**UNIVERSIDADE ESTADUAL DE CAMPINAS**

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# CARACTERIZAÇÃO DA EXPRESSÃO DO ANTÍGENO RECONHECIDO PELO ANTICORPO MONOCLONAL TRA 54 NAS CÉLULAS EPITELIAIS DO EPIDÍDIMO E CANAL DEFERENTE DE CAMUNDONGOS (*Mus musculus*)

Tese apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural na área de Histologia.

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a)
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*Dedico este trabalho,*

*A minha mãe, Santina,*
*por todo amor, ternura e dedicação,*
*por todo colo e todo sorriso,*
*e por me mostrar que é possível*
*levantar a cabeça quando o desejo é mantê-la abaixada...*

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*por todo amor, carinho e dedicação,*
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*por todos os choros e risos que temos*
*compartilhado durante estes dias de*
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*e de minha vida.*

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*Use filtro solar.*
*Se eu pudesse dar um conselho em relação ao futuro, eu diria: usem filtro solar.*
*Os benefícios, a longo prazo, do uso do filtro foram cientificamente provados,*
*Os demais conselhos que dou baseiam-se na minha própria experiência.*
*Eis aqui um conselho:*
*desfrute do poder e da beleza de sua juventude;*
*Oh, esqueça! Você só vai compreender o poder e beleza de sua juventude quando já tiverem desaparecido...*
*Mas acredite em mim: dentro de 20 anos, você olhará suas fotos e compreenderá,*
*de um jeito que não pode compreender agora,*
*quantas oportunidades se abriram pra você, e eram realmente fabulosas...*
*Você não é tão gordo quanto você imagina!!!*
*Não se preocupe com o futuro,*
*ou se preocupe, se quiser, sabendo que a preocupação é tão eficaz quanto tentar resolver um problema de álgebra mascando chiclete.*
*É quase certo que os problemas que realmente têm importância em sua vida*
*são aqueles que nunca passarão em sua mente,*
*como aqueles que tomam conta de você as quatro da tarde, de uma terça-feira ociosa...*
*Todos os dias faça alguma coisa que seja assustadora.*
*Cante.*
*Não trate os sentimentos dos outros de forma irresponsável.*
*Não tolere aqueles que agem de forma irresponsável em relação a você.*
*Relaxe.*
*Não perca tempo com a inveja.*
*Algumas vezes você ganha, algumas vezes você perde.*
*A corrida é longa, e no final, tem que contar só com você.*
*Lembre-se dos elogios que recebe, esqueça os insultos*
*(e se conseguir fazer isso, me diga como!)*
*Guarde todas as suas cartas de amor.*
*Jogue fora seus velhos extratos bancários.*
*Estique-se.*
*Não tenha sentimentos de culpa se não sabe bem o que quer da vida;*
*as pessoas mais interessantes que eu conheço não tinham, aos 22 anos,*
*nenhuma idéia do que fariam na vida...*
*E algumas das pessoas interessantes de 40 anos que conheço ainda não sabem...*
*Tome bastante cálcio. Seja gentil com seus joelhos,*
*você sentirá falta deles quando não funcionarem mais!*
*Talvez você se case, talvez não; talvez tenha filhos, talvez não;*
*talvez se divorcie aos quarenta,*

*talvez dance uma valsinha quando fizer 75 anos de casamento...*
*O que quer que faça, não se orgulhe nem se critique demais.*
*Todas as suas escolhas têm 50% de chance de dar certo,*
*como as escolhas de todos os demais...*
*Curta seu corpo da maneira que puder;*
*não tenha medo dele ou do que os outros pensam dele; ele é seu maior instrumento.*
*Dance.*
*Leia todas a indicações, mesmo que não as siga.*
*Não leia revistas de beleza; a única coisa que elas fazem é mostrar*
*como você é uma pessoa feia.*
*Saiba entender seus pais,*
*você nunca sabe a falta que vai sentir deles.*
*Seja agradável com seus irmãos;*
*eles são seu melhor vínculo com seu passado e aqueles que, no futuro,*
*provavelmente nunca deixarão você na mão...*
*Entenda que amigos vão e vem, mas que há um punhado deles, preciosos,*
*que você tem que guardar com carinho.*
*Trabalhe duro para transpor os obstáculos geográficos e da vida.*
*Porque quanto mais você envelhece, tanto mais precisa das pessoas que conheceram você na juventude.*
*More em Nova York, mas mude-se antes que a cidade transforme*
*você em uma pessoa dura.*
*More no norte da Califórnia, mas mude-se antes de tornar-se uma pessoa muito mole.*
*Viaje.*
*Aceite certas verdades eternas: os preços sempre vão subir, os políticos são todos mulherengos e você também vai envelhecer.*
*E quando envelhecer vai fantasiar que, quando era jovem, os preços eram acessíveis, os políticos eram nobres de alma e as crianças respeitavam os mais velhos.*
*Respeite as pessoas mais velhas.*
*Não espere apoio de ninguém.*
*Talvez você tenha uma aposentadoria, talvez você tenha um cônjuge rico,*
*Mas você nunca sabe quando um ou outro podem desaparecer.*
*Beije.*
*Tenha cuidado com as pessoas que lhe dão conselhos, mas seja paciente com elas:*
*conselho é uma forma de nostalgia;*
*dar conselho é uma forma de resgatar o passado da lata do lixo, limpá-lo, esconder as partes feias, e reciclá-lo por um preço maior do que realmente vale...*
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## RESUMO

O processo de fecundação em mamíferos depende de uma seqüência de eventos que culminam na ativação do oócito pelo espermatozóide. A diferenciação completa das células germinativas testiculares em células com capacidade fecundativa envolve não somente o testículo, mas também o epidídimo, ducto deferente e trato reprodutor feminino. No epidídimo acontece a maturação epididimária dos espermatozóides, pela qual ocorrem alterações no padrão molecular da superfície do gameta, visando a capacidade de fecundação. Diversas proteínas sintetizadas pelas células epiteliais do epidídimo, inicialmente liberadas na luz deste órgão, parecem ser posteriormente localizadas na superfície ou mesmo no interior da vesícula acrossômica do espermatozóide. A aquisição da capacidade de fertilização pelo espermatozóide tem sido correlacionada com a nova organização molecular da membrana adquirida durante o fluxo do mesmo a partir da cabeça em direção à cauda do epidídimo. A obtenção de anticorpos monoclonais que reconhecem os antígenos expressos pelas células germinativas testiculares durante seus processos contíguos de diferenciação, bem como aqueles expressos pelas células dos ductos pelos quais os espermatozóides transitam durante o processo de maturação têm constituído importante estratégia para permitir a geração de um mapa das moléculas que atuam na preparação do espermatozóide para a fecundação. O anticorpo monoclonal (Amc) TRA (t*esticular germ cells immunized to rat - monoclonal antibody*) 54 reconhece um antígeno localizado em espermatócitos e espermátides dos túbulos seminíferos de camundongos C57 BL/6 com idade superior a 24 dias pós-parto (d.p.p.). Resultados preliminares mostraram que o mesmo Amc reconhece um antígeno nas células epiteliais da cabeça do epidídimo, bem como

espermatozóides da luz deste órgão. Este estudo teve por objetivo caracterizar a expressão do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo quanto à ontogenia, características bioquímicas e regulação da expressão. Em conjunto, os dados obtidos com o desenvolvimento deste trabalho poderão auxiliar no esclarecimento do modelo da expressão do antígeno identificado em células germinativas e em células somáticas epiteliais e, adicionalmente, poderão permitir inferências acerca do papel funcional desta molécula na biologia da reprodução.

## ABSTRACT

In mammals, fertilization depends on a sequence of events that culminates in the activation of the oocyte by the spermatozoa. The complete differentiation of the testicular germ cells in cells with fertilization ability involves the testis, epididymis, vas deferens and female reproductive organs. In the epididymis, the membrane surface of the spermatozoa may be modified, through a process known as epididymal maturation. Several proteins synthesized and secreted by the epididymal epithelial cells can be further located on the spermatozoa membrane surface or even though inside its acrosomal vesicle. The acquisition of the fertilization ability by the spermatozoa has been related with a new molecular organization of the membrane provided during the epididymal transit. The production of monoclonal antibodies (mAb) that recognizes antigens exclusively expressed by testicular germ cells or by the cells of the ducts through the spermatozoa passes during its maturation have been important approach to permit the elaboration of the molecular map involved in the spermatozoa preparation. The mAb TRA 54 recognizes an antigen located in spermatocytes and spermatids of the seminiferous tubules of C57 BL/6 mice more than 24 days old. Preliminary results showed that this mAb also recognizes an antigen in the epithelial cells of the caput of the epididymis and in the luminal spermatozoa. This work was performed in order to obtain additional information about the antigen recognized by the mAb TRA 54 in the epididymal epithelial cells for ontogenic expression, biochemical characteristics and expression regulation. The results obtained with the development of this work could contribute to elucidate the expression pattern of the antigen recognized by the antigen identified in both germinative and somatic cells and, additionally, could permit the formulation of hypothesis around the functional role of this molecule in the reproductive biology.

## SUMÁRIO

# CAPÍTULO I

## INTRODUÇÃO

O processo de fecundação em mamíferos depende de uma seqüência de eventos que culminam na ativação do oócito pelo espermatozóide (LÉDARE *et al.*, 1999). Este processo resulta na formação do zigoto, a partir do qual serão desenvolvidas duas populações celulares distintas, células somáticas e germinativas. A partir da diferenciação das células germinativas, são originados novos gametas com potencial para reiniciar o ciclo reprodutivo da espécie (McCARREY, 1993).

Durante o processo de espermatogênese, as células germinativas são submetidas à divisão mitótica, divisão meiótica e, adicionalmente, processos morfogênicos de diferenciação (NISHIMUNE and OKABE, 1993), resultando na formação de uma célula altamente diferenciada, capaz de atingir o trato reprodutor e o gameta femininos com o objetivo de restabelecer a diploidia (SALING and LAKOSKI, 1985; YANAGUIMACHI 2001).

O processo de diferenciação das células germinativas em espermatozóides envolve tanto um período gonadal, ocorrido no túbulo seminífero, como um período extra-gonadal, ocorrido no epidídimo, ducto deferente e trato reprodutor feminino (ORGEBIN-CRIST, 1967; DENIS, 1994). A regulação exata da diferenciação das células germinativas requer a expressão de genes em estágios específicos não somente nas células germinativas, mas também nas células somáticas testiculares e dos ductos pelos quais estas células transitam (DENIS, 1994; TANAKA et al, 1994; KIRCHHOFF et al, 1998). Desta maneira, embora tenham completado o processo de meiose e adquirido muitas das estruturas essenciais para o desenvolvimento da capacidade de fecundação, os espermatozóides do fluido testicular são pouco móveis e incapazes de fecundar (HAMIL et al, 2000). A capacidade de fecundação só é adquirida depois da passagem pelos

ductos eferentes, cabeça, corpo e cauda do epidídimo, bem como trato reprodutor feminino (DACHEUX et al, 1987).

Dentre os ductos pelos quais o espermatozóide passa até atingir o gameta feminino, o epidídimo é o responsável por providenciar um microambiente especializado no qual os espermatozóides desenvolvem importante grau de diferenciação funcional e antigênica. Além de transportar e armazenar os espermatozóides, o epidídimo induz alterações morfológicas e bioquímicas que permitem ao gameta potencializar a motilidade, estabilizar a cromatina e adquirir sítios de reconhecimento, ligação e fusão para as moléculas da zona pelúcida, bem como regulação indutiva para a reação acrossômica (ORGEBIN-CRIST, 1969; BOUÉ et al, 1994; MOORE, 1998; KAUNISTO et al, 1999). A elaboração deste microambiente especializado se deve ao trabalho conjunto dos diferentes tipos celulares que compõem o epitélio epididimário ao longo de toda a extensão do órgão. O epidídimo é subdividido em quatro segmentos principais, cabeça, corpo, cauda e um segmento inicial anterior à cabeça (FLICKINGER et al 1978; GOYAL, 1985; CUMMINS et al, 1986; CALVO et al, 1997; VICENTINI and ORSI, 1987; YEUNG et al, 1994). Cinco tipos celulares distintos compõem o epitélio pseudo-estratificado cilíndrico do epidídimo: células principais, basais, apicais, claras e, por fim, células halo (HAMILTON, 1975). Resumidamente, as células principais são as mais abundantes ao longo de todo o ducto epididimário e apresentam longos estereocílios relacionados com a atividade absortiva (GOYAL, 1985; CUMMINS et al, 1986). As pequenas células basais, mais numerosas no segmento inicial e no corpo, atuam como possíveis elementos de estabilização e renovação do epitélio (CALVO et al, 1997; VICENTINI and ORSI, 1987). As células apicais são aparentadas às principais e parecem estar envolvidas no transporte e digestão de proteínas e carboidratos do lúmen (GOYAL, 1985). Linfócitos intra-epiteliais são denominados células halo e as células claras, que ocorrem

fundamentalmente na cauda do epidídimo, apresentam função ainda controversa (MARTAN, 1969; FLICKINGER et al, 1978). Além do tipo celular, outras variações segmentares incluem a altura epitelial, que diminui da cabeça para a cauda do epidídimo, e os diâmetros tubular e luminal, que mostram comportamento oposto ao da altura epitelial.

O conjunto de eventos sofridos pelo espermatozóide durante a passagem pelo epidídimo é denominado maturação epididimária (DACHEUX et al, 1987). Durante a maturação epididimária, proteínas de superfície do espermatozóide podem ser alteradas, extraídas ou outras podem ser adicionadas, num processo complexo que resulta numa série de modificações essenciais e contribuem para a aquisição da capacidade de fecundação (FLESCH et al, 2000). A síntese e secreção de proteínas epididimárias é bem descrita em diferentes espécies, de camundongos a primatas (BOUÉ et al, 1995). Diversas proteínas sintetizadas pelas células epiteliais do epidídimo, inicialmente liberadas na luz deste órgão, parecem ser posteriormente localizadas na superfície do espermatozóide (VREEBURG et al, 1992; TULSIANI et al, 1993; EDDY and O'BRIEN, 1994; KIRCHHOFF and HALE, 1996) ou mesmo no interior da vesícula acrossômica (COHEN et al, 2000). Algumas dessas proteínas parecem ser envolvidas na ligação inicial do espermatozóide à zona pelúcida (BOUÉ et al, 1994) ou à membrana plasmática do oócito (SALING et al, 1985), bem como na posterior fusão de membranas entre oócito e espermatozóide (COHEN et al, 2000). Muitas destas proteínas secretadas pelas diferentes regiões do epidídimo correspondem a enzimas capazes de alterar as glicoproteínas de superfície do espermatozóide, sugerindo assim uma participação ativa do epidídimo na seqüência específica de modificação dos mesmos. A aquisição da capacidade de fecundação pelo espermatozóide tem sido correlacionada com a nova organização molecular adquirida pela membrana plasmática durante o fluxo do gameta a

partir da cabeça em direção à cauda do epidídimo (JONES et al, 1985; BOUÉ et al, 1995; HAMIL et al, 2000; MATHUR et al, 2000). Interessantemente, muitos estudos têm relatado que o desenvolvimento e a manutenção da função do epidídimo estão sob regulação hormonal primária de andrógenos testiculares e seus metabólitos derivados (JONES and CONNEL, 1982; NITTA et al, 1993; McMAHON et al, 1995; SONEA et al, 1997; SYNTIN et al, 1999; ROBAIRE et al, 2000; PASTOR-SOLER et al, 2002). Várias das proteínas produzidas pela região da cabeça do epidídimo e posteriormente incorporadas pelo espermatozóide apresentam caráter andrógeno-dependente (TEZON et al, 1985; BOUÉ et al, 1995; ELLERMAN et al, 1998; ROBAIRE et al, 2000).

O conhecimento acerca das proteínas expressas pelas células germinativas testiculares durante os processos contíguos de diferenciação, bem como das proteínas expressas pelas células dos ductos pelos quais os espermatozóides transitam durante o amadurecimento, pode amplamente contribuir com o desenvolvimento de pesquisas com fins científicos e clínicos envolvendo o processo fecundativo. Visto que muitas destas proteínas devem ser adquiridas pelo espermatozóide para sua maturação e, portanto, podem ser relevantes durante a fecundação, tais moléculas específicas poderiam ser utilizadas para o desenvolvimento de vacinas com potencial contraceptivo (NAZ et al, 1993; ELLERMAN et al, 1998), ou poderiam estar relacionadas à etiologia da infertilidade idiopática masculina (BOUÉ and SULLIVAN, 1996). A obtenção de anticorpos monoclonais que reconhecem os antígenos especificamente expressos durante o desenvolvimento dos espermatozóides, além de proporcionar ferramentas em potencial na investigação clínica da infertilidade, auto-imunidade e contracepção imunológica (FLORKE-GERLOFF et al, 1985; JASSIM and FESTENSTEIN, 1987; BOUÉ and SULLIVAN, 1996; ELLERMAN et al, 1998; PURI et al, 2000), têm constituído uma importante estratégia para permitir a geração de um mapa completo e integrado das

moléculas que, em conjunto, atuam na preparação do espermatozóide para a fecundação (WATANABE et al, 1992; ENDERS and MAY II, 1994; TSUCHIDA et al, 1995; TANAKA et al, 1997; KIRCHHOFF et al, 1998; PEREIRA et al, 1998).

A série do anticorpo monoclonal (Amc) TRA (testicular germ cells immunized to rat - monoclonal antibody), utilizado no desenvolvimento do presente trabalho, foi obtida no *Department of Science for Laboratory Animal Experimentation do Research Institute for Microbial Diseases* (Universidade de Osaka, Japão), com o intuito de reconhecer os antígenos de maior interesse no estudo da diferenciação das células germinativas testiculares (PEREIRA et al, 1998). Os resultados preliminares mostraram que um dos anticorpos selecionados, posteriormente denominado Amc TRA 54, reconhecia antígenos presentes no corpo cromatóide e posteriormente no acrossomo de espermatócitos e espermátides do estágio 1 a 12 de camundongos C57 BL/6 com idade igual ou superior a 24 dias pós-parto (d.p.p.). Por análise de Western blotting foram identificados para este antígeno 3 complexos de bandas alongadas (pesos moleculares aproximados de 200 kDa, 190 kDa e 85 kDa) (PEREIRA et al, 1998). A análise imunohistoquímica de outros órgãos de camundongos C57 BL6 mostrou que algumas células epiteliais da cabeça do epidídimo também apresentavam o antígeno correspondente ao Amc TRA 54, e que, embora ausente em espermatozóides testiculares, forte imunomarcação podia ser verificada no conteúdo luminal do epidídimo.

No contexto acima e com o intuito de contribuir para a compreensão dos complexos mecanismos que preparam o espermatozóide para a fecundação, o presente trabalho buscou caracterizar o modelo da expressão do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo quanto à ontogenia, características bioquímicas - peso molecular - e regulação da expressão. Em conjunto, os dados obtidos com o desenvolvimento deste trabalho podem grandemente contribuir no esclarecimento

do modelo da expressão de um antígeno identificado em células germinativas testiculares e em células somáticas do trato reprodutor masculino e, adicionalmente, podem permitir inferências acerca do papel funcional desta molécula na biologia da reprodução.

O conjunto dos experimentos conduzidos no desenvolvimento deste trabalho está apresentado nos capítulos subseqüentes sob a forma de dois artigos científicos, dos quais um está submetido para publicação em revista especializada. Cada capítulo apresenta formatação específica, de acordo com as normas editoriais da revista a qual o artigo correspondente está (Capítulo II) ou será (Capítulo III) submetido.

O primeiro trabalho, constante do Capitulo II, intitulado "Orquidopexy reverts the histological alterations in the epididymis and vas deferens caused by cryptorchidism", é resultado de análises morfológicas paralelas ao estudo da expressão do antígeno reconhecido pelo Amc TRA 54, conduzidas em epidídimos de animais submetidos ao criptorquidismo experimental.

O criptorquidismo é uma condição patológica na qual os testículos permanecem alojados na cavidade abdominal do organismo, submetidos desta forma a uma temperatura de 2 a 6 °C superior à temperatura da bolsa escrotal (FORESTA et al, 1996; GUNAY et al, 1998). Nestas condições, de desregulação térmica, a espermatogênese é interrompida (BRONSON and HEIDMAN 1993; FORESTA et al, 1996; BASIMOGLU-KOCA et al, 1998), impossibilitando o desenvolvimento das células germinativas e a chegada de espermatozóides no epidídimo. Resumidamente, o criptorquidismo experimental constituiu um modelo de estudo para verificação da influência da presença das células germinativas testiculares na expressão do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo. Paralelamente, foi observado que a condição criptorquídica, além de causar prejuízos à morfologia e fisiologia testiculares, resultava em

alterações na morfologia epididimária, as quais estão escassamente descritas na literatura quanto à incidência, "fenótipos" e possível reversibilidade. Este artigo científico descreve as lesões epididimárias e testiculares induzidas pelo criptorquidismo bem como a total reversibilidade das lesões em função da orquidopexia. Tal observação sugere que casos de infertilidade masculina pós orquidopexia não devem ter como etiologia alterações morfológicas do testículo e epidídimo.

O capitulo III, intitulado "Characterization of an antigen recognized by monoclonal antibody TRA 54 in mouse epididymal and vas deferens epithelial cells", está constituído pelo artigo que avalia a expressão do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo e ducto deferente em relação a: ontogenia, padrões de pesos moleculares e efeitos promovidos pelas condições experimentais de criptorquidismo e castração (McMAHON et al, 1995; SONEA et al, 1997; ROBAIRE et al, 2000). Estas duas últimas metodologias experimentais foram conduzidas com objetivo de verificar, respectivamente, a influência das células germinativas e de hormônios testiculares sobre a expressão do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo.

O capítulo IV apresenta uma breve conclusão elaborada a partir dos resultados obtidos com o desenvolvimento deste estudo, os quais embasam perspectivas metodológicas futuras para o estudo acerca da caracterização bioquímica e funcional do antígeno reconhecido pelo Amc TRA 54.

## OBJETIVOS

Os objetivos deste trabalho foram:

- Avaliar as alterações morfológicas sofridas pelo epidídimo e canal deferente causadas pela condição experimental de criptorquidismo e verificar a possível reversibilidade das mesmas por cirurgia de orquidopexia.

- Descrever a ontogenia da expressão do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais das diferentes regiões do epidídimo e do canal deferente;

- Determinar o peso molecular do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo e comparar os resultados obtidos com aqueles previamente publicados para o antígeno quando expresso nas células germinativas testiculares (PEREIRA et al, 1998), visando estabelecer a natureza molecular do antígeno expresso em duas populações celulares distintas.

- Verificar se a expressão do antígeno reconhecido pelo Amc TRA 54 pelas células epiteliais do epidídimo é dependente da presença de células germinativas ou de hormônios testiculares, bem como se a expressão do antígeno encontrado no epidídimo e canal deferente está sob mesma via de regulação.

## Orchidopexy reverts the histological alterations in the epididymis and v as deferens caused by cryptorchidism

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### Objectives

To assess the effects of cryptorchidism on the morphology of immature and mature epididymis and vas deferens, and to determine whether these alterations could reversed by orchidopexy.

### Material and Methods

Young (15 days old) and adult (60 days old) C57 BL6 mice were randomized into three g roups: control mice, bi/unilaterally cryptorchidic mice and bilaterally cryptorchidic mice with orchidopexy. The testes, epididymis and vas deferens were pulled from the scrotum via a bilateral laparotomy under anesthesia and the adipose tissue around the testes was attached to the muscular abdominal wall by a surgical knot. Six months later, orchidopexy was done under anesthesia; the adipose tissue was released from the abdominal wall and the organs were returned to the pelvic region. Control group consisted of intact mice. One month after orchidopexy, the testes, epididymis and vas deferens were collected and examined histologically. The testes were evaluated as a control for the cryptorchidic condition.

### Results

The epididymis from experimental cryptorchidic young and adult mice were strait, elongated and flattened. Focal areas of the epididymis showed flattening of the epithelial

cells, narrowing of the duct, disorganization of the tubular arrangement, the presence of abundant interstitial tissue throughout the stroma, and lymphocyte infiltration. The vas deferens did not show any alteration. Epididymis from cryptorchidic mice rendered orchidopexy was histologically similar to normal control epididymis.

**Conclusion**

Reestablishment of a normal testes topology was sufficient to restore normal spermatogenesis and the histological features of epididymis. These findings suggest that persistent male infertility clinically observed after orchidopexy surgery is unrelated to morphological alteration in the testis, epididymis and vas deferens.

**Keywords:** epididymis, cryptorchidism, orchidopexy

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**Introduction**

Cryptorchidism is a pathological condition in which the testes do not descend from the inguinal canal into the scrotum. With the retention of the testicles in the abdominal cavity, the seminiferous tubules become atrophic as a result of the increase in temperature which is unfavorable for spermatogenesis (1,2).

Gross abnormalities have been described in the epididymis and vas deferens in humans with cryptorchidic testes (3). Histological analysis of the epididymis and vas deferens has confirmed the immaturity of these organs, with the possibility that these alterations may adversely affect the potential for fertility (3). In addition, McMahon et al reported that cryptorchidism induced by antiandrogens resulted in epididymal malformations in pig (4). However, it was unclear whether these changes resulted from a non-descending testis or from antiandrogen administration (5).

Some studies have examined the ability of orchidopexy to restore spermatogenesis in experimentally cryptorchidic testis (6), but is unclear whether the histological changes in the epididymis and vas deferens caused by cryptorchidism were reverted (3).

In this study, we examined the changes in epididymal and vas deferens histology following uni and bilateral surgical cryptorchidism and their reversal by orchidopexy. Since testes from cryptorchidic newborn boys never complete a wave of spermatogenesis, we use immature mice to investigate whether previous contact of the epididymis with differentiated sperm cells could influence the pattern of epididymal development.

## Material and Methods

### Animals

Mature (60 days old) and immature (15 days old) C57 BL6 mice were housed in a controlled environment at 22 +/- $2^0$C with lights from 6:00 to 20:00h. The mice had free access to pelleted laboratory chow and tap water. The mice were allocated to the following groups:

Group I. Mature mice

- Subgroup 1: intact 60-days-old mice **(mature control mice)** (*n= 3)*
- Subgroup 2: 60-days-old mice subjected to bilateral (*n= 9)* or unilateral (*n=9)* surgical cryptorchidism **(mature cryptorchidic mice)**
- Subgroup 3: 60-days-old mice subjected to bilateral surgical cryptorchidism followed by orchidopexy (*n= 5)* **(mature orchidopexy mice)**

Group II. Immature mice

- Subgroup 1: intact 15-days-old mice **(immature control mice)** (*n= 3)*
- Subgroup 2: 15-days-old mice subjected to bilateral (*n= 9)* or unilateral (*n=9)* surgical cryptorchidism **(immature cryptorchidic mice)**
- Subgroup 3: 15-days-old mice subjected to bilateral surgical cryptorchidism followed by orchidopexy (*n= 5)* **(immature orchidopexy mice)**

This study was approved by the institutional Committee for Ethics in Animal Experimentation (protocol number 126-1). The NIH guidelines for the care and use of laboratory animals were followed.

*Surgical procedure for experimental cryptorchidism*

Mice were anesthetized with Ketamine (0.26 g/kg, i.p.) and xylazine (0.13 g/kg, i.p.). Surgery was done under aseptic conditions according a procedure described elsewhere (8). Following a lateral laparotomy, the adipose tissue around the epididymis was pulled from the scrotum with tweezers and then sutured to the abdominal muscle wall. For unilateral cryptorchidim, only right testicle was manipulated. The testes, epididymis and vas deferens from mice bilateral or unilateral cryptorchidism were collected 1, 3 and 6 months after the surgical intervention (3 mice per group).

*Surgical procedure for orchidopexy*

Six months after producing cryptorchidism, the mice were anesthetized and a lateral laparotomy was done to allow the adipose tissue of the testis to be released from

the abdominal wall and pushed back into the scrotum (8). The testis, epididymis and vas deferens were collected one month after orchidopexy.

*Collection and Preparation of Tissues*

Mice were sacrificed with an overdose of anesthetic and the testes, epididymis and vas deferens from control and surgically treated mice were immediately removed and fixed in Bouin solution for 12 h, followed by embedding in paraffin and sectioning (5 μm thick). Deparaffinized sections were stained with hematoxylin-eosin and examined by light microscopy.

## Results

*Mature control group*

The testis from control mature mice showed seminiferous tubules with complete spermatogenesis and germ cells in several stages of development, as well as spermatozoa (Figure 1a). The epididymis of mature mice showed a population of fully differentiated cells throughout the distinct segments of the duct (Plate I, Figures 1b - d) as described elsewhere (8, 9, 10, 11). In the caput segment, principal, apical and basal cells could be distinguished composing a columnar pseudostratified epididymal epithelium. The same epithelial cell types occurred along the epididymis corpus and cauda, but the height of the epithelium gradually decreased in these segments. In the caudal segment, the epithelial cells and stereocilia were very short and the luminal cavity was extensive large and contained aggregates of spermatozoa (Plate I, Figure 1d). A thin layer of smooth muscle cells surrounded the entire epididymal duct and the interstitial tissue was apparent (Plate I, Figures 1b - d). The pseudostratified epithelium of the vas deferens was tall and

the epithelial cells showed long stereocilia. The luminal content consisted of abundant mass of spermatozoa and two large layers of smooth muscle (longitudinal outer layer and circular inner layer) surrounded the duct (Plate I, Figure 1e).

*Mature cryptorchidic group*

Analyses of the testis confirmed the characteristic cryptorchidic condition in which the gonad was atrophic and the seminiferous tubules were completely devoid of spermatocytes, spermatids and spermatozoa; this population of maturing cells was replaced by several large stores (Plate I, Figure 2a). The seminiferous epithelium was lined by spermatogonia and Sertoli cells. Leydig cells were abundant in the intertubular tissue.

The epididymidis of experimentally cryptorchidic mice were strait, elongated and flattened when compared with that from control mice. The histological structures of these epididymidis were altered to diverse extents. In bilaterally cryptorchidic mice, the anomalies were bilateral. In unilaterally cryptorchidic mice, the contralateral intact epididymis had histological features similar to control mice, whereas the epididymis of cryptorchidic testis showed the same alterations as epididymis from bilaterally cryptorchidic mice. The alterations were more evident 6 months after surgery, although 1 and 3 months was enough to produce the effects characteristic of cryptorchidic condition. Fine histological alterations were observed in cryptorchidic epididymis (Plate I, Figures 2b-d) when compared with control mature epididymis (Figures 1b - d). The alteration consisted of narrowing of the epididymal duct (Plate I, Figure 2b), flattening of the epithelial cells in focal areas (Plate I, Figure 2c), disorganization of the tubular arrangement (Plate I, Figures 2b - d) and the presence of abundant interstitial tissue

throughout the entire epididymis (Plate I, Figure 2d). Lymphocyte infiltration into the interstitial tissue was sometimes observed (Plate I, Figure 2d). In the vas deferens, the epithelium appeared to be unaltered, but luminal aggregates of spermatozoa were replaced by erythrocytes or degenerated germ cells (Plate I, Figure 2e).

*Mature orchidopexy group*

Analyses of the testis confirmed the recovering of normal spermatogenesis in previously cryptorchidic mice (Plate I, Figure 3a); rare focal areas of hypospermatogenesis were observed (not show).

The epididymis and vas deferens of bilaterally cryptorchidic mice rendered orchidopexy (Plate I, Figures 3b - e) did not show any of the morphological alterations seen in the cryptorchidic group (Plate I, Figures 2b - e). The lumen of the entire epididymal duct was plentiful filled with sperm aggregates.

*Immature control group*

The testis was devoid of sperms and the seminiferous tubules consisted of irregular series of a small number of spermatogonies, spermatocytes and Sertoli cells (Plate II, Figure 1a). Leydig cells were normally placed between the seminiferous tubules.

At this stage of the post-natal development, it was not possible to identify the three epididymal segments. Epithelial cells in the continuous segments, caput, corpus and cauda of the epididymis showed similar, undifferentiated characteristics, including short height and juxtaposition of the cells (Plate II, Figures 1b - d). The epithelial organization of

the vas deferens, and of the stereocilia and muscle layers was poorly defined at this stage of development (Plate II, Figure 1e).

*Immature cryptorchidic group*

The testis from cryptorchidic immature mice showed an early arrest in maturation, once its feature was similar that of control immature testis. The most advanced stage of spermatogenesis in the seminiferous tubules was the spermatocyte stage (Plate II, Figure 2a). Sertoli cells were present in the basal line of the seminiferous epithelium and Leydig cells were present in the intertubular tissue.

The epididymis from these mice (Plate II, Figures 2b - d), although histologycally differentiated than that of control immature epididymis (Plate II, Figures 1b - d) showed different characteristics from those of control adult epididymis (Plate I, Figures 1b - d). The epithelium of the caput segment was columnar and pseudostratified, with principal, apical and basal cells, as well as many focal areas of cuboid cells (Plate II, Figure 2b). In these areas, the basal and apical cells could be not distinguished and the stereocilia were generally poorly defined; the nuclear chromatin of the cells was deeply basophilic. In the corpus segment, the epithelial epididymal cells were close together. Some sections were narrow (Plate II, Figure 2c) and surrounded by abundant interstitial tissue when compared with control adult epididymis corpus (Plate I, Figure 1c). The normal lobular arrangement of the epididymis was not always seen. The caudal segment was the most differentiated segment of the epididymis in this experimental group, but focal areas showed very flattened epithelial cells (Plate II, Figure 2d). The lumen of the entire duct was either empty or filled with fluid containing erythrocytes or degenerated germ cells; no sperm were present (Plate II, Figures 2b - d). The epithelial cells of the vas deferens were well

organized in a pseudostratified cylindrical epithelium; the lumen of this epithelium was either empty or contained maturing or degenerating germ cell (Plate II, Figure 2e).

*Immature orchidopexy group*

The testis from cryptorchidic immature mice rendered orchidopexy showed full development with normal spermatogenesis (Plate II, Figure 3a). Rare focal areas of hypospermatogenesis were observed (not show).

The epididymis and vas deferens were filled with sperm aggregates and showed adult morphological features (Plate II, Figures 3b - e) similar to those of control mature mice (Plate I, Figures 1b - e).

**Discussion**

The aim of this study was evaluate the histological changes in the epididymis and vas deferens following experimental uni- and bilateral cryptorchidism in mature and immature mice, and to determine whether observed alterations could be reversed by orchidopexy.

Immature and mature mice were both used to assess whether previous contact of the epididymis with differentiated sperm cells could influence epididymal development. Spermatogenesis in testis from immature cryptorchidic mice was blocked before the accomplishment of the first wave of spermatogenesis, leaving the epididymis devoid with differentiated sperm cells, as occurs in the epididymis of newborn cryptorchidic boys. The morphology of the epididymis of bilaterally cryptorchidic mice varied according to the pos-natal period in which cryptorchidism was performed. The epididymal anomalies were more

evident in immature cryptorchidic mice, probably because of a retardation of epididymal development (12).

In unilateral cryptorchidism, the contralateral epididymis of mature and immature mice show no alterations compared with normal control epididymis. Cryptorchidism is well known to have a harmful effect on testicular germ cells, since the higher temperature of the abdominal cavity increases the apoptotic index in meiotically dividing cells, thus creating an unfavorable environment for spermatogenesis (13). However, little is known about the mediators or events involved in the epididymal alterations. Our results suggest that the epididymal alterations could be caused by the absence of some testicular epithelial factor derived from developing germ, Sertoli or Leydig cells. According to Jegou and workers (14), testicular Sertoli and Leydig cells are preserved in cryptorchidism, and there is no significant difference between cryptorchidic and control mice. As shown here, the contralateral epididymis of unilaterally cryptorchidic mice was histologically similar to control epididymis, whereas epididymis from the cryptorchidic side had the same alterations as epididymis from bilaterally cryptorchidic mice. It is possible that local rather than systemic concentrations of androgens have a predominant effect on epididymal physiology (15,16).

Another possible hypothesis concerning the alterations seen in epididymal morphology could be that since in experimental cryptorchidism the epididymidis and testis are retained in the abdominal cavity. Thus, the change in location could lead to a local reduction in blood flow, in addition to the increase in temperature. Together, these changes could result in less than ideal condition for metabolism in the epididymis.

Cryptorchidism can be prejudicial to male reproductive physiology since the histological alterations in the epididymis could result in the functional damage to this duct,

e.g. loss of biosynthetic and contractile activity by the principal and muscle cells, respectively. Since the epididymis is important in sperm maturation aimed fecundation (17,18), the possibility of reversing the histological alterations caused by cryptrochidism is of clinical relevance. In according to DePalma et al (3), it is unclear whether changes in the epididymis and vas deferens would disappear with orchidopexy. In addition, cryptorchidism is reported to irreversibly alter the structure of epididymis (5, 12). However, as shown here, the epididymis of experimentally cryptrochidic mice recovered its morphology (mature group) and developed normally (immature group) after reverse surgical intervention. This restoration most likely resulted from the replacement of the testis into the scrotum thereby allowing the reestablishment of the normal physiological temperature and the release of testosterone from the testis to epididymis by paracrine via (15).

The histological structure of the vas deferens was not altered by cryptorchidism, suggesting that the development and maintenance of the vas deferens and epididymis are controlled independently.

In conclusion, returning the testis to its normal topology was enough to restore normal spermatogenesis and the histology of epididymis. These results provide further indirect evidence that persistent male infertility after orchidopexy (3) is unrelated to morphological alterations in the testis, epididymis and vas deferens.

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**Plate Legends**

**Plate I**

Photomicrographs of testis, epididymis (caput, corpus and cauda) and vas deferens of mature control (1a-1e), cryptorchidic (2a-2e) and orchidopexy (3a-3e) mice. Scale bar: 74μm.

Testes from control mature mice showed complete spermatogenesis (1a). The epithelium of the epididymis and vas deferens contained fully differentiated cells. The lumen of the epididymis and vas deferens contained numerous spermatozoa (1b - e). Testes from cryptorchidic mice were devoid of spermatocytes, spermatids and spermatozoa (2a). The epididymis had an altered epithelium and interstitial tissue (2b - d), whereas vas deferens was unaltered (2e). Testes from cryptorchidic mice subjected to orchidopexy showed normal spermatogenesis (3a). The epididymis had a normal histological appearance (3b - d), as did the vas deferens (3e). Both the epididymis and the vas deferens were full of sperm aggregates.

**Plate II**

Photomicrographs of testis, epididymis (caput, corpus and cauda) and vas deferens of immature control (1a-1e), cryptorchidic (2a-2e) and orchidopexy (3a-3e) mice. Scale bar: 142 μm.

Testes from control immature mice were devoid of sperm (1a), and the three main segments of the epididymis (caput, corpus and cauda) were practically undifferentiated (1b - d). The organization of the vas deferens epithelium, the stereocilia and the muscle layers was poorly defined at this stage of development (1e). No spermatozoa were observed are in he lumen of the epididymis and vas deferens. Testes from cryptorchidic mice showed features similar to those of the control immature group (2a) (maturation arrest). The epithelial cells incompletely differentiated throughout the epididymis. The arrangement of the tubular sections was irregular and there was abundant interstitial tissue (2b - d). The epithelial cells of the vas deferens were well organized in a pseudostratified cylindrical epithelium (2e). Testes from cryptorchidic mice subjected to orchidopexy reached a full development with normal spermatogenesis (3a). The epididymis showed normal morphological features, with fully differentiated segments and cell populations (3b - d) (Compare with figure 1a - c). The vas deferens showed a normal morphology (3e). Spermatozoa were abundant in the lumen of the epididymis and vas deferens.

Plate I

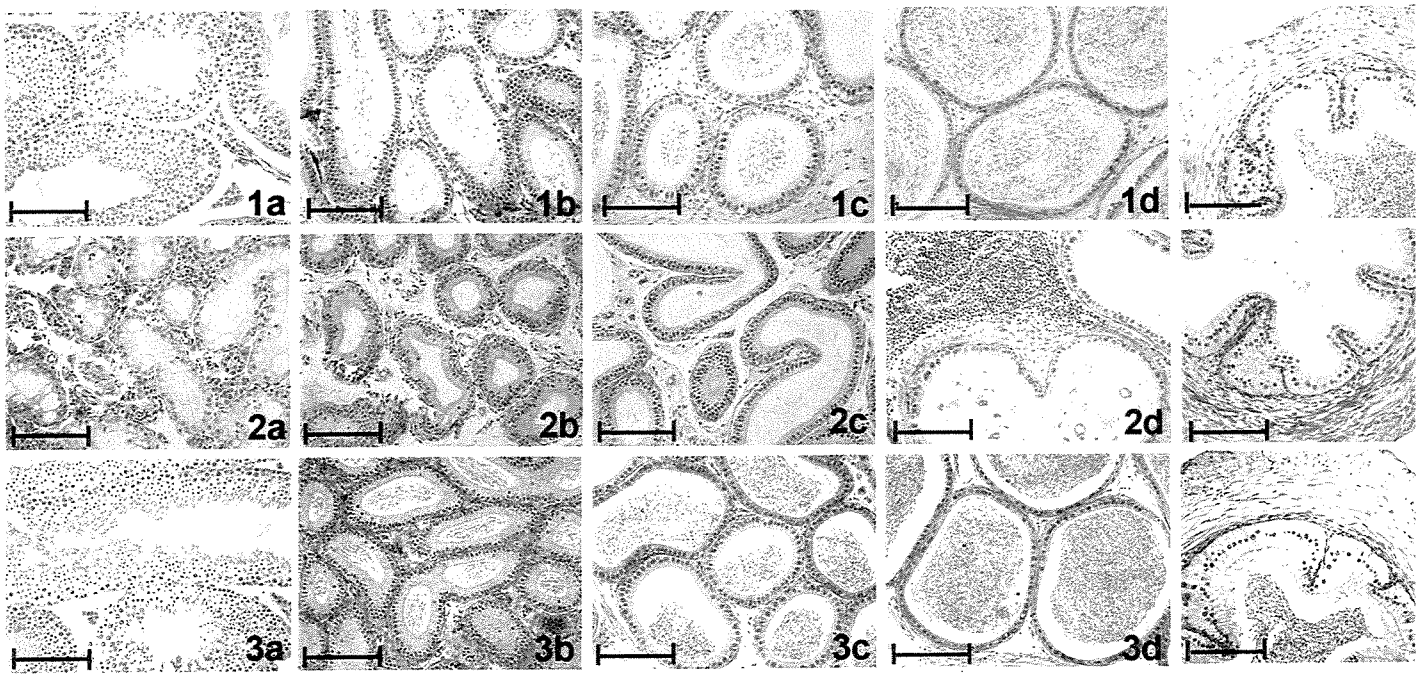
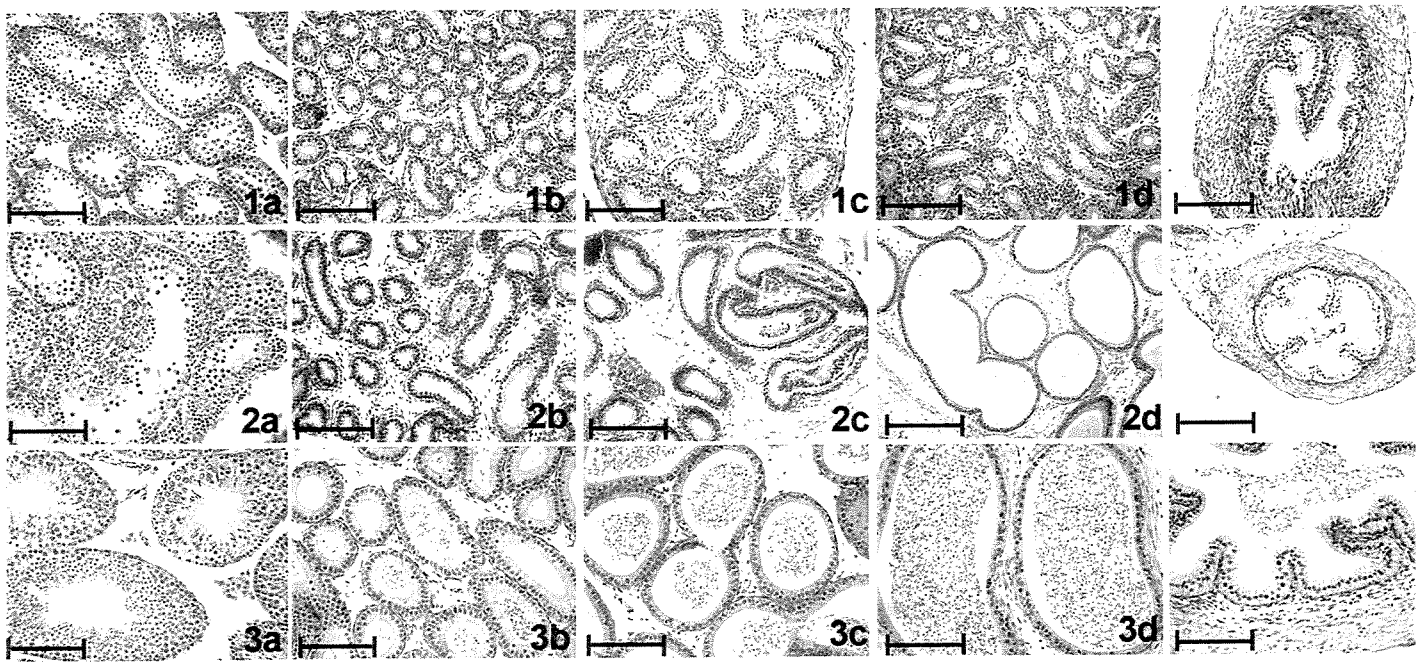



Plate II



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# CHARACTERIZATION OF AN ANTIGEN RECOGNIZED BY MONOCLONAL ANTIBODY TRA 54 IN MOUSE EPIDIDYMAL AND VAS DEFERENS EPITHELIAL CELLS

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Running title: Characterization of an antigen recognized by monoclonal antibody TRA 54.

## ABSTRACT

The epididymis is known to be involved in the biochemical maturation of sperms. The monoclonal antibody (mAb) TRA 54 recognizes an antigen in epididymal epithelial cells of the caput, and in spermatocytes and spermatids of the seminiferous tubules of C57 BL6 mice more than 24 days old. In this study, we investigated the ontogenic expression of this antigen in epididymis. Expression of the antigen in the epithelium of the caput started at around 24 days of age and increased during sexual maturation. Indirect immunohistochemistry with mAb TRA 54 in cross-sections of testis and epididymis from uni- and bilaterally castrated and uni- and bilaterally experimentally cryptorchid mice showed that castration and cryptorchidism altered the epididymal morphology to different extents. Whereas the pattern of antigen expression in the epididymis was unaltered in cryptorchid mice, bilateral castration abolished. Bilaterally castrated mice given hormonal testosterone replacement showed a normal pattern of antigen expression. These observations indicate that the expression of this antigen in epididymis and testis occur independently. A testicular hormonal factor appears to be essential for epididymal maturation and for maintaining antigen expression in the epididymis of cryptorchidic mice. Immunoblotting of testicular and epididymal caput proteins using mAb TRA 54 revealed slightly different patterns of antigen expression in spermatids and epididymal cells, suggesting that the antigen is expressed in two different isoforms with a common epitope. Analysis of the vas deferens suggested that the vas deferens and epididymis could be regulated by the same androgen in adult post-natal life.

## INTRODUCTION

Fertilization in mammals depends on a sequence of events that culminates in the activation of an oocyte by sperm (Saling, 1996). Upon leaving the testis, mammalian

spermatozoa move through the duct system formed by the vasa efferentia, epididymis, and vas deferens. The functions of the epididymis include the absorption of seminiferous fluid and the concentration, transport and storage of spermatozoa (Lédare et al, 1999). The functional and antigenic modification of spermatozoids by the epididymal environment, a process known as epididymal maturation (Dacheuxs, 1987; Kaunisto et al, 1999; Mathur et al, 2000), involves changes in the antigen profile and expression, as addition of new molecules on the surface of gametes (Jones et al, 1985; Toshimori et al, 1988; Vreeburg et al, 1992; Tulsiani et al, 1993; Eddy and O'Brien, 1994; Kirchhoff and Hale, 1996; Kirchhoff, 1998).

The alterations that occur in spermatozoa during epididymal maturation are essential for the success of fertilization (Yanagimachi, 1994). However, relatively little is known about the actual role of the antigens released by the epididymis (Moore, 1998). The study of antigens expressed by cells of the male reproductive organs using monoclonal antibodies (mAb) has contributed to our understanding of their role in sperm formation (Watanabe et al, 1992; Enders and May II, 1994; Tsuchida et al, 1995; Kirchhoff et al, 1998). The mAb TRA 54 (testicular germ cells immunized to rat - monoclonal antibody) recognizes an antigen expressed exclusively in in spermatocytes and spermatids of the seminiferous tubules of C57 BL6 mice more than 24 days old (Pereira et al, 1998).

In this study, we investigated some of the properties and ontogenic expression of this antigen in epididymis and vas deferens. We also examined whether expression of the antigen in epididymis and vas deferens was dependent on the presence of sperm or was regulated by testicular testosterone.

## MATERIAL AND METHODS

### Preparation of Monoclonal Antibody

The monoclonal antibody was produced as described by Pereira et al (1998).

### Animals

C57 BL6 mice were housed under standard co conditions of temperature and light, daylight, with free access to water and food. The mice were sacrificed by cervical dislocation and the testes, epididymides and vas deferens were immediately removed.

### Ontogeny

The ontogenic development of mAb TRA 54 antigen in epididymis and vas deferens was studied in C57 BL6 mice 1, 5, 12, 16, 24, 30 and 60 days old. Testes, epididymides, vas deferens and other organs were immediately removed and fixed in Bouin solution for subsequent immunohistochemical analysis.

### Experimental cryptorchidism

To examine influence of developing germ cells on antigen expression in the epididymis and vas deferens, adult and 15-day-old mice underwent experimental uni- and bilateral cryptorchidism (Nishimune et al, 1978). For unilateral cryptorchidism, the right testicle was manipulated. The mice were sacrificed three months after the operation and the epididymides and vas deferens were removed and fixed in Bouin solution.

**Castration**

Adult and 15-day-old mice were uni- and bilaterally castrated by the abdominal route under anesthesia (Mirosevich et al, 1999, Keenan and Thomas, 1975), and the epididymides were replaced close to the scrotum. For unilateral castration, the right testicle was removed. Three months after the operation, the epididymides and vas deferens were removed and fixed in Bouin solution.

**Hormonal replacement**

The effect of testosterone on the expression of the epididymal antigen recognized by mAb TRA 54 was examined in adult and 15-day-old bilaterally castrated mice with testosterone replacement. The testes were removed via an abdominal incision as described above and 30 days after castration, testosterone was administered (physiological dose 3 μg/g body weight; Deposteron – testosterone cipionate, Novaquímica, Sigma, Brazil) (Referencia da simone). The hormone was diluted in mineral oil and given daily (intraperitoneal injections) for 10, 20 and 30 days. The epididymides and vas deferens were subsequently collected and fixed in Bouin solution for imunohistochemistry. Control mice received mineral oil alone and the epididymis and vas deferens were collected 30 days after the start of treatment.

**Immunohistochemistry**

Immunohistochemistry was done according to Pereira et al (1998). The tissues were fixed in Bouin solution and embedded in paraffin. Non- specific sites in desparaffinized sections were blocked with 20% normal goat serum. The sections were incubated overnight with mAb TRA 54 acitic fluid (diluted 1:4000 in PBS/1%BSA) followed by incubation with biotinylated secondary antibody, and then Strept ABC kit (DAKO A/S,

Glostrup, Denmark). The reaction was developed by incubating the slides with hydrogen peroxide and diaminobenzidine and the sections were counterstained with hematoxylin. Control sections were treated similarly but without mAb TRA 54.

**Immunoblotting**

Testes, epididymis (caput, corpus and cauda segments) and other organs (brain, liver, kidney, spleen and seminal vesicle) from adult C57 BL6 mice were homogenized in 10mM Tris HCl, pH 7.4, containing 10mM EDTA, 10mM sodium pyrophosphate, 100mM sodium fluoride, 10mM sodium orthavonadate, 2mM PMSF and 0,1mg of aprotinina /ml. The homogenates were centrifuged and the protein concentration of the supernatants was determined using a Bradford (Bio-Rad) protein assay kit (Bio-Rad, Richmond, CA, USA). Aliquots of each tissue were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). The proteins were transferred electrophoretically to polyvinylidenedifluoride (PVDF) membrane filters (Millipore, Bedford, MA, USA), which were then blocked with 5% low-fat dry milk and washed with TBS-T. The filters were subsequemtly incubated with mAb TRA 54 (diluted 1:2500 in TBS-T), washed in TBS-T, and finally incubated with an anti-rat Ig conjugated to peroxidase. Reactive bands were detected by incubating the membrane filters with 0.03% hydrogen peroxide and 0,05% diaminobenzidine in 50 mM Tris-HCl, pH 7,2. To determine whether the epitope of the antigen detected by mAb TRA 54 resides in sugar moieties, the immunoblots were treated with periodate as described by Woodward et al (1985).

## RESULTS

### Ontogeny

No immunohistochemical staining was observed in epididymal and vas deferens sections from mice less 24 days old (Figure 1). In contrast, the mAb TRA 54 recognized antigen from 24 days onwards, when the brush border of the epididymal caput and corpus sections were stained (Figure 2 and 2a). The caudal segment contained no antigen (Figure 2b) whereas the stereocilia and apical cytoplasm of vas deferens epithelial cells were stained with mAb TRA 54 (Figure 2c). The initial segment of the epididymis showed no staining at any time (Figure 3), but the apical cytoplasm of caput epithelial cells and the brush border of caput and corpus epithelial cells of adult (60-day-old) mice were strongly stained (Figure 3). The staining of the supranuclear cytoplasm in the caput segment was diffuse and homogenous (Figure 3a), while in the caudal segment only the luminal content was stained (Figure 3b). The stereocilia and cytoplasm of the vas deferens epithelial cells were also strongly stained (Figure 3c).

### Cryptorchidism

The pattern of antigen expression in cryptorchidic mice was similar to that seen in adult control (non- cryptorchidic) mice (Figure 4). Focal areas of stained residual spermatozoa were seen in the lumen of the epididymis and vas deferens.

### Castration

As with cryptorchidism, unilateral castration had no effect on the expression of the antigen compared to adult control (non-castrated mice). Indirect immunohistochemical staining using mAb TRA 54 on cross-sections from 15-day-old and adult epididymis and vas deferens from bilaterally castrated mice showed that there was a loss of the antigen

recognized by mAb TRA 54 since no cell, fluid or other structure was stained (Figures 5a-b). In the vas deferens, weak positive staining was restricted to some focal areas of the remaining fluid in the brush border of the cells (Figure 5c).

**Hormonal replacement**

After 30 days of bilateral castration, daily injections of testosterone for 20 days were sufficient to reestablish the pattern of antigenic expression pattern in the epididymis and vas deferens (Figure 6) seen in adult control mice (Figure 3). In control mice treated with mineral oil alone, no expression was observed in the epididymal duct or vas deferens. Whereas hormonal replacement restored the morphology of the epididymis and vas deferens, in control castrated mice (data not show) the same ducts were similar to very immature epididymis. The results obtained with indirect immunohistochemistry using mAb TRA 54 are summarized in table 1.

**Immunoblot analysis**

Detergent-extracted protein from testes, epididymides and other mouse organs were separated by SDS-PAGE, electrotransferred from the gel to a PVDF membrane and reacted with mAb TRA 54 antibody. The antibody did not cross-react with extracts from brain, liver, kidney or spleen, but immunoreactive bands of 260, 200, 115 and 90 kDa were detected in testis extracts (Figures 7 and 8). Immunoreactive bands of 260 and 200 kDa were also detected in caput, and weakly in the epididymal corpus, cauda and vas deferens whereas the 115 and 90 kDa bands were not (Figure 8). Blot treated with 1 mM sodium metaperiodate were considerably less immunoreactive, and immunoreactivity was completely lost after treatment with 10 or 20 mM sodium metaperiodate (Figure 8). The

molecular mass of the immunoreactive bands was unaffected by reducing conditions (data not show).

TABLE 1. Presence (+) or absence (-) of a positive immunoreaction to mAb TRA 54 in epididymis and vas deferens under different experimental conditions. Caput I, II and III refer to the initial, proximal and distal segments of the epididyma caput I, respectively.

| | **Caput I** (cells/ lumen) | **Caput II** (cells/ lumen) | **Caput III** (cells/ lumen) | **Corpus** *(cells/ lumen)* | **Cauda** (cells/ lumen) | **Vas deferens** (cells/ lumen) |
|---|---|---|---|---|---|---|
| **16 days** | - / - | - / - | - / - | - / - | - / - | - / - |
| **24 days** | - / - | - / + | +/+ | - / + | - / - | +/+ |
| **Adult** | - / - | +/+ | +/+ | - / + | - / + | +/+ |
| **Cryptorchid** | - / - | +/+ | +/+ | - / + | - / - | +/+ |
| **Castrated** | - / - | - / - | - / - | - / - | - / - | - / - |
| **Castrated with testosterone replacement** | - / - | +/+ | +/+ | - / + | - / + | +/+ |

## DISCUSSION

Spermatozoa in testicular fluid have weak forward motility and can not interact with unfertilized eggs. As they pass through the efferent ducts and the initial segment, caput, corpus and cauda of the epididymis, the spermatozoa undergo further maturation to acquire the ability to fertilize eggs. Enzymes that alter the sperm surface are secreted by

different regions of the epididymis, indicating that there are surface modifications during passage through the epididymis (Hamil et al, 2000). To identify new molecules involved in sperm maturation, we have characterized an antigen recognized by mAb TRA 54 in the epididymis of mice, expressed in a highly cell-, region- and age-specific manner.

Pereira et al (1998) reported that an antigen recognized by mAb TRA 54 was present in spermatocytes and spermatids from the seminiferous tubules of mice more than 24 days old. Expression of this antigen was limited to the cytoplasm of a specific cell population along the epididymal and vas deferens ducts of 24-day-old mice. This observation confirmed that the epithelial cells along the epididymis and vas deferens had specific functions and were regulated independently, with region- and cell- specific patterns of gene expression within the epithelium (Robaire et al, 2000, Rodriguez et al, 2001). In addition to epithelial cells, the antigen was also recognized in luminal spermatozoa. Since testicular sperm did not react with mAb TRA 54 (Pereira et al, 1998) whereas luminal epididymal and sperm were strongly stained, we conducted that the antigen recognized by mAb TRA 54 could be a protein secreted by the epididymal caput which then bound to the surface of spermatozoa during their transit through the epididymis. Other immunohistochemical studies have shown that some antigen produced in the columnar epithelium of the epididymis pass into the tubule where they associate with the spermatozoa surface or acrosomal vesicle during maturation in the duct (Poulton et al, 1996, Cohen et al, 2000).

Expression of the antigen recognized by mAb TRA 54 in epididymal epithelial cells and in testicular germ cells occurs independently in these two regions since the pattern of antigen expression was not affected by the cryptorchidism, despite the absence of spermatogenesis in the testis. Interessantly, about 48% of proteins released by the

epididymal caput are dependent of androgens (Syntin et al, 1999; Robaire et al, 2000) such as testosterone released by Leydig cells. Nistal and Jiménez-Heffernan (1997) reported that the interstitial Leydig cells are functional in cryptorchidism conditions. Thus, the release of testicular androgens to the epididymis could be involved in maintaining the antigen recognized by mAb TRA 54 in epidydimal epithelial cells.

Indirect immunohistochemistry in the epididymis and vas deferens of bilaterally castrated adult mice showed that mAb TRA 54 did not recognize the antigen in this condition. Similar results were obtained with 15 day-old-mice, allows suggest that the antigen has never been synthesized. In contrast, unilateral castration did not affect expression of the antigen in right and left epididymis and vas deferens. Together, these results indicate the systemic action of testicular factors in regulating the epididymal and vas deferens expression of the antigen. In agreement with this, the treatment of bilaterally castrated mice with testosterone for 20 days restored normal pattern of antigen expression in epididymis and vas deferens. These results reinforce our suggestion that testosterone is essential for full expression of the antigen recognized by mAb TRA 54 in epididymal epithelial cells.

Immunoblot analysis showed that the antigens recognized by mAb TRA 54 in testis and epididymis were very similar. The presence of different immunoreactive bands probably reflected the occurrence of different isoforms of the same molecule, both with a common epitope recognized by mAb TRA 54. Similar antigenic variation in a given molecule has also been were reported by others (Hall, et al, 1996; Syntin and Cornwakk, 1999). This antigenic epitope of mAb TRA 54 must be carbohydrate since it was sensitive to periodate treatment. Alterations in the carbohydrate chains of glycoconjugates during

spermatogenesis can explain the loss of reactivity in the acrosome of late testicular spermatids, result of antigen masking (Toshimori et al, 1991; Pereira et al, 1998).

The results described here show that the antigen molecule detected by mAb TRA 54 is produced by the epithelium of the epididymal caput and secreted into the epididymal lumen, from to here it moves down to corpus and cauda of the organ. The molecule appears to adhere to the epithelial cilia in the corpus and cauda and to luminal spermartozoa. The vas deferens also synthesizes and releases a similar molecule to the lumen through which spermatozoa transit. Antigen transfer from the caput to cauda occurs in the absence of sperm (cryptorchidism), indicating that the antigen can be transcribed in epididymal caput cells independently of the presence of testicular germ cells. The synthesis and secretion of antigen recognized by mAb TRA 54 in the epididymal caput and vas deferens cells are regulated by androgens, although different isoforms appears to be produced in each tissue. Finally, our results strongly suggest that the antigen must play an important role in testicular spermatogenesis and subsequent epididymal maturation.

## Figure Legends

**Figure 1.** Immunohistochemical staining with mAb TRA 54 in a cross-section of epididymis from a 16-day-old mouse (22x). **1a:** detail of the transition initial segment (*) and caput segment. Scale bar: 50 μm. **1b:** detail of the cauda segment. Scale bar: 50 μm. **1c:** detail of vas deferens. Scale bar: 50 μm (x). Antigen recognized by mAb TRA 54 was found in any cell or structure.

**Figure 2.** Immunohistochemical staining with mAb TRA 54 in a cross-section of epididymis from a 24-day-old mouse (13x). Expression of the antigen recognized by mAb TRA 54 was observed in the distal caput of the duct. **2a:** detail of the caput segment; antigen was found in the apical brush border of the epithelial cells. Scale bar: 50 μm. **2b:** detail of the cauda segment. Scale bar: 50 μm. **2c:** detail of vas deferens. The stereocilia and apical cytoplasm of the cells were stained by mAb TRA 54. Scale bar: 50 μm.

**Figure 3.** Immunohistochemical staining with mAb TRA 54 in a cross-section of epididymis from an adult mouse (8x). The antigen recognized by mAb TRA 54 was strongly expressed, except in the initial segment of the epididymis. **3a:** detail of the caput segment. No antigen was found in tubular sections of the initial segment (arrow), but was present in the apical cytoplasm of the epithelial cells in the caput segment, as well as in the brush border and luminal content of the duct. Scale bar: 50 μm. **3b:** detail of the cauda segment. Antigen was found in luminal spermatozoa and in some focal areas of the brush border. Scale bar: 50 μm. **3c:** detail of vas deferens. The stereocilia and cytoplasm of the cells were strongly with mAb TRA 54. Scale bar: 50 μm.

**Figure 4.** Immunohistochemical staining with mAb TRA 54 in a cross-section of epididymis from a bilaterally cryptorchid 15-day-old mouse. The pattern of the antigen

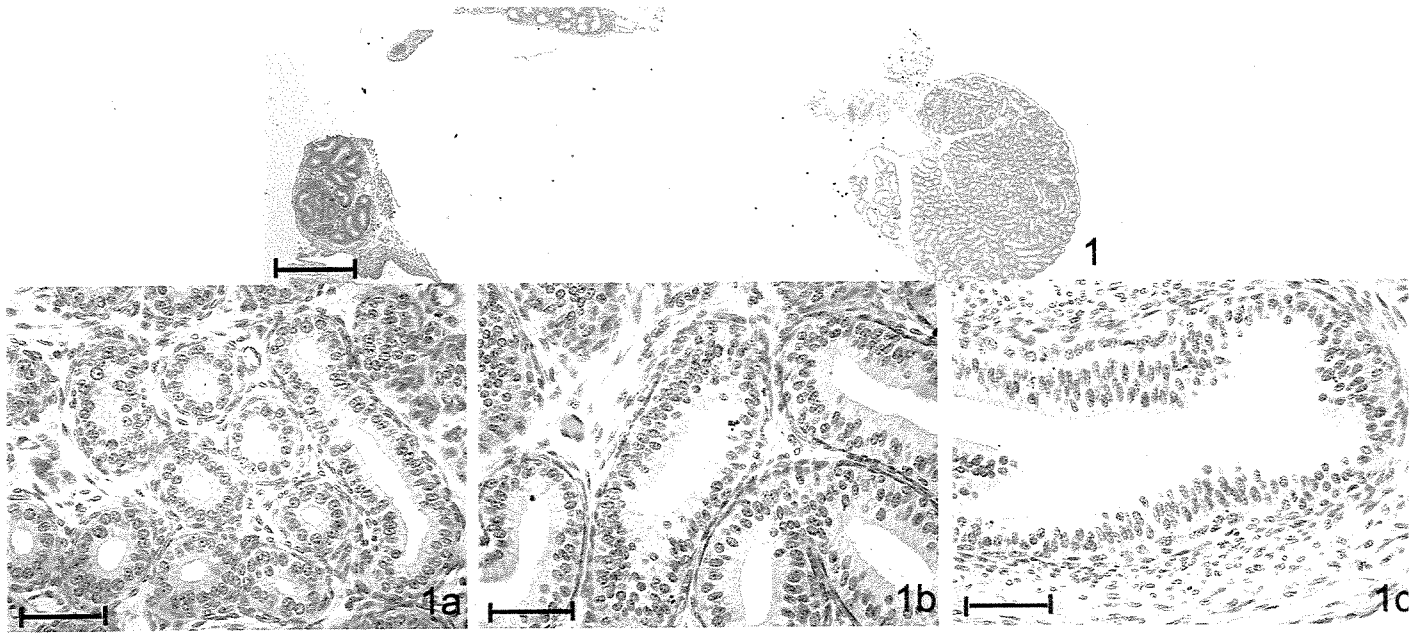
expression was similar to adult control epididymis (6.6x). **4a:** detail of the caput segment. The apical cytoplasm of the epithelial cells and the brush border were stained. Scale bar: 50 μm. **4b:** detail of the distal corpus towards the cauda segment. In the corpus segment, the antigen was found in luminal spermatozoa and in some focal areas of the brush border. The caudal segment (∴) showed no antigen adhered or synthesized. Scale bar: 50 μm. **4c:** detail of vas deferens. The stereocilia and cytoplasm of the cells were strongly stained with mAb TRA 54. Scale bar: 50 μm.

**Figure 5.** Immunohistochemical staining with mAb TRA 54 in a cross-section of epididymis from a bilaterally castrated 15-day-old mouse. The pattern of the antigen expression was severely modified in this condition (16.2x). **5a:** detail of the caput segment. There was no recognition of antigen by mAb TRA 54. Epithelial cells were short and undifferentiated. Scale bar: 50 μm. **5b:** detail of the cauda segment. The epithelial cells were morphologically similar to caput epithelial cells. No antigen was detected. Scale bar: 50 μm. **5c:** detail of vas deferens. Weak positive staining was restricted to some focal areas of the fluid in the brushborder of the cells. Scale bar: 50 μm

**Figure 6.** Immunohistochemical satining with mAb TRA 54 in a cross-section of epididymis from a bilaterally castrated mouse with testosterone replacement. The pattern of the antigen expression was normal throughout the epididymis. As in the normal adult pattern of expression, the initial segment was not stained (9x). **6a:** detail of the caput segment. The apical cytoplasm of the epithelial cells and the brush border were stained. Scale bar: 50 μm. **6b:** detail of the distal corpus segment. In the corpus segment, antigen was detected in the brush border of the epithelial cells and in the luminal content. Scale bar: 50 μm. **6d:** detail of vas deferens. The stereocilia and cytoplasm of the cells were very strongly stained with mAb TRA 54. Scale bar: 50 μm.

**Figure 7.** Western blot stained with mAb TRA 54. Cont: negative control, te: testis, epi: epididymis, br: brain, lv: liver, kd: kidney, spl: spleen proteins. Immunoreactive bands of 260, 200, 115 and 90 kDa were detected in testis extracts (pink head arrows) whereas bands of 260 and 200 kDa were detected in epididymal proteins (blue head arrows).

**Figure 8.** Western blot stained with mAb TRA 54. Cont: negative control. te: testis, cap: epididymal caput, cor: epididymal corpus, cau: epididymal cauda, def: vas deferens. (*), protein extracts with sodium metaperiodate. Immunoreactive bands of 260, 200, 115 and 90 KDa were detected in testis extracts (pink head arrows), whereas bands with 260 and 200 kDa were detected in epididymal caput proteins (blue head arrows). Treatment with sodium metaperiodate resulted in a loss of immunoreactivity of the proteins.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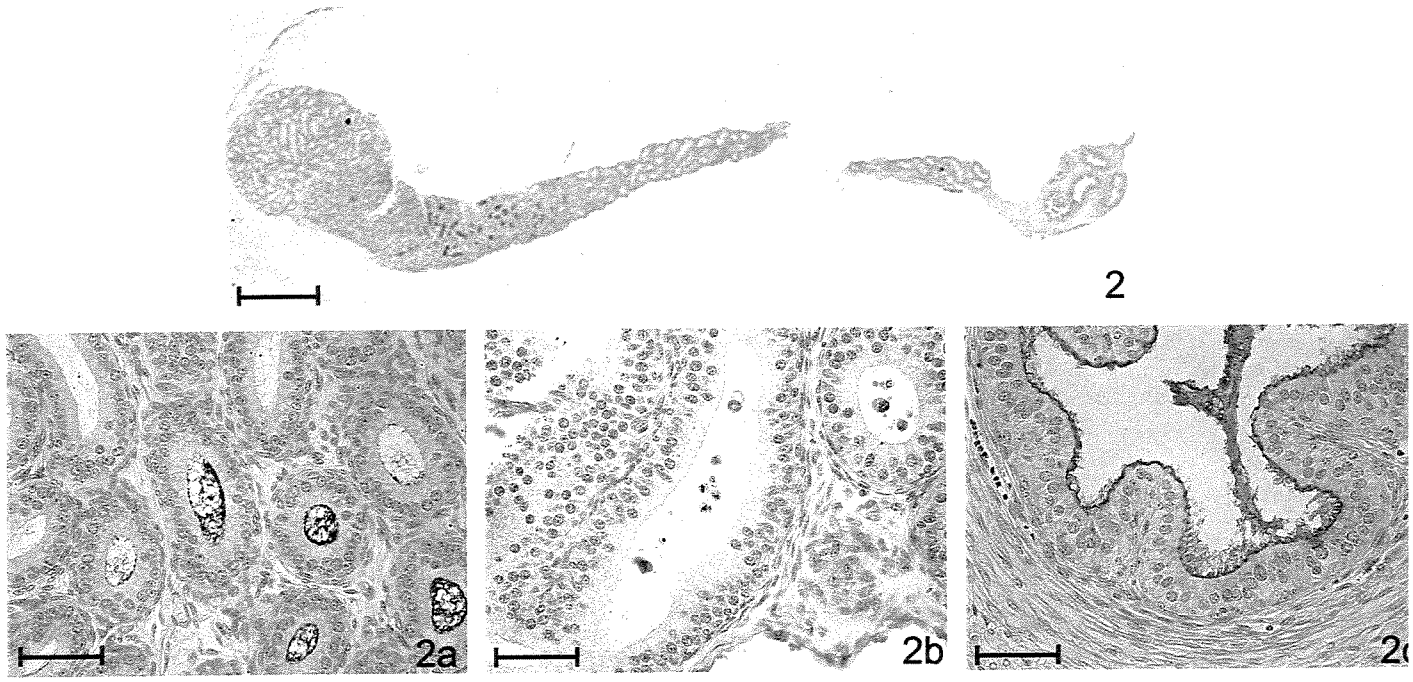
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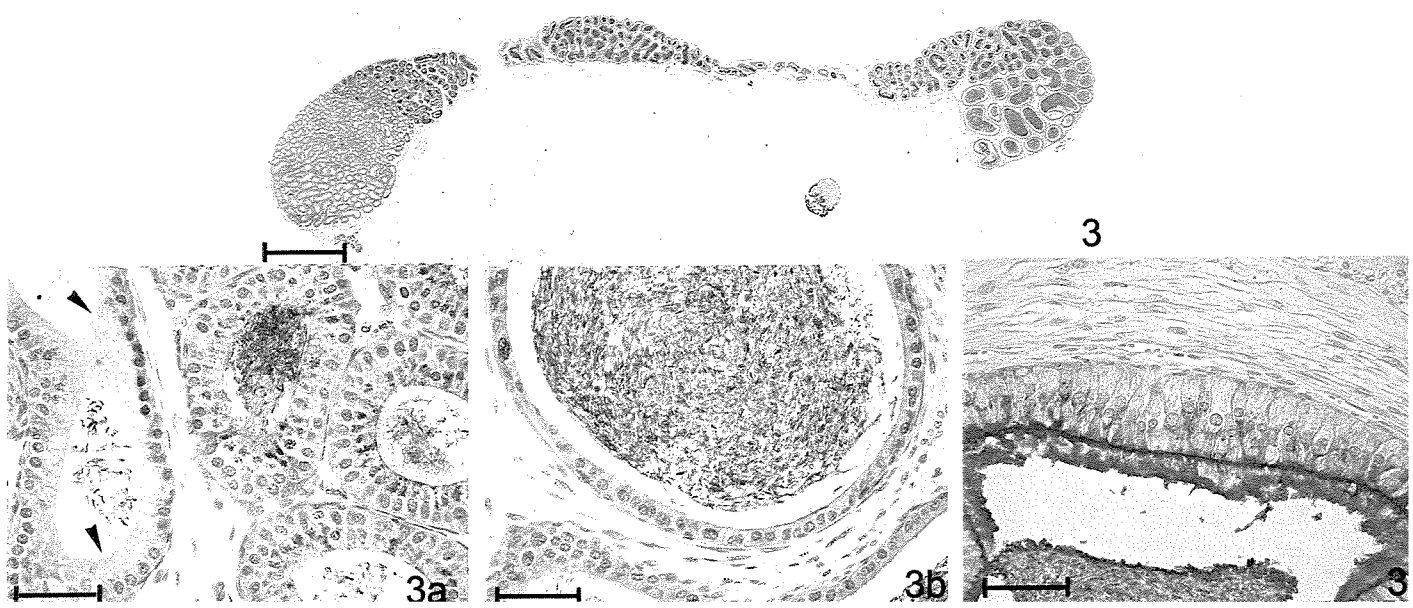
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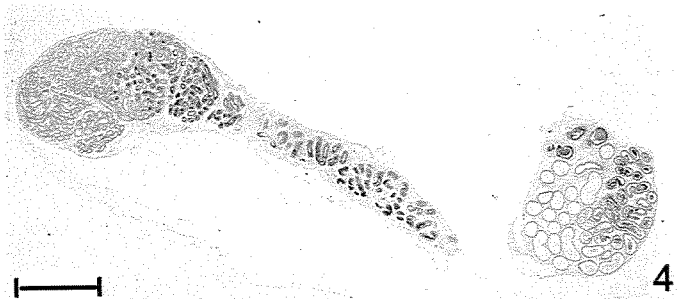

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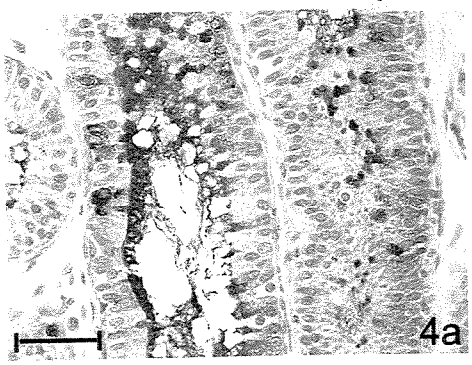

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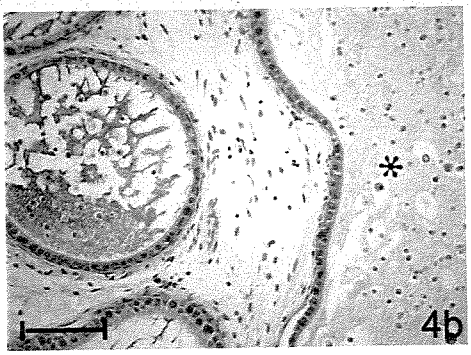

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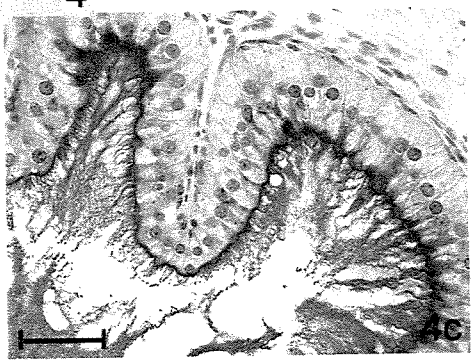

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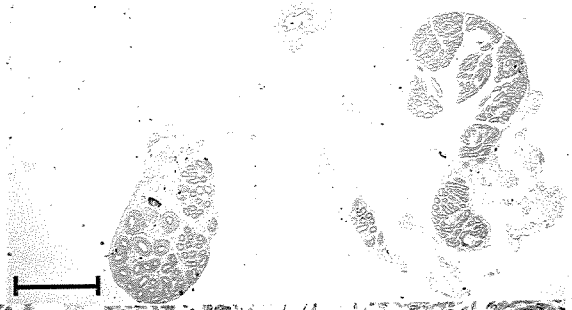

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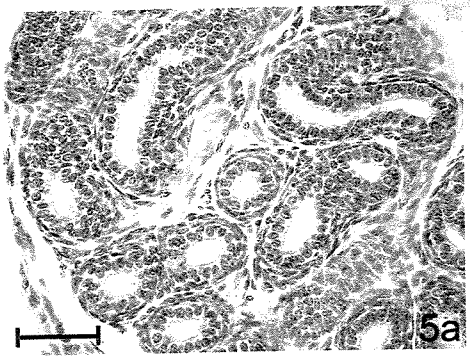

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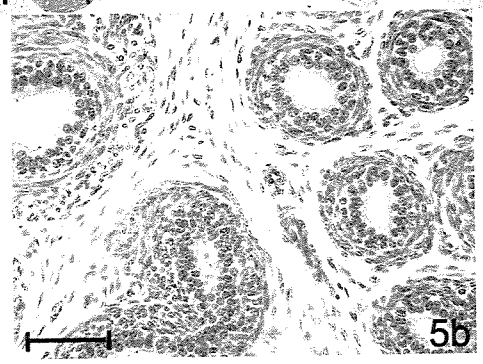

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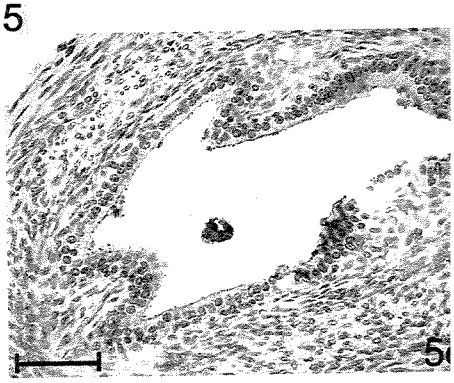

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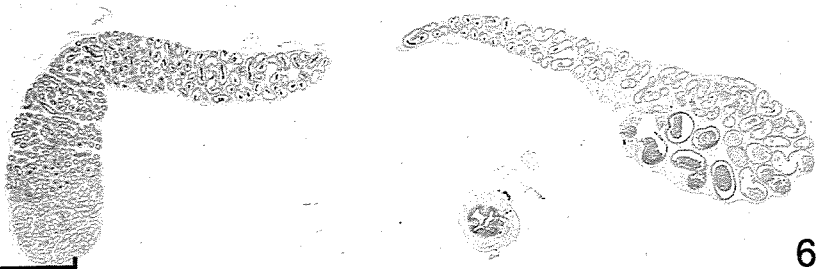

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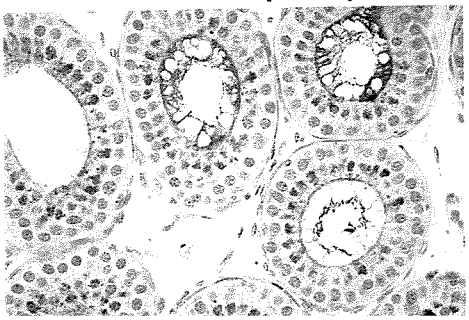

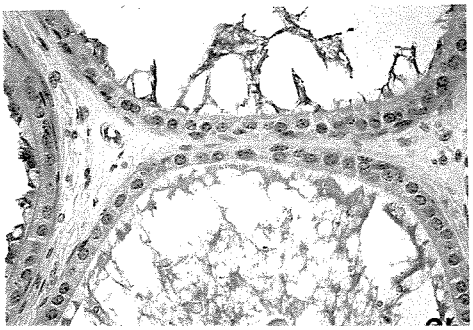

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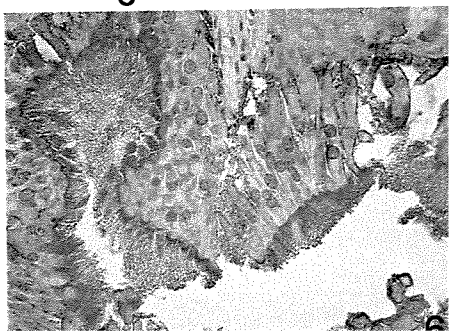

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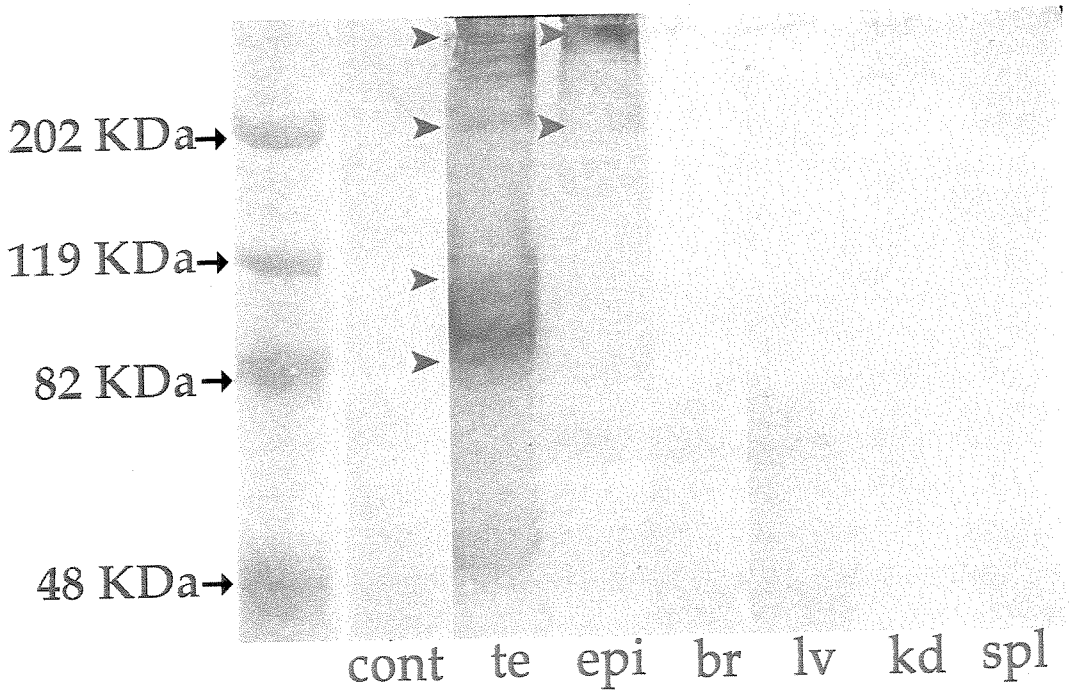
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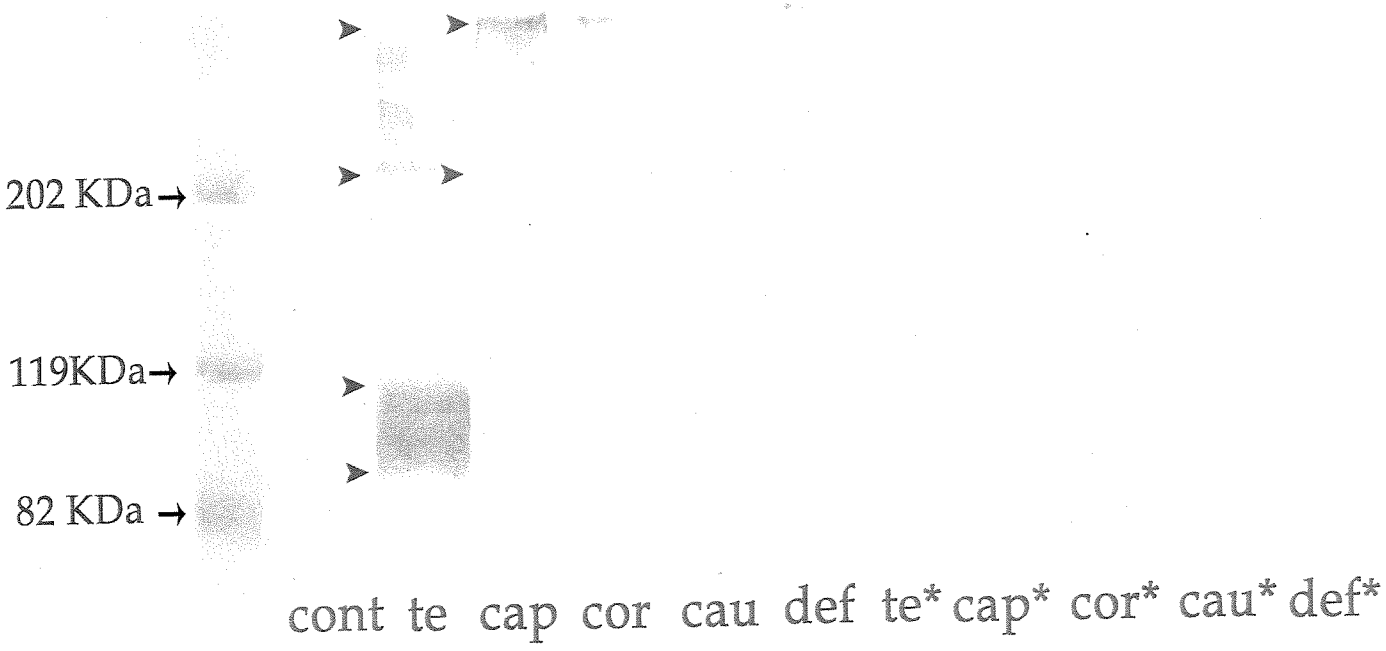
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## CONSIDERAÇÕES E CONCLUSÕES FINAIS

Este trabalho permite as seguintes conclusões:

(1) A molécula antigênica reconhecida pelo Amc TRA 54 é produzida por uma população específica de células germinativas em desenvolvimento e por uma população limitada de células da região da cabeça do epidídimo e do canal deferente a partir de onde é secretada para a luz;

(2) A molécula antigênica secretada pelo epidídimo parece percorrer a luz do órgão a partir da região da cabeça em direção a cauda, e durante este fluxo pode se incorporar aos espermatozóides e se aderir aos estereocílios dos demais segmentos epididimários;

(3) A molécula antigênica é sintetizada pelas células do epidídimo e do canal deferente independentemente da presença de células germinativas testiculares em desenvolvimento ou de espermatozóides na luz do epidídimo; a expressão da molécula antigênica no epidídimo/canal deferente e no testículo constitui eventos independentes.

(4) A síntese e secreção da molécula antigênica reconhecida pelo Amc TRA 54 na cabeça do epidídimo e canal deferente são eventos regulados pela testosterona testicular;

(5) A molécula antigênica expressa tanto em espermatócitos e espermátides testiculares como pelas células epiteliais do epidídimo apresentam natureza similar, representando diferentes isoformas de uma mesma molécula, ambas com um epítopo glicídico comum reconhecido pelo Amc TRA 54.

(6) A manutenção da morfologia do epidídimo e do canal deferente na vida adulta parece estar relacionada com níveis adequados de testosterona atuando de forma sistêmica e parácrina conjuntamente. As alterações encontradas tanto em epidídimo como em canal deferente de animais criptorquídicos aparentam ser menos severas que as encontradas em animais castrados, e podem ser revertidas por processo cirúrgico de reversão (orquidopexia). Infertilidade masculina verificada após realização de cirurgia corretiva para criptorquidismo não deve ser relacionada com alterações não obstrutivas da morfologia do epidídimo e do canal deferente.

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## ANEXO

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