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RESEARCH ARTICLE

New monoclonal antibodies specific for mammalian protamines P1 and P2

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ABSTRACT

The expression of protamines and the binding of these small arginine-rich proteins to DNA complete the process of spermatid chromatin reorganization and the global inactivation of the male's haploid genome that occurs during the final stages of sperm development in mammals. While a number of anti-protamine antibodies have been created during the last 40 years, only a few have proven useful for detecting the presence of the protamines, determining the timing of their expression and deposition in chromatin, and investigating their structure and function in both maturing spermatids and sperm. The aim of this effort was to develop an additional set of monoclonal antibodies (MAbs) that not only recognize new P1 and P2 protamine epitopes but also work well as IHC reagents for detecting and identifying mammalian protamines in testicular tissue and ejaculated sperm. Using a combination of native and synthetic human protamines as antigens, 38 hybridoma clones recognizing human protamine P1 or P2 were generated. Antibodies produced by the 12 best clones were screened for selectivity by enzyme-linked immunosorbent assay, and two were found to recognize only human protamine P1 or P2, while a number of the others bound to both the human and mouse proteins. One MAb recognized every protamine tested. All the antibodies, including one recognizing stallion P1 and another recognizing stallion P2, bound to the native protamines in the chromatin of spermatids or sperm. While the majority labeled only elongating spermatids or sperm, several of the antibodies were found to also bind to the cytoplasm or nuclei of cells that lack protamine, which indicates these MAbs must recognize epitopes present in the protamines that are also found in other proteins. Thirteen overlapping human protamine P1 peptides were synthesized and subsequently used to identify the epitopes recognized by the six best antibodies.

Abbreviations: BSA: bovine serum albumin; ELISA: enzyme-linked immunosorbent assay; HCl: hydrochloric acid; IHC: immunohistochemistry; i.p: intraperitoneal; LIS: lithium diiodosalicylate; MAb: monoclonal antibody; PBS: phosphate buffered saline

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Introduction

The differentiation of spermatocytes into sperm in the testes of mammals is a highly complex process that involves both the physical transformation of the primary spermatocyte into four motile sperm cells and the reprogramming of their haploid genomes to enable the sperm chromatin to function transcriptionally in the manner required for early embryogenesis once the sperm fertilizes an egg. Protamines appear to play multiple roles in this process when they bind to DNA and complete the repackaging of the chromatin in late-stage spermatids and mature sperm. These include compacting the maturing spermatid's DNA (Lee et al. 1995; Brewer et al. 1999; ; Kasinsky et al. 2014; Iuso et al. 2015; Ribas-Maynou et al. 2015; Hutchison et al. 2017; Balhorn 2018a) to optimize the hydrodynamic properties of the sperm head (Martin-Coello et al. 2009; Luke et al. 2014a, 2014b, 2016a, 2016b) and aid its ability to reach and fertilize the egg, protecting the male's genetic material from damage once it leaves the testis (Oliva and Dixon 1991; Aoki et al. 2005b, 2006a; Oliva 2006; Balhorn 2007; Rathke et al. 2010; Bjorndahl and Kvist 2011; Garcia-Peiro et al. 2011; Simon et al. 2011; Ausio et al. 2014; Zatecka et al. 2014; Ribas-Maynou et al. 2015; Luke et al. 2016b), and helping block the activation of all but those genes that need to be turned on immediately after gamete fusion (Hammoud et al. 2009b; Samans et al. 2014).

Two different protamines have been identified and characterized (Kleene et al. 1985; Ammer and Henschen 1987; Krawetz et al. 1987; Ammer and Henschen 1988;

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Hecht 1989; Oliva et al. 1989; Tanhauser and Hecht 1989; Pirhonen et al. 1990; Domenjoud et al. 1991; Queralt and Oliva 1991; Adroer et al. 1992; Retief and Dixon 1993; Retief et al. 1993a, 1993b; Winkfein et al. 1993; Queralt et al. 1995; Retief et al. 1995a, 1995b; Saunders et al. 1996; Corzett et al. 1999; Pirhonen et al. 2002; Van Den Bussche et al. 2002; Yu and Takenaka 2004; Zhao et al. 2005; Oliva 2006; Balhorn 2007; Karanth et al. 2008; Nanassy et al. 2010; Luke et al. 2011, 2016b; The UniProt Consortium 2017; Balhorn 2018b). The smaller of the two proteins, protamine P1, has been found in the sperm of all mammals examined to date. Protamine P1 contains a central very arginine-rich DNA binding domain flanked by amino-terminal and carboxy-terminal sequences (Balhorn 2007) in which a number of amino acids are post-translationally modified by phosphorylation, acetylation and methylation (Brunner et al. 2014; Barrachina et al. 2018). Cysteines located in these two segments also participate in the formation of intra-and inter-protamine disulfide bonds during the final stages of spermatid maturation in eutherian mammals (Balhorn 1989; Balhorn et al. 1991; Vilfan et al. 2004). Unlike protamine P1, protamine P2 is expressed as a larger precursor protein that undergoes processing after being deposited onto DNA (Balhorn et al. 1984; Yelick et al. 1987; Balhorn 1989; Martinage et al. 1990; Carre-Eusebe et al. 1991; Elsevier et al. 1991; Chauviere et al. 1992; Lee et al. 1995; Balhorn 2018b). Protamine P2 is also acetylated and phosphorylated (Brunner et al. 2014; Barrachina et al. 2018) and forms disulfide crosslinks. P2 has only been observed in the sperm of a subset of mammals, which includes primates, perissodactyls, and certain rodents, bats, carnivores and lagomorphs (Yelick et al. 1987; Sautiere et al. 1988; Balhorn 1989; Pirhonen et al. 1989, 1990; Unni et al. 1994; Saunders et al. 1996; Corzett et al. 1999, 2002; Lee and Cho 1999; Yu and Takenaka 2004; Luke et al. 2011, 2014a). The two protamines appear to be unique to spermatids and sperm as they have not been found to be expressed in any other normal cell or tissue (The Human Protein Atlas: Tissue expression of PRM1; The Human Protein Atlas: Tissue expression of PRM2; Uhlen et al. 2015).

The discovery that protamine P2 is synthesized as a precursor protein and is progressively shortened by the removal of ~40% of its amino-terminal sequence after it binds to DNA (Balhorn et al. 1984; Yelick et al. 1987; Sautiere et al. 1988; Balhorn 1989; Martinage et al. 1990; Carre-Eusebe et al. 1991; Elsevier et al. 1991; Chauviere et al. 1992; Lee et al. 1995; Balhorn 2018a), while protamine P1 is not (Yelick et al. 1987), has suggested the two proteins must perform different functions in packaging spermatid and sperm DNA. Since protamine P2 is not present in the sperm of all species of mammals, it has been difficult to understand how this protein might perform a function that is critical for male fertility. In addition, the proportion of the two protamines in sperm chromatin varies markedly between species (Corzett et al. 1999, 2002; Lee and Cho 1999; Luke et al. 2014a). Yet deficiencies in protamine P2 content, such as those observed in sperm obtained from certain infertile human males (Chevaillier et al. 1987; Balhorn et al. 1988; Belokopytova et al. 1993; de Yebra et al. 1993; Khara et al. 1997; Bench et al. 1998; Carrell and Liu 2001; Cho et al. 2001; Steger 2001; Mengual et al. 2003; Steger et al. 2003; Aoki et al. 2005a, 2006a, 2006b; Oliva 2006; Torregrosa et al. 2006; Carrell et al. 2007; Gazquez et al. 2008; de Mateo et al. 2009; Garcia-Peiro et al. 2011; Jodar et al. 2011; Nanassy et al. 2011; Azpiazu et al. 2014; Jodar and Oliva 2014; Ni et al. 2016; Barrachina et al. 2018), and alterations in P2 precursor processing (de Yebra et al. 1993, 1998; Carrell and Liu 2001; Torregrosa et al. 2006; de Mateo et al. 2009, 2011) appear to be positively correlated with male infertility.

Antibodies recognizing protamines P1 and P2 have become valuable tools for studying the progression of spermiogenesis in humans and other mammals (Zhou et al. 2012; Zimmermann et al. 2014) and for detecting the presence of the protamines in specific cell types and at different stages of spermatid differentiation (Djureinovic et al. 2014). But only a few antibodies have been developed that recognize mammalian protamines when they are bound to DNA in their native environment (in spermatids and sperm) (Atlas Antibodies: Anti-PRM1 Antibody (HPA055150); Atlas Antibodies: Anti-PRM2 Antibody (HPA056386); Stanker et al. 1987, 1992, 1993; Nasr-Esfahani et al. 2004; de Mateo et al. 2011). These antibodies have been used to examine how the aberrant timing and level of protamine expression (Lee et al. 1995; Hammadeh et al. 2010) impact sperm development and to investigate the relationship between the protamine content of sperm chromatin and the sperm's fertilizing potential (Carrell and Liu 2001; Aleem et al. 2008) or an individual's fertility (Bench et al. 1998; Cho et al. 2001; Zhang et al. 2006; Aoki et al. 2006a; Ramos et al. 2008; Hammoud et al. 2009a; Yao et al. 2017). They have also been used to study other processes that appear to impact male fertility (Zhao et al. 2004; Yuen et al. 2014; Zimmermann et al. 2014; El Zowalaty et al. 2015), characterize the role other genes and their protein products play in chromatin remodeling (Zhong et al. 1999; Yamauchi et al. 2010; Yanagiya et al. 2010; Gill-Sharma et al. 2011; de Vries et al. 2013; Li et al. 2014; Schneider et al. 2016; Takeda et al. 2016; Barral et al. 2017; Jha et al. 2017; Moretti et al. 2017), study protamine P2 precursor processing (de Mateo et al. 2011), detect and identify processed forms of the protamine P2 precursor (Stanker et al. 1992; de Yebra et al. 1998), as well as determine the

timing of protamine removal from sperm chromatin following fertilization (Jones et al. 2011). In addition, anti-protamine antibodies have been used to assess the impact of oxidative damage on the outcome of fertilization (Noblanc et al. 2013), examine the effect of cancer therapeutics and other compounds on sperm chromatin remodeling (Codrington et al. 2007; Maselli et al. 2012; Zatecka et al. 2014), and evaluate the potential use of protamine expression to shut down cancer cell function (Gunther et al. 2015).

While the protamine antibodies that are currently available have proven their utility in human, rat and mouse reproductive biology studies, it would be helpful to have monoclonal antibodies (MAbs) that not only target different protamine epitopes but also recognize and bind to the protamines of many other species. Here we describe the development of a group of new mouse MAbs that recognize a number of additional P1 and P2 epitopes found in the protamines of humans, horses, and most other species of mammals, marsupials, monotremes, birds and reptiles. These epitopes are sufficiently exposed when the protamines are bound to DNA to enable their use in immunohistochemical (IHC) assays for detecting or identifying the protamines in testicular tissue sections and sperm with applications that maybe developed for chromatin immunoprecipitation (CHIP) experiments to characterize the genes and DNA sequences to which protamines P1 and P2 are bound.

Results and discussion

Using a combination of native human protamines isolated from the sperm of healthy men and a synthetic form of human protamine P2 as antigens, a series of MAbs were generated in mice that recognize human protamines P1 and P2 and also cross-react with the protamines of mouse, bull and stallion. These antibodies have been screened by enzyme-linked immunosorbent assay (ELISA) for protamine binding and selectivity *in vitro*, and they have also been tested for binding to the protamine proteins in their native state in sections of testicular tissue and smears of ejaculated sperm.

Antibody producing hybridomas

Five Balb/c mice were immunized with a mixture of protamine P1 and P2 proteins isolated from ejaculated human sperm using a modified version of an immunization protocol described previously (Stanker et al. 1987, 1992, 1993). Following the fifth boost with the antigen, the serum titers of each mouse (Table 1) were determined by ELISA using the mix of native human protamines P1 and P2 and synthetic human protamines P1 and P2 proteins as antigens. While the sera of all five mice had antibody titers for the native human protamine mix and synthetic human protamine P1, the titers varied considerably from mouse to mouse. No reactivity to synthetic human protamine P2 was observed.

Table 1. Serum titers of five Balb/c mice immunized with human protamine.

Mouse	Native P1/P2	Human P1	Human P2
Serum titer[a] after 5th boost			
3002	1:64,000	1:64,000	<1:1000
3003	1:16,000	1:16,000	<1:1000
3004	1:8,000	1:8,000	<1:1000
3005	1:128,000	1:128,000	<1:1000
3006	1:16,000	1:16,000	<1:1000
Serum titer[a] after 10th boost			
3005	1:512,000	1:256,000	1:256,000

[a]Highest dilution of serum with signal/blank ≥2.1.

The mouse with the second highest titer, mouse 3002, was used to create the first set of hybridoma clones. Splenocytes obtained 4 days after the final boosts were fused with SP2/0 myeloma cells by electrofusion to generate the hybridomas. All fused cells were plated into the wells of ten 96-well plates, grown up, and the conditioned media was collected from each well and screened by ELISA for reactivity to the native mix of human protamines P1 and P2 as well as individual synthetic human protamines P1 and P2. The mouse with the highest titer, mouse 3005, was given four additional boosts of synthetic human protamine P2 and a final boost of the native human protamine P1 and P2 mixture before the spleen of the mouse was removed and used to create a second set of hybridoma clones. Cells from the 19 wells with the most reactive conditioned media from each fusion were subsequently cloned, grown up, and their conditioned media was screened again for protamine antibody activity.

The antibodies produced by the top 19 hybridoma clones obtained from the mouse 3002 fusion all recognized the isolated native human protamines (P1 and P2 mix) as well as synthetic human protamine P1 when tested by ELISA (Table 2). In nearly every case, their reactivity to immobilized native and synthetic protamine in the ELISA assay (as assessed by the measured absorbance at 450 nm per 50 ng protamine at the same dilution) was similar, consistent with the likelihood that these antibodies are recognizing a particular group of amino acids present in both the native and synthetic proteins and not a post-translationally modified epitope that would only be found in the native protamines. Antibodies produced by only four of the clones, 2A11, 2C12, 5D1 and possibly 8H6, showed a very low-level cross-reactivity with synthetic human protamine P2.

The 19 best clones obtained from the mouse 3005 fusion, in contrast, produced antibodies that showed significant reactivity to both the native human protamine P1 and P2 mix as well as synthetic human

Table 2. Reactivity of antibodies produced in undiluted conditioned media by 19 mouse 3002 hybridoma clones to human protamines (50 ng/well) as determined by ELISA.

	Absorbance (450 nm)		
Mouse 3002 derived monoclonal antibody	Native human P1/P2	Synthetic human P1	Synthetic human P2
2A11	2.205	2.317	0.490
2C12	2.205	2.325	0.400
2H5	2.242	2.130	0.253
3A4	2.324	2.165	0.216
3E9	2.335	2.305	0.193
3F11	2.349	2.182	0.235
5A2	2.270	1.942	0.182
5C2	2.290	2.188	0.191
5D1	2.457	2.311	0.358
6F3	2.333	2.209	0.229
7F5	2.323	2.084	0.184
8B9	2.234	1.956	0.279
8F5	2.192	2.264	0.284
8H6	2.363	1.996	0.331
9D6	2.209	1.985	0.200
9F1	2.299	2.077	0.199
9G2	2.314	1.691	0.239
11C1	2.363	2.467	0.297
13F5	2.309	1.816	0.214
Media	0.173	0.185	0.180

protamine P2 (Table 3). The majority appeared to bind only to protamine P2. Antibodies produced by five of the clones (17B8, 20D4, 20F11, 26F2 and 28D10) also bound to synthetic human protamine P1. These results suggest that the four additional boosts of synthetic human protamine P2 had a marked impact on the selectivity of the antibodies produced by the hybridomas obtained from mouse 3005, changing the selectivity of the majority of the antibodies from recognizing only human protamine P1 (Table 2) to preferentially binding to human protamine P2 (Table 3).

Table 3. Reactivity of antibodies produced in undiluted conditioned media by 19 mouse 3005 hybridoma clones to human protamines (50 ng/well) as determined by ELISA.

	Absorbance (450 nm)		
Mouse 3005 derived monoclonal antibody	Native human P1/P2	Synthetic human P1	Synthetic human P2
17B8	1.869	1.168	2.080
18A1	1.315	0.097	0.948
18A8	1.462	0.082	1.263
18C10	1.403	0.111	0.800
18G9	1.307	0.169	1.373
19A2	1.689	0.266	1.473
20D4	1.413	0.721	1.613
20F11	1.111	0.561	1.455
21C4	1.207	0.285	1.358
23E11	1.466	0.128	1.072
24B7	1.579	0.095	1.517
25C5	1.591	0.122	1.174
25E8	1.409	0.200	1.363
26A8	1.318	0.096	1.212
26B11	0.723	0.143	0.803
26F2	1.565	0.738	1.211
26F6	1.520	0.118	1.497
27C9	1.472	0.114	0.975
28D10	1.464	0.507	0.958
Media	0.173	0.185	0.180

Characterization of the purified antibodies

Twelve of the most interesting hybridoma clones (based on their media titers and selectivity in binding to either human protamine P1 or P2) were injected into Balb/c mice to generate ascites and the antibodies were isolated from ascites fluid and purified by Protein A column chromatography. Each of the purified MAbs was then evaluated by ELISA to determine its protamine binding selectivity and extent of cross-reactivity to the human, mouse, stallion and bull protamines. Since the amounts of antigen deposited into each well were the same for each protamine tested (50 ng/well), the absorption values obtained in the ELISA assays at 450 nm could be used to directly compare how well each antibody bound to the different protamines.

As shown in Figure 1, the purified antibodies produced by all 12 hybridoma clones recognized and bound to the mixture of native human protamines P1 and P2 isolated from ejaculated sperm. The 26B11 antibody was specific for human protamine P1, while seven others (2A11, 5A2, 5D1, 6F3, 8F5, 9F1, and 11C1) recognized and bound best to human protamine P1 and, to a lesser extent, mouse P1. 11C1 and 5D1 differed from the others in that they also bound weakly to mouse and stallion P2 and bull P1. The 17B8 antibody was highly selective and bound only to human protamine P2, while 26F6 bound to both human and mouse protamine P2. The antibody produced by clone 18C10 only bound very weakly to mouse protamine P1 and to the native human protamine P1 and P2 mix. 18C10 did not have an affinity for any of the purified protamines from any of the other species tested, which suggests its binding might be influenced by either a post-translationally modified form of a protamine epitope or some other antigen present in the isolated mixture of native human protamines P1 and P2. None of the antibodies recognized calf or human histones (data not shown).

The antibody produced by clone 20D4 was unusual, when compared to the others, in that it recognized and bound to all of the protamines tested. This antibody bound best to the human and mouse P2 protamines. It bound least well to stallion protamine P2. As shown in the ELISA binding curves for 20D4 in Figure 1, the other protamine antigens were recognized in the order mouse P1 = bull P1 > stallion P1 = human P1. The observation of significant binding of 20D4 to both protamines P1 and P2 from each species tested indicate the recognized epitope may involve a peptide sequence that is common to many mammalian protamines.

In addition to 20D4, which exhibited excellent binding to stallion P1 with only weak reactivity to stallion P2, monoclonal 5D1 showed significant

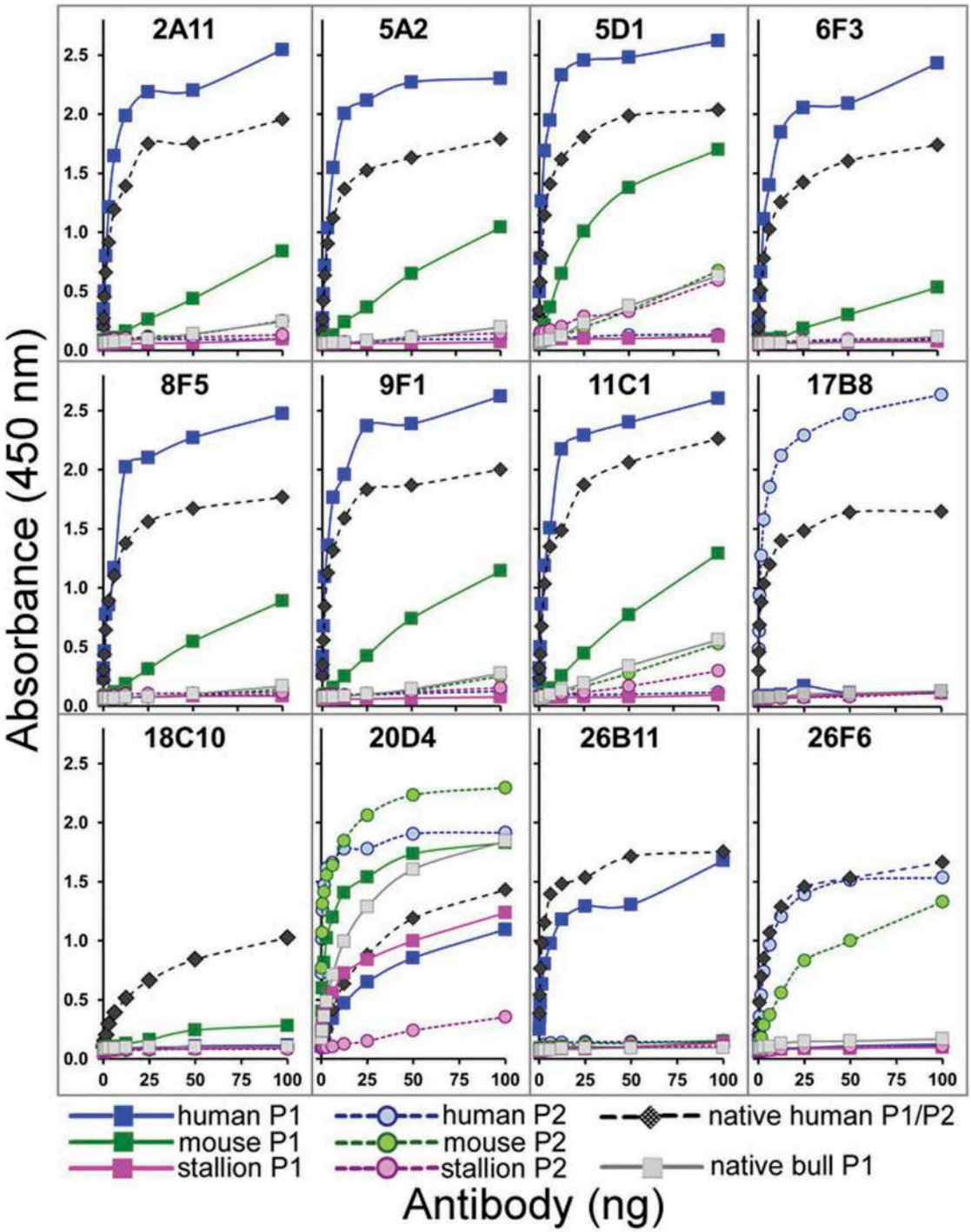

Figure 1. ELISA titration curves showing the binding of twelve purified anti-protamine monoclonal antibodies to human, mouse, stallion and bull protamines. Each titration curve was generated in a 96-well ELISA plate using 50 ng of protamine per well and a range of 0.195–100 ng of antibody. The human, mouse and stallion protamine P1 (filled squares) and P2 proteins (filled circles) were synthesized using an automated peptide synthesizer. The native bull P1 and native human P1/P2 proteins were isolated from ejaculated sperm. Antibody binding to the protamines coating the bottoms of the wells was determined as described in the "Materials and methods" section using a biotinylated secondary antibody and streptavidin conjugated horse-radish peroxidase detection of the biotin using a substrate whose product in the presence of a strong acid absorbs at 450 nm.

cross-reactivity to stallion protamine P2. This is noteworthy, as there have not been any antibodies developed previously, including the Hup 2B antibody, that recognize stallion protamine P2 (Table 4). The Hup1N antibody has been shown to be specific for protamine P1 (Stanker et al. 1987, 1993) and does recognize stallion P1 (Table 5), but not as strongly as the 20D4 antibody.

Reactivities toward cells in testicular tissue

Purified antibodies produced by 11 of the hybridoma clones evaluated for binding to isolated and synthetic protamines (18C10 was not included) were then tested for their utility and selectivity in labeling spermatids and sperm in fixed and paraffin-embedded human (Figure 2) and mouse testes (Figure 3) sections following antigen retrieval.

Antibody 2A11

This antibody, which binds to both human and mouse protamine P1, showed faint labeling of round spermatids (where the expression of protamines is first observed) and strong labeling of elongated spermatids in human testicular tissue sections. Background labeling was low with little labeling observed in the seminiferous epithelium or the inter-tubule tissue. While the antibody recognized mouse protamine P1 in the ELISA assays, it exhibited non-specific labeling in mouse testicular tissue sections and did not label the chromatin in testicular sperm.

Antibody 5A2

Human testicular tissue showed an excellent pattern of cell labeling with the clone 5A2 antibody. While round spermatids did not bind antibody, the elongating spermatids and testicular sperm labeled very well. Background labeling within the seminiferous epithelium and inter-tubule tissue were both low. At high antibody concentrations, additional labeling was observed in the cytoplasm of spermatocytes. No 5A2 antibody binding was observed in mouse testicular tissues.

Table 4. ELISA titration of 5D1, 11C1, 20D4 and Hup2B antibody binding to stallion, human and mouse protamine P2.

	Absorbance (450 nm)					
	5D1	11C1	20D4	Hup2B		
MAb (ng)	Stallion P2			Human P2	Mouse P2	Stallion P2
0.195	0.111	0.076	0.090	0.268	0.430	0.061
0.39	0.131	0.086	0.091	0.370	0.676	0.066
0.78	0.155	0.087	0.094	0.560	1.036	0.068
1.56	0.158	0.088	0.096	0.882	1.573	0.068
3.125	0.160	0.093	0.099	1.130	2.031	0.700
6.25	0.172	0.940	0.104	1.407	2.427	0.072
12.5	0.203	0.105	0.127	1.671	2.724	0.087
25	0.286	0.117	0.151	1.845	2.813	0.093
50	0.331	0.168	0.241	2.001	3.000	0.094
100	0.597	0.298	0.355	2.243	3.051	0.136

Table 5. ELISA titration of 20D4 and Hup1N antibody binding to stallion, human, mouse, and bull protamine P1.

	Absorbance (450 nm)				
	20D4	Hup1N			
MAb (ng)	Stallion P1	Human P1	Mouse P1	Bull P1	Stallion P1
0.195	0.159	0.109	0.208	0.116	0.092
0.39	0.191	0.144	0.279	0.160	0.114
0.78	0.285	0.160	0.470	0.237	0.119
1.56	0.376	0.223	0.732	0.370	0.150
3.125	0.484	0.335	1.037	0.569	0.196
6.25	0.557	0.436	1.385	0.885	0.288
12.5	0.724	0.598	1.449	1.238	0.339
250	0.841	0.760	2.225	1.570	0.451
50	0.999	0.884	2.414	1.882	0.620
100	1.237	1.172	2.579	2.131	0.687

Antibody 5D1

Clone 5D1 antibody labeled elongating spermatids and testicular sperm in sections of human testicular tissue, but additional faint labeling of round spermatid cytoplasm and spermatocytes was also observed at higher antibody concentrations. In addition, this antibody, which also bound to stallion protamine P2, did not bind to the protamines in Bouin-fixed and paraffin-embedded stallion testicular tissue (data not shown).

Antibody 6F3

The antibody produced by clone 6F3 labeled human testicular tissue in a manner very similar to the clone 2A11 antibody. Round spermatids labeled faintly and strong labeling of elongating spermatids and testicular sperm was observed. No labeling was detected within the seminiferous epithelium or inter-tubular tissue. The 6F3 antibody showed some very light non-specific labeling in mouse testicular tissue sections and did not label the chromatin in testicular sperm.

Antibody 8F5

This antibody showed only faint labeling of human testicular tissue, even at a high antibody concentration. Most of the round spermatids were not labeled and the binding of the antibody to elongating spermatids appeared to be localized in the residual bodies. Due to its unusual pattern of labeling of human testes sections, this antibody was not tested for its ability to bind to spermatids or sperm in mouse testis sections.

Antibody 9F1

The labeling observed with this antibody appeared inhomogeneous. In some cases, only very faint labeling of human testicular sections was observed. In others labeling of both spermatid and spermatocyte cytoplasm was observed. These results suggest the antibody does not work well or consistently with fixed and paraffin-embedded tissue, even after antigen retrieval.

Antibody 11C1

Human testicular tissue showed intense labeling of elongating spermatids and testicular sperm with only very faint binding of MAb 11C1 to round spermatids. At high antibody concentrations, elongating spermatids were labeled clearly. Round spermatids were still not labeled, but some cytoplasmic labeling in spermatocytes

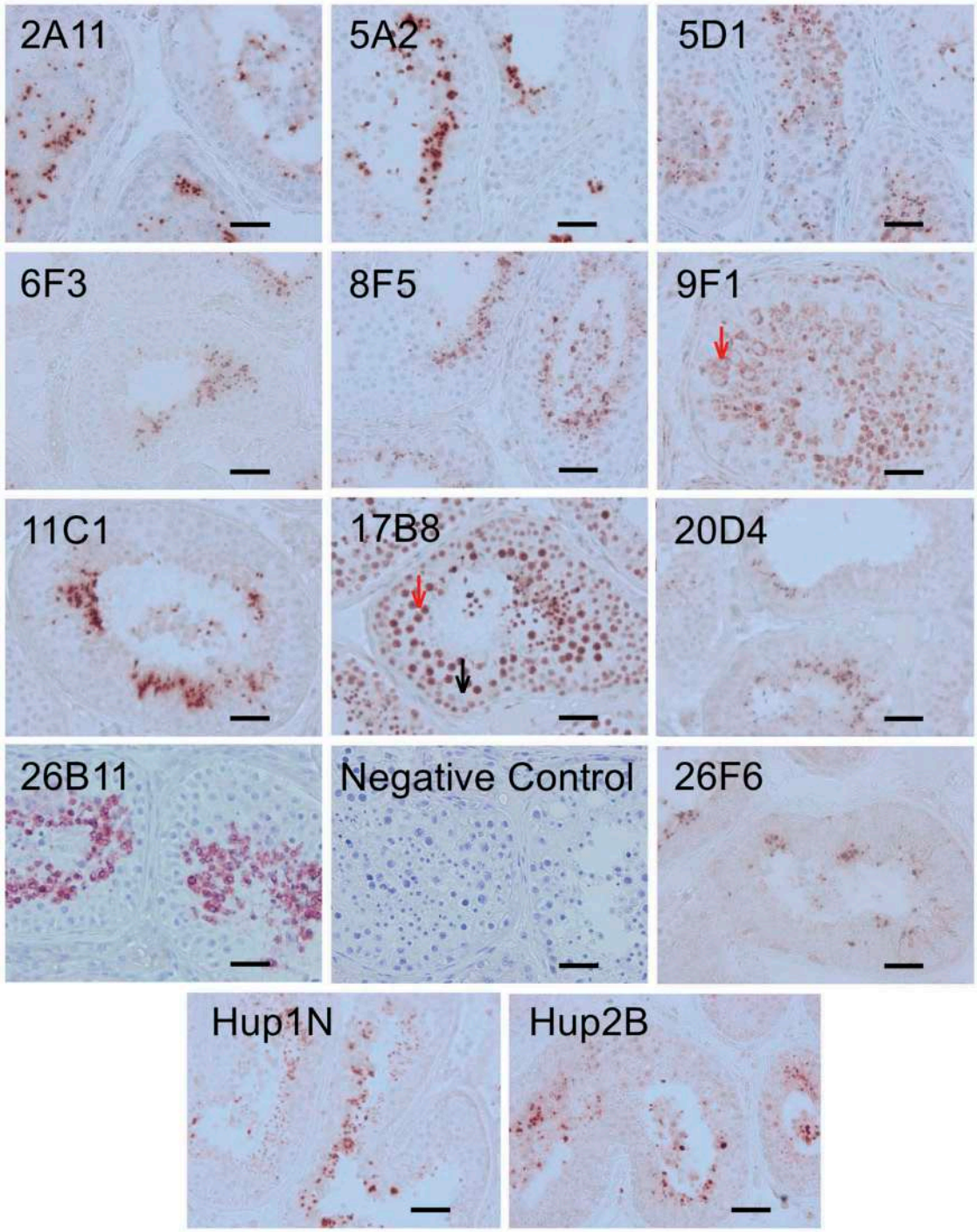

Figure 2. Immunolabeling of fixed and embedded human testicular tissue sections following antigen retrieval. The binding of 11 anti-protamine antibodies and two positive control antibodies that have been previously shown (Stanker et al. 1987, 1993) to bind to human protamine P1 (Hup1N) and human protamine P2 (Hup2B) were visualized as described in the "Materials and methods" section using a biotinylated goat-anti-mouse secondary antibody and detection of the biotin using the ABC amplification system and the substrate 3-amino-9-ethylcarbazole, which leads to the production of an insoluble red precipitate. An example of a human testis section labeled using the same protocol without a primary antibody is also shown as a negative control. Up to three primary antibody concentrations (5, 10 and 20 ng/ml) were tested for each antibody. Immunolabeling of elongating spermatids and sperm is observed in every image except those of negative controls. These images show the labeling observed at the optimal anti-protamine antibody concentrations of 5 ng/ml (MAbs 5A2, 5D1, 6F3, and 26B11), 10 ng/ml (MAbs 2A11, 8F5, 17B8, Hup1N and Hup2B) and 20 ng/ml (MAbs 9F1, 11C1, 20D4 and 26F6). The red arrows show spermatocytes in 9F1 and 17B8 that are also labeled by these two antibodies. The black arrow shows spermatogonia lightly labeled by 17B8. The black scale bar is 50 µm.

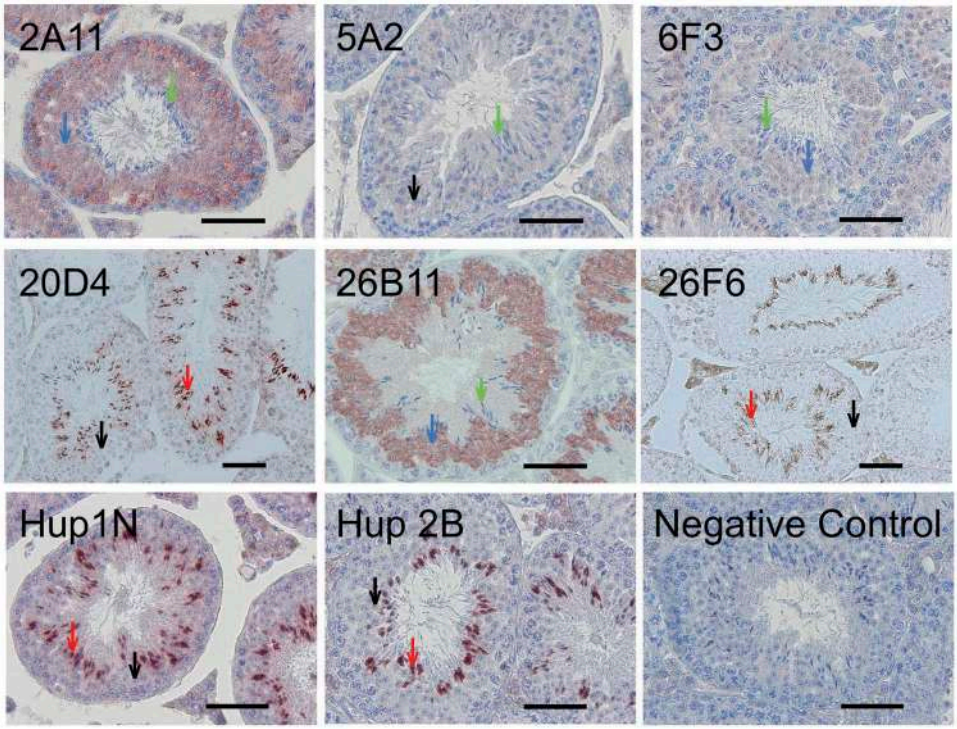

Figure 3. Immunolabeling of fixed and embedded mouse testicular tissue sections following antigen retrieval. The binding of six anti-protamine antibodies was visualized as described in the "Materials and methods" section using a biotinylated goat-anti-mouse secondary antibody and detection of the biotin using the ABC amplification system and the substrate 3-amino-9-ethylcarbazole, which leads to the production of an insoluble red precipitate. Up to three primary antibody concentrations (5, 10 and 20 ng/ml) were tested for each antibody. These images show the labeling observed at the optimal anti-protamine antibody concentrations, which correspond to 5 ng/ml for MAb 26B11 and 10 ng/ml for MAbs 2A11, 5A2, 6F3, 20D4 and 26F6. Red arrows point out elongated spermatids labeled by the antibody. Green arrows point to elongated spermatids that are not labeled by the antibody. Blue arrows point to round spermatids that are labeled by the antibody. Black arrows point to round spermatids that are not labeled by the antibody. Three of the antibodies, MAbs 2A11, 5A2 and 6F3 did not label mouse sperm chromatin, in spite of the fact they each exhibited low-level binding to mouse protamine P1 in the ELISA assays. The black scale bar is 50 μm.

was observed, indicating the antibody begins to show some non-selectivity in binding when used at high concentrations. This antibody did not show any binding to mouse testicular tissue sections (data not shown).

Antibody 17B8

The clone 17B8 antibody, which in the ELISA assays only bound to native or synthetic human protamine P2, labeled every nucleus in the human seminiferous epithelium. In some sections, a black dot was observed in the spermatid nucleus in a region that may be the starting point of the acrosome.

Antibody 20D4

Labeling with this antibody is faint and restricted to elongating spermatids and testicular sperm in human testicular tissue sections. In contrast, very good labeling of elongating spermatids and testicular sperm was obtained with mouse testicular tissue. Round spermatids were not labeled. Labeling of stallion testicular tissue sections was not observed (data not shown), even though stallion protamine P2 was observed to bind to the antibody in ELISA assays.

Antibody 26B11

Labeling of human and mouse testicular tissue sections with the clone 26B11 antibody showed the epitope recognized by the antibody is not specific for human protamine P1. While the antibody bound to both human and mouse testicular tissues, the antibody labeled the cytoplasm, not the nucleus, of the spermatids.

Antibody 26F6

A high concentration of the clone 26F6 antibody provided a nice labeling of the elongating spermatids and testicular sperm in human testicular tissue, but in some cases the spermatogonia near the basal membrane were labeled while in others labeling of the Sertoli cell cytoplasm was observed. When the antibody was tested at lower concentrations, the labeling of human testicular tissue was too light to be useful. Mouse testicular tissue sections labeled

with the clone 26F6 antibody showed binding to the right location in elongating spermatids, but the labeling was very faint and unusual under the conditions used in this study.

These results, summarized in Table 6, show three of the antibodies (2A11, 5A2 and 6F3) bind selectively to the human and mouse P1 protamines and work well when used to label spermatids and testicular sperm in fixed and paraffin-embedded sections of human testes after antigen retrieval. None of these three antibodies exhibited selectivity for spermatid and sperm chromatin in mouse testis sections (Figure 3). Two antibodies, 20D4 and 26F6 (which recognize human and mouse protamine P2), work well for labeling spermatids and testicular sperm in fixed and paraffin-embedded sections of both human and mouse testes after antigen retrieval. A higher concentration of 20D4 was required to label human testis sections, which suggests it may have some problems binding to previously fixed or paraffin-embedded sections. While the labeling observed with both 20D4 and 26F6 was restricted to elongating and testicular sperm (round spermatids did not bind either antibody) in mouse testes, the 26F6 antibody labeling was less intense compared to 20D4. 26F6 also appeared to be less selective when used to label sections of human testes; some Sertoli cells and spermatogonia near the basal membrane were observed to be labeled in addition to the elongating spermatids and sperm. The other antibodies, while recognizing and binding well to protamine proteins in ELISA assays, must recognize epitopes present in the protamines that are also present in other proteins. These antibodies were found to label the cytoplasm of spermatocytes, round spermatids or Sertoli cells.

Neither of the antibodies that recognize synthetic stallion protamine P1 (clone 20D4) or stallion P2 (clone 5D1) labeled fixed and paraffin-embedded stallion testis sections. One explanation could be that these two antibodies may not bind well to stallion tissue that has been fixed with Bouin's and embedded in paraffin using our current protocol, even after antigen retrieval. It is also possible these two antibodies recognize epitopes on protamine P1 and P2 that are no longer accessible for antibody binding in spermatid and testicular sperm chromatin. The latter explanation seems less likely, since 20D4 and 5D1 recognize different epitopes on different protamines and these epitopes remain exposed in human spermatids and testicular sperm chromatin, but it cannot be ruled out based simply on the results obtained from the labeling of testicular tissues. Additional studies need to be conducted using other fixation and labeling protocols. None of the antibodies have been tested on frozen sections or sections preserved using other fixatives or protocols.

Labeling of ejaculated sperm

Each of the antibodies was also tested to determine if they recognize and bind to the protamines in ejaculated human sperm. As shown in Figure 4, all 11 of the antibodies tested bound to lithium diiodosalicylate (LIS) treated sperm heads, and the intensity of labeling appeared to be relatively uniform throughout the head for all but two of the antibodies. The binding of antibodies 2A11 and 26B11 occur primarily around the perimeter of the heads. The interior of the head (the chromatin) does not appear to bind antibody. Because 2A11 showed intense labeling of the chromatin in elongating and testicular sperm in human testes, the result obtained with ejaculated sperm suggests the protamine epitope recognized by the 2A11 antibody may become inaccessible during the final stages of sperm chromatin maturation. This inaccessibility could relate to changes in protamine epitope exposure as a consequence of its binding to DNA or the protamine's interaction with neighboring proteins. Alternatively, it is also possible that the decondensation of the sperm with LIS may have displaced some of the sperm chromatin from the head, which could explain the brightly labeled halo surrounding the heads of ejaculated sperm labeled with the 2A11 antibody. Testicular spermatids appear to have a faint halo around the heads as well, although the intensity of 2A11 labeling is higher in the interior of the spermatid heads.

Table 6. Functional profile of 11 new anti-protamine monoclonal antibodies.

Monoclonal antibody	Isotype	Protamine selectivity (ELISA)	Human sperm staining	Stallion sperm staining	Human testes staining	Mouse testes staining	Stallion testes staining
2A11	IgG1	P1 (human, mouse)	Yes		Good	Not selective	
5A2	IgG1	P1 (human, mouse)	Yes		Good	No	
5D1	IgG2a	P1 (human, mouse, bull) and P2 (mouse, stallion)	Yes	Yes	Good at higher dilutions		No
6F3	IgG1	P1 (human, mouse)	Yes		Good	No	
8F5	IgG1	P1 (human, mouse)	Yes		Unusual staining		
9F1	IgG1	P1 (human, mouse)	Yes		Unusual staining		
11C1	IgG1	P1 (human, mouse, bull) and P2 (mouse)	Yes		Good at higher dilutions		
17B8	IgG2b	P2 (human)	Yes		Not selective		
20D4	IgG2b	P1 (human, mouse, stallion, bull) and P2 (human, mouse)	Yes	Yes	Good	Good	No
26B11	IgG2a	P1 (human)	Yes		Not selective		
26F6	IgG2a	P2 (human, mouse)	Yes		Unusual staining		

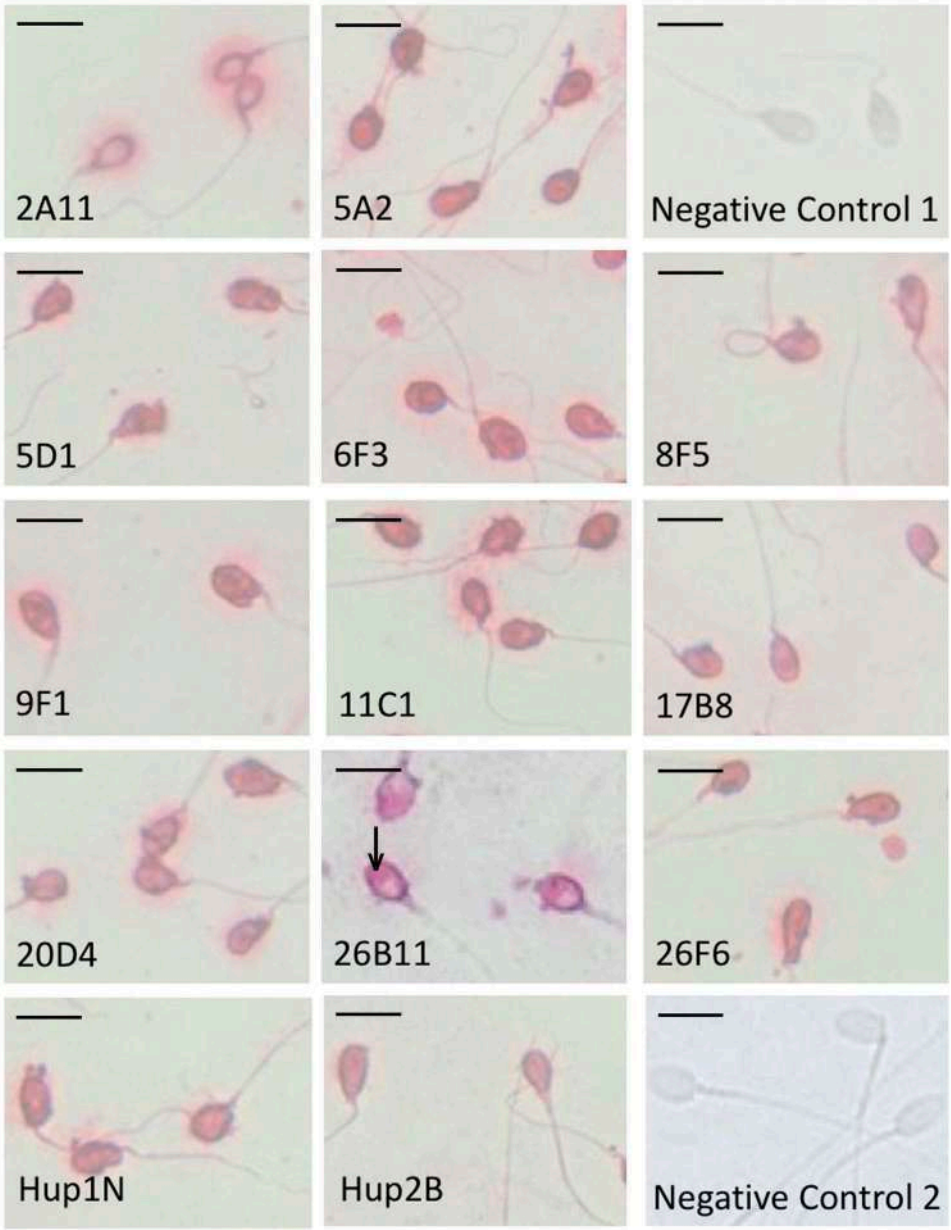

Figure 4. Immunohistochemical labeling of air-dried smears of ejaculated human sperm. The binding of 11 anti-protamine antibodies and two positive control antibodies to lithium diiodosalicylate decondensed human sperm was visualized (red labeling) as described in the "Materials and methods" section using a biotinylated goat-anti-mouse secondary antibody and detection of the biotin using the ABC amplification system and the substrate 3-amino-9-ethylcarbazole. The two antibodies used as positive controls have been shown by others (Stanker et al. 1987, 1993) to bind to human protamine P1 (Hup1N) or human protamine P2 (Hup2B). Each antibody was tested at the same concentration of 10 ng/ml. Two smears processed without the addition of the primary antibody are shown as Negative Control 1 and 2. All but two of the antibodies uniformly labeled the sperm chromatin inside the nucleus. With many of the sperm, this labeling also extended a short distance outside the nucleus as the antibodies bound to the protamines in the LIS decondensed chromatin. Sperm treated with MAb 2A11 had much larger halo of labeling surrounding the nucleus and very little staining inside it. MAb 26B11 labeled only the acrosome (black arrow) or the chromatin underlying it. The black scale bar is 10 μm.

In the case of antibody 26B11, which binds to spermatid cytoplasm in sections of testes but not the nucleus of elongating or elongated spermatids, the lack of chromatin labeling in ejaculated sperm, except in the area occupied by or underlying the acrosome, is consistent with what is observed in testicular sections. The epitope recognized by 26B11 in isolated protamine P1 is clearly not accessible when the protein is bound to DNA in spermatid and sperm chromatin. The results also provide evidence that the 26B11 epitope must be found in other proteins that

are present in spermatid cytoplasm, associated with or near the acrosome in mature sperm, or located in the perinuclear theca or near or in the membrane.

The two antibodies that bound to synthetic stallion protamines in the microtiter plates were also tested for their ability to bind to the protamines in ejaculated stallion sperm. Antibody 20D4, which recognizes and binds well to stallion protamine P1, labeled stallion sperm heads uniformly. Antibody 5D1, which recognizes stallion protamine P2, also labeled stallion sperm heads uniformly. Since both of these antibodies bind to the protamines in ejaculated sperm (Figure 5), the observed lack of labeling of spermatids and sperm in stallion testicular tissue sections rules out the possibility that the epitopes recognized by these antibodies cannot be accessed when the proteins are bound to DNA and suggest some residual effect of the fixation of the stallion testis sections with Bouin's or its embedding in paraffin may prevent antibody binding. The observed weak labeling of spermatids and sperm in human testicular tissue by antibodies 8F5 and 9F1 also indicated the binding of these two antibodies might be affected by the fixation and/or paraffin. Both 8F5 and 9F1 bound well to the protamines in ejaculated human sperm.

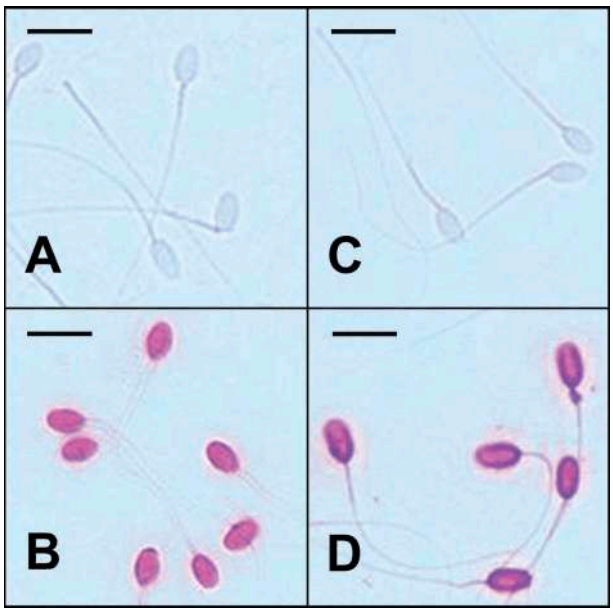

Figure 5. Immunohistochemical labeling of air-dried ejaculated stallion sperm. The binding of the anti-protamine antibodies to lithium diiodosalicylate decondensed stallion sperm was visualized (red labeling) as described in the "Materials and methods" section using a biotinylated goat-anti-mouse secondary antibody and detection of the biotin using the ABC amplification system and the substrate 3-amino-9-ethylcarbazole. The concentration of antibody that was found to work well for labeling stallion sperm with both antibodies 5D1 and 20D4, as shown in these images, was 5 ng/ml. (A) 20D4 negative control (no primary antibody). (B) 20D4 antibody. (C) 5D1 negative control (no primary antibody). (D) 5D1 antibody. The black scale bar is 10 µm.

The sperm and testicular tissue labeling results, taken together with the analyses of antibody binding to isolated protamines, suggest antibodies 17B8 and 26B11 bind to an epitope in protamine that is also found in other proteins. Some cytoplasmic labeling of testicular tissue was also observed with 5D1 and 26F6 when the antibodies were used at high concentrations, but the selectivity of spermatid and sperm labeling obtained with lower concentrations of 5D1 and 26F6 indicate these two antibodies may work well for labeling stallion P2 and human P2, respectfully, once the labeling conditions are optimized. Antibodies 11C1 and 20D4 bound to the correct cell types and cellular locations when used at low concentrations (2.5–5 ng/ml), but their selectivity was lost when the antibody concentration was increased fourfold or more. Such a response often indicates the binding of the antibody, which is selective for a particular epitope, can also bind to closely related amino acid sequences (in this case those that are found in other non-protamine proteins) if the antibody used for labeling is increased to a high enough concentration.

Antibody epitope characterization

Purified antibodies produced by the six most promising hybridoma clones (2A11, 5A2, 5D1, 11C1, 20D4 and 26B11) were tested in an ELISA capture assay for binding to a set of overlapping biotinylated peptides as a first step toward characterizing the epitopes recognized by each antibody. Because all six antibodies bound to human protamine P1, this sequence was used to generate the 13 overlapping peptides (Figure 6). Twelve of the synthetic peptides contained 15 amino acids with an overlap of three residues. The C-terminal peptide contained 14 residues. To minimize the likelihood the antibodies might not bind to amino acids located near the N-terminus of the peptides to which the biotin was linked, the biotin was conjugated to the amino-terminus of each peptide through an aminohexanoic acid linker.

The results of the peptide binding assays (Figure 7) show four of the antibodies 2A11, 5A2, 5D1, and 11C1 appear to recognize and bind to the same sites on human protamine P1. The amino acid sequences within the recognized epitope that contribute to binding are located in both the amino- and carboxy-terminal regions of the protein (Figure 8A). Based on the observed differences in antibody binding to successive overlapping peptides, which include changes in the

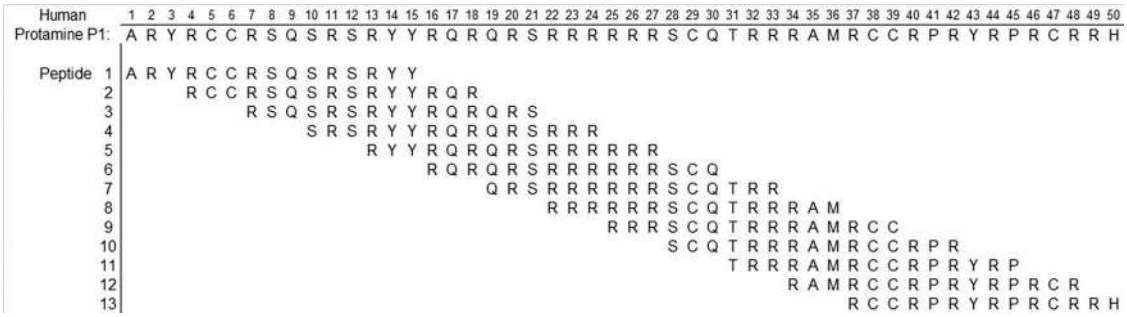

Figure 6. Sequences of the synthetic peptides screened for anti-protamine antibody binding. As an initial approach to identifying the location of the epitopes recognized by six of the anti-protamine monoclonal antibodies, all of which were observed to bind to native and synthetic human protamine P1, the human protamine P1 sequence was used to create the set of overlapping peptides to be tested for antibody binding by ELISA. Using a three amino acid overlap, the 13 peptides cover the entire length of the human protamine P1 sequence. Each peptide contains a biotin conjugated to the amino-terminus of the peptide through an aminohexanoic acid linker and a free carboxyl-group at the carboxy-terminus.

amount of bound antibody (A450 nm) and the magnitude of the loss or increase in bound antibody as each group of three amino acids is lost from or added to the sequence, two sequences in the amino-terminal half of the protamine were found to participate in antibody binding. Amino acids in the sequence SRS were observed to have the largest impact on antibody binding, while those within the sequence RQR also contributed to binding, albeit to a significantly lesser degree (Figure 8A). Amino acid residues located in two sequences, TRR and RCC, were found to contribute to antibody binding to the C-terminal half of human protamine P1. In addition to TRR and RCC, RRH also appears to participate in the interaction, but its contribution to the overall affinity of the antibodies for protamine is only minor. The binding of 2A11, 5A2, 5D1, and 11C1 to the three sequences located in the C-terminal half of human protamine P1 appear to be significantly weaker than its binding to SRS, but both the amino-terminal pair of sequences and the carboxy-terminal set of three sequences were sufficient to independently enable antibody binding. Two of these sequences, SRS and RCC, are also present in mouse protamine P1 and contribute to the binding of these antibodies to mouse P1. The reduced ELISA response observed with mouse P1 is consistent with its containing only two of sequences that contribute to antibody binding to human protamine P1.

It is interesting to note that while the ELISA results identified the same five peptide sequences as contributors to the binding of 2A11, 5A2, 5D1 and 11C1 to human and mouse P1, the observation that 5D1 and 11C1 also exhibited a low affinity for mouse and stallion P2 and bull P1 while 11C1 and 5A2 do not indicates the antigen binding sites on the four antibodies are not identical. In addition, the current results do not explain why the 5D1 and 11C1 antibodies should bind to stallion P2. None of the peptide sequences in human protamine P1 recognized by the 5D1 or 11C1 antibodies are present in stallion protamine P2 (Figure 8B). The most likely explanation may be that the 5D1 and 11C1 paratopes (sites on the antibody to which the antigenic sequences in protamine bind) are more accepting of amino acid sequence variation than are the paratopes in the 2A11 or 5A2 antibodies.

The antibodies produced by clones 20D4 and 26B11 recognize two different sets of epitopes. The epitope recognized by the 26B11 antibody, which binds only to human protamine P1, is located very near the carboxy-terminal end of human protamine P1. Amino acids within the sequence YRP are essential for recognition and binding and appear to contribute the majority of the binding affinity, while other residues located within the neighboring sequence RAM enhance binding slightly but are not sufficient on their own to provide for detectable antibody binding to the protamine. The absence of the YRP sequence in the other protamines tested (Figure 8B) is consistent with their lack of binding to 26B11 and explains the antibody's selectivity for human protamine P1.

Antibody 20D4 turned out to be the most unusual of the antibodies as it bound to all of the P1 and P2 protamines tested to date. Two peptide sequences, the N-terminal sequence ARY and amino acids in the sequence RSRRRRRRSCQ located near the center of human P1 were both found to contribute to 20D4 binding to protamine (Figure 8A). The amino-terminal ARY sequence, which is found in the majority of known mammalian P1 protamines, contributes to the recognition and binding of 20D4 to P1 protamines independent of the presence or absence of the RSRRRRRRSCQ sequence.

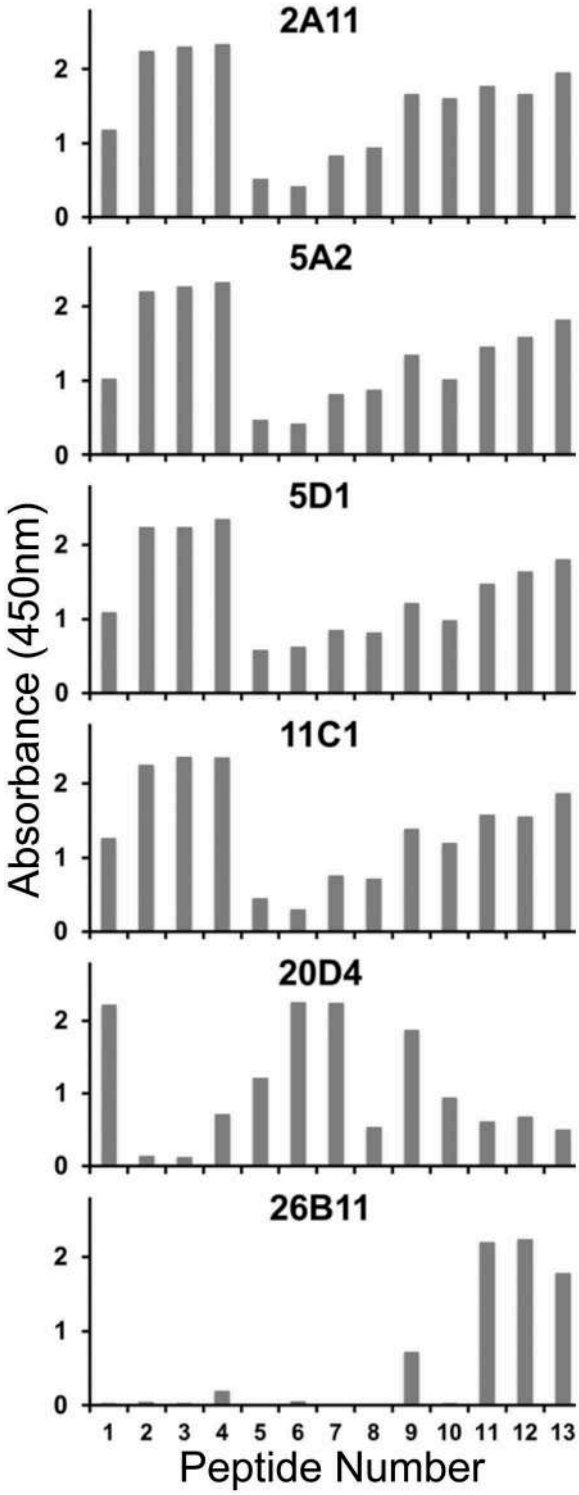

Figure 7. Binding of six purified anti-protamine monoclonal antibodies to immobilized biotinylated human protamine P1 peptides as determined in an ELISA capture assay. The sequences of the 13 peptides and their location in the human protamine P1 protein are shown in Figure 6. Streptavidin-coated ELISA plates were used to immobilize 500 ng of each peptide on the bottom of microtiter plate wells, in duplicate, and antibody binding to each peptide was assessed using 100 ng of the antibodies as described in the "Materials and methods" section. The absorbance values plotted for each peptide correspond to the average absorbance measured at 450 nm for the two (duplicate) wells after subtracting the absorbance measured for the corresponding background wells. Because the overlap between neighboring peptides is three amino acids, the observed changes in antibody binding can only pinpoint the amino acid residues recognized by the antibodies with a resolution of 3 amino acids.

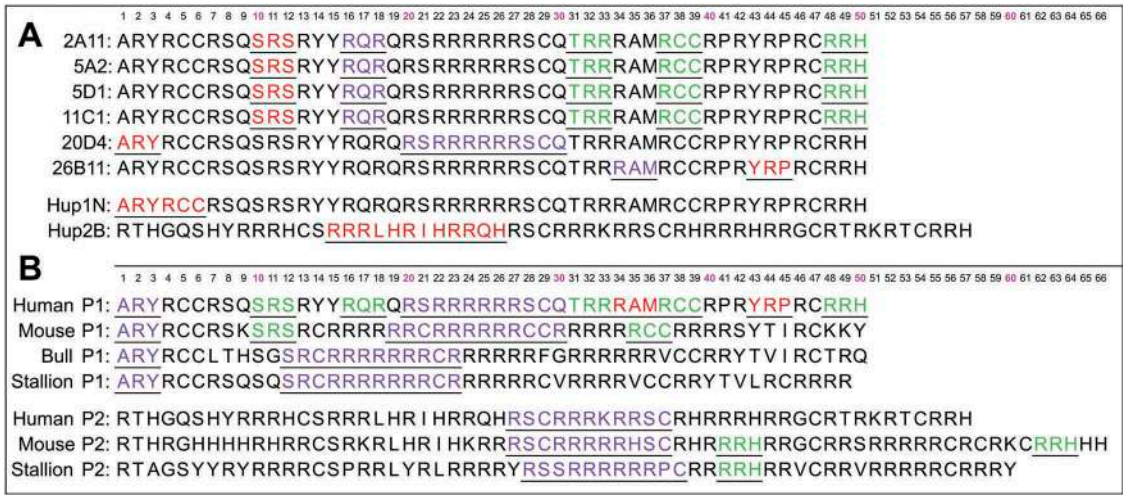

Figure 8. Epitopes recognized by monoclonal anti-protamine antibodies. (A) Amino acid residues in human protamine P1 that contribute to antibody binding. Due to the use of a three amino acid overlap in the human protamine P1 peptides tested in the assay, the resolution of the contact is limited to regions containing three amino acids. Top (2A11, 5A2, 5D1, 11C1, 20D4 and 26B11): Amino acid contacts in human protamine P1 peptides identified through the analysis of antibody binding to a set of overlapping peptides (see Figure 7). Sequences highlighted in red contribute the most to binding as determined by the magnitude of the change in antibody binding (see results shown in Figure 7) when the sequence is no longer present in the next (adjacent) peptide; purple sequences are moderate binders and the green are the weakest contributors to antibody binding. Bottom (Hup1N and Hup2B): The epitope in human protamine P1 recognized by Hup1N and the epitope in human protamine P2 recognized by Hup2B are highlighted in red. (B) Location of the amino acid sequences in human, mouse bull and stallion protamines that should be recognized by the six anti-protamine antibodies 2A11, 5A2, 5D1, 11C1, 20D4 and 26B11 based on the epitopes identified experimentally in human protamine P1. Green: 2A11, 5A2, 5D1 and 11C1 contact sequences. Purple: 20D4 contact sequences. Red: 26B11 contact sequences.

The presence of the central RSRRRRRRSCQ sequence, or a related sequence variant, in both the P1 and P2 protamines of human, mouse, stallion is consistent with the observed binding of 20D4 to each of these protamines.

The epitopes recognized by these six MAbs are different from those recognized by the Hup1N and Hup2B protamine antibodies that were developed ~30 years ago (Figure 8A). The protamine epitope recognized by Hup1N (Stanker et al. 1993), ARYRCC, includes the N-terminal ARY sequence recognized by 20D4. But the two cysteine residues in the Hup1N epitope are required for Hup1N binding. The Hup2B epitope RRRLHRIHRRQH (Steger et al, unpublished results), which is located immediately adjacent to the RSRRRRRRSCQ sequence recognized by 20D4, is found only in the protamine P2 molecules of primates and rodents. While the overlapping peptide method we used to identify regions of the protamine that bind to these antibodies does not have the resolution to determine the individual contributions each amino acid makes to antibody binding, the approach has enabled us to quickly compare these six new protamine antibodies, locate the regions of the proteins that contribute to antibody binding, and identify a group of new antibodies that recognize an additional set of mammalian protamine epitopes.

Selectivity and potential utility of the new monoclonal antibodies

In our initial assessment of these antibodies we have used a combination of ELISA experiments conducted with purified protamines and histones and the IHC labeling of testicular tissue sections and ejaculated sperm to evaluate the selectivity of the MAbs binding to a small set of P1 and P2 protamines. Unlike Western blotting, in which it is very difficult to accurately quantify the binding of an antibody to specific protamines or histones in an acid-urea gel, the ELISA experiments have made it possible to very quickly obtain binding curves for 11 different purified MAbs to the P1 and P2 protamines of human, mouse, horse and bull under conditions that allow us to not only determine if the antibodies bind, but to also compare how well each MAb binds to the different proteins and how well the different MAbs bind to the same protamine. The IHC experiments conducted in parallel, which evaluate the binding of the antibodies to the entire proteome of proteins present in testicular tissue and sperm (not just the basic nuclear proteins typically analyzed in Western blots of sperm chromatin extracts), have provided additional information regarding the MAbs selectivity for protamine by showing whether or not the

MAbs bind only to the nuclei in those cells that are known to contain the protamines and not to non-protamine proteins located in other subcellular compartments (cytoplasm, membrane or tail) or cell types. However, the current results do not completely rule out the possibility that other cells may contain proteins with these epitopes that also bind the MAbs. This could potentially be tested more rigorously using whole cell extracts fractioned by an approach that does not denature the proteins and expose epitopes that are not generally accessible in the native proteins, but such an effort is beyond the scope of this study. Analyses of fully structured proteins are critical when assessing cross-reactivity for IHC applications because a large percentage of potential epitopes in a properly folded and functional protein are not exposed and accessible for antibody binding.

One limitation of these studies is that we have not yet determined how any of the antibodies will perform as reagents for detecting or identifying the protamines by Western blotting. We suspect, based on the knowledge that isolated protamines in solution have been shown to be unstructured (Nakano et al. 1989; Hud et al. 1994; Roque et al. 2011) and do not adopt any significant conformation until they bind to DNA (Hud et al. 1994), that these antibodies may also work well in Western blotting protocols applied to sperm chromatin protein extracts. The observation that all the antibodies bind well to the isolated protamines in ELISA assays and that they also recognize and bind to very short amino acid sequences in the linear epitopes we identified using 15-mer peptides support this expectation. However, even small folds located in the C- and N-terminal domains of the protamines, such as those that develop in protamine P1 in solution upon the formation of intra-molecular disulfide bonds (Balhorn et al. 1992; Vilfan et al. 2004) or in protamine P2 upon zinc binding (Gatewood et al. 1990b; Bianchi et al. 1992), could have a significant impact epitope recognition and antibody binding. In order to fully assess the utility of these new anti-protamine MAbs, it will be important to further examine their performance and specificity under the conditions used for Western blotting.

While we have only tested the binding of MAbs 2A11, 5A2, 5D1 and 11C1, 20D4 and 26B11 to protamines, testicular tissues and sperm from a few species, the ability of all the MAbs to bind to the short peptides used to identify their epitopes indicate these antibodies are capable of recognizing and binding to any protamine containing the identified sequences providing the amino acid residues are accessible and the neighboring amino acids surrounding the epitopic sequence do not prevent antibody binding. This 'neighborhood' or position-dependent effect of neighboring amino acids on antibody binding to its epitope has been reported by others (Leone et al. 1993; Qiu et al. 2017), and it may explain why the 2A11, 5A2, 5D1 and 11C1 MAbs did not bind to a second RCC sequence present near the amino-terminus of human protamine P1 (residues 4–6), as shown by the nearly identical binding of the antibodies to peptide 2 containing RCC and peptide 3 lacking it, or the lack of binding to these antibodies to stallion protamine P1 containing a single RCC sequence in the same amino-terminal location.

Other than this evaluation of the utility of the best of these MAbs for their recognition and binding to the isolated protamines and the protamines in their native environments (bound to DNA in testicular spermatids and sperm), how well these MAbs will perform in future studies of spermatogenesis, sperm chromatin organization or protamine function in other mammalian species remains to be determined. The observation that all but the 26B11 epitope is recognized both in isolated protamines (which in solution adopt an unstructured random coil conformation in solution (Hud et al. 1994) and in sperm and spermatid chromatin when the proteins are bound to DNA is consistent with the current theory that protamines bind to DNA in an extended conformation with the majority of the sequence exposed in such a manner that the N- and C-terminal domains of the protein can be post-translationally modified or participate in the formation of intra- and inter-protamine disulfide crosslinks (Hud et al. 1994; Balhorn et al. 1999; Vilfan et al. 2004; Oliva 2006; Balhorn 2007). The inability of MAb 26B11 to bind to the YRP epitope located in the C-terminal domain of protamine P1 in spermatids and sperm suggests this sequence must not be fully exposed when the protein is incorporated into spermatid or sperm chromatin. This region of P1 protamine does not bind to DNA (Brewer et al. 2003) and is thought to participate in the formation of inter-protamine interactions that develop between neighboring protamine molecules following their binding to DNA (Balhorn et al. 1991; Vilfan et al. 2004; Balhorn 2018b).

The epitopes recognized by MAbs 2A11, 5A2, 5D1 and 11C1, 20D4 and 26B11 would appear to provide a much broader range of reactivity to protamines from different species of mammals than either Hup1N or Hup2B. Since all but seven of the 265 known mammalian P1 protamines contain either an SRS or an RCC sequence (Supplemental Figure S1), we expect MAbs 2A11, 5A2, 5D1 and 11C1 may recognize and bind to all known mammalian

protamine P1s except for those present in the sperm and spermatids of the short-finned pilot and false killer whales and a few species of dolphins. The SRS sequence is also found in the protamine P1 equivalents of the echidnas and platypus (Monotremes), two birds (chicken and Japanese quail) and three reptiles (American alligator, Western painted turtle and Green anole) (Supplemental Figure S2). The presence of a single SRS, RQR, RCC or TRR epitope in many of the known protamine P2 molecules (Supplemental Figure S3) suggest the P2 protamines of most primates and at least one bat (Little brown bat) should be recognized by these four antibodies as well, although the strength of MAb binding is likely to be weak. While protamine P2 gene sequences have been reported for the domestic bull, Eurasian wild pig and the European red deer, which also contain a single TRR sequence, these proteins do not appear to be present in mature sperm (Maier et al, 1990).

The ARY epitope recognized by MAb 20D4 is also found in the P1 protamines of the majority of known species of mammals, including marsupials (Supplemental Figure S1). Two exceptions are cetaceans and lagomorphs. The other 20D4 epitope RSRRRRRRSCQ, or a conserved sequence variant, is present in the protamine P1 sequences of many bats, carnivores, perissodactyls, artiodactyls, primates and the one known lagomorph (New Zealand White rabbit) P1 sequence, but it is not found in cetaceans or marsupials. While the ARY epitope is not found in any protamine P2 protein (Supplemental Figure S3), the majority of P2 protamines do contain the RSRRRRRRSCQ epitope or one of several conserved sequence variants. The nearly universal presence of 20D4 epitopes in protamines P1 and P2 and the observed selectivity in 20D4 binding to elongating spermatids and sperm in testes and semen samples, suggest this antibody is likely to prove useful as a reagent for detecting or identifying the majority of mammalian protamines except those of whales, porpoises and dolphins (cetaceans). Based on the very limited sequence information currently available (Supplemental Figure S2), 20D4 would not be expected to recognize the protamines of monotremes, but it should recognize the protamines of some birds and reptiles.

Because protamines containing only three sequence variations of the RSRRRRRRSCQ epitope in human protamine P1 recognized by MAb 20D4 (RSCRRRKRRSC in human P2, RSCRRRRRHSC in mouse P2 and RSSRRRRRRPC in stallion P2) were tested in this study and observed to also bind the antibody in the absence of 20D4's other epitope ARY, we cannot identify with certainty the full range of variant sequences MAb 20D4 will recognize in the protamines of other species. Those variants that we have predicted to be recognized by MAb 20D4 in Supplemental Figures S1 and S3 are based on very limited conservative changes such as those present in human and mouse protamine P2. Interestingly, certain amino acid insertions (H or R between residues 27 and 28) and/or substitutions (K for R26) in the mouse and human protamine P2 sequences corresponding to RSRRRRRRSCQ (Figure 9) appear to improve antibody binding to these protamines. The substitution of proline at residue 28 in the stallion protamine P2 sequence reduces 20D4 binding substantially.

The YRP epitope recognized by MAb 26B11 has not been found in any protamine other than human P1. This includes the protamines of all other primates for which protamine P1 and P2 sequences are known (Supplemental Figure S1). Thus MAb 26B11 should be human specific in its recognition of protamine P1. The only other reported human protamine P1 specific MAb for which data have been published is Hup1M (Stanker et al. 1993). The epitope Hup1M recognizes has not been determined.

The presence of the epitopic sequences recognized by these antibodies in all but a few of the known mammalian protamine P1 and P2 sequences (Supplemental Figures S1–S3) should enable the application of MAbs recognizing protamines to reproductive biology studies in almost every mammalian species, including cetaceans and marsupials. Because the antigens used to create these antibodies included the processed form of protamine P2, all of the antibodies that recognize P2 epitopes are expected to recognize and bind well to the intact protamine P2 precursor as well as all the partially processed forms of protamine P2. One notable species, for which there is considerable interest, is the horse. The binding of the MAbs 20D4 and 5D1 to stallion protamines P1 and P2,

20	21	22	23	24	25	26	27		28	29	30		A450 nm
R	S	R	R	R	R	R	R	-	S	C	Q	Human P1	1.1
R	S	C	R	R	R	R	R	H	S	C	R	Mouse P2	2.3
R	S	C	R	R	R	K	R	R	S	C	R	Human P2	1.9
R	R	C	R	R	R	R	R	R	C	C	R	Mouse P1	1.8
S	R	C	R	R	R	R	R	R	R	C	R	Bull P1	1.8
S	R	C	R	R	R	R	R	R	R	C	R	Horse P1	1.2
R	S	S	R	R	R	R	R	R	P	C	R	Horse P2	0.3

Figure 9. Variation in the sequence of the RSRRRRRRSCQ human P1 epitope recognized by 20D4 in mouse, human, bull and stallion protamines. The sequences of the corresponding epitopes in these other protamines (with differences highlighted in green and yellow) are listed in the order of the strength of their binding to antibody 20D4 (magnitude of absorbance at 450 nm) as determine by ELISA with the mouse P2 sequence showing the strongest binding and stallion P2 showing the weakest binding. The proline in the stallion P2 epitope (highlighted in yellow) may contribute to the observed weak binding.

respectively, as isolated proteins and when bound to DNA in sperm suggest these two MAbs should prove useful for studying spermatogenesis and infertility in horses and other perissodactyls.

A small percentage (5–15%) of the DNA in human sperm retains its nucleosome organization (Tanphaichitr et al. 1978; Gusse et al. 1986; Gatewood et al. 1990a; Bench et al. 1996; Wykes and Krawetz 2003) and is not packaged by protamines. CHIP and other studies have shown the sperm DNA that retains the histone packaging is not comprised of random genes or DNA sequences, but instead contains imprinted genes, promoters for transcription factors, regulators and cell signaling and other proteins important to early embryonic development (Arpanahi et al. 2009; Hammoud et al. 2009b; Brykczynska et al. 2010; Samans et al. 2014; Barrachina et al. 2018). Since all of the new MAbs except 26B11 recognize the protamines when they are bound to DNA in spermatids and sperm, a number of these MAbs could be used in CHIP experiments to capture and identify those genes in sperm that are selectively packaged by protamines. The use of the protamine P1 specific antibodies 2A11, 5A2 or 6F3 and the protamine P2 specific antibody 26F6 (and possibly 17B8) might even enable the identification of specific gene (or sub-gene) nucleic acid sequences bound by P1 and P2. The ability to pull out gene sequences bound to individual or clusters of protamine P1 or P2 molecules by CHIP could provide valuable insight into how the two protamines are organized or distributed along DNA as well as information that might lead to our discovering differences in how the two protamines function.

Materials and methods

Testicular tissue and semen samples

Pooled bull semen was purchased from the American Breeders Service (DeForest, WI). The pooled human semen used to isolate the native human protamine (mix of P1 and P2) for the immunizations and ELISA screens was purchased from Innovative Research (Novi, MI). Individual human semen samples used for the analysis of antibody binding were obtained from healthy donors at the Department of Urology, Pediatric Urology and Andrology, Justus Liebig University, Giessen, Germany (Ethics committee approval 146/06). Semen samples from stallions were obtained from the Pferdezentrum Bad Saarow, Veterinary Faculty of the University, Berlin, Germany. Semen was collected with an artificial vagina from stallions mounting a dummy mare. After removal of the gel fraction the ejaculate was subsequently divided into aliquots, shock frozen in liquid nitrogen and stored at −80°C.

Human testicular tissue samples were obtained from patients with obstructive azoospermia at the Department of Urology, Pediatric Urology and Andrology, Justus Liebig University, Giessen, Germany (Ethics committee approval 75/00). Tissue specimens were Bouin-fixed and paraffin-embedded using standard protocols.

Bouin-fixed and paraffin-embedded testicular tissue samples from mouse and stallion were obtained from the Institute of Veterinary Anatomy, Histology and Embryology, Justus Liebig University, Giessen, Germany.

Preparation of antigens

Native human and bull protamines were isolated from ejaculated sperm as described previously (Balhorn et al. 1977; Corzett et al. 2002). The native human protamine antigen used to immunize and boost the mice and to screen the hybridoma cell supernatants and purified antibodies was the mixture of protamines P1 and P2 as isolated and purified from ejaculated sperm. The bull protamine used in the ELISA assays was bull protamine P1 purified by high performance liquid chromatography as described previously (Mazrimas et al. 1986; Corzett et al. 2002). The synthetic human protamine P1 and P2 and stallion protamine P1 and P2 proteins were synthesized and purified by Peptide 2.0 Inc. (Chantilly, VA). The synthetic mouse protamine P1 and P2 proteins and the N-terminally biotinylated human protamine P1 peptides were synthesized and purified by Atlantic Peptides (Lewisburg, PA). The biotin was attached to the amino-terminus of each human protamine P1 peptide through an aminohexanoic acid linker, and the C-terminus of the peptide was synthesized to contain a free carboxyl-group. The final purities of the synthetic protamines following HPLC purification were the following: Human P1, 95.5%; Human P2, 95.2%; Mouse P1, 96.7%; Mouse P2, 95.2%; Stallion P1, 95.9%; and Stallion P2, 77.8%. The purities of the biotinylated human protamine P1 peptides were the following: Peptide 1, 89.0%; Peptide 2, 80.8%; Peptide 3, 95.3%; Peptide 4, 78.5%; Peptide 5, 85.7%; Peptide 6, 84.0%; Peptide 7, 89.6%; Peptide 8, 90.3%; Peptide 9, 80.3%; Peptide 10, 95.8%; Peptide 11, 94.6%; Peptide 12, 96.4%; and Peptide 13, 88.9%. The calf thymus histone (Cat# 16736) and human (HeLa) core histones (Cat# 11010) used in the ELISA assays were purchased from Cayman Chemical (Ann Arbor, MI).

Mouse immunization, cell fusion, and hybridoma production

Five female Balb/c mice were immunized with the native human protamines P1 and P2 mixture (50 μg/

mouse) as described previously (Stanker et al. 1987, 1993) with the following protocol modifications. After the initial immunization on day 0, antigen boosts were administered weekly for 5 weeks (on days 7, 14, 21, 28 and 50). Test bleeds were performed on days 21 and 50 and the sera were screened by ELISA (as described in the next section) for titers against the native human protamine P1 and P2 mixture and the individual synthetic protamines P1 and P2. The spleen was removed from the mouse with the second highest serum titer (mouse 3002) on day 54, and the splenocytes were fused with SP2/0 mouse myeloma cells by electrofusion. The cells from the fusion were then plated into ten 96 well plates, cultured and the conditioned media from each hybridoma was screened by ELISA (as described in the next section) for the production of antibody recognizing human protamines P1 or P2 using both the native and synthetic human protamines to identify positive (antibody producing) clones.

The mouse with the highest serum titer, mouse 3005, was given four additional boosts of synthetic human protamine P2 1 week apart (on days 57, 64, 71, and 78). Seven days after a final boost of the native human protamine P1 and P2 mixture administered on day 85, its spleen was removed, fused with SP2/0 cells, the fused cells cultured, and their conditioned media screened by ELISA as described above for the first four mice in the group. Nineteen clones exhibiting the highest antibody titers from each mouse (3002 and 3005) fusion were subcloned twice by limiting dilution to ensure the resulting clones were derived from a single parental cell, expanded and aliquots were frozen and stored in liquid nitrogen. Undiluted conditioned media from each final clone was screened by ELISA (see next section) to determine the selectivity of each MAb for binding to native human protamine and synthetic human protamines P1 and P2. These results were used to identify the antibodies to move forward for production, purification and further testing.

Analysis of antibody binding to protamines and peptides by ELISA

In those assays performed on mouse sera from the test bleeds after the 5th and 10th antigen boosts, conditioned media obtained from the hybridoma clones and the final purified antibodies, the native and synthetic protamines were dissolved in 100 mM 2-mercaptoethanol at a concentration of 1 mg/ml and then aliquots were diluted into distilled water to a final concentration of 1 µg/ml. 50 µl aliquots of the proteins (1 µg/ml) were added to each well in a 96 well plate and allowed to dry on the plate at 37°C for 12 h. Phosphate buffered saline (PBS; 50 µl) containing 3% ovalbumin and 10 µg/ml calf thymus DNA (Life Technologies Cat# 15633-019, Carlsbad, CA) was added to each well and incubated at 25°C for 30 min to block reactive sites on the plastic and to neutralize the charge on the protamine molecules. Mouse sera being analyzed for protamine antibody titers and conditioned media from the parental hybridoma clones being tested for human protamine selectivity (native vs. synthetic, P1 vs. P2) were not diluted. Bovine serum albumin (1% in PBS) was used to dilute an aliquot of each purified antibody to a concentration of 1 µg/ml, and then the primary antibody was diluted further to generate a series of 10 antibody concentrations ranging from 1 µg/ml to 1.95 ng/ml. After removing the supernatants from each well, 100 µl of the primary antibody, conditioned media or mouse sera was added to each well and the plate was incubated at 37°C for 1 h. The wells were then washed three times with PBS pH 7.4 and 100 µl (33 ng/ml) of the secondary antibody (Peroxidase-Affinipure Goat-Anti-Mouse IgG Fcy fragment specific antibody (Jackson ImmunoResearch, West Grove, PA) was added to each well and incubated at 37°C for 30 min. After washing the plates three times in PBS pH 7.4, 100 µl of peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB Reagent, Cat.# M00078, GenScript, Piscataway, NJ) was added to each well and allowed to incubate at 25°C for 13 min. 100 µl of stop buffer (1 M HCl) was then added to each well and the absorbance was read at 450 nm using a plate reader. Each protein (native human protamine, purified native bull P1 or synthetic human, mouse or horse protamines P1 and P2, and human histones) along with a negative control (buffer containing 1% bovine serum albumin (BSA) without primary antibody) and the Hup1N and Hup2B antibodies used as positive controls (data not shown) were run concurrently in each assay. The data points presented are the absorbance measured at 450 nm in each well (a single well per data point or antibody concentration) of the same ELISA plate or group of plates analyzed in a typical experiment. The titer identified for each set of antibodies and antigens was defined to be the highest dilution of mouse serum that had a signal/blank ≥2.1.

The analyses of antibody binding to the biotinylated peptides were performed in a capture mode with the biotinylated peptides (500 ng per well) being immobilized on streptavidin-coated plates. The wells containing the peptides were not pretreated with BSA containing calf thymus DNA prior to the addition of the primary and secondary antibodies. Each assay was performed in duplicate using 100 ng of primary antibody and incubation and washing conditions similar to those used in the assays conducted with the

protamines. Blank wells contained only the buffer. Each peptide was run in duplicate and the A450 nm results reported are the average obtained for the two wells.

Antibody production and purification

In preparation for ascites production, female Balb/c mice were injected intraperitoneally (i.p.) with 0.5 ml of paraffin oil per mouse 7 days prior to inoculation with hybridoma cells. Each of the 12 hybridoma clones selected for antibody production was grown up in a tissue culture flask, the cells were harvested, centrifuged at 1000 *g* for 5 min at room temperature, washed in sterile PBS, pelleted again by centrifugation, and then resuspended in sterile PBS at a concentration of 2×10^6 cells/ml. For each clone, 1×10^6 cells were injected i.p. into four paraffin oil treated Balb/c mice, the cells were allowed to grow for 7–10 days, and the resulting ascites were then harvested. The ascites was incubated in the collection tube at 37°C for 1 h, cooled to 4°C and stored overnight, and then centrifuged at 12,000 *g* for 10 min. The supernatant was transferred to a fresh tube and the antibody was purified by Protein A column chromatography.

Tissue sectioning and semen processing

Testicular tissue sections were deparaffinized in xylene and hydrated through a graded series of ethanol. For chromatin decondensation, slides were treated with a decondensing mix (25 mM dithiothreitol, 0.2% Triton X-100, 200 IU heparin/ml in PBS, pH 7.4) for 10 min at 37°C. For antigen retrieval, slides were incubated in citrate buffer (pH 6.0) for 20 min at 98°C. Slides were then treated with 3% hydrogen peroxide in methanol for 20 min followed by incubation in 3% BSA in PBS (pH 7.4) for 20 min.

Ejaculates were washed in PBS (pH 7.4), spread on a slide and air-dried. For chromatin decondensation, slides were treated with a 10 mM DTT in Tris-HCl, pH 7.4 for 10 min at 37°C followed by incubation in 10 mM LIS in 10 mM DTT in Tris–HCl, pH 7.4 for 2 h at room temperature. Subsequently, sperm cells were fixed with 4% paraformaldehyde in PBS for 1 h and incubated in 0.1% BSA in PBS (including 2% Triton X-100) for 15 min at room temperature.

Immunohistochemical labeling of tissue sections and ejaculated sperm

All antibodies were diluted to a concentration of 1 µg/ml with PBS. Incubations with the different protamine antibodies diluted 1:50 (20 ng/ml), 1:100 (10 ng/ml) or 1:200 (5 ng/ml) were performed overnight in a humidified chamber at 4°C. Antibody binding was visualized by applying a biotinylated goat-anti-mouse secondary antibody (Dako/Agilent, Hamburg, Germany) for 60 min and detecting the presence of the biotin using the ABC system (Vector Laboratories, Burlingame, CA) for 60 min at room temperature. Immunolabeling was performed using 3-amino-9-ethylcarbazole substrate (Dako/Agilent, Hamburg, Germany). A negative control (no primary antibody was added) and a positive control (using Hup1N or Hup2B documented by others to recognize protamine P1 or protamine P2, respectively, as the primary antibodies) were included for each set of tissue sections or ejaculate smears labeled. At least two testicular tissue sections were tested for labeling with each antibody. The mouse testicular tissue was obtained from two different mice.

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Disclosure statement

R. Balhorn and M. C. Balhorn are founders of Briar Patch Biosciences, LLC, the company that provided the funding for the development of the antibodies characterized in this study. K. Steger, M. Bergmann, H.-C. Schuppe and S. Neuhauser report no declarations of interest.

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Notes on contributors

Isolated or prepared antigens, developed strategy for antigen immunizations and antibody development, analyzed data, wrote the paper: RB; Directed and analyzed IHC labeling of human, mouse, and stallion testicular tissues and human and stallion semen, contributed to writing the paper: KS; Tissue fixation and embedding, histological evaluation of human

tissue samples: MB; Obtained ethics committee approval for the use of the tissues included in this study and performed semen analyses according to the WHO recommendations: HCS; Collected stallion semen samples: SN; Analyzed data, contributed to the writing and editing of the paper, and prepared the final forms of the figures: MCB.

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