degenerative diseases and cancer (Jo et al., 2014).

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Sox9 expression in feline mammary hypertrophy

Introduction

Feline mammary hypertrophy (FMH) is a progesterone-responsive non-neoplastic proliferation of the mammary gland of the cat characterized by sudden and rapid onset (Hayden et al., 1981). This pathological condition has been also described with the name of fibroadenomatous change or benign mammary hypertrophy (Nimmo and Plummer, 1981). At about 20% of benign lesions of the cat mammary gland is represented by FMH lesions (Gorlinger et al., 2002). FMH occurs mainly in young females, during pregnancy or false pregnancy and due to progestin treatments. In older males this condition is always related to the last one (Hayden et al., 1989; Loretti et al., 2004; Sontas et al., 2008). Physical features of FMH are primarily characterized by a rapid enlargement of one or all mammary glands without milk production (Gorlinger et al., 2002). Histologically FMH can be classified in intraductal and solid fibroepithelial type. Intraductal fibroepithelial hyperplasia was further subdivided into papillary and circumferential types, where neoplastic proliferation occurs into the lumen of a very dilated duct. Solid form, instead, shows a compact and ring-shaped pattern that infiltrates stromal cells (Hayden et al., 1989). FMH treatments include progestin administration interruption, ovariectomy, mastectomy in case of failure of the previous choices, or anti-progestins administration (Leidinger et al., 2011).

Since 1973 (Allen et al., 1973) an hormonal cause was suspected and subsequent studies confirmed a correlation between progesterone treatment and FMH occurrence (Hayden et al., 1989; Loretti et al., 2004), but mechanisms that drive these changes are still debated. Progesterone receptors have been detected in both epithelial and stromal cells of FMH cases (Rutteman et al., 1991; Martin de las Mulas et al., 2000; Gracanin et al., 2012) while estrogen receptors were present only in 50% of cases (Martin de las Mulas et al., 2000). Progesterone is an important regulator for mammary stem and progenitor cells: this hormone leads staminal cells close to the progesterone expressing cells to proliferate and differentiate (Gracanin et al., 2012). Moreover, through a cascade of signals progesterone-induced involving growth hormone (GH) and subsequently insulin-like growth factor (IGF), epithelial and stromal cells proliferate. The progesterone-induced GH production in the mammary gland leads to local production of IGF, so this growth-promoting effect is modulated by autocrine and/or paracrine mechanisms. This local stimulation may be the reason why FMH in a number of cases progresses even after ovariectomy and progesterone absence (Gorlinger et al., 2002; Ordás et al. 2004; Zegers, 2011).

High Ki-67 proliferative index has been reported in FMH in several studies (Millanta et al., 2002). However, many pathogenic aspects have not been clearly elucidated yet. Recent studies indicated that SOX9 transcription factor, in cooperation with Slug, controls the mammary stem cell state (Guo et al., 2012) and that increased ductal branching may be observed in transgenic mice overexpressing SOX9 in mammary epithelium (Wang et al., 2013).

Aim of the study

The aim of this study is to gain insight into the role of SOX9 in FMH development, analyzing by immunohistochemistry SOX9 expression in FMH, non-FMH hyperplastic/dysplastic mammary lesions and normal mammary tissue of cat.

Materials and methods

Formalin fixed paraffin embedded tissue blocks of surgical biopsy were retrieved from archives as follows: samples of 10 FMH, 6 non-FMH hyperplastic/dysplastic mammary lesions and 3 normal mammary gland of female cats.

The diagnosis of each tumor was revised according to the criteria of the World Health Organization classification.

Signalment and anamnestic data of the patients were collected.

Tissue samples were examined for SOX9 expression by

ABC immunostaining method, using a polyclonal rabbit serum produced with a polypeptide with 97% identity with *Felis catus* Sox9 (Sigma-Aldrich, Prestige antibodies, HPA001758).

Serial sections (4 μm) on poli-L-lysine coated glass were dewaxed, treated with hydrogen peroxide 0.5% in methanol for 20 min, and rehydrated. Antigen retrieval was carried out by microwave in 0,01 M citrate buffer, pH 6.0 (10 min, 650 W). Slides then were rinsed in TRIS and incubated in normal serum goat for 30 min at room temperature. The sections then were incubated with primary anti-Sox9 antibody diluted 1:200 and incubated at 4°C overnight.

After incubation with the secondary biotinylated anti-rabbit diluted 1:200 (Vector Laboratories, Inc.) for 30 min, the avidin–biotin–peroxidase complex method (Vector Laboratories, Inc.) was performed. The peroxidase reaction was developed by 3,3-diaminobenzidine (DAB) (Vector Laboratories, Inc.), and sections were counterstained with Mayer's hematoxylin. Negative controls were obtained replacing the primary antibody with normal goat serum.

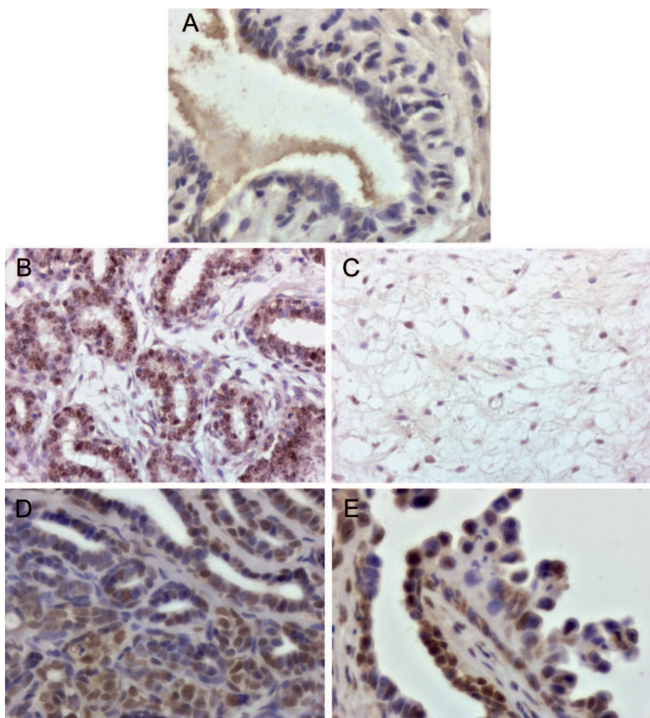

Figure 1. Sox9 immunohistochemistry on feline mammary gland, haematohylin counterstain. (A) Normal mammary gland with moderate Sox9 response (obj. 40 x). FMH lesions with strong positivities in ducts (B) (obj. 20 x) and moderate immunostaing in stromal cells (C) (obj. 20 x). (D), (E) Hyperplastic/dysplastic lesions with strong immunolabeling (obj.40 x).

Evaluation of immunohistochemical staining

Immunohistochemical stained tissue slides were examined using standard light microscopy. Immunolabeling of epithelial cells and myoepithelial cells of mammary gland (ducts) were counted separately from stromal myofibroblasts. Cutaneous adnexa in healthy skin, when present, were employed as internal positive control.

Intensity was scored as negative=0; weak=1; moderate=2; intense=3. Percentage of reacting cells was evaluated by counting 1000 cells. Immunostaining was localized and assessed as nuclear or cytoplasmic, or both.

Results

The 3 control-cats of the study, with normal mammary gland, were aged 7-12 years (average 9,7); the average age of 10 FMH cases was 4,9 years (animals aged 4 months-14 years); 6 subjects with other dysplastic lesions were aged 6 – 13 years (average 10 years).

In one case, normal mammary gland tissue came from castrated cat. Dysplastic lesions were all from sterilized animals, whilst FMH samples were from female cats (8 cases), castrated (1 case) and unknown (1 case).

Positive Sox9 immunostaining of variable intensity and percentage was seen in all the samples analyzed and was essentially nuclear or in a few cases nuclear and weakly cytoplasmic. In normal mammary gland (Table I) Sox9 was detected both in epithelial and in myoepithelial cells. Percentage of positive cells ranged from 33.2% to 55.4% (mean value 40.5%) (Fig. 1A). Sox9 positive staining was seen only in a few stromal cells. Intensity was strong in one case and moderate and in 2 cases.

All FMH samples showed moderate to strong Sox9 expression both in glandular and in stromal tissue (Table II); positivity ranged from 81.6% and 94.5% (mean value 86.5%) in ductal cells (Fig. 1B) and from 51.4% and 86% (mean value 75.1%) in stromal fibrocytes (Fig. 1C).

In non-FMH hyperplastic/dysplastic lesions staining was intense and percentage of positive cells varied from 60.3% to 71.9% (mean value 66.4%) (Fig. 1D; 1E); Sox9 positive staining was infrequent in stromal fibrocytes (Table III).

Discussion

Up to now a number of studies reported that FMH occurs mainly in young female cats (Hayden et al., 1989; Gorlinger et al., 2002; Loretti et al., 2004; Sontas et al., 2008); our results confirm this previously described trend, with

Tabella I. Sox9 immunohistochemical results in normal mammary glands.

Normal mammary gland case	Intensity	Localization	Score	Percentage	Notes
1	Intense	Nuclear	3	54,9%	--
2	Moderate	Nuclear	2	33,2%	Intense hair follicle positivity
3	Moderate	Nuclear	2	33,3%	Moderate hair folli-cle positivity

Table II. FMH Sox9 immunohistochemical results.

FMH case	Intensity	Localization	Score	Percentage
1	Intense	Nuclear	3	Ducts: 93,1% Stroma:76,0%
2	Intense	Nuclear	3	Ducts: 86,6% Stroma: 60,0%
3	Intense	Nuclear	3	Ducts: 87,0% Stroma:84,6%
4	Moderate	Nuclear/ Cytoplasmic	2	Ducts:82,1% Stroma: 75,9%
5	Intense	Nuclear	3	Ducts:82,2% Stroma:85,1%
6	Intense	Nuclear	3	Ducts: 94,5% Stroma: 77,2%
7	Intense	Nuclear	3	Ducts: 84,0% Stroma: 86,0%
8	Moderate	Nuclear/ Cytoplasmic	2	Ducts: 87,8% Stroma: 73,5%
9	Moderate	Nuclear	2	Ducts: 81,6% Stroma: 51,4%
10	Intense	Nuclear	3	Ducts: 86,3% Stroma: 81,0%

Table III. Other dysplastic lesions Sox9 immunohistochemical results.

Other displastic le-sions	Intensity	Localization	Score	Percentage	Notes
1	Intense	Nuclear	3	60,3%	Ductal hyperplasia
2	Intense	Nuclear	3	62,8%	Hyperplasia ed ectatic ducts
3	Intense	Nuclear	3	64,6%	Multiple ductal cyst
4	Intense	Nuclear	3	68,5%	Lobular hyperplasia
5	Intense	Nuclear	3	70,2%	Lobular hyperplasia and ductal cysts
6	Intense	Nuclear	3	71,9%	Lobular and ductal hyperplasia

most of FMH cases recorded in non-castrated female cats with an average age of 4,9 years.

This study provide evidence that in feline mammary FMH both glandular and stromal cells express high levels of SOX9. Different levels of this transcription factor have been found among FMH, other dysplastic lesions and healthy mammary gland. According to previous reports on Sox9 expression in normal and neoplastic tissue, Sox9 immunolabeling was in most of cases nuclear, and sometimes both nuclear and cytoplasmic. The significance of this finding is still debated. Considered by some Authors as not specific, cytoplasmic immunostaining in Chakravarty et al. (2011a,b) studies is associated to a poor prognosis.

Sox9 role in normal mammary gland and mammary gland neoplasms needs to be clarified yet but together with Slug, Sox9 act on mammary epithelial cells and morph them into stem cells able to regenerate mammary ductal tree (Guo et al., 2012). Sox9 moreover is expressed in mammary basal-like o triple-negative carcinomas (Riemenschnitter et al., 2013; Wang et al., 2013); cytoplasmic (Chakravarty et al., 2011a,b) or nuclear (Guo et al., 2012), its presence is always associated to poor prognosis. FMH is a non-neoplastic benign lesion; the high positivity rate of ductal cells and stromal cells may be related to the presence of normal mammary cell turnover. During developmental processes of mammary gland, as in case of mammary ductal side-branching morphogenesis and alveologenesis (Lain et al., 2013), Sox9 is widely present in epithelial cells of the branching ductal structures (Chakravarty et al., 2011b; Guo et al., 2012). The mammary ductal side-branching occurs in puberty (following hormonal/estrogenic stimuli) and pregnancy (progesterone induced) (Lain et al., 2013). Mammary ductal tree growth comes from the interaction among progesterone and its receptors in mammary epithelial cells. Subsequently, in FMH pathogenesis, GH and IGF production are induced (Gorlinger et al., 2002; Zegers, 2011), but there are many other transcription factors involved and that play important roles in ductal epithelial cells proliferation. Up to now only few of them have been identified. Among them Anphiregulin (Ciarloni et al., 2007; Booth et al., 2010), Msx2 (Fleming et al., 2012), Wnt4 (Brisken et al., 2000; Robinson et al., 2000) and RANKL (Beleut et al., 2010). It is possible that other Wnt proteins contribute to hormone response, even if their role has not elucidated yet (Roarty et al., 2012).

Sox9 is part of Msx2 and Wnt signaling pathway, where Sox9 regulates the expression of Low Density Lipoprotein Receptor-related Protein 6 (LRP6) and of T-cell Factor 4 (TCF4), both representative elements of the Wnt/β–catenin signaling pathway (Wang et al., 2013). In general, therefore, it seems that both progesterone and Sox9 act on undifferentiated mammary epithelial cells inducing encoding of transcription factors involved in mammary gland side-branching. However it is still to clarify if and how progesterone and/or its receptors can activate Sox9 transcription determining its overexpression in FMH.

Various studies suggest now that SOX9 plays multiple important roles in branching morphogenesis of several organs (Furuyama et al., 2011; Rockich et al., 2013), it can be hypothesized therefore that also in FMH SOX9 drives branching morphogenesis by controlling proper balance between proliferation and differentiation.

Sox9 expression in feline mammary carcinomas

Introduction

Mammary gland neoplasms in cat are usually aggressive carcinomas (Hayden and Nielsen, 1971; Hayes, 1981; Hayes and Mooney, 1985; Amorim, 2006; Zappulli et al., 2013), with a ratio among malignant and benign tumors that accounts for 4.1 (Misdorp et al., 1999) to 9:1 (Hayes et al., 1981). Feline mammary carcinomas (FMC)constitute approximately 17% of all feline neoplasms (Zappulli et al., 2015); they show high infiltration and local relapse rate and frequent metastases (Seixas et al., 2007). They seed metastases in 22,7% (Hayes et al., 1981) to 70,6% (Weijer et al., 1972) of cases, especially in regional lymph nodes and lungs (Weijer et al., 1972; Hayden e Nielsen, 1971). FMC usually occurs in queens, with a risk to develop neoplastic lesions 7 times higher than in castrated animals (Morris, 2013). In only 1-5% of cases, FMC are reported in male cats (Skorupski et al., 2005). Adult cats aged 10-12 years represent the target population for this lesion, but FMC have been signalled in cats from 9 months to 23 years of age. It seems there is a breed predisposition to FMC development in Siamese cat (Morris, 2013). FMC are induced by hormonal fluctuations associated to repeated estrus cycles. Repeated progestins treatments, indeed, increase the risk of developing FMC, even in males. Oncogenic effect of sexual steroid leads to tumor initiating. Hormonal fluctuations modify mammary tissues; estrogen and progesterone receptors are found in normal mammary tissues and in benign tumors but are often lost in malignant tumors and metastases (Morris, 2013). Mammary tumors appear as a single subcutaneous mass or nodule within the mammary gland, but multiple mammary masses involving several glands are common. Sometimes they are ulcerated in appearance, in few cases cystic (Morris, 2013).

In accordance with World Health Organization (WHO) classification of tumors of domestic animals, FMC are divided in non-infiltrating *in situ* carcinomas and in infiltrative simple carcinomas of different histologic subtypes (Misdorp et al., 1999; Zappulli et al., 2013). Recently Zappulli et al. (2013) proposed a revision of feline mammary neoplasms, assimilating the WHO classification. In contrast to canine mammary neoplasms, feline ones are less heterogeneous; simple adenocarcinoma, arising from luminal epithelium of ducts and alveoli, is the most common form of FMC and shows an aggressive behavior (Zappulli et al., 2013, 2015). The typical myoepithelial feature of canine complex tumors is missing (Zappulli et al., 2013). According to this interpretation, the term "complex" should be avoided in cats, and Authors propose to classify feline mammary tumors (FMT) with a myoepithelial component as ductal adenoma/carcinoma and intraductal papillary adenoma/carcinoma, respectively (Zappulli et al., 2013). Survival rate is determined by means of prognostic parameters that include stage based on tumor size, lymph node status, and metastases; histologic grade; molecular markers, gross appearance, histological type, molecular markers, tumor grading and lymph node/lymphovascular invasion (Webster et al., 2011; Zappulli et al., 2015). Mastectomy represent the most employed therapeutic approach for malignant tumors; neoplastic tissue and regional lymph nodes are surgically removed (Gimenez et al., 2010). Chemotherapy results gave conflicting results, but generally positive (Lana et al., 2001; Novosad et al., 2006).

The purpose of this study was to determine the immunohistochemical expression of Sox9 transcription factor in FMC in order to evaluate its possible involvement in the pathogenesis of this tumor.

Materials and methods

39 cases of FMC were selected from the archive of the Department of Veterinary Medicine as follows: 22 cases of tubulopapillary carcinomas, 11 cases of cribriform carcinomas, 4 solid carcinomas and 4 complex ductal carcinomas. Normal skin tissue surrounding the tumor was also included in the study. Age and breed of the subjects were collected. The diagnosis of each tumor was revised according to the criteria of the World Health Organization clas-

sification.

For immunohistochemical examination, 4 μm sections from routine formalin-fixed, paraffin-embedded samples were prepared on poly-L-lysine-coated glass slides. Sections were deparaffinized in xylene and rehydrated through graded alcohols. Endogenous peroxidase was blocked using 3% hydrogen peroxide in distilled water for 5 min, then, antigen retrieval for formalin-fixed samples was accomplished by microwave irradiation in 0,01 M citrate buffer, pH 6.0, for 10 min. Sections were incubated in normal goat serum for 30 min at room temperature. Later they were incubated for 18 h at 4°C with anti-Sox9 rabbit polyclonal antibody (HPA001758; Sigma-Aldrich, St. Louis, MO, USA) diluted 1:200. The antibody recognizes a Sox9 specific peptide of 117 amino acids with 97% identity with *Felis catus*. The sections were incubated for 30 min with 1:200 goat anti-rabbit biotin (Vector Laboratories, Burlingame, CA, USA; BA 1000), and stained for 30 min with streptavidin-biotin peroxidase kit (Vectastain Elite ABC, Vector Laboratories, PK-6100). The peroxidase reaction was developed by 3,3-diaminobenzidine (DAB) (Vector Laboratories, Inc.), and sections were counterstained with Mayer's hematoxylin. Negative controls were obtained by omission of the primary antibody.

Evaluation of immunohistochemical staining

The immunohistochemically stained tissue slides were examined using standard light microscopy. Sox9 expression was classified into 4 groups according to the proportion of positive cells: 0 = no positive cells; 1 = <10% positive cells; 2 = 10-50% of positive cells; 3 = >50% of positive cells. Intensity was scored as negative = 0; weak =1; moderate = 2; intense = 3. A total score was conferred multiplying the positive cells percentage score and the intensity of immunolabeling score. Cutaneous adnexa in healthy skin, when present, were employed as internal positive control.

Results

Immunohistochemical results and signalment data are summarized in table I.

Mammary samples were obtained by 39 female cats; 27 were European cats, 9 Siamese cats, 2 Persian and 1 unknown breed. Animals were aged from 6 to 15 years (age not reported in one case), with an average age of 11 years and 5 months.

Sox9 nuclear immunostaining was detected in all samples (figure 1), but one (case n. 25). This solid carcinoma showed weak rare Sox9 positivities in hair follicles, demonstrating sample reactivity. In 10 FMCs, immunoreactivity was present in less than 10% of cells, in 9 cases in 10-50% of cells and in 19 samples in more than 50% of cells. Signal intensity was scored as weak in 10 FMCs, moderate in 14, and intense in 14 cases. Total score obtained were 9 (10 cases), 6 (9 cases),4 (3 cases), 3 (4 cases), 2 (8 cases), 1 (4 cases), 0 (1 case).

Discussion

In this investigation, 39 FMCs have been investigated for Sox9 expression. According to our signalament data, prevalence of FMC in male cats are lower than the reported data of 1-5%. Sox9 was expressed in almost all FMC, but one. Sox9 detection was variable according to the number of positive cells and immunostaining intensity. Results obtained can be divided in 3 groups: negative or low positivity FMCs (total scores 0-2: 13 cases), intermediate FMC positivity (total scores 3, 4: 7 cases), high FMC positivity (total scores 6-9: 19 cases). It emerges that almost half of the FMC of this study shows high Sox9 immunoreactivity.

Sox9 has been suggested to be implicated in breast cancer development and progression (Guo et al., 2012; Riemenschnitter et al., 2013; Wang et al., 2013; Fazilaty et al., 2015; Pomp et al., 2015). This transcriptor factor is often detected in mammary basal-like o triple-negative carcinomas (Riemenschnitter et al., 2013; Wang et al., 2013), and its expression, nuclear (Guo et al., 2012; Fazilaty et al., 2015; Pomp et al., 2015) or cytoplasmic (Chakravarty et al., 2011a,b), is associated to metastases and a poor prognosis. Up to now no data about this issue are available in veterinary medicine. With regard to our previous work on normal feline mammary gland, feline mammary hypertrophy (FMH) cases and other cat dysplastic/hyperplastic lesions (data reported above in this thesis), results demonstrate that Sox9 is present, with variable pattern, even in non-neoplastic diseases; Sox9 indeed regulates mammary stem cells activity in normal mammary gland too (Guo et al., 2012; Ye et al., 2015). Intense and diffuse Sox9 signal was observed in all FMH cases, a non-neoplastic proliferation of the mammary gland of the cat characterized by sudden and rapid onset (Hayden et al., 1981). This observation may support the hypothesis that, in mammary pathologies, Sox9 high expression alone is not able to induce a neoplastic condition, either benign or malignant. Sox9 exhibit its regulatory functions forming complexes with other transcription factors, with context-dependent mechanisms, according to specific tissue and target cells (Jo et al., 2014). Among various signaling pathways cooperating with Sox9, as Wnt/β-Catenin or Notch, Snail family with the transcription factors Snail and Slug

in particular, activate different EMT program in MaSCs (Guo et al. 2012; Ye et al., 2015). Slug overexpression has been previously associated to poor prognosis in human breast cancer lines (Guo et al. 2012), but Snail expression results in even a higher number of tumor initiating cells and metastases (Ye et al., 2015); this findings leads to new and different considerations of prognostic significance of Slug in mammary neoplasms. High levels of Slug reported were related to basal differentiation, classical feature of aggressive tumor (Ye et al., 2015).

The findings of this investigation complement those of the earlier study on FMH and other cat dysplastic/hyper-

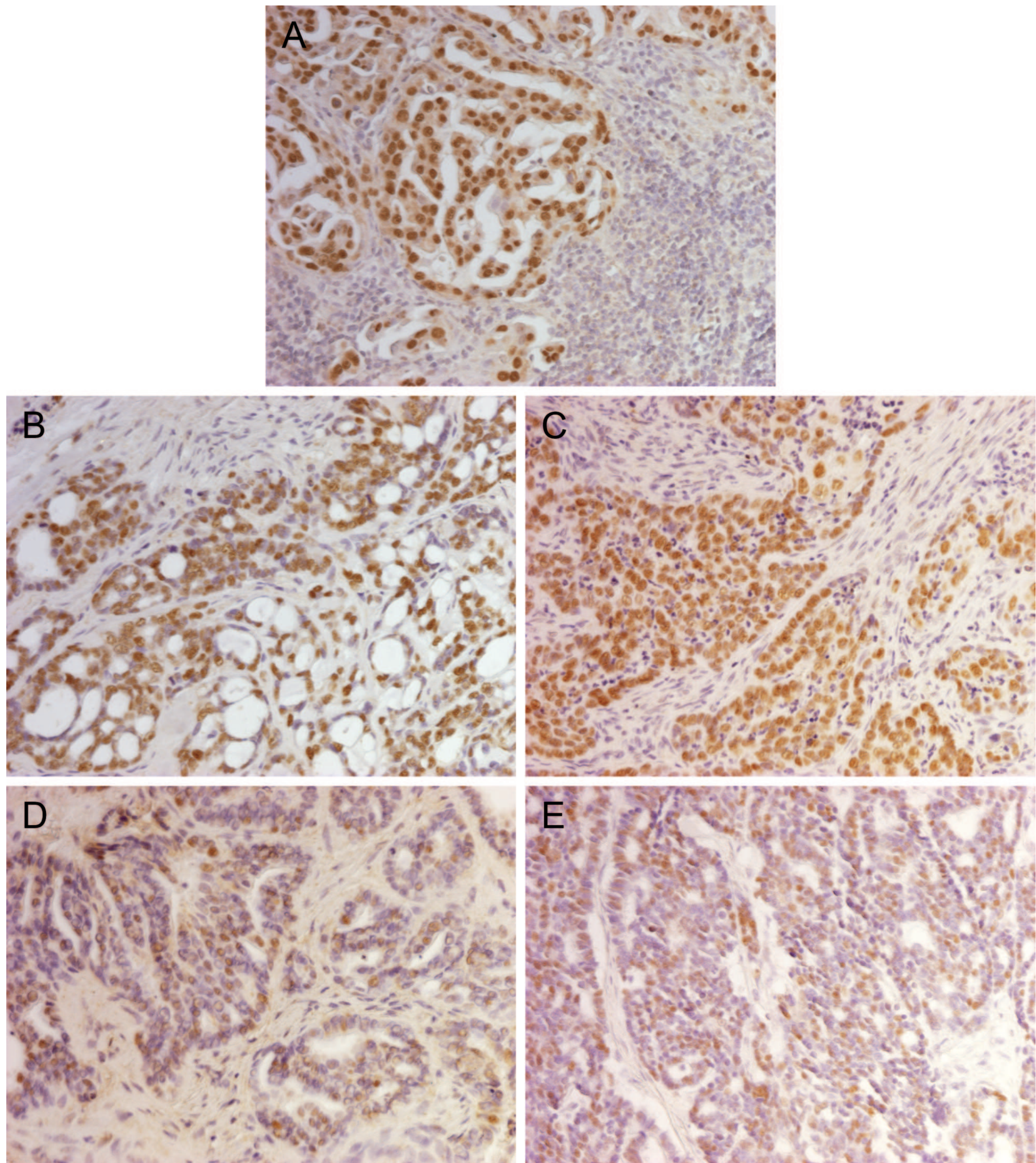

Figure 1. Sox9 immunohistochemical staining in FMC. Intense positivity detected in tubulopapillary (A), cribriform (B) and solid (C) carcinomas, with total scores 9. (D) Weak Sox9 positivity (total score 3) in a tubulopapillary carcinoma. (E) Solid carcinoma with moderate Sox9 immunostaining (total score 6). Haematoxylin counterstain (obj. 20 x).

Table I. Sox9 immunohistochemical results and signalment of cats. NA = not available.

Case	FMC	Breed	Age	Percentage	Intensity	Total Score
1	Tubulopapillary	Siamese	10	2	3	6
2	Tubulopapillary	Siamese	10	3	3	9
3	Tubulopapillary	European	8	3	3	9
4	Tubulopapillary	European	14	3	3	9
5	Tubulopapillary	European	12	1	2	2
6	Tubulopapillary	European	11	1	1	1
7	Tubulopapillary	European	15.5	3	2	6
8	Tubulopapillary	European	10	3	3	9
9	Tubulopapillary	European	10	3	3	9
10	Tubulopapillary	Siamese	13	3	3	9
11	Tubulopapillary	European	NA	3	2	6
12	Tubulopapillary	Persian	14	3	2	6
13	Tubulopapillary	Siamese	10	3	2	6
14	Tubulopapillary	European	6	2	2	4
15	Tubulopapillary	Siamese	13	3	3	9
16	Tubulopapillary	European	8	1	1	1
17	Tubulopapillary	Siamese	11	1	2	2
18	Tubulopapillary	Persian	14	2	1	2
19	Tubulopapillary	European	13	1	1	1
20	Tubulopapillary	NS	15	1	2	2
21	Tubulopapillary	Siamese	10	3	1	3
22	Tubulopapillary	European	13	1	3	3
23	Solid	Siamese	13	3	3	9
24	Solid	European	14	3	2	6
25	Solid	Siamese	11	0	0	0
26	Solid	European	9	3	1	3
27	Cribriform	European	14	3	3	9
28	Cribriform	European	12	2	2	4
29	Cribriform	European	9	3	3	9
30	Cribriform	European	13	2	3	6
31	Cribriform	European	15	2	1	2
32	Cribriform	European	9	1	1	1
33	Cribriform	European	12	2	1	2
34	Cribriform	European	6	1	2	2
35	Cribriform	European	14	1	2	2
36	Cribriform	European	10	3	1	3
37	Cribriform	European	13	3	2	6
38	Complex ductal	European	9	2	3	6
39	Complex ductal	European	12	2	2	4

plastic lesions, but further characterization of FMC is needed, investigating a possible correlation between triple negative phenotype (negativity for estrogen receptor, progesterone receptor and HER-2) and Sox9 expression in FMC, in order to define its prognostic significance. It would be interesting, moreover, to assess the expression of Slug and Snail in normal tissue, neoplastic and displastic lesions of mammary gland, to better understand the complex interactions of Sox9 with its regulators.

Sox9 expression in canine mammary neoplasms

Introduction

Master regulatory genes are transcription factors coordinating the main phases of organism development. Sox9, as previously explained, is involved in a wide variety of embryonic developmental processes and in differentiation of several tissues and organs, cartilage included (Akiyama et al., 2004; Vidal et al., 2005; Sakai et al., 2006; Seymour et al., 2007; Davis and zur Nieden, 2008; Akiyama, 2008; Barrionuevo and Scherer, 2010; Antoniou et al., 2010; Pritchett et al., 2011). Sox9 indeed has been demonstrated to be of primary importance in the early phases of cartilage differentiation from mesenchymal stem cells. It is widely expressed in all differentiated chondrocytes until the cells reach hypertrophy in the growth plate (Akiyama and Lefebvre, 2011). Although Sox9 expression has been reported in several adult organs such as intestine, pancreas, testis and hair follicle (Nowak et al., 2008) its function in postnatal tissues has not been completely elucidated. In veterinary literature there is few data concerning the role of Sox9 and Runx2, another master regulatory gene, in dogs. Csaki et al. (2007) demonstrated that canine mesenchymal stem cells, treated with suitable induction media, differentiate in chondrocytes and osteoblast, exhibiting their own specific transcription factors too, represented respectively by Sox9 and Runx2.

Benign mixed tumor is one of the most common neoplasm in female dogs. This neoplasm is composed by neoplastic epithelial cells morphologically similar to the normal epithelial component of mammary gland, and by mesenchymal cells giving rise to cartilage, bone or adipose tissue, sometimes associated to fibrous tissue. Its malignant counterparts, carcinoma and sarcoma in benign tumor, recur too; in these neoplasms, foci of respectively epithelial or mesenchymal neoplastic cells are present (Misdorp et al., 1999). The origin of chondroid and osteoid component in these neoplasms is still debated. Recent works suggest a role of basal cells/myoepithelial cells or of the mammary stem cells (Tateyama et al., 2001; Espinosa de Los Monteros et al., 2002; Gama et al., 2003; Gama et al., 2004), but mechanisms involved in osteochondrogenic process of canine mixed mammary tumor are still unclear.

The objective of this work is to investigate the immunohistochemical localization of the master chondrogenic transcription factor Sox9 in mammary tumors of dog, to better assesses its role in the histogenesis of the chondroid component in mixed tumors.

Materials and Methods

Case selection

Different histotypes of canine mammary tumors were retrieved from the files of the Department of Veterinary Medicine. Samples of simple tubular adenoma (n=2), complex adenoma (n=4), benign mixed tumor (n=7), carcinoma in mixed tumor (n=4), complex adenocarcinoma (n=6), simple adenocarcinoma (n=4), anaplastic carcinoma (n=1), solid adenocarcinoma (n=1) and osteochondroma (n=1) were selected. Normal mammary tissue surrounding the tumor was also included in the study. The diagnosis of each tumor was revised according to the criteria of the World Health Organization classification of Mammary Tumors of the Dog and the Cat.

Immunohistochemical staining

Four-micrometer thick sections from routinely formalin-fixed, paraffin-embedded tissue blocks were prepared on poly-L-lysine–coated glass slides for immunohistochemistry. The sections were de-paraffinized in xylene and rehydrated through graded alcohols. Endogenous peroxidase was blocked using 3% hydrogen peroxide in distilled water for 5 min, then antigen retrieval for formalin-fixed samples was accomplished by microwave irradiation in citrate buffer (pH 6.0) for 10 minutes. Sections were incubated for 18 hours at 4°C with anti-Sox9 antibody rabbit polyclonal antibody (Sigma, Product Number HPA001758) diluted 1:200 followed by staining with streptavidin-biotin peroxidase kit (Vectastain Elite ABC, Vector Laboratories, Cat. # 6102 and 6105). Positive staining was visualized

with 3.3 - diaminobenzidine-4 HCl (Vectastain, Vector) and nuclei were counterstained with Mayer's haematoxylin. Sox9 reactivity of follicular outer root sheath present in normal cutaneous tissue of each tumor sample was used as internal positive control, while sections incubated under identical conditions with normal rabbit serum were used as negative control.

Evaluation of immunohistochemical data

Immunohistochemical reaction of different neoplastic cell types was evaluated: epithelial cells (EC), myoepithelial cells (MEC), elongated or round solid myoepithelial cells (SMEC), myxoid matrix with spindle or stellate cells (MyxC), chondroblast/chondrocyte (Ch), osteoblast/osteocyte (Ost). Sox9 expression was classified into 4 groups according to the proportion of positive cells: 0= no positive cells; 1= <10% positive cells; 2= 10-50% of positive cells; 3= >50% of positive cells. Intensity was scored as negative=0; weak=1; moderate=2; intense=3. A total score was conferred multiplying the positive cells percentage score and the intensity of immunolabeling score. The percentage of Sox9-positive cells and the intensity of labelling were

Table 1. Total scores (percentage of positive cells x signal intensity) in different neoplastic cell types. NP = not present, EC = epithelial cells , MEC = myoepithelial cells, SMEC = elongated or round solid myoepithelial cells, MyxC = myxoid matrix with spindle or stellate cells, Ch = chondroblast/chondrocyte, Ost = osteoblast/osteocyte.

	Case	EC	MEC	SMEC	MyxC	Ch	Ost
Simple tubular adenoma	1	0	NP	NP	NP	NP	NP
	2	1	NP	NP	NP	NP	NP
Complex adenoma	3	0	0	0	0	NP	NP
	4	1	0	0	1	NP	NP
	5	1	0	NP	1	NP	NP
	6	1	0	0	0	NP	NP
Benign mixed tumor	7	3	0	3	2	2	NP
	8	2	2	NP	4	0	NP
	9	2	0	0	1	0	NP
	10	0	0	0	0	NP	NP
	11	0	0	0	0	NP	0
	12	0	0	NP	1	0	NP
	13	0	0	0	0	0	NP
Carcinoma in mixed tumor	14	2	1	1	1	2	NP
	15	1	NP	2	NP	1	0
	16	0	0	0	0	0	NP
	17	6	0	6	NP	6	0
Complex adenocarcinoma	18	1	1	1	1	NP	NP
	19	0	0	1	0	NP	NP
	20	0	0	NP	0	NP	NP
	21	4	4	4	NP	NP	NP
	22	1	0	0	0	NP	NP
	23	1	1	1	1	NP	NP
Simple adenocarcinoma	24	9	NP	NP	NP	NP	NP
	25	6	NP	NP	NP	NP	NP
	26	9	NP	NP	NP	NP	NP
	27	6	NP	NP	NP	NP	NP
Anaplastic carcinoma	28	0	NP	NP	NP	NP	NP
Solid carcinoma	29	0	NP	NP	NP	NP	NP
Osteochondroma	30	NP	NP	NP	NP	0	0

independently assessed by two researchers.

Results

Results of Sox9 immunolabeling in the different type of cells are summarized in table 1.

One of two cases of simple tubular adenoma showed weak Sox9 immunolabeling in neoplastic EC (total score 1).

In complex adenomas weak Sox9 positivity was present in neoplastic ECs (3 out of 4 cases) and MyxCs (2 out of 4 cases) with total scores 1.

ECs in benign mixed tumor were Sox9 positive in 3 of 7 cases (total scores ranging from 2 to 3) (Fig. 1A), MECs and SMECs in 1 out of 7 cases (total scores of 2 and 3 respectively), MyxC in 4 of 7 cases (total scores from 1 to 4) and Ch in 1 of 7 cases (total score 2).

In carcinoma in mixed tumor cases, Sox9 was detected in ECs, SMECs and Ch (3/4 of cases) (Fig. 1B) with total scores from 1 to 6; in MECs and MixCs in 1/4 of cases (total score 1).

Complex adenocarcinomas were positive for Sox9 in neoplastic ECs and SMECs (4/6 of cases) (Fig. 1C) with total scores from 1 to 4, in MECs in 3/6 cases (total scores from 1 to 4) and in MixCs in 2/6 cases (total scores 1).

Simple adenocarcinoma showed strong Sox9 immunolabeling in ECs (4/4 cases) (Fig. 1D) with total scores ranging from 6 to 9.

Osteochondroma, anaplastic and solid carcinoma were

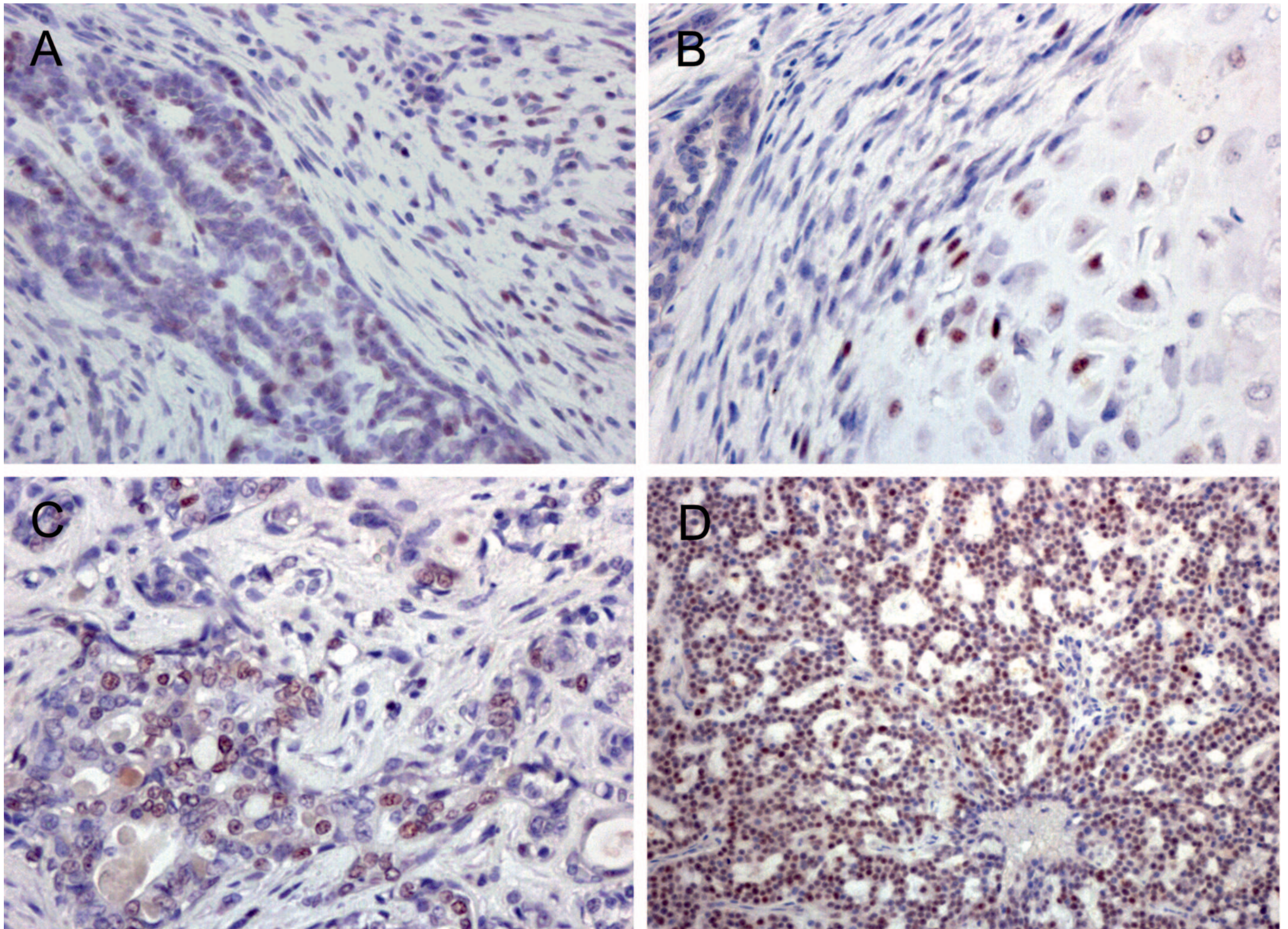

Figure 1. Sox9 immunohistochemistry in canine mammary gland neoplasms, haematoxylin counterstained. (A) Benign mixed tumor with moderate and scattered immunolabeling in ECs and MECs, diffuse in MyxC (obj. 20 x). (B) Carcinoma in mixed tumor with rare positivities of MyxCs next to the fibrocartilaginous area and of few undifferentiated chondrocytes (obj. 20 x). (C) Complex adenocarcinoma with moderate positivity especially in ECs (obj. 20 x). (D) Simple adenocarcinoma with strong immunolabeling in neoplastic ECs (obj. 10 x).

all Sox9 negative.

Most of Sox9 positivities were detected in ECs. Among benignant and malignant mixed tumors, Sox9 showed Ch immunolabeling in 4/11 cases (case n 17 showed the highest total scores).

Ectatic ducts and lobular hyperplasia were also present at the periphery of neoplastic tissue of in cases n°30 and n°8 respectively. ECs and MECs of ectatic ducts showed few cells with moderate Sox9 positivity, while in lobular hyperplasia an intense and diffuse immunolabeling was observed.

Discussion

The aim of the study was to examine the expression of the transcription factor Sox9 in canine mammary tumors to establish if Sox9 plays a role in histogenesis of chondroid tissue of mixed tumor. Obtained data is somewhat surprising and unexpected.

According to the literature, the highest Sox9 immunolabeling was expected in chondrocytes and various cell types (solid myoepithelial cells, spindle/stellate cells in myxoid areas) involved in cartilage histogenesis.

Sox9 was detected in many different mammary tumors, but especially in the epithelial cells of simple carcinoma show a diffuse and strong Sox9 expression. Similar results have been recently described in human medicine by Chakravarty et al. (2011a,b) and Guo et al. (2012) and similar results also come out from the evaluation of sox9 expression in feline mammary carcinomas reported in the previous part of this thesis. They demonstrated a Sox9 over-expression in the most malignant breast carcinomas. A possible explanation for this, as suggested by Guo et al. (2012), might be that Sox9 is involved in epithelial-mesenchymal transition mechanisms, that are associated to high-grade malignancy, including motility, invasiveness and increased resistance to apoptosis.

Anyway, Sox9 immunohistochemical detection was found in a large number of both malignant and benign tumors; higher Sox9 positivity and immunolabeling intensity were present especially in the malignant ones.

Sox9 expression in tumors with solid groups of spindle/round myoepithelial cells or spindle/stellate cells in myxoid matrix, was variable, from undetectable to weak/moderate in few scattered cells. It may be that even this low Sox9 expression leads cells to chondroid differentiation. An analogous Sox9 expression pattern has been observed in benign mixed tumor chondrocytes ad in carcinomas in benign mixed tumor. A possible explanation for this finding might be that, in these neoplasms, other chondrogenic transcription factors as Sox5, Sox6, Bmp2 and Bmp7 contribute to cartilage formation (Csaki et al., 2007; Akiyama, 2008; Nishimura et al., 2012).

As expected, osteocytes and osteoblasts were unresponsive to Sox9. A possible role of other transcription factors (i.e. TGF-b, Bmp2, FGF2 and Runx2) in bone-osteoid histogenesis has to be considered (Davis and zur Nieden 2008; Komori 2010; Chen et al. 2012).

These findings, while preliminary, suggest that:

1) Sox9 expression is often detected, in variable intensity, in epithelial luminal cells of dog mammary tumor.

2) Sox9 protein expression is pronounced in simple carcinoma, tumor characterized by high malignancy. Sox9 may be investigated as an interesting malignancy marker. To confirm this data, further studies, with a wider number of cases need to be undertaken.

3) A low grade of Sox9 expression is detectable in benign mixed tumors and carcinoma in benign mixed tumor, where often cartilagineous tissue is found. In this case too, further research should be done; the present finding, indeed, is comparable to the results obtained by Kusafuka et al. (2008) in four cases of brest matrix-producing carcinoma in human medicine.

Sox9 in nervous tissue and neoplasms

Immunohistochemical panel evaluation for differential diagnosis of horse spindle cells tumors

Introduction

SoxE and nervous tissue

Sox E proteins, as previously said, include Sox8, Sox9 and Sox10 (Wright et al., 1995); regarding nervous tissue formation, they play multifunctional roles in neural crest formation and development regulation (Haldin and LaBonne, 2010). In addition to promote neural crest formation, they play a central role in the formation of various neural crest derivates, as skin pigment cells, glial cells, oligodendrocytes, cartilage and components of peripheral and enteric nervous system (Chew and Gallo, 2009; Haldin and LaBonne, 2010).

After the neural tube closure, multipotent neural crest stem cells (NCSCs) arise from the neural plate, a transient structure present at the junction of epidermal and neural ectoderm (Kipanjula et al., 2014).

NCSCs can be considered as a transient migratory population of stem cells that originates from cells in the dorsal neural folds at the border of the neural plate and epidermal ectoderm (Cheung and Briscoe, 2003). Following epithelial to mesenchymal transition (EMT), they migrate throughout the early embryo and give rise to a wide set of derivatives (Cheung and Briscoe, 2003; Haldin and LaBonne, 2010; Kipanjula et al., 2014), notably neurons and myelinating glia of the peripheral nervous system, as well as the entirety of enteric nervous system (Haldin and LaBonne, 2010). They are responsible for neuroendocrine cells formation, differentiation of melanocytes of the skin, mesenchymal tissues and cartilage (Haldin and LaBonne, 2010; Kipanyula et al., 2014). SoxE factors are versatile transcription factors; they maintain cell multipotency and at the same time instruct the differentiation of multiple neural crest derivates (Haldin and LaBonne, 2010).

In vivo and *in vitro* studies suggest that formation, migration, survival, differentiation and specialization of NCSCs are under control of: environmental factors, related to the presence of specific signaling molecules, and intrinsic signals, mediated by specific transcription factors and the related target genes and proteins (Kipanyula et al., 2014).

Despite the complexity of the signaling mechanism, emerging experimental evidence suggest that induction of neural crest formation and its subsequent delamination can be ascribed to the interaction of SoxE with (i.e.) bone morphogenetic protein (BMP), fibrocyte growth factor (FGF) and proto-oncogenic molecules of Wnt e Hedgehog (Hh) families for its formation; Slug, Snail and FoxD3 for cell migration (Kipanyula et al., 2014).

Sox9 and nervous tissue

Sox9 is required for both formation and maintenance of nervous staminal cells during embryogenesis and for glial cells determination in central nervous system (Haldin and LaBonne, 2010). Sox9 expression in staminal cells and in neuroepithelial cells precedes the onset of gliogenesis (Stolt et al., 2003) and participates to EMT process prior to neural crest delamination (Cheung et al., 2005).

In case of Sox9 absence, neural epithelial cells forming neural crest undergo apoptosis prior to or shortly after delamination (Cheung et al., 2005). Hierarchical correlation among various genes involved in neural crest formation need to be clarified yet, but it seems that Sox9 plays a pivotal role in EMT regulation through interaction and activation of Snail2. This transcription factor inhibits E-cadherin expression, encouraging in this way cellular differentiation (Cano et al., 2000; Sakai et al., 2006). Sox9 is important not only for the generation of neural crest, but even for gliogenesis (Stolt et al., 2003). In the central nervous system, myelin-forming oligodendrocytes and non-myelinating astrocytes represent the two main types of glia (Stolt et al., 2003; Scott et al., 2010). These neuronal subtypes arise from two different compartment of the ventricular zone (Stolt et al., 2003). Astrocytes preserve Sox9 expression till adulthood; a different behavior is observed in oligodendrocytes. Oligodendrocytes progenitors are Sox9 positive; Sox9 expression, that together with Olig2 is essential for oligodendrocytes specification, is main-

tained in migrating and proliferating oligodendrocytes. Sox9 disappears in terminal differentiation. Its expression in oligodendrocytes, thus, is transient: in adult nervous tissue Sox9 is found neither in oligodendrocytes, nor in neurons (Stolt et al., 2003). Experimental studies demonstrated that the absence of Sox9 causes a reduction in the number of glial cells and an increase in the number of neurons; this because Sox9, together with Sox8, modifies neuroepithelial progenitors features in the ventricular zone and is involved in the neuron-glia switch in the developing spinal cord (Wegner and Stolt, 2005; Pritchett et al., 2011). Forced Sox9 or SoxE genes expression induces an ectopic differentiation of neural crest, at the expense of central nervous system neuronal generation (Cheung and Briscoe, 2003).

Sox10 and nervous tissue

Sox10 has been studied first in human medicine as related to congenital anomalies (Pingault et al., 1998). Sox10 mutations cause several different neurocristopathies; in particular, an important association with central and peripherical myelin deficiency and with Waardenburg syndrome has been described. This genetic disorder is characterized by heterochromia of the irises, hypopigmentation of the skin, deafness, and sometimes is concurrent with Hirschsprung's disease (aganglionic megacolon). It is often defined as PCWH (Peripheral demyelinating neuropathy-Central dysmyelinating leukodystrophy-Waardenburg syndrome-Hirschsprung disease) (Pingault et al., 1998; Kelsh, 2006). Sox10 is involved in NCSCs formation, specialization and subsequent differentiation (Kelsh, 2006), in addition to be essential for early development of Schwann cells from neural crest (Svaren and Meijer, 2008).

Sox10 expression has been detected in the emerging neural crest (Kuhlbrodt et al., 1998). Sox10 is essential for maintenance of stem cell state: it is able to inhibit overt differentiation, preserve proliferative abilities and NCSCs multipotency (Kim et al., 2003). Sox10 seems to be induced by Sox9 prior that neural crest cells start migration (Cheung et al., 2005; Stolt and Wegner, 2015) and, once induced, it is maintained transiently in migrating cells (Kelsh, 2006). Sox10 remains expressed in NCSCs and in cells that are destined for glial, schwannian, and melanocytic differentiation (Shin et al., 2012; Stolt and Wegner, 2015), while its expression, instead, is extinguished in non glial-cells, as in cells oriented to neuronal or smooth muscle differentiation (Kim et al., 2003; Shin et al., 2012).

In the central nervous system Sox10, together with Sox8, plays a crucial role in terminal differentiation of oligodendrocytes, while Sox9 acts during the early stages (Stolt et al., 2003).

Sox10 in NCSCs mediates peripheral glial fate, melanocytic fate, autonomic and enteric neurons fate in a context dependent way, by means of interactions with different intrinsic signaling molecules and specific environmental signals for fine-tuning lineage decisions (Kelsh, 2006; Kipanyula et al., 2014). One of the glial components of the peripheral nervous tissue is represented by Schwann cells, whose precursors differentiation involves interactions of Sox10 with factors as Notch1 and NRG. Maturation of Schwann cells in non-myelinating Schwann cells and in myelinating Schwann cells is mainly directed respectively by Oct-6, EGR2/Krox-20 and Pax3 signaling (Kipanyula et al., 2014).

In normal adult tissues, SOX10 expression was reported in Schwann cells of the peripheral nerves, melanocytes of the epidermis, oligodendrocytes of the cerebral cortex, enteric ganglia, sympathetic ganglia, sensory ganglia, mast cells, myoepithelial cells of the submucosal bronchial glands and breast, acinar and myoepithelial cells of the salivary glands (Kuhlbrodt et al., 1998; Wegner and Stolt, 2005; Ordóñez, 2013).

Sox9, Sox10 and nervous tissue neoplasms

Neoplastic cells often show characteristics similar to those of the progenitor cells they come from; for this reason master regulatory genes as Sox9 and Sox10 are often detected in neoplastic lesions related to tissues they guide the development of, nervous tissue included (Matheu et al., 2012).

In a immunohistochemical study performed on samples of Schwannomas, neurofibromas, and malignant peripheral nerve sheath tumors (MPNSTs), Sox9 and Sox10 have been widely detected in all tumor types considered (Pytel et al., 2010). These 3 tumors represent neoplastic proliferations of cells with Schwannian differentiation, so that Sox9 and Sox10 appear to be sensitive markers of Schwannian differentiation (Pytel et al., 2010).

In another study by Miller et al., (2009) Sox9 was found to be overexpressed in peripheral nerve sheat tumors (PNST) of patients with neurofibromatosis1 (NF1). Another study suggested that even if Sox9 is not a specific feature of neurofibromatosis 1 tumors, it could play a role in the development of MPNST in patients with neurofibromatosis 1 (Carbonnelle-Puscian et al., 2011).

Sox9 was expressed at higher levels in MPNST than NF1-derived primary benign neurofibroma Schwann cells. Malignant transformation seems to be related to a higher Sox9 transcriptional activity in MPNST cells associated

to a reduced expression of Sox10 (Miller et al., 2009).

Reduced expression of Sox10 in NF1 tumors suggests that this decreased expression may be necessary for tumor formation. Low Sox10 presence in Schwann cell tumors is also consistent with failure of complete differentiation (Miller et al., 2009).

Strong immunohistochemical positivity for Sox9 and Sox10 has been detected in central neuroepithelial tumors at high grade of malignancy, while reactive astrogliosis was characterized by an increase of Sox9 only (Kordes and Hagel, 2006).

Sox10, because of its restricted expression in normal tissues, is commonly expressed in melanomas, tumors with Schwann cell differentiation, and in salivary gland neoplasms (Ordóñez, 2013). Gershon et al. (2005) demonstrated Sox10 presence in tumors arising from neural crest, especially for the melanocytic ones. Subsequent studies confirmed Sox10 as a marker for benign and malignant melanocytic lesions of several subtypes to distinguish them from fibrohistiocytic and histiocytic proliferations (Shin et al., 2012). Among epithelial neoplasms, Sox10 was found strongly positive in myoepithelial or basal cell–related neoplasms, especially in salivary gland benign and malignant tumors (Ohtomo et al., 2013; Miettinen et al., 2015). Sox10 has been investigated as a marker for brest cancer too (Dravis et al., 2015).

There is few literature concerning the expression of Sox10 in tumors of the nervous system, but published results indicate that this gene is commonly expressed in Schwannomas (100%), neurofibromas (98% to 100%) and, less frequently, in MPNSTs (50% to 55%) (Ordóñez, 2013). As previously said, Sox9 and Sox10 are considered good markers for Schwann cell differentiation (Pytel et al., 2010). Comparing Sox10 and S-100 for melanomas and peripheral nerve sheath tumors diagnosis, it has emerged that Sox10 nuclear expression was found in 97% of melanomas, 49% of MPNST, whereas S-100 protein was expressed in 91% of melanomas and 30% of MPNST. Sox10 moreover was diffusely expressed in schwannomas and neurofibromas. Sox10 is considered as a more sensitive and specific marker for the diagnosis of melanocytic and schwannian tumors, especially of MPNST, than S100 (Nonaka et al., 2008; Karamchandani et al., 2012). In a recent study in human medicine Sox10 resulted a specific marker (93% positivity), but not so sensitive (67% positivity) for the diagnosis of MPNST and for differential diagnosis with sinovial sarcomas (Kang et al., 2014).

Horse spindle cells tumors

Horse spindle cells tumors are comprehensive of a wide range of mesenchymal neoplasms and not only, that include tumors of fibroblastic origin, as sarcoids, muscular neoplasms, vascular neoplasms, arising from peripheral nerve sheats and even melanocytic (Scott et al. 2011).

In the present study, the most common skin spindle cell neoplasm of the horse, the sarcoid, and neoplasms where a differential diagnosis is often needed, as peripheral nerve sheath tumor (PNST), malignant peripheral nerve sheat tumors (MPNST) and, rarely, other soft tissue sarcomas (Scott et al., 2011) have been investigated. Currently differentiation of these neoplasms is mainly based on their histopathological phenotype but, in horse as in dog, several immunohistochemical markers have been proposed especially to distinguish fibroblastic tumors from PNSTs (Bogaert et al., 2011; Meyer and Klopfleisch, 2014).

PNST

Benign peripheral nerve sheath tumor (PNST) represent an heterogeneous group of neoplasms originating from Schwann cells, fibroblasts or perineural cells (Sturgeon et al., 2008). PNST are relatively common in human beings, but occur infrequently in domestic animals (Quinn et al., 2005).

In human medicine PNSTs are classified in 4 types: schwannoma (or neurilemmoma), neurofibroma, perineurioma, and ganglioneuroma. They show characteristic diagnostic features, and different prognosis and treatment (Schöniger et al., 2011). In addition to these forms, neurofibromatosis type1 (NF1) has been identified; this is an inherited disorder characterized by the presence of multiple dermal or plexiform neurofibromas. These patients are at higher risk for neoplastic differentiation than the general population, especially for MPNST development (Thway and Fisher, 2014).

In veterinary medicine, according to the World Health Organization International Histological Classification of tumors of domestic animals, PNST are divided in benign forms (schwannoma -PNST- and neurofibroma) and malignant forms (malignant schwannoma -MPNST- and neurofibrosarcoma) according to their histogenesis (Koestner et al., 1999).

Nevertheless, in veterinary literature, the terms benign PNST, schwannoma, and neurofibroma are often used interchangeably (Schöniger et al., 2011). It seems that all PNSTs show a similar clinical behavior, but the criteria to distinguish among them are not well established for animals yet (Schöniger et al., 2011). Some Authors believe in an increased complexity of benign PNST in animals and are trying to improve diagnostic criteria for their subclas-

sification (Koestner et al., 1999, Schöniger et al., 2011; Schöniger and Summers 2009).

PNSTs are most often reported in dogs and cattle (Schöniger and Summers 2009), while are said to be rare in horse: they account for 1,1 to 5% of the equine cutaneous neoplasms in various surveys (Scott et al., 2011; Schöniger et al., 2011).

Schwannoma

Schwannomas arise from the myelinating cell of the peripheral nervous system and are composed almost entirely of Schwann cells; they typically grow within a capsule that remains peripherally attached to the parent nerve (Wippold II et al., 2007).

Antoni A and Antoni B tissue patterns are characterized respectively by densely packed neoplastic cells and poorly cellular areas of fusiform neoplastic Schwann cells in a stroma that is either collagenous and scant, or is myxoid and abundant (Schöniger and Summers 2009). In Antoni A areas nuclear palisading and Verocay bodies may be detected (Schöniger et al., 2011).

Neurofibroma

Neurofibromas contain a mixture of all the cellular elements of a peripheral nerve, including Schwann cells, fibroblasts, perineurial cells, and axons. Tumor cells grow diffusely within and along nerves, causing the nerves to expand radially while entrapping native neural elements within the substance of the tumor (Wippold II et al., 2007).

MPNST

Malignant peripheral nerve sheath tumor (MPNST) are soft tissue neoplasms that usually arise from peripheral nerves and show variable histological patterns according to the cellular components of the nerve sheath (Schwann cells, fibroblasts, and perineural cells) they arise from (Thway and Fisher, 2014). In veterinary medicine these tumors occur infrequently (Quinn et al., 2005), but they are most commonly seen in dogs. They usually involve the brachial or lumbar plexus, often compressing and invading the spinal cord and brain (Koestner et al., 1999). In horses, they have been described extradurally within the cranium and invading the cervical spinal cord, mediastinum, gastrointestinal tract, skin, periocular tissues and heart (Quinn et al., 2005). Histologically, cells are arranged in fascicles, whorls, or sheets, and range from spindle-shaped to plump fusiform cells with variable amount of fibrillar collagenous stroma to epithelioid arrangements. These differentiated, pleomorphic, anaplastic populations of cells infiltrate adjacent tissues or metastasize to other organs (Stoica et al., 2001). MPNSTs indeed represent a heterogeneous group of neoplasms with a wide range of morphology; in human medicine they are often aggressive tumors with a tendency to recur and metastasize (Thway and Fisher, 2014), while in animals these tumors commonly recur after excision, but metastasis is rare (Koestner and Higgins, 2002). Due to their morphologic heterogeneity and the lack of specific immunohistochemical or molecular markers, histologic diagnosis is challenging (Thway and Fisher, 2014). Schwannomas lacking these classical morphologic patterns are often difficult to differentiate from other spindle cell tumors such as fibroma, canine hemangiopericytoma, fibrous histiocytoma, melanosarcoma, and leiomyoma/leiomyosarcoma (Stoica et al., 2001). MPNSTs show positive immunohistochemical staining for Vimentin, NSE, S-100 and variable for GFAP (Kirchhof et al., 1996; Quinn et al., 2005; Maxie and Sameh, 2007).

Sarcoid

Sarcoid is a common, locally aggressive, typically non-regressing, fibroblastic cutaneous neoplasm of the horse. It is the most common skin neoplasm of the horse, accounting for 35,3 to 90% of the total in numerous surveys (Scott et al., 2011).

Sarcoids can occur anywhere on the body, but they are detected especially on the head, lips, legs, and ventral trunk (Koestner and Higgins, 2002) and in areas subjected to traumatisms as wounds, tack or insects (Scott et al., 2011).

The pathogenesis of equine sarcoids is the result of a nonproductive infection with bovine papillomavirus types 1 and 2 (Koestner and Higgins, 2002; Scott et al., 2011). Histologically, most lesions are composed of a thickened epidermis with prominent epithelial pegs that extend into a dermal proliferation of fibroblasts arranged in arranged in whorls, tangles, and/or herringbone patterns and containing small amounts of collagen (Bogaert et al., 2011; Scott et al., 2011).

The gross appearance of sarcoids can be quite variable, but six broad categories are recognized: occult, verrucous, nodular, fibroblastic, mixed and malignant (Scott et al., 2011).

Because of sarcoid variability of dermal configuration, especially in case of ulceration with loss of the distinctive epidermal component, differential diagnosis with fibroma, fibropapilloma, fibrosarcoma, neurofibroma, neurofibrosarcoma, Schwannoma can be challenging (Koestner and Higgins, 2002; Scott et al., 2011). A useful tool to improve sarcoids diagnosis is represented by polymerase chain reaction for bovine papillomavirus DNA (Bogaert et al.,

2011).

Other differential diagnosis

Other differential diagnosis for equine spindle cells tumors are represented by fibromas, fibrosarcomas, leiomyomas, leiomyosarcomas, rhabdomyosarcomas, hemangiomas, hemangiosarcomas, hemangiopericytomas and other soft tissues sarcomas (Maxie and Sameh, 2007).

Aim of the work

The aim of this work is to evaluate the usefulness of a panel of immunohistochemical markers for neural differentiation, including Sox9 and Sox10, for differential diagnosis of equine spindle cells tumors.

Materials and methods

Tissue samples

For this retrospective preliminary study, samples of paraffin blocks of formalin-fixed horse spindle cell tumors were retrieved from the archives of the Department of Veterinary Medicine in a period going from 1998 to 2015. Tumors identified with a diagnosis of: PNST (schwannoma, neurofibroma) (n=18), MPNST (malignant schwannoma, neurofibrosarcoma) (n=2), undifferentiated sarcoma (n=1) and sarcoid (n=6) were selected. A limited number of sarcoids (n=6) was selected for comparison. The diagnosis of each tumor was revised according to the criteria of the World Health Organization classification.

Four-micrometer thick sections were prepared on poly-

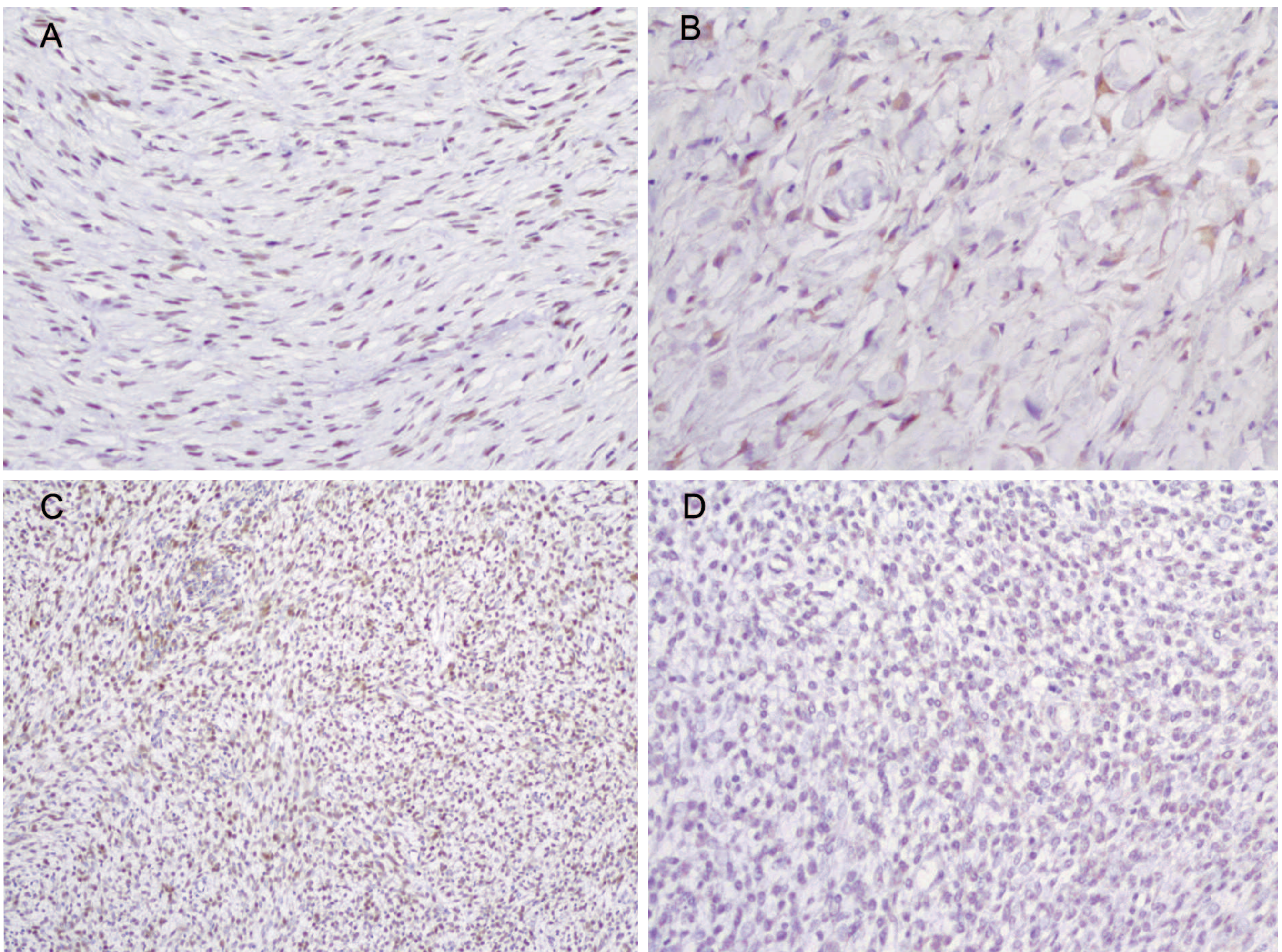

Figure 1. Immunohistochemical expression of Sox9 (A, C) and Sox10 (B, D) in PNSTs (A, B) and MPNSTs (C, D). Haematoxylin counterstain (A, B, D = obj. 20 x; C = obj. 10 x).

L-lysine–coated glass slides for immunohistochemistry. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method (Vectastain ABC Kit, Vector Laboratories Inc., Burlingame, CA, USA).

The immunohistochemical panel included rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP, 1 : 25000, Dako Denmark A/S, Glostrup, Denmark), S-100 protein (1 : 10000, Dako) , neuron-specific enolase (NSE, 1:25, Dako), Sox9 protein (1:200, HPA001758; Sigma-Aldrich, St. Louis, MO, USA), goat polyclonal antibody against Sox10 protein (1:200, (N-20): sc-17342 Santa Cruz Biotechnology, Inc.), and mouse monoclonal antibodies against Vimentin (clone 3B4, 1:1000, Dako). The Sox9 antibody employed recognizes a specific peptide with 98% identity with Equus caballus; Sox10 antibody is recommended for detection of Sox-10 in a variety of species, including equine.

Concerning GFAP, NSE, S-100 and Vimentin (VIM), tissue sections were immersed in a pre-heated solution at 94°C of Dewax and HIER Buffer H (Thermo Fischer Scientific, Lab Vision Corporation, Fremont, CA, USA) diluted 1:15 with deionized water for 40 minutes. This solution is designed to simultaneously dewax and perform heat induced epitope retrieval.

Sox9 and Sox10 sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was accomplished by microwave irradiation in 0,01 M citrate buffer, pH 6.0, for 10 min. Endogenous peroxidase

Table I. Immunohistochemical results divided for tumor type. + = positive; - = negative; A = % of positive cells; B = immunolabeling intensity; C = product of A and B.

						SOX9			SOX10		
Histological diagnosis	**Case n.**	**VIM**	**S-100**	**GFAP**	**NSE**	**A**	**B**	**C**	**A**	**B**	**C**
PNST	1	+	+	+	-	3	2	6	0	0	0
	2	+	+	-	-	2	2	4	4	2	8
	3	+	+	-	-	3	2	6	4	2	8
	4	+	+	-	+	3	1	3	4	2	8
	5	+	+	-	+	1	1	1	3	2	6
	6	+	+	+	+	1	1	1	3	2	6
	7	+	+	-	-	4	3	12	4	3	12
	8	+	+	+	+	4	2	8	4	2	8
	9	+	+	+	+	1	1	1	2	1	2
	10	+	+	-	-	2	2	4	0	0	0
	11	+	-	-	-	1	2	2	2	2	4
	12	+	+	+	-	3	1	3	4	3	12
	13	+	+	-	+	4	2	8	4	3	12
	14	+	+	+	-	2	3	6	4	1	4
	15	+	+	-	-	4	3	12	4	3	12
	16	+	+	-	+	4	3	12	3	1	3
	17	+	+	-	+	3	2	6	4	3	12
	18	+	+	-	-	3	3	9	0	0	0
Sarcoid	1	+	+	-	-	4	2	8	3	2	6
	2	+	+	-	+	1	1	1	0	0	0
	3	+	+	-	-	2	2	4	3	1	3
	4	+	-	-	+	0	0	0	0	0	0
	5	+	-	-	+	0	0	0	3	2	6
	6	+	-	-	+	2	3	6	3	2	6
MPNST	1	+	-	-	-	2	3	6	0	0	0
	2	-	+	+	+	4	3	12	3	2	6
Undifferentiated sarcoma	1	+	+	+	+	2	2	4	2	1	2

was blocked using 1% hydrogen peroxide in Tris buffer for 45 min. Sections were incubated for 18 hours at 4°C with primary antibodies. After incubation with the secondary biotinylated immunoglobulin (diluted 1:200; Vector Laboratories, Inc.) for 30 min, the avidin–biotin–peroxidase complex method (Vector Laboratories, Inc.) was performed. Positive staining was visualized with 3.3-diaminobenzidine-4 HCl (Vectastain, Vector Laboratories, SK-4100) and nuclei were counterstained with Mayer's hematoxylin. Negative control sections were produced by omission of the primary antibody.

Evaluation of immunohistochemical data

The immunohistochemically stained tissue slides were examined using standard light microscopy.

Table II. Immunohistochemical results divided for type of tumor and antibody employed.

Histological diagnosis	Antibody	Positivity	%
PNST n=18	VIM	18/18	100%
	S-100	17/18	94%
	GFAP	6/18	33%
	NSE	8/18	44%
	SOX9	18/18	100%
	SOX10	15/18	83%
Sarcoid n=6	VIM	6/6	100%
	S-100	3/6	50%
	GFAP	0/6	0%
	NSE	4/6	67%
	SOX9	4/6	67%
	SOX10	4/6	67%
MPNST n=1	VIM	1/2	50%
	S-100	1/2	50%
	GFAP	1/2	50%
	NSE	2/2	100%
	SOX9	2/2	100%
	SOX10	1/2	50%
Undifferentiated sarcoma n=1	VIM	1/1	100%
	S-100	1/1	100%
	GFAP	1/1	100%
	NSE	1/1	100%
	SOX9	1/1	100%
	SOX10	1/1	100%

GFAP, NSE, S-100, Vimentin immunoreaction was evaluated as positive (+) or negative (-). Sox9 and Sox10 protein expression were assessed by categorizing immunoreaction of each tumor into five groups according to the proportion of positive cells: 0, no positive cells; 1, from 1% to 25% positive cells; 2, from 25% to 50%; 3, from 50% to 75%; 4, from 75% to 100%. The intensity of labeling was graded as: W, weak positive staining; M, moderate positive staining; I, intense positive staining, and scored as follows: W=1, M=2, I=3. A global score was conferred multiplicating the positive cells percentage score and the intensity of immunolabeling score.

Results

Immunohistochemical results are reported in the tables above (Table I and II).

Sarcoid

The 6 sarcoids selected were positive for vimentin (100%), 50% of samples showed immunoreactivity for S-100, 67% for NSE, but GFAP immunolabeling was absent. No one of the samples showed a complete immunoreactivity for all the neural markers employed, but they were positive for at least one of them. Four sarcoids were Sox9 positive (67%) with variable signal intensity and cell positivity. Four sarcoids were Sox10 immunoreactive too (67%): 3 cases with total score 6 and 1 case with total score 1. Positive samples had a percentage of positive cells varying from 50 to 75% and a weak to moderate signal intensity.

PNST

PNSTs were all positive for vimentin (100%), and most of them for S-100 (97%). 33% of cases was GFAP positive, 44% NSE positive. Three cases showed positive immunolabeling for vimentin, GFAP, NSE, S-100; 7 cases for vimentin and at least two neural markers. Sox9 was detected in 100% of samples (Fig. 1A). Sox9 total scores obtained were: 12 (3 cases) (Fig. 1B), 9 (1 case), 8 (2 cases), 6 (4 cases), 4 (2 cases), 3 (2 cases), 2 (1 case), 1 (3 cases). Percentage of positive cells was variable: 0-25% in 4 cases, 25-50% 3 cases, 50-75% 6 cases, 75-100% 5 cases. Samples showed intense (5 cases), moderate (8 cases) and weak (5 cases), immunolabeling. Concerning Sox10, total score distribution was: 12 (5 cases), 8 (4 cases), 6 (2 cases), 4 (2 cases), 3 (1 case), 2 (1 case). Percentage of positive cells varies from 25-50% (2 cases with intensity from weak to moderate), 50-75% (3 cases with intensity from weak to moderate), 75-100% (10 cases with intensity from moderate to intense).

MPNST

One of the 2 samples was positive for vimentin, S-100 and GFAP; both were NSE positive. Sox9 showed positive immunolabeling in both cases with a percentage of positive cells varying from 25 to 75% and intense immunostaining, with total scores of 6 and 12 (Fig. 1C).

Sox10 was expressed in 1 case, with 50-75% of positive cells and moderate intensity (total score 6) (Fig. 1D).

Undifferentiated sarcoma

The case selected was positive for vimentin, GFAP, NSE, S-100. Sox9 and Sox10 were detected in 25-50% of cells with moderate and weak intensity respectively.

Discussion

Spindle cell tumors of the horse are comprehensive of a wide group of mesenchymal neoplasms that often represent a challenge for the pathologist. In most of cases they are benign neoplasms, but due to gross and histological similarities among sarcoids and PNST, immunohistochemical examination is often needed to accurately diagnose those neoplasms. Markers as S-100, GFAP, NSE, vimentin are commonly employed in veterinary medicine for this purpose. The present study employed this ordinary immunohistochemical panel with the support of Sox9 and Sox10, markers of neural differentiation recently introduced in diagnostic oncology, in order to evaluate the application of this "enlarged panel" for the diagnosis of the most common neoplasms of the neural crest.

In human medicine, Schwann cell tumors express both Sox9 and Sox10 (Pytel et al., 2010). In particular, there is a growing interest in these transcription factors because morphological features of neurofibromas and low-grade MPNSTs, that may arise within neurofibromas, are often overlapping; moreover frequently morphological and immunohistochemical features distinguishing between MPNSTs and other sarcomas are frequently lacking (Pytel et al., 2010).

According to the present results, Sox9 and Sox10 are expressed in horse PNST and MPNST with variable intensities. Sox9 immunolabeling is detectable in all PNST cases, while Sox10 was present in 13/15 cases of PNST: 3 samples were negative for this marker despite variable immunostaining of the same cases to neural markers. Anyway Sox9 seems to be expressed in a more uniform way than Sox10, but with variable total score distribution. Sox10, instead, when present, shows high positivity values and intensity. These data may support Pytel et al. (2010) statement, that Sox10 represents a more sensitive marker than Sox9 for PNST diagnosis. In one case (n=11) neural markers resulted negative, mildly positive for Sox9 and Sox10, positive for Vimentin: this immunohistochemical response is similar to the one observed for sarcoids in this study. Therefore this case should be better classified as sarcoid.

Aa regards MPNST, one case was negative for Sox10 and S-100. Sox10 is considered a more reliable marker than S-100 for neural crest tumors, especially regarding PNST (Karamchandani et al., 2012; Kang et al., 2014). High grade differentiation MPNST, in addition to be S-100 negative, as in the present case, express Sox10 in 54% of cases only (Ordóñez, 2013, Miettinen et al., 2015). In the second MPNST case, Sox9 immunolabeling shows a higher intensity than Sox10 one. Previous studies have suggested that malignant transformation of PNST may be related to a Sox10 downregulation and to an increase of Sox9 expression (Miller et al., 2009; Pytel et al., 2010); due to the low number of cases, no reliable hypothesis can be advanced.

The undifferentiated sarcoma, previously diagnosed on histological basis, without any immunohistochemical aid, was positive to the whole immunohistochemical panel: vimentin, neural markers, Sox9 and Sox10. In literature, a case of undifferentiated sarcoma positive for Sox10 is reported (Ordóñez, 2013), but in the light of these results this tumor should be diagnosed MPNST.

All the sarcoids were vimentin-positive and variously positive for neural markers. What is surprisingly is that 4 neoplasms were positive for Sox9 and Sox10 too. It has been demonstrated that Sox9 is expressed in pathological processes characterized by fibrosis (Pritchett et al., 2011); Sox9 immunohistochemical positivity in mesenchymal neoplastic cell may be related to its involvement in mechanisms of cellular proliferation. Sarcoid Sox10 immunolabeling in 4/6 cases instead, is unclear and unexplained. Sox10 is detected in schwannomas, melanocytic neoplasms and in some myoepithelial neoplasms, but it is usually absent in other mesenchymal tumors (Miettinen et al., 2015). Total scores were anyway lower than PNST ones.

To my knowledge, this is the first time that Sox9 and Sox10 have been employed in horse. They seem to be promising markers for the diagnosis of PNST/MPNST in horse, however further studies with a wider number of records need to be carried out in order to better understand the role of Sox9 and Sox10 in sarcoids and to validate definitively these markers in veterinary medicine for PNST/MPNST differential diagnosis.

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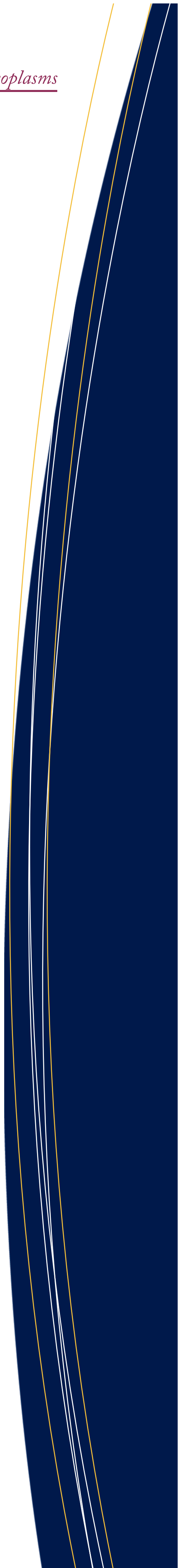

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Cover page:
Equine skin adnexa, SOX9 immunolabelling.
Haematoxylin counterstain (obj. 20 x).

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