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Implication des claudines et de la barrière hémato-épididymaire dans la fertilité masculine humaine

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RÉSUMÉ

L'infertilité masculine est un problème de santé qui affecte un grand nombre de couples consultant pour des problèmes de fertilité. Plusieurs évidences suggèrent qu'un dysfonctionnement au niveau de l'épididyme serait impliqué. En effet, au cours de leur passage dans l'épididyme, les spermatozoïdes subissent une maturation qui leur permet d'acquérir leur motilité et leur pouvoir fécondant. Cette maturation est possible grâce à la présence d'un microenvironnement spécifique dans la lumière de l'épididyme avec lequel les spermatozoïdes interagissent. Ce microenvironnement est en partie créé par la barrière hémato-épididymaire. Cette barrière permet également de protéger les spermatozoïdes contre les attaques du système immunitaire. La barrière hémato-épididymaire est composée de jonctions serrées entre les cellules principales. Ces jonctions comprennent différentes protéines, dont les claudines (CLDNs). Les CLDNs sont responsables de l'étanchéité de la barrière, mais aussi de la formation de pores qui permettent un transport sélectif d'ions au travers de la barrière. Très peu d'informations existent sur la barrière hémato-épididymaire humaine. Nous avons d'abord identifié les différentes composantes de cette barrière à l'aide de microréseaux. Nos résultats ont démontré que la barrière hémato-épididymaire humaine est composée d'un grand nombre de protéines jonctionnelles. Nous avons confirmé la localisation de plusieurs de ces protéines aux jonctions serrées par immunohistochimie. Nous avons ensuite regardé si la barrière est compromise en cas d'infertilité masculine, plus particulièrement chez des patients souffrant d'azoospermie non obstructive. Chez ces patients, plusieurs protéines jonctionnelles sont exprimées, mais certaines, par exemple CLDN10, ne le sont pas exclusivement aux jonctions serrées, suggérant que la barrière est présente, mais qu'elle est dysfonctionnelle. De plus, chez ces patients, plusieurs protéines de canaux d'eau et d'ions sont affectées suggérant que le transport transcellulaire est altéré en plus du transport paracellulaire. Nous avons ensuite développé des lignées cellulaires, les premières à ce jour de l'épididyme humain adulte, afin d'étudier le rôle de certaines CLDNs dans l'étanchéité de la barrière à l'aide de petits ARN interférants. Cette étude a permis de montrer que les CLDN1, 3, 4 et 7 sont essentielles pour la fonction de barrière

des jonctions serrées. Nous avons ensuite regardé l'état de la barrière chez des patients azoospermiques obstructifs par RT-PCR en temps réel et par immunohistochimie. L'expression des *CLDN1, 4* et *10,* de CDH1 et de TJP1 est affectée chez ces patients. Il est cependant très difficile d'obtenir des tissus épididymaires provenant de patients azoospermiques obstructifs. Nous avons donc développé des lignées cellulaires afin d'approfondir l'étude de ce type d'infertilité. Les résultats obtenus ont montré que la formation et le maintien des jonctions serrées sont altérés en cas d'azoospermie obstructive. Ces altérations s'étendent même jusqu'aux jonctions adhérentes et lacunaires. Dans l'ensemble, ces résultats démontrent l'importance de la barrière hémato-épididymaire dans le développement et dans le maintien de la fertilité masculine.

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ADN	acide désoxyribonucléique
AF-6	afadine
ARN	acide ribonucléique
ARNm	acide ribonucléique messager
bp	paire de bases
C	carboxyle
Caco-2	lignée cellulaire dérivée d'un carcinome humain du côlon
CAR	*coxsackievirus and adenovirus receptor*
Cdc42	*cell-division control protein 42*
CDH	cadhérine
CLDN	claudine
CO2	dioxyde de carbone
CRISP	protéine de sécrétion riche en cystéines
CTNN	caténine
Cx	connexine
DSP	desmoplakine
EPPIN	*epididymal protease inhibitor*
ESAM	*endothelial cell adhesion molecule*
FHCE1	*fertile human caput epididymal cell line 1*
h/hrs	heures
HE1	*human epididymal protein 1*
IHCE1	*infertile human caput epididymal cell line 1*
IMCD3	*inner medullary collecting duct cells*
JAM	*junctional adhesion molecule*
K-RAS	*v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog*
LTAg	antigène T
MAGI	*membrane- associated guanylyl kinase inverted*
MAGUK	*membrane associated guanylate kinase*
MDCK	*Madin-Darby canine kidney*
min	minute
MUPP1	*multi-PDZ domain protein 1*
N	aminée
PAR	*partitioning defective protein*
PALS1	*protein associated with Lin-7*
PATJ	*PALS1 associated tight junction*
PCDH	protocadhérine
PDZ	*postsynaptic density/discs large/ zonula occludens*

PCR	réaction en chaîne par polymérase
PI3K	phosphatidylinositol 3-kinase
PKA	protéine kinase A
PKC	protéine kinase C
PP2A	protéine phosphatase 2A
PSA	antigène spécifique de la prostate
PTEN	*phosphatase and tensin homolog*
RT	transcriptase inverse
s	seconde
TJP	*tight junctional protein*
µ	micro
YY1	*Yin-Yang-1*
ZO	*zonula occludens*
ZONAB	*ZO-1 associated nucleic acid protein*

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INTRODUCTION

L'infertilité masculine affecte un grand nombre de couples consultant pour des problèmes de fertilité. Les causes de l'infertilité masculine humaine sont multiples et, dans certains cas, il est difficile d'en définir l'origine [1]. Plusieurs données suggèrent que des anomalies dans le fonctionnement de l'épididyme pourraient contribuer à ce syndrome [2].

L'épididyme joue un rôle crucial dans le développement de la fertilité masculine. Le transit épididymaire permet aux spermatozoïdes d'acquérir leur motilité et leur pouvoir fécondant en leur procurant un microenvironnement luminal spécifique nécessaire à leur maturation [3]. La formation de microenvironnements dans différents systèmes biologiques est essentielle pour le développement de cellules spécialisées. Dans l'épididyme, ce microenvironnement est en partie créé grâce à la barrière hémato-épididymaire qui assure un transport sélectif de molécules entre la lumière épididymaire et la circulation sanguine. Cette barrière est formée de jonctions serrées. Une famille de protéines a été identifiée au niveau de ces jonctions: les claudines (CLDNs). Ces protéines sont à l'origine de l'étanchéité des jonctions serrées. Une altération de la barrière et une mauvaise expression des CLDNs ont été associées à différentes pathologies [4]. L'importance des CLDNs dans le maintien de diverses barrières épithéliales a également été confirmée par des manipulations génétiques d'animaux de laboratoire [5-7].

Chez les rongeurs, plusieurs éléments des jonctions serrées épididymaires ont été identifiées et la composition de ces jonctions varie d'une région à l'autre [8-11]. La composition en CLDNs des jonctions serrées est un élément-clé de la création et du maintien du microenvironnement présent dans la lumière de l'épididyme. En effet, la composition en CLDNs détermine la sélectivité du transport paracellulaire [12]. De plus, une étude, chez le rat, a démontré que la barrière hémato-épididymaire est compromise durant le vieillissement, entraînant une baisse de fertilité [13]. Les changements observés au niveau de la fertilité des rats sont similaires à ceux observés chez l'humain au cours du

vieillissement (baisse de la spermatogenèse, baisse de la stéroïdogenèse, baisse de la qualité du sperme) [14]. Chez l'humain, il existe encore très peu d'informations sur la barrière hémato-épididymaire. Il est donc essentiel d'identifier les protéines composant la barrière hémato-épididymaire humaine et leur rôle afin de mieux comprendre l'implication de cette barrière dans la fertilité. Ces données pourraient éventuellement contribuer à comprendre l'infertilité masculine, un problème important et coûteux pour le réseau de la santé.

Compte tenu de l'importance des CLDNs dans le maintien de différentes barrières cellulaires, l'hypothèse de ce projet était que les claudines sont un élément important des jonctions serrées, qui forment la barrière hémato-épididymaire humaine, et qu'un changement au niveau de ces protéines pourrait conduire à une baisse d'intégrité de la barrière hémato-épididymaire humaine et ainsi affecter la fertilité masculine. Ce projet comportait donc trois volets. Nous avons d'abord voulu déterminer les composantes de la barrière hémato-épididymaire humaine. Nous avons, par la suite, vérifier si l'expression des CLDNs change en cas d'infertilité masculine. Finalement, afin de nous aider à répondre à ces objectifs, nous avons développé et caractériser des lignées cellulaires de l'épididyme humain.

La première partie de cette thèse comporte une revue de littérature présentant l'état des connaissances sur le rôle de l'épididyme humain dans le développement de la fertilité. Un intérêt particulier est porté au rôle des jonctions serrées dans ce processus. La deuxième partie de cette thèse est composée de quatre manuscrits rédigés à partir des résultats obtenus dans le cadre de ce projet de doctorat. Les deux premiers articles ont été publiés dans la revue scientifique *Biology of Reproduction* en 2007 et en 2008 tandis que les deux derniers sont acceptés. Finalement, cette thèse comprend également une discussion générale et la contribution de ces travaux aux domaines de la reproduction et des jonctions cellulaires.

CHAPITRE 1: REVUE DE LITTÉRATURE

1. L'épididyme

L'épididyme, du grec *epi* (sur) et *didymoi* (testicules), est situé sur la partie dorsolatérale du testicule (Figure 1). Cet organe est responsable du transport, de la maturation, de la protection et de l'entreposage des spermatozoïdes dans un état latent. À la sortie du testicule, les spermatozoïdes ne sont pas physiologiquement matures. Le transit des spermatozoïdes dans l'épididyme leur permet de devenir matures par l'acquisition de leur motilité et de leur pouvoir fécondant. L'épididyme a surtout été étudié chez des espèces animales. Très peu d'études se sont intéressées à l'épididyme humain à cause de la difficulté d'obtenir des tissus, de l'absence de cancer et du développement de techniques de fécondation *in vitro* qui permettent de contourner son rôle dans le développement de la fertilité. Cependant il est important de mieux comprendre le fonctionnement de l'épididyme humain afin d'élucider les causes de l'infertilité masculine humaine.

1.1 Structure de l'épididyme

L'épididyme est constitué d'un long tubule unique et contourné, reliant les canaux efférents et déférents, qui peut mesurer de quelques mètres chez les rongeurs à 80 mètres chez l'étalon [15]. Chez l'homme, il mesure environ 6 mètres [16]. Cet organe est présent chez tous les mammifères, les oiseaux, les reptiles et les poissons cartilagineux [3]. Chez la plupart des espèces, on distingue généralement quatre régions anatomiques: le segment initial, la tête (*caput*), le corps (*corpus*) et la queue (*cauda*) [3, 17]. Des études chez le rat et la souris ont identifié jusqu'à 19 segments distincts par microdissection [18, 19]. L'épididyme humain ne comporte pas de segment initial. De plus, la tête de l'épididyme humain, qui ressemble au segment initial, comprend des vas efférents (Figure 2A) [20].

Le tubule épididymaire est composé d'une lumière bordée par un épithélium pseudostratifié cylindrique entouré de cellules musculaires lisses (Figure 2B).

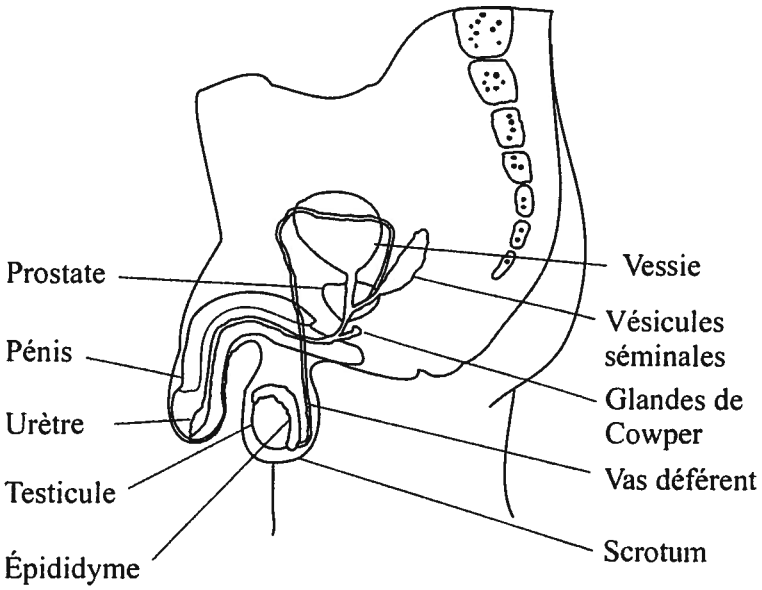

Figure 1: Le système reproducteur mâle. Il est constitué des testicules, d'un réseau de canaux (les vas efférents, les épididymes, les vas déférents et l'urètre), de glandes sexuelles annexes (les vésicules séminales, la prostate et les glandes de Cowper) et de structures de soutien. Créée par Evemie Dubé.

L'épithélium comprend plusieurs types cellulaires: les cellules principales, les cellules basales, les cellules apicales, les cellules claires, les cellules étroites et les cellules en halo [3, 17, 21-25]. Chacun des segments diffère par sa composition cellulaire. D'autres caractéristiques, comme l'épaisseur de l'épithélium, les propriétés d'absorption et la sécrétion de protéines, peuvent aussi varier le long de l'épididyme [3, 17].

Les cellules principales, qui composent 65 à 80% des cellules le long de l'épididyme, sont impliquées dans la sécrétion et dans l'absorption [3, 17, 21, 25]. Il s'agit de cellules ayant une forme allongée, possédant des microvillosités et différents organelles cellulaires. Cependant, leur composition en organelles cellulaires varie d'une région à l'autre. De plus, la hauteur de ces cellules décroît le long de l'épididyme, alors que la taille de la lumière augmente [3, 17, 25]. Les cellules basales forment un réseau sous les cellules principales, près de la membrane basale de l'épithélium. Le rôle des cellules basales n'est pas très bien connu, mais il serait possible qu'elles jouent un rôle immunitaire ou régulateur des fonctions des cellules principales [3, 17, 23, 25]. Il a récemment été montré que les cellules basales possèdent de longues projections pouvant s'étendre jusqu'à la lumière de l'épididyme. Les cellules basales pourraient ainsi participer à la régulation des cellules principales en captant l'environnement intraluminal [26]. Les cellules apicales sont présentes le long de l'épididyme, mais leur nombre diminue vers la queue du tubule [22]. Ces cellules jouent un rôle dans l'acidification du fluide intraluminal [3, 17, 25, 27]. Quant aux cellules en halo, elles agissent comme des cellules immunitaires et sont décrites comme des lymphocytes ou des macrophages [3, 17, 25, 28]. Deux autres types cellulaires, les cellules étroites et les cellules claires, sont présents dans l'épididyme. Chez les rongeurs, les cellules étroites se retrouvent au niveau du segment initial et de la zone intermédiaire, une région se situant entre le segment initial et la tête de l'épididyme. Ces cellules seraient impliquées dans l'acidification du milieu intraluminal et dans l'endocytose [17, 24, 25]. Les cellules étroites sont présentes dans la tête, le corps et la queue de l'épididyme. Ces cellules seraient également impliquées dans l'acidification du milieu intraluminal [3, 17, 25].

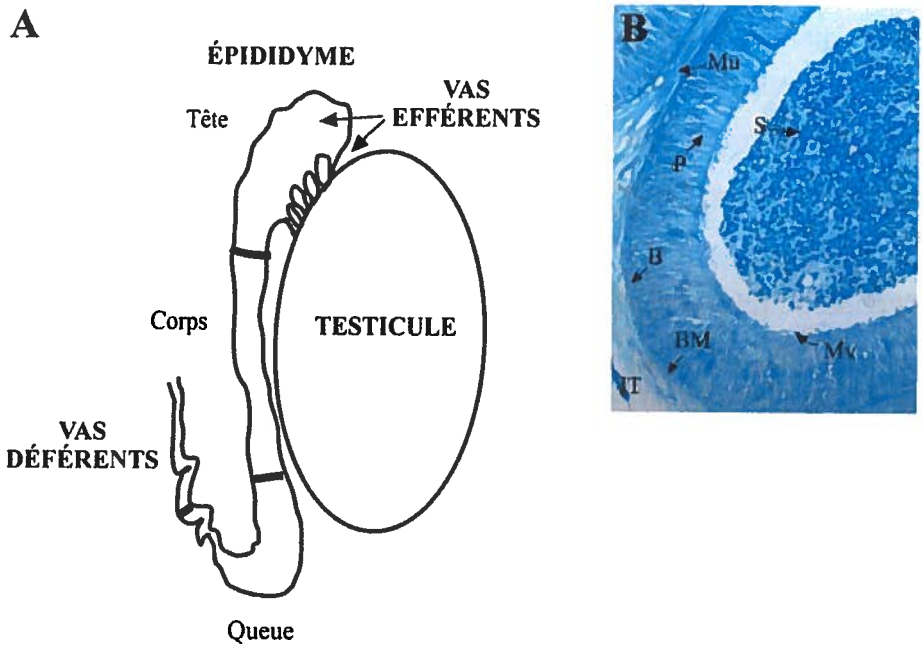

Figure 2: Structure de l'épididyme humain. (A) L'épididyme humain est généralement divisé en trois régions anatomiques: la tête, le corps et la queue. La tête de l'épididyme humain comprend également des vas efférents. (B) L'épithélium pseudostratifié, qui entoure la lumière de l'épididyme, comprend notamment des cellules principales et des cellules basales. Les tubules sont entourés d'une couche de cellules musculaires lisses. S, spermatozoïde; P, cellules principale; B, cellules basale; Mv, microvilli; Mu, couche musculaire; BM, membrane basale; IT, interstitium. Créée par Evemie Dubé.

1.2 Fonctions de l'épididyme

L'épididyme joue un rôle important dans le développement de la fertilité. Cet organe possède plusieurs fonctions qui peuvent être attribuées, soit à la lumière du tubule, soit à l'épithélium. Dans son ensemble, l'épididyme assure le transport, la maturation, le maintien, la protection et l'entreposage des spermatozoïdes.

1.2.1 Transport des spermatozoïdes

Le transit épididymaire prend en moyenne de 10 à 13 jours chez la plupart des espèces [17]. Dans la tête de l'épididyme, le transport des spermatozoïdes, qui sont alors peu motiles, est assuré par l'activité péristaltique du tubule et par la pression hydrostatique à l'intérieur du tubule. De plus, l'activité musculaire spontanée est augmentée par l'action de fibres nerveuses, d'angiotensines, de vasopressine et d'ocytocine, provenant du sang, et par l'action de produits paracrines de l'épithélium [3, 17].

1.2.2 Maintien et protection des spermatozoïdes

Le microenvironnement présent dans la lumière de l'épididyme est essentiel à la survie et à la maturation finale des spermatozoïdes. Le fluide intraluminal est le résultat de l'absorption d'une grande partie du liquide testiculaire en provenance du *rete testis* [29], du transport sélectif des constituants sériques [30] et de la sécrétion de molécules par les cellules, surtout principales, de l'épithélium épididymaire [31]. De plus, la composition de ce liquide varie le long de l'épididyme [32]. Les gamètes sont également protégés du système immunitaire par la présence de la barrière hémato-épididymaire [33, 34].

1.2.3 Maturation des spermatozoïdes

Chez la plupart des mammifères, une maturation post-testiculaire des spermatozoïdes est nécessaire afin de leur permettre d'acquérir leur pouvoir fécondant et leur motilité [3, 17]. Les premiers spermatozoïdes motiles sont observés au niveau du corps de l'épididyme [35]. La motilité est acquise suite à des modifications des mitochondries et de la composition lipidique de la membrane plasmique des spermatozoïdes. Ces changements permettent un meilleur contrôle des éléments nécessaires à la production d'énergie pour la contraction des bras de dynéine et le glissement des fibres de l'axonème [36]. Young et ses collègues ont été les premiers à démontrer que l'acquisition du pouvoir fécondant se fait de manière graduelle le long de l'épididyme. [37-39]. En effet, en effectuant des inséminations intra-utérines, ils se sont rendu compte que les taux de fécondation pouvaient varier entre 0% et 71 % en utilisant des spermatozoïdes provenant soit de la tête, soit de la queue de l'épididyme. Cependant, la durée de la maturation et le site d'acquisition du pouvoir fécondant varient d'une espèce à l'autre [15]. De plus, afin de féconder un ovocyte, les spermatozoïdes doivent d'abord être capables de se fixer sur la zone pellucide. Cette capacité de liaison à la zone pellucide dépend de protéines présentes au niveau de l'acrosome du spermatozoïde. Les spermatozoïdes interagissent avec les sécrétions épididymaires afin d'acquérir de nouvelles protéines ou de modifier celles qui existent déjà sur le gamète mâle à la sortie du testicule [40]. La maturation des spermatozoïdes implique également la mise en place de l'hélice mitochondriale, de la forme définitive de l'acrosome ainsi que la disparition de la gouttelette cytoplasmique présente au niveau de la région arrière de leur tête [3, 17, 41]. Les spermatozoïdes qui parviennent à la queue de l'épididyme sont donc motiles et capables de féconder l'ovocyte. Ils sont alors entreposés dans cette région de l'épididyme dans un milieu propice à leur survie.

1.2.4 Entreposage des spermatozoïdes

Les spermatozoïdes sont entreposés dans la queue avant d'être éjaculés. Cette capacité de réservoir varie d'une espèce à l'autre. Chez l'homme, cette fonction est quasi inexistante, alors que chez le rongeur elle est très utilisée. Les spermatozoïdes sont alors maintenus dans un état métabolique latent grâce à différents facteurs: une faible concentration en sodium dans la lumière, une concentration élevée en spermatozoïdes (grâce à la fonction d'absorption de l'épididyme), la présence de protéines qui limitent les mouvements des spermatozoïdes, la sécrétion de facteurs décapacitants et l'agrégation des spermatozoïdes qui empêchent toute activation précoce des gamètes mâles en vue de la fécondation [15, 17].

1.3 Implication de l'épididyme humain dans la maturation

Chez l'humain, la durée du transit épididymaire varie de 2 à 10 jours [17, 42]. Des études montrent qu'une durée de 2 à 4 jours est fréquente [43, 44]. Ce temps de transit est très court si on le compare à d'autres espèces (10 à 13 jours). La seule autre espèce ayant un temps de transit épididymaire de quelques jours est le chimpanzé [17]. Ces données suggèrent que le temps de maturation est très rapide ou que les spermatozoïdes subissent une faible maturation. Plusieurs études ont remis en doute la nécessité d'une maturation post-testiculaire des spermatozoïdes humains, puisque des spermatozoïdes d'origine testiculaire ou épididymaire peuvent être utilisés avec succès en fécondation *in vitro* [45-49]. Cependant, de meilleurs taux de fécondation sont obtenus en utilisant des spermatozoïdes provenant de la queue, plutôt que de la tête de l'épididyme [50]. D'autres études ont démontré que le transit épididymaire permet aux spermatozoïdes d'acquérir une motilité progressive, une meilleure aptitude à subir la réaction acrosomale et à se lier à l'ovocyte, mettant en évidence que les spermatozoïdes subissent bien une maturation fonctionnelle dans l'épididyme humain [51-56]. L'épithélium épididymaire participe activement à la maturation des spermatozoïdes par la sécrétion, l'absorption et le transport sélectif de protéines dans la lumière [32, 33]. Plusieurs protéines sécrétées par

l'épididyme humain ont été identifiées et ont un rôle potentiel à jouer dans la maturation, l'entreposage ou la protection des spermatozoïdes. On peut entre autres citer P34H, HE1, HE2, HE5, EPPIN et CRISP1 [57-61]. De plus, la création et le maintien de ce microenvironnement sont étroitement régulés par la présence de la barrière hémato-épididymaire.

2. La barrière hémato-épididymaire

La présence de jonctions serrées dans l'épididyme a été décrite pour la première fois chez le rat par Friend et Gilula [62]. Ce complexe jonctionnel hautement développé est composé de jonctions serrées présentes à l'apex de cellules principales. Ces jonctions serrées apparaissent comme des points de fusion entre les membranes plasmiques des cellules adjacentes [63]. L'existence d'une barrière hémato-épididymaire a été confirmée chez la souris [64], le singe [65], le vison [66], l'étalon [67] et le chien [68].

2.1 Formation de la barrière hémato-épididymaire

Chez le rat, le développement de la barrière hémato-épididymaire est progressif, car elle n'est pas étanche dès la naissance. Agarwal et Hoffer [69] ont montré que, chez le rat Sprague-Dawley, la barrière commence à se former dès le jour post-natal 18, mais ce n'est qu'au jour 21 que les jonctions serrées deviennent étanches et la barrière imperméable. Il a récemment été observé que, chez le rat Wistar, la barrière est complétée dès le jour post-natal 7 [10]. Suzuki et Nagano [64, 70] ont démontré que, chez la souris, des brins de jonctions serrées apparaissent dès le jour embryonique 12 et que la complexité de ces jonctions augmente jusqu'au jour postnatal 37. Il a aussi été observé que, chez le vison, la barrière hémato-épididymaire est déjà fonctionnelle à l'âge embryonique [66].

2.2 Fonctions de la barrière hémato-épididymaire

Dans les organismes multicellulaires, les barrières cellulaires sont essentielles pour créer des compartiments fonctionnels. Les jonctions serrées, qui composent ces barrières, ont plusieurs rôles: 1) une fonction de barrière physique et étanche entre deux compartiments, 2) une fonction de frontière entre le domaine apical et basolatéral de la membrane plasmique qui permet de créer et de maintenir la polarité cellulaire, 3) une perméabilité sélective qui assure le contrôle du mouvement de l'eau, des ions et des protéines entre la circulation sanguine et, dans ce cas-ci, la lumière épididymaire afin de créer des microenvironnements spécifiques [71].

La fonctionnalité de la barrière hémato-épididymaire chez le rat a été mise en évidence pour la première fois par Hoffer et Hinton [72]. Ils ont montré que ni le lanthanum ni l'inuline ne pouvaient traverser cette barrière au niveau de la tête de l'épididyme. L'intégrité de la barrière hémato-épididymaire a également été confirmée chez le chien en utilisant le lanthanum comme traceur [68]. Par contre, d'autres composés, tel que l'inositol, ont une concentration plus élevée dans la lumière de l'épididyme que dans la circulation sanguine [3, 73]. De plus, la composition du microenvironnement intraluminal varie dans les différentes régions de l'épididyme [32, 33] suggérant que les caractéristiques de la barrière hémato-épididymaire changent également. En effet, chez le rat, le complexe jonctionnel varie en nombre et en organisation géométrique le long du tubule épididymaire avec un plus grand nombre de brins jonctionnels dans la tête que dans la queue [70]. Cependant, il a été montré que le niveau de perméabilité d'une barrière n'est pas corrélé au nombre de jonctions serrées [74]. De plus, Chan et collaborateurs [75] ont démontré, en mesurant *in vitro* la résistance transépithéliale, que la barrière hémato-épididymaire était plus perméable au niveau de l'épididyme proximal que de la queue, ce qui pourrait être important pour l'entreposage des spermatozoïdes dans un état latent. Cyr et collaborateurs [34] ont également démontré que la longueur du complexe jonctionnel présent le long de la membrane plasmique des cellules principales est réduite dans le corps et la queue par rapport à la tête, alors que le nombre de desmosomes augmente le long de l'épididyme. Cette étude a aussi mis en évidence que les jonctions serrées sont des structures dynamiques et que le renouvellement des

protéines jonctionnelles se fait par l'intermédiaire du *turnover* des membranes plasmiques. La barrière hémato-épididymaire humaine a été très peu étudiée, même si la présence de jonctions serrées entre les cellules principales de l'épididyme humain a été mise en évidence *in vitro* [76-78].

2.3 Composition de la barrière hémato-épididymaire

Au cours des dernières décennies, plusieurs familles de protéines transmembranaires, cytoplasmiques et régulatrices ont été identifiées au niveau des jonctions serrées (Figure 3).

2.3.1 Protéines transmembranaires des jonctions serrées

Plusieurs types de protéines transmembranaires se retrouvent au niveau des jonctions serrées. Le premier groupe de protéines transmembranaires comprend l'occludine, la tricelluline et les CLDNs. Ces protéines sont composées de quatre domaines transmembranaires avec deux boucles extracellulaires dont les extrémités terminales aminée (N) et carboxyle (C), sont localisées dans le cytoplasme. Le second type de protéines est constitué des molécules d'adhérence de jonctions (JAM) de la superfamille des immunoglobulines, qui ne possèdent qu'un seul domaine transmembranaire [79].

2.3.1.1 L'occludine

L'occludine a été la première protéine intégrale des jonctions serrées à être identifiée [80]. Elle est exprimée dans différents tissus épithéliaux et endothéliaux [71]. Cette protéine est présente le long de l'épididyme chez la souris [81], le rat [13], le lapin [82] et le chien [83]. Cependant l'occludine n'est pas localisée de la même manière le long de l'épididyme des différentes espèces. Chez le rat, l'occludine est localisée exclusivement aux jonctions serrées présentes entre les cellules principales adjacentes. Chez la souris, l'occludine a une localisation similaire le long de l'épididyme à l'exception du segment

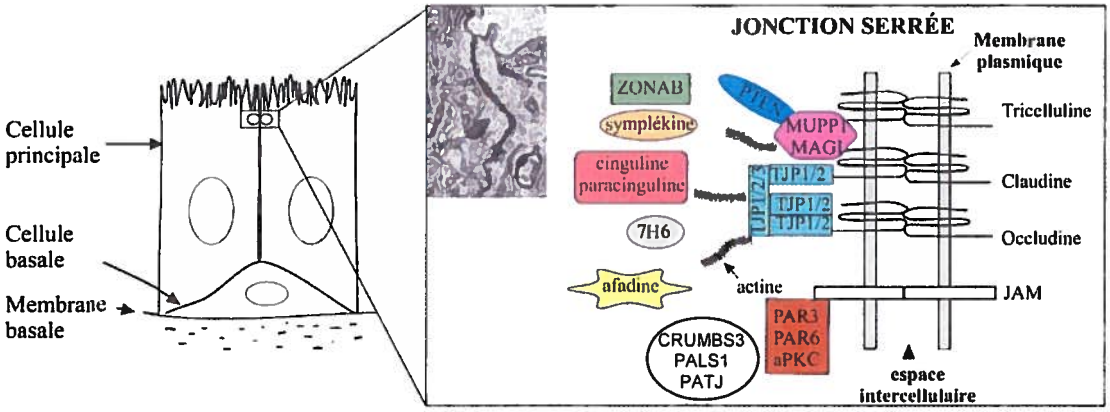

Figure 3 : Organisation des jonctions serrées. Le complexe jonctionnel présent à l'apex des cellules principales, tel que montré dans la photographie de microscopie électronique, est composé de différentes protéines transmembranaires et cytoplasmiques. Créée par Evemie Dubé.

initial. Dans ce segment, l'occludine est associée aux cellules étroites. De plus, dans l'épididyme murin en développement, même si l'occludine est exprimée dès le jour embryonique 13,5, ce n'est qu'au jour embryonique 18,5 que la protéine se retrouve au niveau de la région apicale entre les cellules principales adjacentes [81].

Par l'intermédiaire de son extrémité C-terminale, l'occludine interagit avec plusieurs protéines cytoplasmiques, telles que les protéines TJP1, 2, 3, la cinguline ainsi qu'avec les filaments d'actine [84-88]. Son extrémité C-terminale contient également plusieurs sites de phosphorylation qui peuvent être modifiés par des kinases ou des phosphatases, telles c-yes, PKC ou PP2A [89-91]. Il a été montré que l'occludine non phosphorylée est présente dans le cytoplasme et le long des membranes basolatérales entraînant une baisse de la perméabilité paracellulaire, alors que la protéine phosphorylée est localisée aux jonctions serrées [92].

Le rôle de l'occludine est encore incertain. Des études surexprimant soit la protéine intacte, soit la protéine mutée, soit un peptide correspondant à la deuxième boucle extracellulaire de l'occludine, suggéraient que l'occludine était importante pour la fonction de barrière des jonctions serrées [93-95]. Cependant, des cellules souches embryoniques n'exprimant pas l'occludine sont capables de se différencier en cellules épithéliales polarisées formant des jonctions serrées [96]. De plus, une étude a montré que des souris n'exprimant pas l'occludine ne montrent aucune anomalie au niveau de la barrière intestinale. Des phénotypes complexes ont toutefois pu être observés dans d'autres organes (inflammation, hyperplasie de la muqueuse gastrique, calcification du cerveau et atrophie testiculaire) suggérant un autre rôle pour l'occludine au niveau du complexe jonctionnel [97]. Plusieurs études ont proposé un rôle pour l'occludine dans la signalisation cellulaire, étant donné qu'elle interagit avec différents acteurs de voies de signalisation impliqués dans la régulation des jonctions cellulaires. On peut entre autres citer Raf-1, PI3K et RhoA [98]. Des données récentes suggèrent qu'une autre protéine jonctionnelle, la tricelluline, pourrait compenser le rôle de l'occludine dans la fonction de barrière des jonctions serrées [99].

2.3.1.2 La tricelluline

La tricelluline a été découverte par Ikenouchi et ses collègues [100] comme protéine cible du facteur de transcription SNAIL. Une des particularités de la tricelluline est sa localisation exclusive aux jonctions tricellulaires dans les cellules. L'inhibition de l'expression de la tricelluline par des petits ARNs interférents affecte la structure des jonctions tricellulaires, mais aussi celle des jonctions bicellulaires [100]. Il a aussi été démontré par Ikenouchi et al. [99] que l'inhibition de l'expression de l'occludine a des effets sur la localisation de la tricelluline. Celle-ci est alors beaucoup plus exprimée au niveau des jonctions bicellulaires suggérant un mécanisme de compensation qui serait à l'origine des controverses sur le rôle de l'occludine. De plus, la tricelluline comporte une séquence C-terminale similaire à 32% à l'occludine [100]. La tricelluline est exprimée dans différents tissus, le petit intestin, le rein, l'estomac [100] et l'oreille interne [101], et dans différentes lignées cellulaires telles que la lignée MDCK II, HT-29/B6, Caco-2 et T84 [102]. L'ARNm de la tricelluline est exprimé le long de l'épididyme murin surtout au niveau de la tête distale [18]. À ce jour, il n'existe aucune donnée sur la localisation de la tricelluline dans l'épididyme.

Riazuddin et collaborateurs [101] ont montré que des mutations du gène TRIC codant pour la tricelluline seraient à l'origine de la surdité non syndromique DFNB49 chez l'humain. Ces mutations causeraient la perte totale ou partielle d'une région C-terminale conservée contenant un domaine de liaison à la protéine cytoplasmique TJP1. Toutefois, l'interaction de la tricelluline avec les CLDNs *in vitro* dans les cellules MDCK est indépendante de TJP1 [99]. Il existe plusieurs isoformes de la tricelluline créés par épissage alternatif qui n'auraient peut-être pas tous les mêmes propriétés de liaison à TJP1 [101]. De plus, le rôle de la tricelluline semble être dépendant de sa localisation [102]. Lorsque la tricelluline est exprimée aux jonctions serrées bi- et tri-cellulaires, il y a une augmentation de la résistance transépithéliale et une baisse de la perméabilité paracellulaire aux ions et aux macromolécules. Par contre, lorsque la tricelluline est

surexprimée uniquement aux jonctions tri-cellulaires, seule la perméabilité aux macromolécules est affectée.

2.3.1.3 Les claudines

La présence de jonctions serrées en l'absence de l'occludine a conduit à la découverte d'un autre groupe de protéines transmembranaires, les CLDNs [103]. À ce jour, 24 gènes codant pour des CLDNs ont été identifiés [104]. De plus, plusieurs CLDNs, telles que les CLDNs 10 et 18, ont plusieurs isoformes créés par épissage alternatif [105-107]. Il n'existe aucune similarité entre la séquence des CLDNs et celle de l'occludine [103]. Par l'intermédiaire de leur extrémité C-terminale, les CLDNs interagissent avec des protéines cytoplasmiques telles que TJP1 ou MUPP1 [104, 108, 109]. Leur interaction avec les protéines TJPs permet un lien indirect avec l'actine ce qui permet de stabiliser la jonction serrée [110]. Au cours des dernières décennies, il est devenu évident que les CLDNs constituent la base moléculaire des jonctions serrées. En effet, l'expression exogène de CLDNs peut induire la formation de jonctions serrées dans des fibroblastes [103] tout en participant à la création de pores cellulaires qui permettent une diffusion sélective des ions et des molécules à travers l'espace paracellulaire [111-113]. La perméabilité sélective de ces pores dépend de la concentration et du type de CLDN exprimé par la cellule. En effet, les CLDNs sont exprimées dans différents tissus épithéliaux et endothéliaux, mais leur distribution varie selon le tissu et l'espèce [114]. De plus, il a été démontré que la première boucle extracellulaire des CLDNS est impliquée dans la fonction de barrière et la perméabilité sélective des jonctions serrées [115]. Plusieurs modèles animaux et maladies humaines ont également contribué à mieux comprendre le rôle des CLDNs [4, 5]. Il est important de préciser que certaines CLDNs sont surtout impliquées dans la fonction de barrière des jonctions serrées alors que d'autres jouent un rôle important dans la perméabilité sélective aux ions. Mais ces propriétés dépendent des CLDNs exprimées dans une même jonction et de la manière dont elles sont polymérisées [116].

Les jonctions serrées épididymaires comportent un grand nombre de CLDNs. Les Cldns 1 à 9 sont exprimées dans l'épididyme du rat et de la souris, suggérant un certain degré de conservation dans la composition des jonctions serrées et dans le rôle de chacune de ces Cldns [8-11]. La Cldn11 est également exprimée le long de l'épididyme du rat [19] alors que les Cldns 10 et 16 sont exprimées uniquement dans le segment initial [9, 10]. Les différences dans la composition en CLDNs existant entre les jonctions serrées des différentes régions de l'épididyme suggèrent des différences de caractéristiques de la barrière hémato-épididymaire le long du tubule. Ces variations pourraient être un élément-clé dans la composition du milieu intraluminal qui change le long de l'épididyme. La localisation des Cldns varie également dans l'épididyme du rat et de la souris. Dans l'épididyme du rat, les Cldns 1, 2, 3, 4, 6, 7, 8 et 10 sont localisées aux jonctions serrées présentes entre les cellules principales alors que Cldn5 est exprimée par les cellules endothéliales [8-11]. Cependant les Cldns 1, 7 et 10 ne sont pas localisées exclusivement aux jonctions serrées dans l'épididyme du rat, on les retrouve également le long des membranes plasmiques entre les cellules principales et entre les cellules principales et basales [10, 11]. Il a également été observé que la présence de Cldn7 le long des membranes plasmiques des cellules principales n'était pas dû à la formation de brins jonctionnels [11]. Ces protéines pourraient, soit constituer une réserve de CLDNs pour les jonctions serrées apicales, soit être impliquées dans l'adhésion cellulaire. L'expression et la localisation des CLDNs dans l'épididyme varient également au cours de développement [9, 10].

2.3.1.4 Les protéines JAMs

Les protéines JAMs appartiennent à la superfamille des immunoglobulines et sont exprimées dans une variété de tissus. À ce jour, six protéines JAMs ont été identifiées JAM-1 (JAM-A), JAM-2 (JAM-B), JAM-3 (JAM-C), CAR, ESAM et JAML (aussi connue sous le nom de JAM-4 chez la souris). *JAM-2*, *-3*, *-4* et *ESAM* sont exprimés le long de l'épididyme de rat et de souris, mais leur patron d'expression varie dans les différentes régions des deux espèces [18, 19]. Le rôle précis des protéines JAMs dans les

jonctions serrées n'est pas connu. Ces protéines interagissent avec des protéines associées aux jonctions serrées, telles que TJP1, MUPP1 et MAGI-1 [117], ce qui suggèrent que ces protéines pourraient être impliquées dans la régulation des jonctions serrées. Cette régulation pourrait se faire par le ciblage d'autres protéines au complexe jonctionnel. De plus, les protéines JAMs se lient au complexe de polarité constitué de PAR-3, -6 et aPKC qui est impliqué dans la formation des jonctions serrées [118, 119]. À l'exception de JAM-1, il existe très peu d'information sur le rôle de ces protéines dans la formation des jonctions serrées. Il a été montré que l'expression de JAM-1 entraîne la localisation de TJP1 et de l'occludine aux points de contact intercellulaires [120, 121]. Plusieurs études suggèrent également que les protéines JAMs seraient impliquées dans la fonction de barrière des jonctions. En effet, l'inhibition de JAM-1 affecte la formation des jonctions serrées et la résistance transépithéliale [122, 123]. Il reste encore à déterminer si les protéines JAMs jouent un rôle dans la barrière hémato-épididymaire.

2.3.2 Protéines cytoplasmiques des jonctions serrées

Les protéines transmembranaires des jonctions serrées sont le noyau d'un échafaudage moléculaire qui est relié à de nombreuses protéines cytoplasmiques. Ces protéines cytoplasmiques peuvent être soient des adapteurs reliant les protéines transmembranaires au cytosquelette d'actine, ce qui permet de stabiliser la structure de la jonction, soient être des protéines régulatrices impliquées dans différents processus comme la transcription, la polarité cellulaire ou la signalisation cellulaire. Certaines protéines comportent des domaines PDZ qui reconnaissent un motif particulier situé à l'extrémité C-terminale des protéines transmembranaires. On peut entre autres citer les protéines TJP1, 2, 3 (aussi connues sous le nom de *zonula occludens proteins* ZO-1, 2, 3), les protéines MAGUK MAGI-1, MAGI-2 et MAGI-3, la protéine MUPP1, les protéines PAR-3 et PAR-6, les protéines PALS1 et PATJ. D'autres protéines, comme la cinguline, la paracinguline, la symplékine, cdc42 (*cell-division control protein 42*), PKCa (PKC atypique) et ZONAB (ZO-1 associated nucleic acid protein), ne possèdent pas de domaines PDZ. Ces protéines sont plus éloignées de la membrane que TJP1 [71, 79]. Des études de microréseaux ont

permis de mettre en évidence l'expression de plusieurs de ces protéines dans l'épididyme [18, 19].

2.3.2.1 Les protéines TJPs

TJP1 a été la première constituante des jonctions serrées à être identifiée. Cette protéine de 220kDa est localisée dans le cytoplasme des cellules endothéliales et épithéliales à proximité des jonctions serrées [124-126]. Par la suite, TJP2 et TJP3 ont été découvertes grâce à leur interaction avec TJP1 [86, 127]. TJP1, 2 et 3 sont exprimées dans l'épididyme de la souris [18]. De plus, TJP1 est exclusivement localisée aux jonctions serrées dans l'épididyme du rat [13]. Par l'intermédiaire de leur extrémité N-terminale, les protéines TJP1, 2, et 3 interagissent avec l'occludine [84] et les CLDNs [109], alors que leur extrémité C-terminale leur permet d'interagir avec le cytosquelette d'actine [128]. TJP1 interagit également avec les protéines JAMs [79]. Cependant, TJP1 et TJP2, contrairement à TJP3, sont importantes pour le ciblage des CLDNs aux jonctions serrées, pour la formation de brins jonctionnels ainsi que pour la fonction de barrière des jonctions serrées [110, 129]. TJP1 interagit également avec des protéines des jonctions adhérentes et lacunaires, ces interactions seront discutées ultérieurement dans les sections 3 et 4.

2.3.2.2 Les protéines MAGI

Les protéines MAGI-1 et MAGI-3 sont exprimés dans l'épididyme du rat et de la souris, [18, 19]. Ces protéines interagissent avec diverses protéines transmembranaires des jonctions serrées tel que JAM-4 [79, 130]. Même si MAGI-1 est localisée aux jonctions serrées [131], cette protéine se lie également à la β-caténine ce qui suggère un rôle pour MAGI-1 dans la formation des jonctions adhérentes et serrées [132]. De plus, deux protéines de liaison à l'actine, la synaptopodine et la α-actinine 4, sont capable de lier MAGI-1, assurant ainsi un lien au cytosquelette [133]. Finalement, les protéines MAGI jouent un rôle dans différentes voies de signalisation cellulaire: MAGI-1 a en effet été

identifiée comme une protéine liant K-RAS, alors que MAGI-2 et -3 ont été identifiées comme des protéines liant PTEN [134-136].

2.3.2.3 La protéine MUPP1

MUPP1, qui est exprimé le long de l'épididyme murin [18], a tout d'abord été identifiée par son interaction avec le récepteur 5-hydroxytryptamine de type 2C [137]. Ce n'est que plus tard qu'il a été découvert que MUPP1 était localisée aux jonctions serrées grâce à son interaction avec CLDN1 et JAM-1 [108]. D'autres études ont mis en évidence son interaction avec CLDN8 et PALS1 [138, 139] mais son rôle au niveau des jonctions serrées est encore incertain. En effet, la perte de MUPP1 n'affecte ni la formation ni le maintien des jonctions serrées dans les cellules EpH4 [140] alors que cela entraîne une baisse de la résistance transépithéliale dans les cellules IMCD3 [139].

2.3.2.4 La cinguline, la paracinguline et la symplékine

La cinguline est exprimée dans l'épididyme du rat et de la souris [18, 141]. Cette protéine interagit avec les protéines TJPs, JAMs, l'actine et la myosine [121, 142, 143]. Des études récentes suggèrent que la cinguline joue un rôle dans la régulation transcriptionnelle et dans la prolifération cellulaire. En effet, une étude portant sur des souris n'exprimant pas la cinguline a mis en évidence plusieurs altérations dans les niveaux d'expression des ARNm de plusieurs protéines de jonction serrée, telles que les CLDNs. Cependant les études, basées sur l'utilisation de cellules exprimant peu ou pas de cinguline et formant des jonctions serrées, suggèrent que la cinguline ne joue pas un rôle direct dans la structure et dans les fonctions des jonctions [144, 145]. La paracinguline a été identifiée comme une protéine similaire à la cinguline par sa séquence, identique à 40%, et par sa structure. La paracinguline est exprimée dans l'épididyme du rat [19]. Cependant la paracinguline n'est pas exclusive aux jonctions serrées et se retrouve également aux jonctions adhérentes [146]. Il a récemment été montré que la paracinguline régule les activités de Rac1 et de RhoA pendant la formation des jonctions, suggérant un

rôle dans la signalisation cellulaire [147]. Quant à la symplékine, qui est exprimée dans l'épididyme du rat et de la souris [18, 19], il a été montré que cette protéine s'associe aux jonctions serrées dans les cellules épithéliales polarisées [148] et qu'elle régule l'expression du facteur de transcription ZONAB suggérant un rôle dans la répression transcriptionnelle [149].

2.4 Régulation des jonctions serrées

Les fonctions de l'épididyme sont régulées par de nombreuses hormones, incluant les androgènes [150]. Une étude réalisée par Suzuki et Nagano [64] démontre que la formation et le maintien des jonctions serrées dans l'épididyme proximal est régulée par des facteurs testiculaires. En effet, l'orchidectomie de souris adultes entraîne une baisse du nombre de brins de jonctions serrées dans la tête de l'épididyme. Il a d'ailleurs été démontré que Cldn1 est régulée par les androgènes et par les hormones thyroïdiennes dans le segment initial de l'épididyme du rat [8, 151]. L'expression des gènes codant pour les Cldn5, 8, 11 et 14 diminue également dans l'épididyme proximal du rat suite à une ligation des vas efférents suggérant que ces gènes sont régulés par des facteurs testiculaires [152]. Par contre, la barrière hémato-épididymaire n'est pas régulée par les androgènes dans la queue de l'épididyme [153].

Les jonctions serrées sont également les cibles de plusieurs voies de signalisation, dont celle des protéines kinases PKA et PKC, de plusieurs phosphatases telle que la phosphatase 2A (PP2A), de certains effecteurs de Ras, en particulier des MAP-kinases (mitogen activated protein kinases) [154, 155]. Ces différentes molécules régulatrices jouent un rôle au niveau de l'assemblage, du maintien des jonctions serrées et de leurs fonctions. Plusieurs facteurs de transcription sont aussi impliqués dans la régulation des jonctions serrées. On peut entre autres citer SNAIL, un des facteurs de transcription les plus étudiés en lien avec les jonctions serrées, qui est exprimé dans l'épididyme de rat et de souris [18, 19]. En effet, il a été démontré que SNAIL diminue l'expression de plusieurs protéines, telles que la cadhérine E (CDH1), les CLDNs et l'occludine

entraînant ainsi une augmentation de la perméabilité paracellulaire et une perturbation de la fonction de barrière [100, 156, 157]. Dufresne et Cyr [158] ont démontré que deux autres facteurs de transcription, SP1 et SP3, sont impliqués dans la régulation de Cldn1 dans une lignée cellulaire dérivée de la tête de l'épididyme de rat. Ces deux facteurs sont exprimés de manière ubiquitaire et sont connus pour réguler de nombreux gènes [159], incluant CLDN3 et CLDN4 dans les cellules ovariennes cancéreuses [160, 161], CLDN4 dans les cellules MDCK [162] et CLDN19 dans des lignées cellulaires rénales [163]. Un autre suppresseur de tumeur, qui pourrait jouer un rôle dans la régulation des jonctions serrées épididymaires, est p63. En effet, p63, qui est exprimé par les cellules basales de l'épididyme humain et du rat, est impliqué dans la régulation de Cldn1 dans des cultures primaires de kératinocytes de souris [164-166].

La régulation de la barrière hémato-épididymaire est donc complexe et multifactorielle. Cette régulation pourrait également impliquée des interactions avec les jonctions adhérentes et lacunaires.

3. Interactions entre les jonctions serrées et adhérentes

La formation des jonctions adhérentes facilite celle des jonctions serrées dans divers tissus [167], ce qui se reflète par les nombreuses interactions entre les composantes des deux types de jonctions. Deux complexes multiprotéiques se retrouvent au niveau des jonctions adhérentes: le complexe nectine-afadine et le complexe cadhérine-caténine (Figure 4). L'expression spécifique de certaines nectines et de certaines cadhérines détermine les propriétés d'adhésion de la jonction adhérente [168].

3.1 Le complexe nectine-afadine

Les nectines constituent une famille de molécules d'adhésion appartenant à la superfamille des immunoglobulines. Cette famille comporte quatre membres, les nectines 1 à 4, ainsi que leurs isoformes générés par épissage alternatif. Ces protéines sont

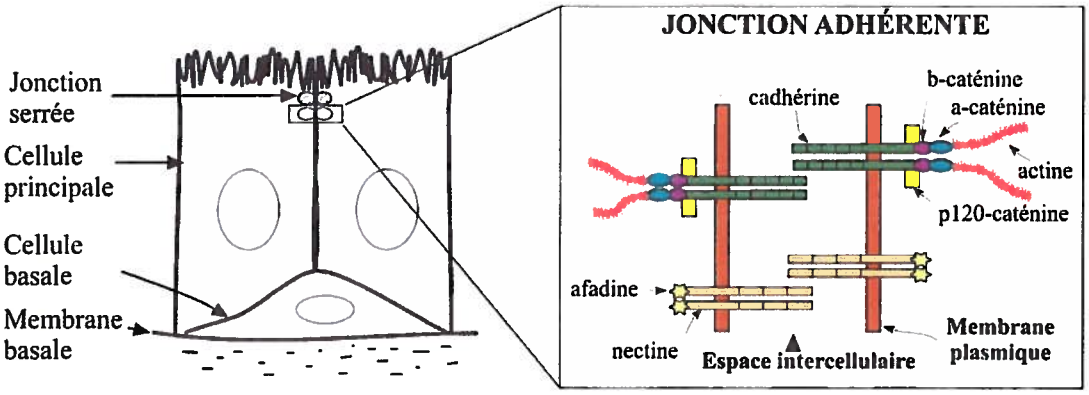

Figure 4: Organisation de jonctions adhérentes. Deux complexes multiprotéiques composent les jonctions adhérentes, le complexe nectine-afadine et le complexe cadhérine-caténine. Créée par Evemie Dubé.

composées d'un domaine extracellulaire, d'une seule région transmembranaire et d'une région C-terminale cytoplasmique possédant des sites de liaison aux domaines PDZ. Les nectines interagissent de manière homo- ou hétérophiliques avec d'autres nectines ou des récepteurs similaires aux nectines [168]. Elles se lient entre autres à l'afadine (AF-6) qui constitue un lien direct au cytosquette d'actine [169] et qui est impliquée dans la signalisation [170]. Dans les cellules épithéliales, les nectines et l'AF-6 sont localisées aux jonctions adhérentes [171]. Très peu d'informations existent sur les nectines et l'AF-6 dans l'épididyme, mais des études récentes de microréseaux ont permis de mettre en évidence l'expression des gènes codant pour les nectines 1, 2, 3, 4 et l'AF-6 dans l'épididyme du rat et de la souris [18, 19]. Les nectines jouent un rôle dans l'adhésion cellulaire dépendante des cadhérines et dans la formation des jonctions serrées. En effet, les nectines participent au recrutement des cadhérines au site d'adhésion cellulaire basée sur le complexe nectine-afadine par l'intermédiaire de l'AF-6 et des caténines [172], par la suite elles recrutent les protéines JAMs, les CLDNs et l'occludine aux jonctions adhérentes pour finalement participer à la formation des jonctions serrées [173, 174]. Par exemple, le recrutement de JAM-1 se fait par son interaction avec l'AF-6 et TJP1. De plus, les nectines induisent l'activation de cdc42 et des petites protéines G Rac qui participent à la formation des jonctions adhérentes basées sur les cadhérines suite à la réorganisation du cytosquelette d'actine [175]. Il a d'ailleurs été démontré que l'inhibition des nectines et de l'AF-6 empêche la formation des jonctions adhérentes et serrées dans des cellules épithéliales, ainsi que la formation des jonctions adhérentes dans des fibroblastes [176-178].

3.2 Le complexe cadhérine-caténine

Les cadhérines appartiennent à une large famille de protéines comprenant plus de 100 membres qui sont des molécules d'adhésion cellulaire dépendantes du calcium et possédant un seul domaine transmembranaire. Les cadhérines peuvent être classées en différents groupes: les cadhérines classiques (ou de type I), les protocadhérines, les cadhérines desmosomales, les cadhérines atypiques (ou de type II) et les protéines

apparentées aux cadhérines. L'expression des cadhérines est ubiquitaire et ces protéines sont impliquées dans différents processus incluant l'initiation et la stabilisation de l'adhésion cellulaire, la régulation du cytosquelette d'actine, la signalisation intracellulaire et la régulation de la transcription [179].

Plusieurs cadhérines classiques et protocadhérines sont exprimées dans l'épididyme du rat, de la souris et de l'humain [18, 19, 151]. Contrairement aux cadhérines classiques, les protocadhérines ont très peu de propriétés adhésives, mais ces protéines pourraient, par contre, être impliquées dans la régulation des propriétés adhésives des cadhérines classiques [180]. Les études dans l'épididyme se sont surtout concentrées sur la cadhérine classique CDH1 (aussi appelée cadhérine E). Les cadhérines classiques sont composées d'une partie extracellulaire, constituée de cinq domaines de type immunoglobuline, capable d'interagir avec une molécule identique portée à la surface de la cellule voisine afin d'initier l'adhésion entre deux cellules adjacentes [179]. Dans l'épididyme du rat, CDH1 est exprimée par les cellules principales avec des taux d'expression variant d'une région à l'autre. Son expression est, en effet, élevée dans le corps et dans la tête, moyenne dans la queue et faible dans le segment initial [181]. CDH3 (ou cadhérine P) est aussi exprimée dans l'épididyme du rat [182]. De plus, CDH1 est régulée durant le développement de l'épididyme au niveau de l'ARNm et de la protéine [181, 182]. Une étude récente sur les effets de la ligation des vas efférents sur l'épididyme du rat démontre que plusieurs gènes codant pour des cadhérines (*CDH1, CDH15*) et pour des protocadhérines (*PCDHGA1, PCDHGA2, PCDHGA10, PCDHGA12, PCDHGB5, PCDHGB7, PCDHGA8, PCDHGA7, PCDHGA11, PCDHGA9, PCDHGC3*) sont régulés par des facteurs testiculaires dans l'épididyme proximal [152]. A l'exception de CDH15 (aussi connue sous le nom de cadhérine des myotubules), l'expression de ces gènes diminue en l'absence de facteurs testiculaires. Dans l'épididyme humain, CDH1, et non pas CDH2 (ou cadhérine N), est localisée à la surface des cellules principales [183]. Il a également été observée que CDH1 est exprimée dans le canal de Wolff (le canal précurseur de l'épididyme) de fœtus d'humain et de rat contrairement à CDH2 [184]. Le domaine cytoplasmique des cadhérines classiques est hautement conservé et permet de se

lier à plusieurs protéines périphériques, telles que les caténines β et p120. La caténine β interagit ensuite avec la caténine α créant ainsi un lien entre la cadhérine et le cytosquelette d'actine [185]. De plus, la caténine β peut être transloquée au noyau et participe à la voie de signalisation Wnt [186]. Il a également été démontré que la baisse d'expression de CDH1 retarde la localisation respective de TJP1, des caténines α et β [187]. Par contre, la caténine p120 semble être stable dans le cytosol lorsqu'elle n'est pas liée à une cadhérine. Il a été proposé que l'association entre la caténine p120 et CDH1 permet de stabiliser cette dernière à la membrane plasmique lors de la formation des points de contacts et d'empêcher sa dégradation [188]. De plus, TJP1 peut se lier à la caténine α et se co-localiser avec CDH1 aux premiers points de contacts cellulaires pour, par la suite, migrer aux jonctions serrées lors de leur formation [189]. D'ailleurs, la région de la caténine α qui se lie à TJP1 semble être importante pour une forte adhésion cellulaire. DeBellefeuille et ses collègues [190] ont démontré que plusieurs caténines étaient exprimées dans l'épididyme du rat. Les caténines β, α et p120 sont exprimées le long des membranes plasmiques des cellules épithéliales avec une expression plus élevée dans le corps et dans la queue de l'épididyme pour les caténines α et ß. Une étude d'immunoprécipitation a confirmé que CDH1, les caténines p120 et α s'associent à la caténine β dans l'épididyme du rat adulte. De plus les auteurs ont observé que les caténines α et β, contrairement à la caténine p120, sont régulées par les androgènes dans l'épididyme du rat adulte ainsi que durant le développement postnatal. Ctnnal1, aussi connue sous le nom de alpha-catuline, est exprimée dans l'épididyme du rat et est également régulée par des facteurs testiculaires dans la région proximale de ce tubule [152]. Ctnnal1 est également impliquée dans les voies de signalisation Rho et NF-kappa B, qui pourraient donc jouer un rôle dans la régulation des jonctions épididymaires [191, 192]. Des analyses de microréseaux ont monté que d'autres cadhérines étaient exprimées dans l'épididyme murin telles que *CDH3, CDH10, CDH11, CDH13, CDH15* et *CDH16* [18]. CDH16, contrairement aux autres cadhérines, ne peut pas interagir avec les caténines à cause de sa queue cytoplasmique tronquée. Mais il a été montrée qu'elle interagit avec la protéine alpha-B-crystalline impliquée dans la régulation du

cytosquelette d'actine [193]. De plus, CDH16 est exprimée dans l'épithélium des conduits sexuels embryoniques du lapin [194] et est régulée par SNAIL [195].

De nombreuses études ont démontré le rôle des cadhérines dans la signalisation intracellulaire au niveau du cytoplasme et du noyau [196]. De nombreuses études ont démontré que les jonctions adhérentes contribuent à la mise en place d'autres types de jonctions, mais de plus en plus de données suggèrent que des composantes des jonctions serrées influencent aussi les jonctions adhérentes. En effet, la baisse d'expression de TJP2 retarde la formation des jonctions adhérentes et serrées [197] alors que l'expression exogène de l'extrémité N-terminale de TJP3 affecte la localisation de CDH1, de la caténine β et de TJP1 [198]. Lioni et ses collègues [199] ont également démontré que CLDN7 serait impliquée dans la régulation de CDH1 alors que l'inhibition de PALS1 affecte les jonctions adhérentes et serrées en altérant l'exocytose des cadhérines [200].

4. Interactions entre les jonctions serrées, adhérentes et lacunaires

La communication cellulaire se faisant grâce aux jonctions lacunaires joue un rôle dans une multitude de processus incluant la croissance et la différenciation cellulaire [201]. Les jonctions lacunaires sont formées de deux connexons (Figure 5), homo- ou hétéromériques, qui comprennent un oligomère de 6 connexines (Cxs). Ces canaux de diamètre très petit, permettent le passage d'ions et de molécules de moins de 1kDa entre deux cellules adjacentes. À ce jour, au-moins 20 Cxs ont été identifiées chez l'humain et les rongeurs [202]. Toutes les Cxs ne peuvent pas interagir entre elles. Par exemple, Cx26/GJB2 forme des canaux fonctionnels avec Cx30/GJB6 et Cx32/GJB1, mais ne peut pas en former avec Cx40/GJA5 [203].

Des études ont démontré l'existence de jonctions lacunaires entre les cellules principales de l'épididyme du rat [62, 204]. Dans l'épididyme du rat, Cx43/Gja1 est localisée le long des membranes plasmiques entre les cellules principales et basales, et non pas entre les cellules principales, excepté dans le segment initial [205]. De plus, la localisation de

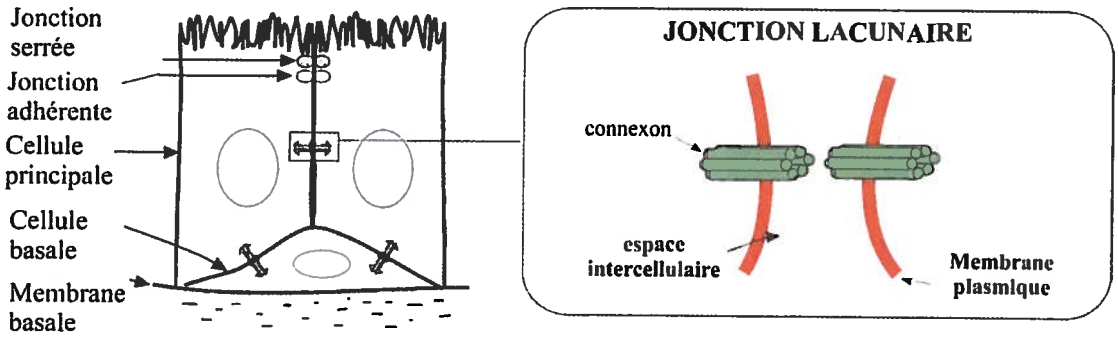

Figure 5: Organisation des jonctions lacunaires. Les jonctions lacunaires sont constituées de deux connexons. Chaque connexon est lui-même composé de six connexines. Créée par Evemie Dubé.

Cx43/Gja1 dans le segment initial est régulée par les androgènes. St-Pierre et ses collègues [206] ont démontré que Cx43/Gja1 est aussi régulée par les hormones thyroïdiennes dans le segment initial, dans la tête et dans le corps de l'épididyme du rat. Les autres Cxs exprimées dans l'épididyme du rat comprennent Cx26/Gjb2, Cx30.3/Gjb4, Cx31.1/Gjb5, et Cx32/Gjb1 mais leur expression varie selon la région et l'âge [207]. Cx26/Gjb2 est exprimée dans la tête et dans le corps de l'épididyme de jeunes rats, alors que Cx30.3/Gjb4, Cx31.1/Gjb5, et Cx32/Gjb1 sont présents dans l'épididyme de rats adultes. De plus, Cx26/Gjb2 et Cx32/Gjb1 sont localisées le long des membranes plasmiques entre les cellules épithéliales dans l'épididyme de rats adultes. Il a été suggéré que Cx26/Gjb2 et Cx43/Gja1 sont impliquées dans la différenciation de l'épithélium épididymaire, et que la communication, via les jonctions lacunaires, serait importante pour les fonctions épididymaires.

Plusieurs évidences suggèrent que les Cxs participent à la signalisation intracellulaire et interagissent avec les composantes des autres types de jonctions [208]. L'augmentation du nombre de points de contact, via CDH1, entraîne même une hausse du nombre de jonctions lacunaires [209]. Des études ont démontré l'association entre Cx43/GJA1et CDH2 [210], entre la caténine p120, Cx43/GJA1 et CDH2 [211] ainsi qu'entre la caténine β et Cx43/GJA1 [212]. De plus, l'inhibition de CDH2 prévient la formation des jonctions adhérentes et la communication cellulaire, via les jonctions lacunaires [213]. Il a été suggéré que la caténine β interagit avec la caténine α, TJP1 et Cx43/GJA1 pendant la mise en place des jonctions lacunaires. En effet, TJP1 interagit avec un grand nombre de Cxs, telles que Cx43/GJA1 [214, 215], Cx47/GJC2 [216], Cx31.9/GJD3 [217] et Cx40/GJA5 [218]. L'extrémité C-terminale de Cx43/GJA1 se lie également à TJP2 [219]. Cx45/GJC1 se lie à TJP1 et à TJP3, mais pas à TJP2 [220], alors que Cx36/GJD2 co-immunoprécipite avec TJP2 et TJP3 [221]. Les protéines TJPs pourraient donc être impliquées dans l'organisation ou dans la régulation des jonctions lacunaires. Une étude récente révèle que la protéine de jonction serrée CAR régulerait la communication cellulaire [222]. En effet, l'inhibition de CAR affecte l'expression et la localisation de Cx43/GJA1. De plus, Cx32/Gjb1 interagit avec l'occludine et co-localise avec Cldn1,

l'occludine et Tjp1 dans des hépatocytes de rat [223, 224]. Ces nombreuses interactions suggèrent que les connexines jouent peut-être un rôle dans les fonctions des jonctions serrées. L'expression de Cx32/GJB1 dans une lignée cellulaire d'hépatocytes a d'ailleurs provoquée une hausse du nombre de jonctions serrées et de l'expression de plusieurs protéines, incluant l'occludine, les CLDNs 1 et 2 et MAGI-1 [223-225]. Dans les cellules Calu-3, la surexpression de Cx26/GJB2 a même empêcher l'ouabaïne (un inhibiteur de la of Na+/K+ATPase) d'altérer les fonctions de barrière des jonctions serrées [226].
Les interactions entre les différentes jonctions mettent en jeu une multitude de protéines essentielles au bon fonctionnement de chaque type de jonction. Dans l'épididyme, certains liens ont été mis en évidence mais il est encore incertain à quel point ces interactions sont importantes pour la formation et le maintien de la barrière hémato-épididymaire et, par conséquent, pour la fertilité masculine.

5. La barrière hémato-épididymaire et l'infertilité masculine humaine

L'infertilité masculine humaine affecte 25% des couples consultant pour des problèmes de fertilité [1]. Les causes de l'infertilité masculine humaine sont multiples et incluent notamment des désordres génétiques ou endocrinologiques, un dysfonctionnement testiculaire, des infections, des problèmes immunologiques et des problèmes éjaculatoires. Cependant, il est impossible de diagnostiquer la cause de l'infertilité dans 18% des cas, on parle alors d'infertilité idiopathique [1]. Plusieurs données suggèrent que des anomalies dans le fonctionnement de l'épididyme pourraient contribuer à l'infertilité masculine. On peut, entres autres, parler de la présence d'anticorps dirigés contre les spermatozoïdes présents chez 9% des couples infertiles [227]. De plus, certains de ces anticorps sont dirigés contres des protéines sécrétées par l'épithélium épididymaire [228]. Suite à la vasectomie, une infiltration de l'épithélium épididymaire par les cellules immunitaires et de plus hauts taux d'anticorps dirigés contre les spermatozoïdes ont également été observés [229]. Une étude récente a mis en évidence des changements d'expression des gènes codant pour les CLDN8 et CLDN10 chez des hommes vasectomisés [230]. Une altération des jonctions serrées pourrait entraîner l'exposition

des spermatozoïdes au système immunitaire et, par conséquent, la production d'antigènes anti-spermatiques. Il a également été démontré que l'intégrité de la barrière peut être compromise avec l'âge suite à des modifications de différentes protéines jonctionnelles, surtout au niveau du corps de l'épididyme [13]. Plusieurs maladies sont d'ailleurs associées à des anomalies de jonctions serrées [231]. Hermo et ses collègues [232] ont montré que, dans des souris déficientes en cathepsine A, une expression plus faible et une mauvaise localisation des Cldn1, 3, 8 et 10 sont associées à une motilité altérée des spermatozoïdes. L'infertilité masculine causée par des anomalies épididymaires pourrait donc être le résultat d'une perturbation de la barrière hémato-épididymaire, du transport paracellulaire et transcellulaire et de différentes voies de signalisation impliquées dans la régulation des fonctions de l'épididyme.

6. Hypothèse de recherche

La revue de littérature présentée ci-dessus permet de constater à quel point la barrière hémato-épididymaire est importante pour la maturation post-testiculaire des spermatozoïdes ainsi que pour leur survie en contribuant à la création et au maintien du milieu intraluminal. De plus, les jonctions serrées sont étroitement liées aux jonctions adhérentes et lacunaires. Cependant, la formation, le maintien et la régulation de cette barrière constituent des processus complexes et multifactoriels, qui ont principalement été étudiés dans des modèles animaux. Malgré de nombreuses similitudes avec les rongeurs, l'épididyme humain présente certaines particularités, tels que la présence de canaux efférents dans la tête de l'épididyme, un temps de maturation de quelques jours et une fonction de réservoir quasiment nulle. Il est donc important de mieux comprendre son fonctionnement afin d'élucider les causes de l'infertilité masculine humaine.

Il n'existe, dans la littérature, aucune information sur la composition des jonctions serrées dans l'épididyme humain. Ce projet de doctorat repose sur l'hypothèse que les CLDNs sont un élément important des jonctions serrées épididymaires et qu'un changement au niveau de ces protéines pourrait conduire à une baisse d'intégrité de la barrière hémato-

épididymaire et affecter la fertilité masculine. Pour vérifier cette hypothèse de recherche, le premier objectif était de déterminer la composition de la barrière hémato-épididymaire humaine en regardant l'expression de gènes codant pour différentes protéines jonctionnelles, et de vérifier si ces protéines étaient localisées aux jonctions serrées. Nous avons, par la suite, vérifier si l'expression et la localisation de ces protéines changent en cas d'infertilité masculine. Nous nous sommes intéressés plus particulièrement aux hommes souffrant d'azoospermie car ce type d'infertilité touche près de 10 à 15 % des hommes infertiles. Il a été démontré que ces patients présentent un taux élevé d'anticorps dirigés contre les spermatozoïdes suggérant que la barrière hémato-épididymaire est compromise. L'azoospermie peut être due à une obstruction des canaux du système reproducteur mâle ou à un problème de production des spermatozoïdes. Ainsi, nous avons voulu comparer les niveaux d'expression des CLDNs dans l'épididyme chez les patients fertiles et infertiles souffrant d'azoospermie non obstructive et obstructive. Le développement de lignées cellulaires de l'épididyme humain nous a finalement permis d'identifier les CLDNs essentielles pour le maintien de la barrière ainsi que les voies de signalisation impliquées dans la formation et le maintien des jonctions serrées. Les résultats obtenus constituent quatre chapitres dans la deuxième partie de cette thèse et ont été publiés ou acceptés dans des journaux scientifiques sous forme de quatre manuscrits différents.

CHAPITRE 2 : ARTICLES SCIENTIFIQUES

SECTION 1: GENE EXPRESSION PROFILING AND ITS RELEVANCE TO THE BLOOD-EPIDIDYMAL BARRIER IN THE HUMAN EPIDIDYMIS

Dubé E, Chan PTK, Hermo L, Cyr DG
Biology of Reproduction, 76(6) ,1034-1044, 2007.

1.1 Résumé de l'article en français

Le microenvironnement présent dans la lumière de l'épididyme est important pour la maturation post-testiculaire des spermatozoïdes. Cet environnement spécifique est créé par la barrière hémato-épididymaire, elle-même formée par les jonctions adhérentes et serrées. Il existe cependant très peu d'informations sur la barrière hémato-épididymaire humaine et sur les protéines qui la composent. Nos objectifs étaient d'étudier les profils d'expression des gènes dans les différents segments de l'épididyme humain et d'identifier les protéines composant la barrière hémato-épididymaire. En utilisant la technique des microréseaux, nous avons identifié 2980 gènes qui sont différentiellement exprimés par un ratio minimum de 2 entre les différents segments de l'épididyme. Ces gènes incluent plusieurs codant pour des protéines d'adhésion (cadhérines et caténines) et des protéines de jonctions serrées (CLDNs, TJPs). Des analyses par RT-PCR ont confirmé les résultats des microréseaux. L'immunolocalisation des CLDN1, 3, 4, 8 et 10 démontrent que la localisation des CLDNs varie le long de l'épididyme. Les CLDN1, 3 et 4 sont localisées aux jonctions serrées, le long des membranes plasmiques latérales entre les cellules principales adjacentes et entre les cellules principales et basales. CLDN8 est localisée aux jonctions serrées le long de l'épididyme, mais aussi le long des membranes plasmiques latérales entre les cellules principales adjacentes dans la tête, et entre les cellules principales et basales dans le corps de l'épididyme. CLDN10, TJP1 et l'occludine sont exclusivement localisées aux jonctions serrées dans les trois régions de l'épididyme. Ces résultats montrent que l'épididyme a un patron d'expression de gènes très complexe, qui incluent plusieurs impliqués dans la formation de la barrière hémato-épididymaire et suggèrent ainsi une régulation complexe de cette barrière.

1.2 Contribution de l'étudiante

L'étudiante a réalisé toutes les expériences présentées dans cet article, a rédigé l'article, a participé au choix du journal de publication et aux corrections nécessaires à la publication de la version finale de l'article.

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Gene Expression Profiling and Its Relevance to the Blood-Epididymal Barrier in the Human Epididymis[1]

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ABSTRACT

The luminal environment along the epididymal duct is important for spermatozoal maturation. This environment is unique and created by the blood-epididymal barrier, which is formed by tight and adhering junctions. For the human epididymis, little information exists on the proteins that comprise these junctions. Our objectives were to assess the gene expression profiles in the different segments of the human epididymis and to identify the proteins that make up the blood-epididymal barrier. Using microarrays, we identified 2980 genes that were differentially expressed by at least 2-fold between the various segments. Of the many genes involved in diverse functions, were those that encoded adhesion proteins (cadherins and catenins) and tight junctional proteins (claudins [CLDN] and others). PCR analyses confirmed the microarray data. Immunolocalization of CLDNs 1, 3, 4, 8, and 10 revealed that the localization of CLDNs differed along the epididymis. In all three segments, CLDNs 1, 3, and 4 were localized to tight junctions, along the lateral margins of adjacent principal cells, and at the interface between basal and principal cells. CLDN8 was localized to tight junctions in all three segments, in addition to being localized in the caput along the lateral margins of principal cells, and in the corpus, at the interface between principal and basal cells. CLDN10, tight junction protein 1, and occludin were localized exclusively to tight junctions in all three epididymal segments. These data indicate that the epididymis displays a complex pattern of gene expression, which includes genes that are implicated in the formation of the blood-epididymal barrier, which suggests complex regulation of this barrier.

cadherin, catenin, claudin, epididymal junctions, epididymis, gene regulation, genomics, male reproductive tract

INTRODUCTION

The epididymis is a highly specialized tissue that is involved in the maturation, transport, protection, and storage of mammalian spermatozoa. This organ is a long, single, convoluted tubule that is morphologically divided into three main segments: the caput, corpus, and cauda epididymidis (Fig. 1). During epididymal transit, spermatozoa acquire progressive motility and the abilities to bind and fertilize the oocyte [1–4]. Spermatozoal maturation involves the remodeling of the sperm plasma membrane by the addition, removal or modification of cell surface molecules, which is in part due to the interaction of spermatozoa with molecules found in the luminal microenvironment [5–7]. The luminal microenvironment of the epididymis is comprised of specific ions, small organic molecules, and proteins that are secreted or absorbed by the epididymal epithelium [1, 8, 9]. The blood-epididymal barrier, which is composed of apical tight junctions between principal cells, forms an impenetrable seal and forces the movement of molecules across these cells by specific receptors, ion and water channels, and solute carrier proteins [1, 10, 11]. Thus, the barrier creates specific environments between the lumen and circulation and within the epithelial cells [12, 13].

The ultrastructure of the blood-epididymal barrier was first described by Friend and Gilula [14], who also reported that the tight junctions varied in terms of the number of strands and complexity along the epididymis, being more extensive in the caput. To date, tight junctions have been shown to be composed of a variety of peripheral membrane proteins, including tight junction proteins 1, 2, and 3 (TJP1, 2, and 3, also known as ZO proteins), symplekin, cingulin, 7H6 antigen, cytoskeletal elements (fodrin and actin), as well as integral transmembrane proteins, such as occludin and claudins (CLDNs) [15–18]. In particular, CLDNs form a multigene family that contains at least 20 members with variable tissue distributions [19–22]. CLDNs are transmembrane proteins that are essential for both the barrier function of tight junctions [23] and specific paracellular ion transport [24–26]. In the rat epididymis, occludin, TJP1, and several CLDNs are present in the tight junctions [13, 27–31]. However, there are few studies that deal with the localization and functions of CLDNs in the human epididymis.

Several genes unrelated to junctional protein genes have been characterized and shown to be expressed differentially along the epididymis [32, 33]. However, the roles of these genes and their products in the formation of the complex luminal fluid and in sperm maturation are not well understood. It is known that the epididymis has a highly region-specific gene expression pattern [34]. However, the molecular mechanisms that contribute to the formation of a specific luminal microenvironment that is crucial for sperm maturation via segment-specific gene expression are unknown [35]. Recently, microarrays have enabled the global study of gene expression and the rapid discovery of novel genes throughout the body. In the human male reproductive tract, gene profiling studies have been performed with microarray analyses of the spermatozoa from fertile men [36], the epididymis of one fertile man [37], testicular germ cell tumorigenesis [38], FSH-stimulated Sertoli cells [39], and testicular development [40]. Several studies of epididymal gene and protein expression patterns have also been carried out in rodents, and it has been reported that gene

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expression in the epididymis can be affected by androgens and ageing [41–44]. A number of regionally expressed genes have been identified that are unique to the epididymis, which suggests specific roles in epididymal function, whereas other genes are not tissue-specific, which implies broader functions. The latter encode secretory proteins (such as proteases, protease inhibitors, antioxidant enzymes, modifying enzymes, growth factors, neuropeptides, and transporters), intracellular proteins (transcription factors, signaling molecules, receptors, and kinases), as well as proteins of unknown function. Together, these studies provide important information on the molecular events underlying posttesticular sperm maturation. Nevertheless, gene profiling of the human epididymis has been limited due to difficulties in obtaining sufficient biological material. Elucidation of the transcriptional profiles of the different human epididymal segments is a critical step towards understanding not only the process of sperm maturation but also the causes of clinical idiopathic male infertility.

The objectives of the present study were to investigate the global gene expression patterns in different segments of the epididymis, to determine the intrasegmental differences in gene expression, particularly of genes associated with adherens and tight junctions, and to assess the localization of different tight junctional proteins in cell type- and segment-specific manners along the human epididymis.

MATERIALS AND METHODS

Tissue Preparation

Human epididymides were obtained from four patients (29–50 years of age) who were undergoing radical orchidectomy for localized testicular cancer (confined within the testicular tunica albuginea). Informed consent was obtained from each patient. This study was conducted with the approval of the Ethics Committee for Research on Human Subjects of McGill University. Epididymides were subdivided into three separate segments (caput, corpus, and cauda epididymidis). All patients had active spermatogenesis. Tissues were received in cold culture medium that contained antibiotics (Dulbecco modified Eagle medium and Ham F-12 with penicillin-streptomycin) and were transported to the laboratory within 1 h of surgery. Tissues were either frozen in liquid nitrogen for RNA preparation or fixed for light and electron microscopy.

Electron Microscopy

Small pieces of tissue (1 mm^3) from each segment were fixed immediately at the time of surgery by immersion in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h, after which they were washed in cacodylate buffer, and then postfixed in potassium ferrocyanide-reduced osmium tetroxide for 1 h, to enhance the staining of membranes as described by Hermo and Jacks [45]. Subsequently, the epididymal tissue was rinsed several times in cacodylate buffer, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Thin sections were cut with a diamond knife, mounted on copper grids, counterstained with uranyl acetate and lead citrate, and examined under the FEI Tecnai 12 electron microscope.

Microarray Processing

Total cellular RNA was isolated using the Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA) according to the manufacturers instructions. The quality of the total RNA was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies). Gene expression profiling was performed with commercially available Human oligonucleotide microarrays (20 174 human genes; Agilent Technologies). Amplification and labeling of 500 ng of total RNA were performed using the Low RNA Input Linear Amplification Kit (Agilent Technologies). The cRNA was labeled with either cyanine 3 or cyanine 5 (Perkin Elmer, Woodbridge, Canada). Arrays were hybridized according to the manufacturers instructions using the In Situ Hybridization kit Plus (Agilent Technologies). Following hybridization, microarrays were scanned with a ScanArray Express scanner (Perkin Elmer). Fluorescence ratios for array elements were extracted using the ScanArray Express Software (Perkin Elmer) and imported into the GeneSpring 6.1 software (Agilent Technologies) for further analysis.

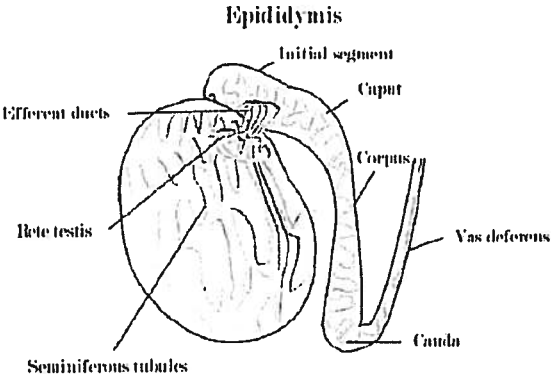

FIG. 1. Schematic representation of the human epididymis. The epididymis is a long convoluted tubule that connects the efferent ducts from the rear of each testicle to the vas deferens. The typical pattern of epididymal segmentation, as used in the present study, is shown.

Microarray Analysis

Expression analysis of all the microarray experiments was performed with GeneSpring 6.1 (Agilent Technologies). The data were normalized using a locally weighted regression Lowess method. Genes were considered to be enriched in a specific segment of the epididymis if their expression was 2-fold lower or higher in that segment than in other segments for at least three patients. Statistical analyses were performed with one-way ANOVA (significance level set at $P < 0.05$). Analyses were carried out in accordance with the MIAME standards.

Real-Time PCR

Real-Time PCR was used to confirm the differences in transcription that were observed in the microarray analysis. Total RNA (500 ng) was reverse-transcribed using an oligo$(dT)_{16}$ primer. Forward and reverse primers for the genes of interest were designed using the Oligo Primer Analyses software (Molecular Biology Insights, Cascade, CO) based on sequences published in GenBank. The primers are listed in Table 1. Real-Time PCR was performed with a Rotor-Gene RG3000. A 2-μl aliquot of the RT reaction was amplified in a 15-μl solution that contained 1× Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Burlington, ON, Canada) and 0.3 μM of each of the reverse and forward primers. The PCR cycling protocols were optimized to maximize reaction efficiency and to ensure that only the target product contributed to the SYBR Green fluorescence signal. For each quantification, a standard curve was created using the appropriate cDNA. Amplification consisted of 40 cycles at 95°C for 15 sec, melting temperature (Tm) for 30 sec, and 72°C for 30 sec. Primers for the housekeeping gene, *GAPDHS*, were used to normalize the values for each sample. Samples were assayed in duplicate and identical samples were run in each assay, to calibrate for interassay variations. Following PCR amplification, melting curve analysis was performed to ensure the accuracy of quantification.

Reverse Transcription-Polymerase Chain Reaction

RT-PCR was used to confirm the presence of transcripts for the different *CLDN* genes. Total RNA (500 ng) was obtained from two different individuals using Biochain (Hayward, CA) and reverse-transcribed using an oligo$(dT)_{16}$ primer. Forward and reverse primers for the genes of interest were designed with the Oligo Primer Analyses software (Molecular Biology Insights) based on the GenBank sequences. The primers are listed in Table 1. PCR amplification was carried out as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, Tm for 30 sec, 72°C for 1 min, and a final step of cooling to 4°C. The PCR products were separated on a 2% agarose gel and visualized with ethidium bromide using a Fluor-S Multi-Imager densitometer (Bio-Rad Laboratories, Mississauga, ON, Canada).

Immunocytochemistry

Small pieces of epididymal tissue were fixed at the time of surgery by immersion in Bouin fixative (Fisher Scientific, Pittsburgh, PA) for 24 h,

TABLE 1. Sequences of the primers used in PCR.

Gene	GenBank accession no.	Primer set (5′–3′)[a]	Annealing temp. (°C)	Amplicon size (bp)
GAPDHS	AF261085	F: GAA GGT GAA GGT CGG AGT CAA R: GGA AGA TGG TGA TGG GAT TTC	56	227
CRISP1	NM_001131	F: ACA TAG AGA AAG GCT TGG TTC R: TAC ATT TGG CAA GTC GGT	54	180
SPINLW1	NM_181502	F: CCT GCA AGA ATA AAC GCT TTC R: TGT TCT GGG AAG GGC TAA G	54	161
DEFB126	NM_030931	F: AAA GAA TGG TTG GGC AAT GTG R: GGG GTA GGA GCC ATC GAA G	54.2	183
CLDN1	NM_021101	F: GTG ATA GCA ATC TTT GTG GC R: CCT CTG TGT CAC ACG TAG TC	65	370
CLDN2	NM_020384	F: GCC ACC CAC AGA CAC TTG TAA GG R: CAC AAT GCT GGC ACC GAC ATA AGA	58.5	269
CLDN3	NM_001306	F: TCG GCA GCA ACA TCA TCA CGT CGC R: GGT TGT AGA AGT CCC GGA TAA TGG	61.3	342
CLDN4	NM_001305	F: CTT CTA CAA TCC GCT GGT GG R: TTA CAC GTA GTT GCT GGC AG	65	193
CLDN5	NM_003277	F: CTC TGC TGG TTC GCC AAC ATT GTC R: GAA GCT GAG GTC GGG ACG G	61.6	183
CLDN6	NM_021195	F: ATG TGG AAG GTG ACC GCT TTC R: GCA AGC AGC CTC CGC ATT A	60	877
CLDN7	NM_001307	F: CCT CCC GGC GTA TCC TAC R: GGG CCT TCT TCA CTT TGT CGT	59.4	582
CLDN8	NM_199328	F: CTG TGG ATG AAT TGC GTG AGG R: GGT GAC TTC TTT CCG GTG TGA	57	500
CLDN9	NM_020982	F: GTG TGA CAG CGG GGT GGT AAG R: GGT CAT GCC CAG CAG TTC TAA GCC	61.3	359
CLDN10	BC010920	F: CCA CGC TGC CCA CCG ACT A R: TGA GCA CAG CCC TGA CAG TAT GAA	58.9	326
CLDN11	NM_005602	F: CTG CAG GTG GTG GGC TTC GTC R: GCA ACA GAG TGA GGC CCT ATT	57.9	1035
CLDN12	NM_012129	F: TCG TGC CCA TAT ACA ATC R: CAT AAG GCT AAA GAC CCA ATA	51.0	745
CLDN14	NM_012130	F: CTG AGG AGC GGC GTG AC R: AAC AGC CAC ATC CGC AAG GTT	59.9	1134
CLDN15	NM_138429	F: TGG CTT CTT CAT GGC AAC TGT R: GGT ATG GCA CTG TGG ACG TG	60.5	854
CLDN16	NM_006580	F: GGA GGC TGG TTG CTT CGG ATA A R: AGG GAG GAA TTT CAC GCA GTC AAG	58.3	552
CLDN17	NM_012131	F: AGG GCT CTG GAT GAA TTG CAT R: TCA CCG GAA TCA GAA CGA AG	57.7	269
CLDN18	NM_016369	F: CAC CGA ATG CAG GCC CTA TT R: GAG CCG CAC CAA ATG TGT ACC	58.5	344
CLDN19	NM_148960	F: GAT GTG GCC ACC TGA CCC TAA R: ACC TCC ACC TCC CGA GTT CA	58.9	553
CLDN23	NM_194284	F: CCC TGC GAC TCC GAC CTC TA R: AAA ACC AAA CCC AAC CTG AAC TAA	58.7	194

[a] F, Forward; R, reverse.

dehydrated, and embedded in paraffin. Thick sections (5 μm) were cut and mounted on glass slides. For immunostaining, the tissue sections were rehydrated through graded ethanol, including 70% alcohol with 1% lithium carbonate for 5 min, to remove residual picric acid. The sections were then incubated in 300 mM glycine for 5 min to block free aldehydes, and washed in 1 M PBS (pH 7.4). Heat-induced epitope retrieval (HIER) was performed by boiling the slides for 10 min in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate). Immunolocalization was performed with the DAKO Catalyzed Signal Amplification System (DAKO, Carpenteria, CA). The primary antibodies used in this study were: rabbit polyclonal anti-CLDN1 (5 μg/ml; Zymed Laboratories, San Francisco, CA); rabbit polyclonal anti-CLDN3 (2.5 μg/ml; Zymed Laboratories); mouse monoclonal anti-CLDN4 (2.5 μg/ml; Zymed Laboratories); rabbit polyclonal anti-CLDN8 (3 μg/ml; Genetex, San Antonio, TX); rabbit polyclonal anti-CLDN10 (2 μg/ml; Abcam, Cambridge, MA); rabbit polyclonal antibody anti-TJP1 (2.5 μg/ml; Zymed Laboratories); and rabbit polyclonal anti-occludin (2.5 μg/ml; Zymed Laboratories). Incubations with the primary antibodies were done either overnight at 4°C (CLDN8 and CLDN10) or for 30–60 min at room temperature (CLDN1, CLDN3, CLDN4, TJP1, and occludin). Omission of primary antibodies served as negative controls. Epididymal sections were counterstained for 2 min with 0.1% methylene blue, dehydrated in ethanol, immersed in Histoclear (Fisher Scientific), and mounted in Permount (Fisher Scientific). Sections were examined under a Leica DMRE microscope.

RESULTS

Ultrastucture of the Human Epididymis

The human epididymis was found to consist of a pseudostratified epithelium underlying a basement membrane and muscular coat that comprised several layers of smooth muscle cells (Fig. 2, A–F). The epithelium was composed mainly of tall columnar principal cells with long microvilli and occasional apical blebs that extended into the lumen (Fig. 2, A–F). The basal cells resided basally (Fig. 2, B, D, and F), while the halo cells were scattered sporadically in the epithelium (Fig. 2D). Principal cells had large oval or elongated nuclei located near the base of the epithelium, with dispersed chromatin clumps (Fig. 2, B, D, and F). In their cytoplasm, pale endosomes, dense lysosomes, mitochondria, endoplasmic reticulum, and the Golgi apparatus were evident (Fig. 2, A, C, E, and F). Apically, tight junctional complexes connected adjacent principal cells to one another (Fig. 2, A, C, and E). Basal cells were round to ovoid in appearance and rested on the basement membrane (Fig. 2, B, D, and F). The lateral

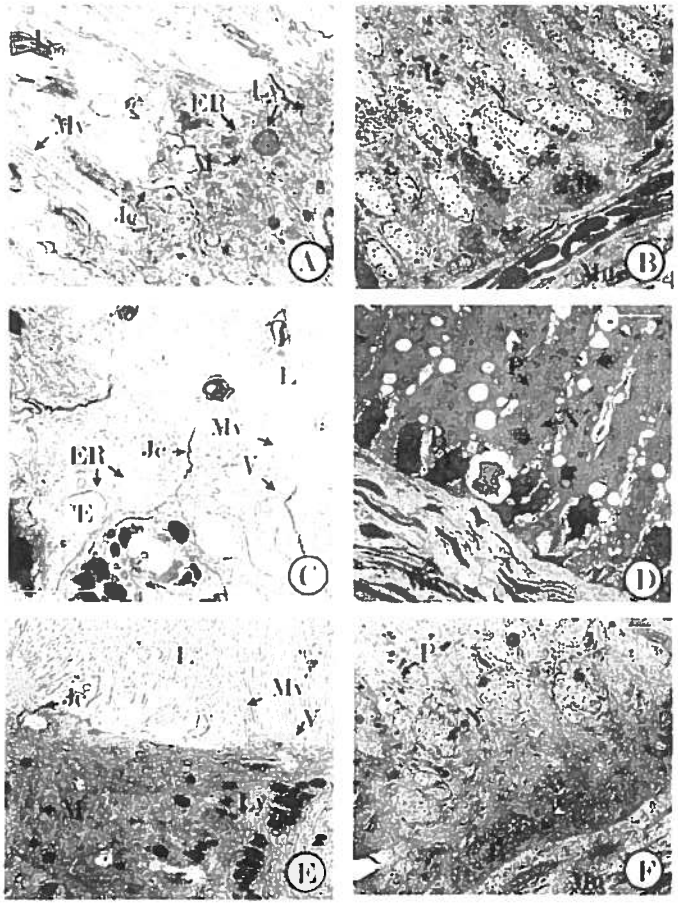

FIG. 2. Electron micrographs of the human epididymis. A) Apical region of the caput epididymidis. B) Basal region of the caput epididymidis. C) Apical region of the corpus epididymidis. D) Basal region of the corpus epididymidis. E) Apical region of the cauda epididymidis. F) Basal region of the cauda epididymidis. Mu, Muscular coat; P, principal cells; B, basal cells; N, nucleus; L, lumen; Mv, microvilli; Jc, junctional complex; ER, endoplasmic reticulum; M, mitochondria; E, endosomes; Ly, lysosomes; V, vesicles. Original magnification ×9900 (A, C), ×1700 (B, F), ×4200 (D), and ×6000 (E).

intercellular spaces between adjacent cells were at times dilated, suggestive of movement of fluid through the epithelium (Fig. 2D). These results confirm that the tissues that were subsequently used for gene analysis were normal.

Segmental Gene Expression Profiling

The human oligonucleotide microarray used in the present study consisted of 20 174 genes. The majority of these genes encoded matrix/structural proteins, as well as proteins that are involved in signal transduction, synthesis/translational control, energy/metabolism, and transcription/chromatin. The array was hybridized with probes representing two different epididymal segments, to identify genes that were highly enriched in each. Genes were considered to be expressed when they were detected in at least three individuals. The number of genes detected in each segment varied. While 98% (19 875) of the genes studied were expressed in the caput epididymidis, 79% (16 036) were expressed in the corpus epididymides, and 88% (17 573) in the cauda epididymidis (Fig. 3). Only 138 genes had no detectable signal, as evidenced by their negative intensity values with either of the probes (data not shown).

Several genes that were expressed in the epididymis were highly characterized with respect to their levels of expression in each epididymal segment. We selected three of these genes for Real-Time PCR analysis, to authenticate the results from the microarray. The expression patterns of *CRISP1* (cysteine-rich secretory protein 1), *DEFB126* (β-defensin 126), and *SPINLW1* (serine peptidase inhibitor-like, with Kunitz and WAP domains 1, also known as *EPPIN*) all showed consistency between the microarray and Real-Time PCR analyses (Fig. 4).

Segment Specificity of Gene Expression

All three segments of the epididymis showed expression of the highly expressed genes (Fig. 3). Of the 20 174 genes studied, 6.6% (1331) were expressed exclusively in the caput, whereas only 0.1% (22) and 0.6% (119) were expressed exclusively in the corpus and cauda epididymides, respectively (Fig. 3). Overall, these 1475 segment-specific genes comprised 7.3% of the transcripts expressed in the epididymis (Fig. 3). Genes expressed in a segment-specific manner were classified into diverse entities based on biological function (Table 2). The products of these genes were primarily involved in fundamental processes, such as apoptosis, signal transduction, transcription, protein modification, host defense, cell growth and differentiation, cell adhesion, and cell signaling. Several genes that have been detected previously in other parts of the male reproductive system were found to be expressed in the epididymis, i.e., *NPM2* (nuclear chaperone), *MOV10L1* (RNA helicase), *DNMT3L* (DNA methyltransferase), *ADAM18*

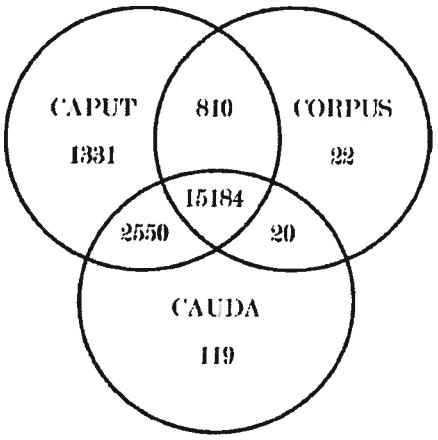

FIG. 3. Regional distributions of the genes detected in each segment of the human epididymis. Overall, 99% (19875 out of 20036) of the genes studied are expressed in the caput epididymidis, 80% (16036 out of 20036) in the corpus epididymidis, and 87.7% (17573 out of 20036) in the cauda epididymidis. Different subsets of genes have been defined subsequently. Of the genes detected, 75% (15184 out of 20036) are expressed in all segments, whereas some genes are expressed in more than one, but not all, of the segments, i.e., 4% (810 out of 20036) in the caput and the corpus, 0.1% (20 out of 20036) in the corpus and the cauda, and 12.7% (2550) in the cauda and caput. Some genes are also segment-specific, i.e., 6.6% in the caput, 0.1% in the corpus, and 0.6% in the cauda. Only 138 genes have no detectable signal.

and *ADAM20* (a disintegrin and metalloprotease), *HIST1H1T* (histone), *OVOL1* (zinc finger protein), *DAZL*, *DMRTC2*, and *DMRT3* (DNA methyltransferase) in the caput, and *KRTAP4–7* (keratin-associated protein) in the corpus. Most of these genes have not been reported previously in the human epididymis. In the cauda epididymidis, no uniquely expressed genes related to processes implicated in fertility were detected. In the human epididymis, 15.3% of the expressed genes were present in only two segments (Fig. 3). Indeed, 4% (810) of the genes were expressed only in the caput and corpus, while 0.1% (20) were found only in the corpus and cauda, and 11.2% (2250) in the caput and cauda (Fig. 3).

Differential Gene Expression in the Human Epididymis

The majority of the genes exhibited an expression ratio of 1.0 when comparisons were made between two segments, which suggests that their expression levels are similar in all the epididymal regions (data not shown). A number of genes were differentially expressed 2-fold. In the corpus, 971 genes exhibited a 2-fold difference in expression level compared to their expression in the caput. Of those, 596 genes exhibited an increase, whereas 375 genes showed a decrease in expression level. In the cauda, 280 genes exhibited an increase in expression level as compared to the corpus, whereas 593 genes showed a decrease. Comparison of the cauda and caput revealed 1136 genes with a 2-fold difference in expression level; 484 genes had higher expression in the caput, whereas 652 genes were more highly expressed in the cauda.

In order to acquire additional information on the selective regional gene expression pattern along the epididymis, genes that showed at least a 4-fold difference in expression in one segment compared to an adjacent segment were examined (Supplementary Tables, available online at www.biolreprod.org). In the caput, the expression levels of 65 genes were

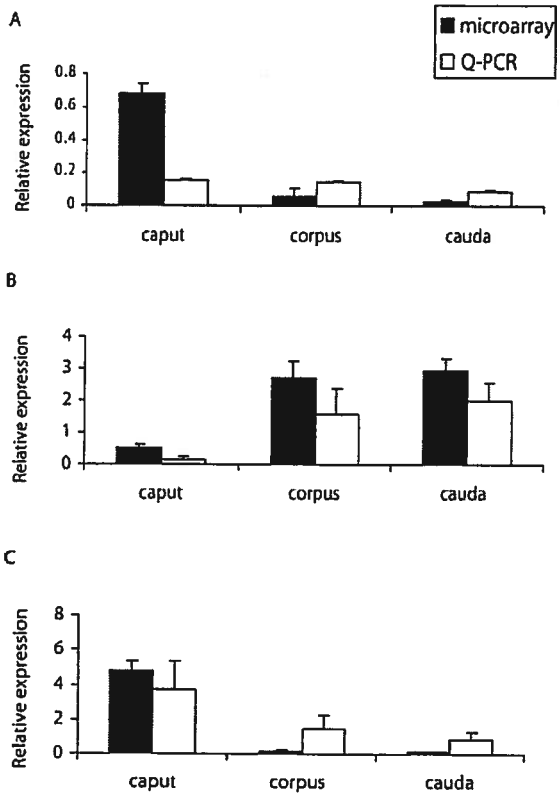

FIG. 4. Confirmation of microarray results by quantitative Real-Time PCR using selected genes that are known to be expressed in the epididymis. The mRNA expression levels of *CRISP1* (A), *SPINLW1* (B), and *DEFB126* (C) in the three segments of the human epididymis were investigated and compared using both microarray and Real-Time PCR (Q-PCR) data. The data are expressed as the ratios of the mRNA levels in relation to *GAPDHS*. Values represent the mean relative expression ± SEM; n = 4.

enriched by a 4-fold difference as compared to the corpus epididymidis. These genes included *DMRTB1*, *RSBN1*, *FAM12A (HE3 ALPHA)*, *FAM12B (HE3 BETA)*, *GGTL4*, *RNASE9*, *DEFB129*, and *CRISP1*, as well as genes involved in transcriptional regulation, cell signaling, and cell growth. In the corpus, the expression levels of 90 genes were enriched 4-fold as compared to the caput. Most of these genes were implicated in transcriptional regulation, cell adhesion, metabolism, and ion transport. Several genes that have previously been detected in the male reproductive tract were also expressed, including *CST9L*, *AQP9*, *CLDN10*, *SPAG11 (HE2)*, *CLDN2*, *DEFB119*, *DEFB123*, *DEFB106A*, *SULT1E1*, *PTGDS*, and *OSTbeta*. In the cauda, the expression levels of 27 genes were enriched 4-fold as compared to the corpus epididymidis. These genes included *DMRTB1* and *RSBN1*. Other genes implicated in transport, transcriptional regulation, cell adhesion, and cell signaling were also expressed.

Genes Implicated in Adhesion and Tight Junctions

Of the genes expressed in the human epididymis, several encoded tight junctional proteins, such as CLDNs 1 to 12, 14 to

TABLE 2. Functional classification of region-specific genes in the human epididymis.

Biological function	No. of genes[a]		
	Caput	Corpus	Cauda
Unknown	615	12	65
Other	323	4	22
Fertility	9	0	0
Hormone	6	0	1
Apoptosis	12	0	0
Cell cycle	15	0	1
Cell proliferation	28	0	2
Cell differentiation	7	0	0
Cell adhesion	45	1	4
Cell-cell signalling	18	0	0
Intracellular signalling	22	1	1
G-Protein signaling pathway	64	1	6
Protein folding	13	0	0
Proteolysis	26	0	1
Transcription	96	0	8
Immune system	32	3	2

[a] Genes were grouped based on their known biological function.

19, and 23, and TJPs 1, 2, and 3, and adhesion proteins, such as E-cadherin (*CDH1*), P-cadherin (*CDH3*), α-catenin (*CTNNA1*) and β-catenin (*CTNNB1*). Of the twenty *CLDN* genes on the array, with the exceptions of *CLDN16* and *CLDN22*, all were expressed in all three segments of the human epididymis (Fig. 5). *CLDN16* was expressed only in the caput and cauda epididymidis and *CLDN22* was not expressed at all. *CLDN4* and *CLDN7* were highly expressed, with relative intensities of more than 1000 (Fig. 5A), whereas *CLDNs 2, 5,* and *10* had relative intensities of 500–1000 (Fig. 5B). All the other *CLDN* genes were weakly expressed, with relative intensities of less than 300 (Fig. 5C). While most *CLDN* genes were expressed at similar levels along the human epididymis, there were some notable exceptions, e.g., *CLDNs 2, 8, 10, 16,* and *23* (Fig. 5). *CLDN2* and *CLDN10* were mainly expressed in the cauda epididymidis (Fig. 5B), whereas *CLDN8* and *CLDN23* were mainly expressed in the caput epididymidis (Fig. 5C). RT-PCR, which was performed for two different individuals using specific primers for each human *CLDN*, confirmed the microarray data (see Fig. 7). *TJPs 1, 2,* and *3* were all expressed at similar levels along the human epididymis. *TJP2* was expressed at higher levels, with relative intensities of 150–200, as compared to *TJP1* and *TJP3*, which had relative intensities of 50–100 (Fig. 6A). Of the different cadherin genes on the array, *CDH1* and *CDH3* were expressed in all three segments of the human epididymis. *CDH1* was expressed at higher levels than *CDH3* with at least a 3-fold difference (Fig. 6B). Three other cadherin genes, *CDH16, CDH22,* and *CDH24*, were highly expressed (Fig. 6B) at similar levels along the epididymis, except for *CHD16*, which had lower expression in the caput epididymidis. Several catenin genes were also detected. *CTNNA1* and *CTNNB1* were expressed in all three segments of the human epididymis at similar levels (Fig. 7C). However, *CTNNA1* was expressed at higher levels than *CTNNB1* and the two p120 catenins (*CTNND1, CTNND2*) by more than a 4-fold difference (Fig. 6C). The α-catulin gene (*CTNNAL1*) was expressed at similar levels to *CTNNA1* (Fig. 6C).

Immunolocalization

Immunocytochemistry performed with antibodies against CLDNs 1, 3, 4, 8, and 10 revealed that these proteins were localized to the area of tight junctions between adjacent principal cells in all three segments (Figs. 8–10). The intensity of CLDN10 immunoreactivity was higher in the corpus and cauda epididymidis than in the caput (Fig. 10, A, D, and G). Furthermore, in all three segments of the epididymis, CLDNs 1, 3, 4, and 8 showed various reactions in other cellular domains. In all three segments, CLDNs 1, 3, and 4 were localized along the lateral margins of adjacent principal cells, as well as between basal and principal cells (Figs. 8 and 9). In contrast, CLDN8 in the caput epididymidis was localized to the lateral margins of principal cells (Fig. 9B), while in the corpus, it was localized to the interface between principal and basal

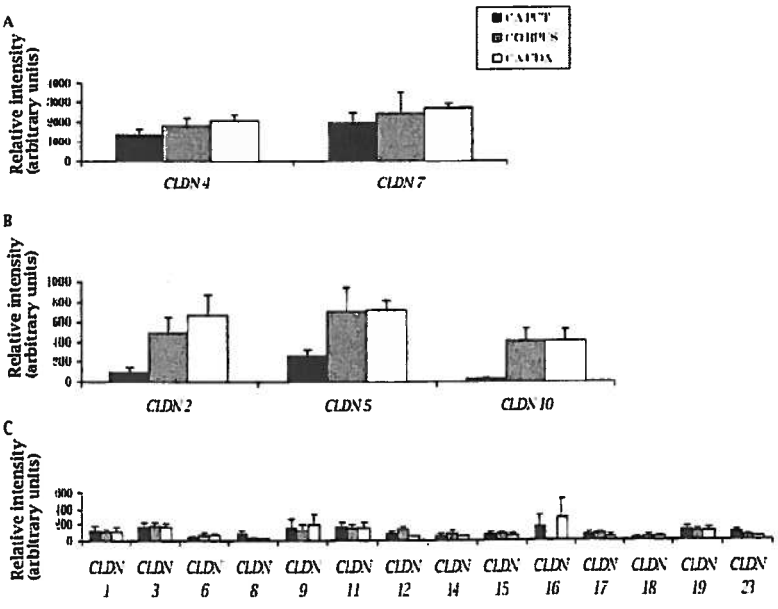

FIG. 5. Expression patterns of *CLDN* genes in the human epididymis. The *CLDN* genes are classified into three groups: highly expressed (A), moderately expressed (B), and weakly expressed (C). Values represent the mean relative intensity ± SEM; n = 4.

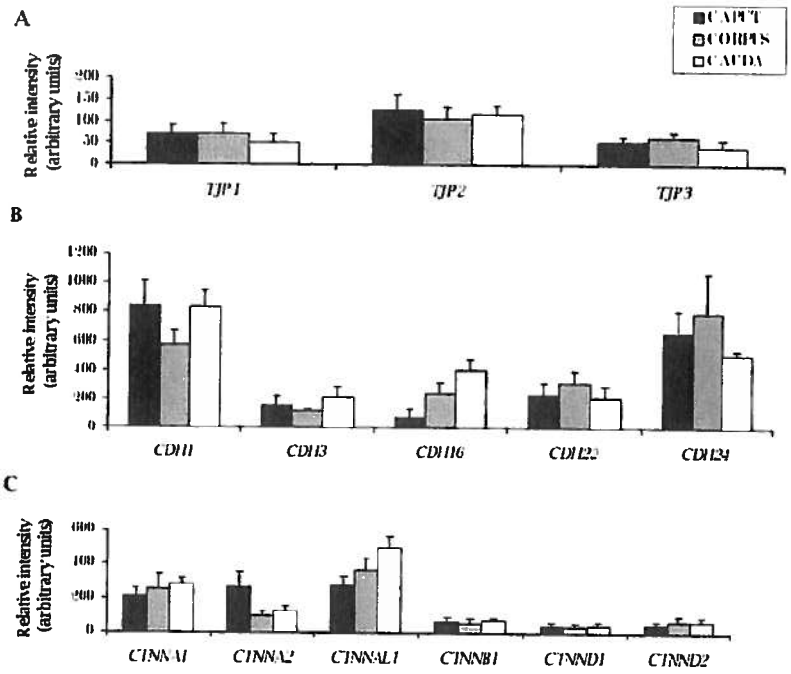

FIG. 6. Expression patterns in the human epididymis of members of the tight junction protein family (A), cadherin family (B), and catenin family (C). Values represent the mean relative intensity ± SEM; n = 4.

cells (Fig. 9D). Immunocytochemistry for TJP1 and occludin revealed that both proteins were localized exclusively to the apical tight junctional complex (Fig. 10).

DISCUSSION

Microarrays constitute a powerful and efficient tool to establish segment-specific gene expression and to highlight genes that may be important in epididymal functions. While several studies have been done on gene profiling in the epididymis, most of them have focused on rodents [41–44, 46, 47].

In the present study, the samples originated from four healthy males with histological confirmation of normal spermatogenesis within the seminiferous tubules and a structurally intact epididymis, as revealed by electron microscopy (Fig. 2), with results that were comparable to those described in the literature [1, 48, 49]. Thus, our present results can be considered to be representative of relatively normal physiological conditions. The microarray data generated in the present study were validated by Real-Time PCR (Fig. 4) and by the published expression patterns of well-studied epididymal transcripts, such as *CRISP1* [50], *SPINLW1* [51, 52], and *DEFB126* [53, 54]. However, in the microarray analysis, there was a difference in the regional distribution of *CRISP1* in the caput epididymidis relative to other regions. These differences were not confirmed by Real-Time PCR. The reasons for these differences are unknown but they may be related to the sensitivities of the different methods used.

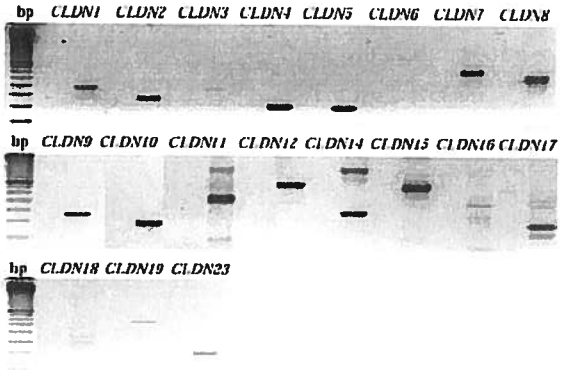

FIG. 7. RT-PCR analysis of different *CLDN* genes in the human epididymis. The primers and PCR conditions are listed in Table 1. Left lane, water control; right lane, total epididymal RNA obtained from a normal 24-yr-old man; bp, 100-bp ladder.

The epididymis displays a complex pattern of gene expression. Indeed, many of the genes detected in the present study are either highly or differentially expressed along the human epididymis. Approximately 15% of the genes differed by at least 2-fold in their levels of expression in different segments. Similar findings have been reported in the mouse [47] and human epididymis [37]. These data suggest that the events in the epididymis that are related to sperm maturation, transport, and storage are complex. Epididymal secretion varies along the length of the duct, resulting in sequential changes to sperm as they move down the duct [7]; this may explain the continuous alterations in the protein composition of the epididymal fluid. Furthermore, the caput epididymidis is known to be the most active segment for protein synthesis and secretion in several species [1, 9, 55]. Therefore, it is not surprising that many of the reproductive and somatic genes were either exclusively expressed (Table 2) or exhibited the highest level of expression in this segment (Supplementary Table 1, available online at www.biolreprod.org). Interestingly, the caput and the cauda epididymidis expressed the largest number of differentially regulated genes. Similar observations

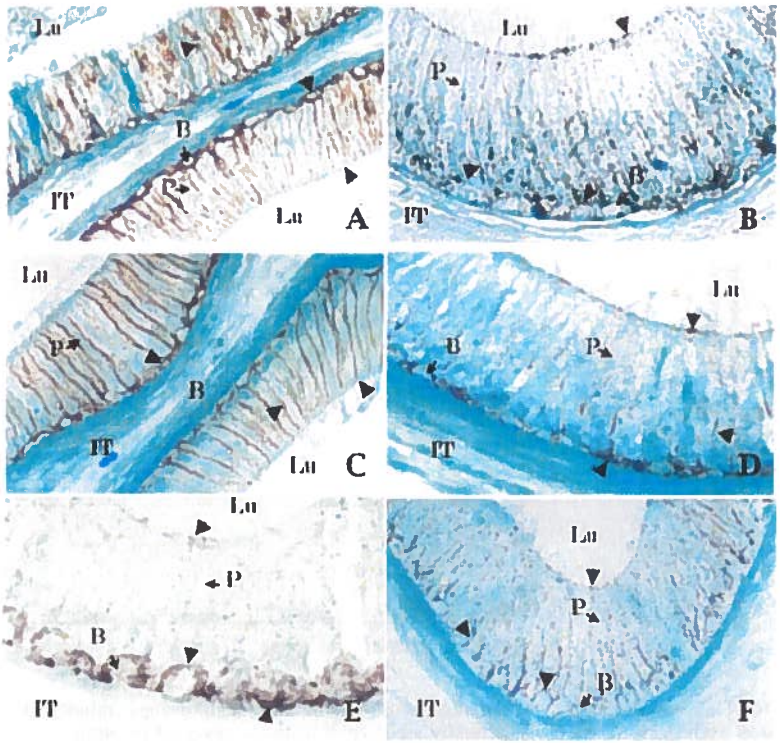

FIG. 8. Immunolocalization of CLDN1 (A, C, E) and CLDN3 (B, D, F) in the human epididymis. CLDN1 and CLDN3 are localized (arrowheads) to the apical tight junctional complex and along the lateral margins of epithelial cells, as well as between the basal and principal cells in the caput (A, B), corpus (C, D), and cauda (E, F) epididymidis. P, Principal cells; B, basal cells; IT, intertubular space; Lu, lumen. Original magnification ×640.

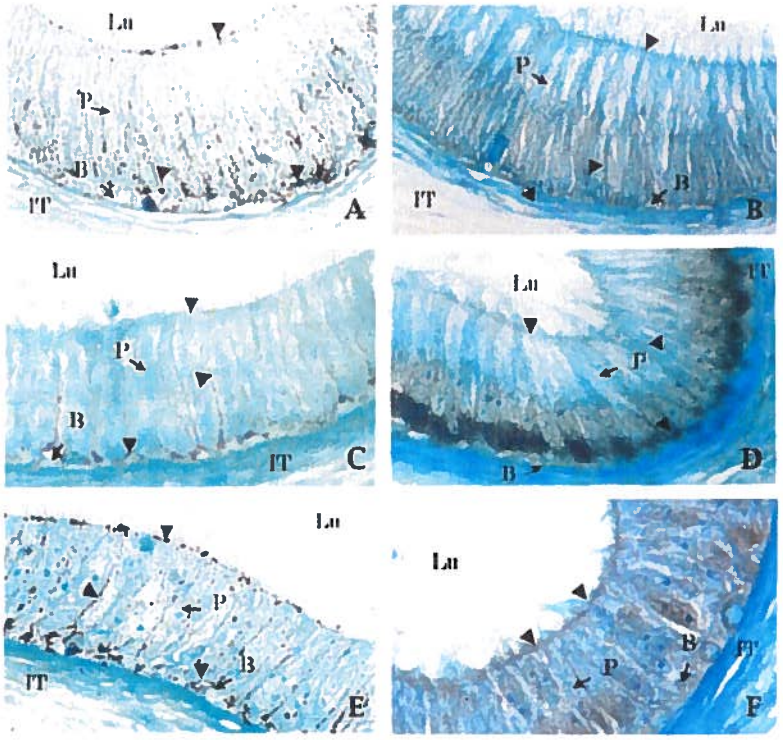

FIG. 9. Immunolocalization of CLDN4 (A, C, E) and CLDN8 (B, D, F) in the human epididymis. CLDN4 is localized (arrowheads) to the apical tight junctional complex and along the lateral margins of epithelial cells, as well as between the basal and principal cells in the caput (A), corpus (C), and cauda (E) epididymidis. CLDN8 is localized to the lateral margins of principal cells and to the apical tight junctions in the caput epididymidis (B). In the corpus epididymidis, CLDN8 is localized to the tight junctional complex and to the interface between principal and basal cells (D). In the cauda epididymidis (F), CLDN8 is localized exclusively to apical tight junctions. P, Principal cells; B, basal cells; IT, intertubular space; Lu, lumen. Original magnification ×640.

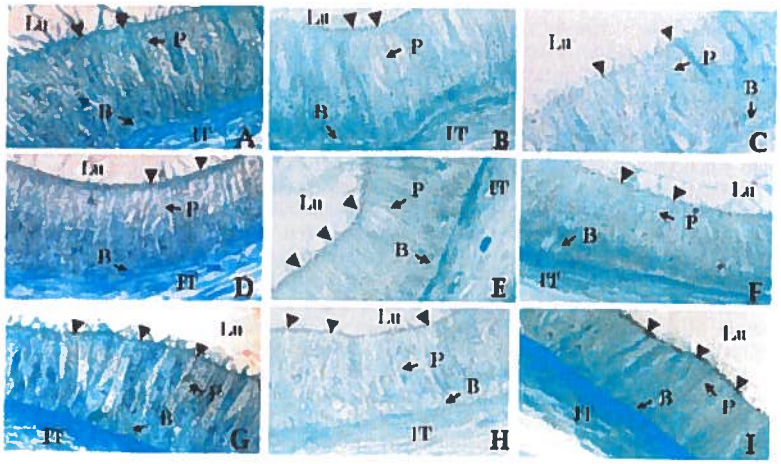

FIG. 10. Immunolocalization of CLDN10 (A, D, G), TJP1 (B, E, H), and occludin (C, F, I) in the human epididymis. CLDN10, TJP1, and occludin are exclusively localized (arrowheads) to the apical tight junctional complex in the caput (A, B, C), corpus (D, E, F), and cauda (G, H, I) epididymidis. P, Principal cells; B, basal cells; IT, intertubular space; Lu, lumen. Original magnification ×640.

have been made by Johnston et al. [47] and Zhang et al. [37] in the mouse and human epididymal transcriptomes, respectively. For example, the gene that encodes 5α-reductase type 2, which is involved in the conversion of testosterone to 5α-dihydrotestosterone (DHT), an essential metabolite for spermatozoal maturation, was expressed at higher levels (2-fold difference; data not shown) in the caput epididymidis as compared to the other segments. Indeed, the caput and cauda epididymidis are morphologically very different and have specialized roles in sperm maturation and storage, respectively, as suggested by our microarray results. Therefore studies on epididymal gene expression profiles can help us elucidate specific functions along the various segments of the epididymis by analyzing the differential expression of specific genes. However, differences were noted between the results obtained in the present study and those obtained by Zhang et al. [37] for specific transcripts, such as *AQP1* and *AQP9*. Zhang et al. [37] have shown that *AQP1* and *AQP9* are most abundant in the cauda and caput epididymidis, respectively, in contrast to our present findings that *AQP9* is mostly expressed in the corpus epididymidis and that *AQP1* is not differentially expressed along the epididymal duct. These differences can be due to genetic variability and low sample size, since their results were generated with samples from a single individual.

Genes expressed along the human epididymis include several that encode adhesion proteins (cadherins, protocadherins, and catenins) and tight junctional proteins (CLDNs and TJPs 1, 2, and 3), which are involved in the formation and integrity of the rodent blood-epididymal barrier [27, 56–58]. *Cdh1* and *Cdh3* are also expressed in the rat epididymis [27, 59, 60], which suggests a degree of conservation in the composition of epididymal adhering junctions and similar roles for these particular cadherins, especially since *Cdh1* is also expressed in the mouse epididymis [47]. The two other cadherins, *CDH22* (also known as rat PB-cadherin) and *CDH24*, which are highly expressed in the human epididymis, have been shown to have two transcripts. The longer isoforms are active in cell adhesion and retain their catenin-binding sites for both p120 catenin and β-catenin, whereas the shorter isoforms do not [61]. The identification of catenins that bind to the cytoplasmic domains of cadherins have been shown to be important for the formation of cadherin-catenin complexes, which are essential for cadherin-mediated cell adhesion and intracellular signaling [62, 63]. *CDH16* (also known as kidney-specific cadherin), which is the only cadherin to be highly differentially expressed along the human epididymis, has been shown to be expressed in the mouse epididymis during embryonic development [64]. Furthermore, in the adult mouse, *Cdh16* is also differentially expressed along the epididymis but with the highest levels of expression in the caput and proximal corpus epididymidis [47]. Interestingly, it has a short intracellular domain and lacks the β-catenin-binding site, which is crucial for the adhesive functions of classical cadherins [65]. It has been proposed that *Cdh16*, in concert with other cadherins, may be required for the differentiation of kidney, lung, and sex duct epithelia, as well as in maintaining the integrity of collecting duct epithelia [64]. In the human epididymis, as in the mouse and rat [47, 57], different catenins are expressed. However, the high-level expression of α-catulin, which is involved in the signal transduction pathway of small G-proteins, such as Rho, suggests that these signaling pathways may be important for the regulation of cadherin-mediated intracellular signaling and epididymal functions. Interestingly, in the mouse epididymis, *Ctnnb1*, and not *Ctnnal1*, is the most highly expressed catenin [47], which suggests the existence of different mechanisms of regulation of cadherin-mediated intracellular signaling in mice and humans.

Tsukita and Furuse [66] have suggested that the combination and mixing ratio of CLDNs may determine the physiological nature of tight junctions. In the human, rat, and mouse epididymis, similar *CLDN* genes are expressed [29- 31, 47], suggesting a high degree of conservation of the composition of epididymal tight junctions and similar roles for each particular CLDN within the tight junctional complex. However, some differences exist between rodents and human in terms of the expression patterns of certain *CLDN* genes. In the rat, *Cldn16* (also known as paracellin-1) and *Cldn10* are expressed exclusively in the initial segment of the epididymis [31]. In the mouse, *Cldn16* is expressed all along the epididymis, albeit at very low levels. In the human epididymis, some *CLDN* genes (*2*, *8*, *10*, *16*, and *23*) also show segment-specific expression, evoking different roles for these *CLDN* genes along the duct. Similar observations have been made in the mouse epididymis for several *Cldn* genes (*2*, *3*, *8*, and *23*), although their expression patterns are different in the mouse than in the human epididymis [47]. Interestingly, these CLDN proteins seem to be part of the cation barrier [31, 30]. Indeed, CLDN2 in MDCK cells increases the permeability of sodium [67], and CLDN16 is implicated in paracellular transport of magnesium, potassium, and sodium [68]. Furthermore, it has

been suggested that the declining sodium:potassium ratio in the epididymis is important for sperm maturation [11].

Immunohistochemistry revealed that not only the expression pattern, but also the localization of CLDNs differed along the human epididymis. All the CLDNs studied were expressed in the apical tight junctional complex. Thus, it appears that CLDN1, CLDN3, and CLDN4, are implicated in epididymal tight junctions and cell adhesion based on their localizations along the lateral plasma membranes between principal cells, as well as between principal and basal cells. This immunostaining pattern resembles that of cell adhesion molecules [69, 70]. Similar observations have been made for CLDN1 in the rat epididymis [29]. Previous studies have also shown that CLDN4 is associated with decreased membrane permeability to sodium ions in MDCK cells [67], which suggests that its increased expression is related to a decrease in cation permeability across the blood-epididymal barrier. In contrast to CLDNs 1, 3, 4, and 10, CLDN8 exhibited segment-specific localization. The localization of CLDN8 suggests that, like CLDN1, this protein may mediate the adherence of principal cells to basal cells in the corpus segment of the human epididymis, whereas in the caput and cauda epididymidis, CLDN8 plays a role primarily in epididymal tight junctions. Recently, it has been reported that CLDN8 expression in MDCK cells reduces the paracellular permeability of protons, ammonium, and bicarbonate, suggesting a role in limiting the passive leakage of these ions via paracellular routes [71]. In the rat epididymis, the decline in pH is dependent upon bicarbonate and sodium reabsorption and the lowest pH is seen in the corpus epididymidis [11]. CLDN8 may play an indirect role in the control of intraluminal pH by regulating bicarbonate permeability. Furthermore, bicarbonate plays a key role in triggering modification of the architecture of the sperm plasma membrane during capacitation, a process that eventually leads to sperm death [72]. Therefore, initiation of premature capacitation in vivo would represent a threat for sperm survival during storage in the epididymis. The establishment of a low bicarbonate concentration in the lumen of the epididymis may thus contribute to maintaining an optimal environment for sperm storage and viability.

The localizations of TJP1 and occludin in the human epididymal tight junctional complex, as in the rat and mouse [27, 28], suggest similar roles for these proteins in humans and rodents. However, in the human epididymis, the expression of *TJP2* and *TJP3* reveals that the junctional complex may be different from that in the rat. Johnston et al. [47] have found that in the mouse epididymis, *Tjp1*, *Tjp2*, and *Tjp3* are expressed at similar levels along the duct, as in the human. Previous studies have suggested that TJP1 is involved in the targeting of CLDN proteins to the area of tight junctions in the epididymis, indicating that the regulatory mechanisms for the formation of the blood-epididymal barrier include not only the expression of specific genes but also their targeting to the tight junctional complex. In the rat epididymis, TJP1 also interacts with β-catenin, especially during the formation of epididymal tight junctional strands [57].

In conclusion, the human epididymis displays a complex gene expression pattern, including several genes that are implicated in the formation and integrity of the blood-epididymal barrier, suggesting complex regulation of this barrier. The fact that human epididymal adhering and tight junctions resemble those found in rodents indicates a high degree of conservation of composition. However, regional differences in the expression and localization of CLDNs along the human epididymis reveal different roles for each CLDN in the formation of the luminal environment by contributing to the creation of specific ionic balances. Further studies on the regulation of these genes in fertile versus infertile patients will allow us to understand better how these genes may be altered in infertile patients.

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REFERENCES

1. Robaire B, Hermo L. Efferent duct, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E, Neill J (eds.), Physiology of Reproduction. New York: Raven Press; 1988:999–1080.
2. Turner TT. On the epididymis and its role in the development of the fertile ejaculate. J Androl 1995; 16:292–298.
3. Yeung C-H, Cooper TG. Acquisition and development of sperm motility upon maturation in the epididymis. In: Robaire B, Hinton BT (eds.), The Epididymis: from Molecules to Clinical Practice. New York: Plenum Press; 2002:417–434.
4. Robaire B, Hinton B, Orgebin-Crist MC. The epididymis. In: Neill J (ed.), Knobil and Neill's Physiology of Reproduction, 3rd ed. New York: Elsevier; 2006:1071–1148.
5. Sullivan R. Male fertility markers, myth or reality. Anim Reprod Sci 2004; 82–83:341–347.
6. Dacheux JL, Dacheux F, Paquignon M. Changes in sperm surface membrane and luminal protein fluid content during epididymal transit in the boar. Biol Reprod 1989; 40:635–651.
7. Dacheux JL, Belghazi M, Lunson Y, Dacheux F. Human epididymal secretome and proteome. Mol Cell Endocrinol 2006; 250:36–42.
8. Hermo L, Oko R, Morales CR. Secretion and endocytosis in the male reproductive tract: a role in sperm maturation. Int Rev Cytol 1994; 154: 106–189.
9. Dacheux JL, Dacheux F. Protein secretion in the epididymis. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York, Plenum Press; 2002:151–168.
10. Hermo L, Robaire B. Epididymal cell types and their functions. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York, Plenum Press; 2002:81–102.
11. Turner TT. Necessity's potion: Inorganic ions and small organic molecules in the epididymal lumen. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York: Plenum Press; 2002:131–150.
12. Cyr DG, Robaire B, Hermo L. Structure and turnover of junctional complexes between principal cells of the rat epididymis. Microsc Res Tech 1995; 30:54–66.
13. Cyr DG, Finnson KW, Dufresne J, Gregory M. Cellular interactions and the blood-epididymal barrier. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York: Plenum Press; 2002:103–118.
14. Friend DS, Gilula NB. Variations in tight and gap junctions in mammalian tissues. J Cell Biol 1972; 53:758–776.
15. Fanning AS, Mitic LL, Anderson JM. Transmembrane proteins in the tight junction barrier. J Am Soc Nephrol 1999; 10:1337–1345.
16. Mitic LL, Van Itallie CM, Anderson JM. Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. Am J Physiol Gastrointest Liver Physiol 2000; 279:G250–254.
17. Denker BM, Nigam SK. Molecular structure and assembly of the tight junction. Am J Physiol 1998; 274:F1–F9.
18. Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. Annu Rev Physiol 2006; 68:403–429.
19. Morita K, Furuse M, Fujimoto K, Tsukita S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci U S A 1999; 96:511–516.
20. Rahner C, Mitic LL, Anderson JM. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. Gastroenterology 2001; 120:411–422.
21. Furuse M, Tsukita S. Claudins in occluding junctions of humans and flies. Trends Cell Biol 2006; 16:181–188.
22. Hewitt KJ, Agarwal R, Morin PJ. The claudin gene family: expression in normal and neoplastic tissues. BMC Cancer 2006; 6:186–194.
23. Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol 1998; 143:391–401.

24. Van Itallie C, Rahner C, Anderson JM. Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. J Clin Invest 2001; 107:1319–1327.
25. Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, Fromm M. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. J Cell Sci 2002; 115:4969–4976.
26. Muller D, Kausalya PJ, Claverie-Martin F, Meij IC, Eggert P, Garcia-Nieto V, Hunziker W. A novel claudin 16 mutation associated with childhood hypercalciuria abolishes binding to ZO-1 and results in lysosomal mistargeting. Am J Hum Genet 2003; 73:1293–1301.
27. Levy S, Robaire B. Segment-specific changes with age in the expression of junctional proteins and the permeability of the blood-epididymis barrier in rats. Biol Reprod 1999; 60:1392–1401.
28. Cyr DG, Hermo L, Egenberger N, Mertineit C, Trasler JM, Laird DW. Cellular immunolocalization of occludin during embryonic and postnatal development of the mouse testis and epididymis. Endocrinology 1999; 140:3815–3825.
29. Gregory M, Dufresne J, Hermo L, Cyr D. Claudin-1 is not restricted to tight junctions in the rat epididymis. Endocrinology 2001; 142:854–863.
30. Guan X, Inai T, Shibata Y. Segment-specific expression of tight junction proteins, claudin-2 and –10, in the rat epididymal epithelium. Arch Histol Cytol 2005; 68:213–225.
31. Gregory M, Cyr DG. Identification of multiple claudins in the rat epididymis. Mol Reprod Dev 2006; 73:580–588.
32. Krull N, Ivell R, Osterhoff C, Kirchhoff C. Region-specific variation of gene expression in the human epididymis as revealed by in situ hybridization with tissue-specific cDNAs. Mol Reprod Dev 1993; 34: 16–24.
33. Clauss A, Lilja H, Lundwall A. A locus on human chromosome 20 contains several genes expressing protease inhibitor domains with homology to whey acidic protein. Biochem J 2002; 368:233–242.
34. Cornwall GA, Hsia N. Cres (cystatin-related epididymal spermatogenic) gene regulation and function. Zhonghua Nan Ke Xue 2002; 8:313–318.
35. Rodriguez CM, Kirby JL, Hinton BT. Regulation of gene transcription in the epididymis. Reproduction 2001; 122:41–48.
36. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. Lancet 2002; 360:772–777.
37. Zhang JS, Liu Q, Li YM, Hall SH, French FS, Zhang YL. Genome-wide profiling of segmental-regulated transcriptomes in human epididymis using oligo microarray. Mol Cell Endocrinol 2006; 250:169–177.
38. Skotheim RI, Monni O, Mousses S, Fossa SD, Kallioniemi OP, Lothe RA, Kallioniemi A. New insights into testicular germ cell tumorigenesis from gene expression profiling. Cancer Res 2002; 62:2359–2364.
39. McLean DJ, Friel PJ, Pouchnik D, Griswold MD. Oligonucleotide microarray analysis of gene expression in follicle-stimulating hormone-treated rat Sertoli cells. Mol Endocrinol 2002; 16:2780–2792.
40. Rodriguez S, Jafer O, Goker H, Summersgill BM, Zafarana G, Gillis AJ, van Gurp RJ, Oosterhuis JW, Lu YJ, Huddart R, Cooper CS, Clark J, et al. Expression profile of genes from 12p in testicular germ cell tumors of adolescents and adults associated with i(12p) and amplification at 12p11.2-p12.1. Oncogene 2003; 22:1880–1891.
41. Hsia N, Cornwall GA. DNA microarray analysis of region-specific gene expression in the mouse epididymis. Biol Reprod 2004; 70:448–457.
42. Chauvin TR, Griswold MD. Androgen-regulated genes in the murine epididymis. Biol Reprod 2004; 71:560–569.
43. Ezer N, Robaire B. Gene expression is differentially regulated in the epididymis after orchidectomy. Endocrinology 2003; 144:975–988.
44. Sipila P, Pujianto DA, Shariatmadari R, Nikkila J, Lehtoranta M, Huhtaniemi IT, French FS, O'Rand M. Differential endocrine regulation of genes enriched in initial segment and distal caput of the mouse epididymis as revealed by genome-wide expression profiling. Biol Reprod 2006; 75:240–251.
45. Hermo L, Jacks D. Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. Mol Reprod Dev 2002; 63:394–410.
46. Jervis KM, Robaire B. Dynamic changes in gene expression along the rat epididymis. Biol Reprod 2001; 65:696–703.
47. Johnston DS, Jelinsky SA, Bang HJ, DiCandeloro P, Wilson E, Kopf GS, Turner TT. The mouse epididymal transcriptome: transcriptional profiling of segmental gene expression in the epididymis. Biol Reprod 2005; 73: 404–413.
48. Yeung CH, Cooper TG, Bergmann M, Schulze H. Organization of tubules in the human caput epididymidis and the ultrastructure of their epithelia. Am J Anat 1991; 191:261–279.
49. Yeung CH, Nashan D, Sorg C, Oberpenning F, Schulze H, Nieschlag E, Cooper TG. Basal cells of the human epididymis–antigenic and ultrastructural similarities to tissue-fixed macrophages. Biol Reprod 1994; 50:917–926.
50. Jalkanen J, Huhtaniemi I, Poutanen M. Mouse cysteine-rich secretory protein 4 (CRISP4) exclusively expressed in the epididymis in an androgen-dependent manner. Biol Reprod 2005; 72:1268–1274.
51. Richardson RT, Sivashanmugam P, Hall SH, Hamil KG, Moore PA, Ruben SM, Poulanen M. Cloning and sequencing of human Eppin: a novel family of protease inhibitors expressed in the epididymis and testis. Gene 2001; 270:93–102.
52. Sivashanmugam P, Hall SH, Hamil KG, French FS, O'Rand MG, Richardson RT. Characterization of mouse Eppin and a gene cluster of similar protease inhibitors on mouse chromosome 2. Gene 2003; 312:125–134.
53. Perry AC, Jones R, Moisyadi S, Coadwell J, Hall L. The novel epididymal secretory protein ESP13.2 in Macaca fascicularis. Biol Reprod 1999; 61: 965–972.
54. Rodriguez-Jimenez FJ, Krause A, Schulz S, Forssmann WG, Conejo-Garcia JR, Schreeb R, Motzkus D. Distribution of new human beta-defensin genes clustered on chromosome 20 in functionally different segments of epididymis. Genomics 2003; 81:175–183.
55. Viger RS, Robaire B. Gene expression in the aging brown Norway rat epididymis. J Androl 1995; 16:108–117.
56. Cyr DG, Hermo L, Robaire B. Developmental changes in epithelial cadherin messenger ribonucleic acid and immunocytochemical localization of epithelial cadherin during postnatal epididymal development in the rat. Endocrinology 1993; 132:1115–1124.
57. DeBellefeuille S, Hermo L, Gregory M, Dufresne J, Cyr DG. Catenins in the rat epididymis: their expression and regulation in adulthood and during postnatal development. Endocrinology 2003; 144:5040–5049.
58. Troxell ML, Chen YT, Cobb N, Nelson WJ, Murrs JA. Cadherin function in junctional complex rearrangement and posttranslational control of cadherin expression. Am J Physiol 1999; 276:C404–C418.
59. Cyr DG, Robaire B. Developmental regulation of epithelial- and placental-cadherin mRNAs in the rat epididymis. Ann N Y Acad Sci 1991; 637: 399–408.
60. Cyr DG, Hermo L, Blaschuk OW, Robaire B. Distribution and regulation of epithelial cadherin messenger ribonucleic acid and immunocytochemical localization of epithelial cadherin in the rat epididymis. Endocrinology 1992; 130:353–363.
61. Katafiasz BJ, Nieman MT, Wheelock MJ, Johnson KR. Characterization of cadherin-24, a novel alternatively spliced type II cadherin. J Biol Chem 2003; 278:27513–27519.
62. Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion. Annu Rev Biochem 1990; 59:237–252.
63. Behrens J. Cadherins and catenins: role in signal transduction and tumor progression. Cancer Metastasis Rev 1999; 18:15–30.
64. Wertz K, Herrmann BG. Kidney-specific cadherin (cdh16) is expressed in embryonic kidney, lung, and sex ducts. Mech Dev 1999; 84:185–188.
65. Aberle H, Schwartz H, Kemler R. Cadherin-catenin complex: protein interactions and their implications for cadherin function. J Cell Biochem 1996; 61:514–523.
66. Tsukita S, Furuse M. Pores in the wall: claudins constitute tight junction strands containing aqueous pores. J Cell Biol 2000; 149:13–16.
67. Van Itallie CM, Fanning AS, Anderson JM. Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins. Am J Physiol Renal Physiol 2003; 285:F1078–F1084.
68. Hou J, Paul DL, Goodenough DA. Paracellin-1 and the modulation of ion selectivity of tight junctions. J Cell Sci 2005; 118:5109–5118.
69. Tiwari-Woodruff SK, Buznikov AG, Vu TQ, Micevych PE, Chen K, Kornblum HI, Bronstein JM. OSP/claudin-11 forms a complex with a novel member of the tetraspanin super family and beta1 integrin and regulates proliferation and migration of oligodendrocytes. J Cell Biol 2001; 153:295–305.
70. Berman AE, Kozlova NI, Morozevich GE. Integrins: structure and signaling. Biochemistry (Mosc) 2003; 68:1284–1299.
71. Angelow S, Kim KJ, Yu AS. Claudin-8 modulates paracellular permeability to acidic and basic ions in MDCK II cells. J Physiol 2006; 571:15–26.
72. Gadella BM, Van Gestel RA. Bicarbonate and its role in mammalian sperm function. Anim Reprod Sci 2004; 82–83:307–319.

Supplementary Table 1: Highly expressed genes in the caput epididymidis. Gene listed have an average ratio of the medians CT: CS of 4 or higher. For each gene, the GenBank accession number is shown along with the function. CT, caput; CS, corpus.

Gene code	Ratio	Gene name	GenBank	Biological function
A_23_P46105	100	*HAO2*	NM_001005783	electron transport
A_23_P58706	47,17	*SPINK5L3*	XM_376433	unknown
A_23_P253791	43,86	*CAMP*	NM_004345	immune response
A_23_P133722	29,67	*CRISP1*	NM_001131	fusion of sperm to egg plasma membrane, spermatogenesis
A_23_P165598	25,64	*similar to LOC92196*	NM_001017920	unknown
A_23_P155711	20,49	*NEIL3*	NM_018248	DNA repair
A_23_P36521	19,01	*ARF3*	NM_001659	intracellular protein transport
A_23_P417821	17,86	*DMRTB1*	NM_033067	regulation of transcription; sex differentiation
A_23_P150555	17,33	*SCGB1D2*	NM_006551	cancer development
A_23_P368225	15,65	*EME1*	NM_152463	ATP synthesis coupled proton transport
A_23_P385295	15,57	*AP1S3*	NM_178814	endocytosis; intracellular protein transport
A_23_P85509	15,15	*IVL*	NM_005547	keratinocyte differentiation
A_23_P102172	14,1	*CPO*	NM_173077	proteolysis and peptidolysis
A_23_P431284	13,81	*FLJ32658*	NM_144688	unknown
A_23_P203231	12,79	*ABCG4*	NM_022169	transport
A_23_P217498	12,55	*GDPD2*	NM_017711	glycerol metabolism
A_23_P14414	12,18	*FAM12B/HE3b*	NM_022360	spermatid cell development
A_23_P209559	11,53	*REG3G*	NM_001008387	nuclear factor kappaB signaling
A_23_P117662	11,21	*HDC*	NM_002112	amino acid metabolism
A_23_P258310	11,18	*FLJ25471*	NM_144651	unknown
A_23_P102694	10,93	*DEFB129*	NM_080831	defense response to bacteria
A_23_P160336	10	*LEFTY1*	NM_020997	cell growth; TGF-B receptor signaling pathway
A_23_P86470	9,901	*CH25H*	NM_003956	lipid metabolism
A_23_P76872	9,091	*RNASE9*	NM_001001673	protection of spermatozoa
A_23_P4387	9,009	*KRT24*	NM_019016	unknown
A_23_P1691	8,772	*MMP1*	NM_002421	collagen catabolism
A_23_P42386	8,264	*CGA*	NM_000735	cell-cell signaling; signal transduction
A_23_P74701	8,696	*COL24A1*	NM_152890	cell adhesion; phosphate transport; transcription
A_23_P145644	8	*DDC*	NM_000790	amino acid metabolism; catecholamine biosynthesis
A_23_P166847	7,693	*LTF*	NM_002343	immune response
A_23_P132048	7,576	*GATA5*	NM_080473	positive regulation of transcription
A_23_P5853	7,407	*SAG*	NM_000541	cell surface receptor linked signal transduction
A_23_P166360	7,299	*PRAME*	NM_206956	unknown
A_23_P1331	6,803	*COL13A1*	NM_005203	phosphate transport
A_23_P42855	6,493	*MGC26647*	NM_152706	unknown
A_23_P25813	6,289	*FAM12A/HE3a*	NM_006683	sperm displacement
A_23_P127781	6,25	*SCGB1D1*	NM_006552	unknown
A_23_P6041	6,061	*DEFB126*	NM_030931	defense response to bacteria
A_23_P118476	5,882	*RUTBC1*	NM_014853	unknown
A_23_P204079	5,681	*NPFF*	NM_003717	neuropeptide signaling pathway
A_23_P397910	5,586	*CBLC*	NM_012116	modulation of cell signalling
A_23_P57199	5,154	*GGTLA4*	NM_080920	unknown
A_23_P338410	5,154	*WFDC9*	NM_147198	unknown
A_23_P518	5,025	*B7-H4*	NM_024626	immune system
A_23_P120809	4,975	*GGTL4*	NM_080839	protection of spermatozoa

Gene code	Ratio	Gene name	GenBank	Biological function
A_23_P502320	4,807	*AGRP*	NM_001138	hormone-mediated signaling
A_23_P153932	4,762	*ACVR2*	NM_001616	protein amino acid phosphorylation
A_23_P122163	4,651	*IL9*	NM_000590	cell-cell signaling
A_23_P52362	4,587	*SLC18A3*	NM_003055	acetylcholine transport
A_23_P401076	4,566	*SUSD3*	NM_145006	unknown
A_23_P68488	4,545	*BMP7*	NM_001719	growth; skeletal development
A_23_P54291	4,545	*DUOX1*	NM_017434	electron transport; thyroid hormone synthesis, epithelial host defense
A_23_P29735	4,525	*BSN*	NM_003458	synaptic transmission
A_23_P145104	4,425	*TFAP2B*	NM_003221	neurogenesis; regulation of transcription
A_23_P362046	4,425	*LOC93081*	NM_138779	unknown
A_23_P106874	4,237	*PMFBP1*	NM_031293	regulation of transcription
A_23_P218190	4,219	*CAPN3*	NM_000070	muscle development; proteolysis and peptidolysis
A_23_P130743	4,132	*DKKL1*	NM_014419	acrosome assembly or function
A_23_P112004	4,132	*LRRC6*	NM_012472	unknown
A_23_P158165	4,132	*CA8*	NM_004056	one-carbon compound metabolism
A_23_P142974	4,115	*ARHGAP25*	NM_001007231	negative regulation of Rho family GTPases
A_23_P137957	4,065	*RSBN1*	NM_018364	transcriptional regulation in spermatogenesis
A_23_P210253	4,016	*DGKD*	NM_152879	cell growth

Supplementary Table 2: Highly expressed genes in the corpus epididymidis. Gene listed have an average ratio of the medians CS: CT of 4 or higher. For each gene, the GenBank accession number is shown along with the function. CT, caput; CS, corpus.

Gene code	Ratio	Gene name	GenBank	Biological function
A_23_P120588	37,13	*CST11*	NM_130794	unknown
A_23_P164504	29,76	*DSG1*	NM_001942	cell adhesion
A_23_P51015	29,03	*SCN2A2*	NM_021007	cation transport
A_23_P422809	25,83	*HIPK3*	NM_005734	protein amino acid phosphorylation
A_23_P135903	25,37	*EBF*	NM_024007	development; regulation of transcription
A_23_P208222	24,78	*ZNF480*	NM_144684	regulation of transcription
A_23_P106042	23,07	*CKLFSF5*	NM_181618	chemotaxis
A_23_P143443	22,58	*OR4K15*	NM_001005486	G-protein coupled receptor protein signaling pathway
A_23_P36234	22,33	*TEAD1*	NM_021961	regulation of transcription
A_23_P155786	22,1	*SULT1E1*	NM_005420	steroid metabolism, regulation of estrogen homeostasis
A_23_P74892	20,53	*LIN28*	NM_024674	regulation of transcription
A_23_P33539	20,17	*ADCY2*	NM_020546	cAMP biosynthesis; intracellular signaling cascade
A_23_P106362	20,14	*AQP9*	NM_020980	excretion; immune response; metabolism; response to osmotic stress; water homeostasis and transport
A_23_P413089	20,07	*DEFB119*	NM_153323	defense response to bacteria
A_23_P393598	19,57	*B7H3*	NM_025240	cell proliferation; immune response
A_23_P200240	18,17	*PCSK9*	NM_174936	cholesterol metabolism; lipid metabolism; negative regulation of enzyme activity; proteolysis and peptidolysis
A_23_P216468	14,84	*SLC1A1*	NM_004170	L-glutamate transport; dicarboxylic acid transport; synaptic transmission
A_23_P138524	13,51	*CPXM2*	NM_198148	cell adhesion; proteolysis and peptidolysis
A_23_P314835	13,11	*C20orf85*	NM_178456	unknown
A_23_P200030	11,27	*FPGT*	NM_003838	fucose metabolism
A_23_P100696	11,19	*HELZ*	NM_014877	unknown
A_23_P356694	10,63	*DEFB123*	NM_153324	defense response to bacteria
A_23_P363255	10,28	*SE57-1*	NM_025214	unknown
A_23_P48350	9,15	*CLDN10*	NM_182848	cell adhesion
A_23_P219126	9,048	*SPAG11/HE2*	NM_016512	spermatogenesis; sperm maturation
A_23_P218669	8,246	*WFDC5*	NM_145652	unknown
A_23_P31922	8,031	*ASS*	NM_054012	arginine biosynthesis; urea cycle
A_23_P125692	7,414	*DLG3*	NM_021120	negative regulation of cell proliferation
A_23_P254363	7,387	*PRRG1*	NM_000950	unknown
A_23_P253012	7,169	*GRAMD1C*	NM_017577	unknown
A_23_P162192	7,133	*OVTN*	NM_198185	proteolysis and peptidolysis
A_23_P86540	6,911	*ARMC3*	NM_173081	unknown
A_23_P211561	6,879	*MGC40042*	NM_152513	meiotic chromosome synapsis
A_23_P372496	6,8	*FLJ40427*	NM_178504	microtubule-based movement
A_23_P254654	6,756	*CLIC3*	NM_004669	chloride transport; ion transport; signal transduction
A_23_P308720	6,705	*MGC16372*	NM_145038	unknown
A_23_P100240	6,583	*CDH16*	NM_004062	cell adhesion
A_23_P217009	6,209	*C9orf24*	NM_032596	unknown
A_23_P342000	6,201	*RBM11*	NM_144770	unknown
A_23_P120902	6,197	*LGALS2*	NM_006498	unknown
A_23_P425815	6,122	*DEFB106*	NM_152251	defense response to bacteria
A_23_P80545	5,948	*IL12A*	NM_000882	unknown
A_23_P162589	5,818	*VDR*	NM_001017535	mineral metabolism
A_23_P93584	5,788	*PRDM1*	NM_001198	regulation of transcription
A_23_P122445	5,651	*HIST1H1C*	NM_005319	nucleosome assembly
A_23_P49448	5,614	*FA2H*	NM_024306	metabolism
A_23_P91520	5,534	*SH3BGR*	NM_007341	protein complex assembly

Gene code	Ratio	Gene name	GenBank	Biological function
A_23_P128319	5,495	*ATP2B1*	NM_001682	ion transport; metabolism
A_23_P33961	5,447	*BRS3*	NM_001727	G-protein coupled receptor protein signaling pathway
A_23_P108200	5,391	*RAB3A*	NM_002866	exocytosis; neurotransmitter secretion; protein transport; small GTPase mediated signal transduction
A_23_P125562	5,293	*CLDN2*	NM_020384	cell adhesion
A_23_P141074	5,223	*ABCC11*	NM_033151	transport
A_23_P56022	5,199	*ZNF529*	NM_020951	regulation of transcription
A_23_P168771	5,159	*KIAA1505*	NM_020879	unknown
A_23_P92122	5,121	*similar to S-100*	XM_059578	unknown
A_23_P142738	5,12	*MGC33926*	NM_152390	unknown
A_23_P36738	5,099	*ANKRD13*	NM_033121	unknown
A_23_P158725	5,079	*SLC16A3*	NM_004207	monocarboxylic acid transport; organic anion transport
A_23_P61987	5,061	*MGC4659*	NM_025268	unknown
A_23_P103981	5,046	*HIST2H2AA*	NM_003516	nucleosome structure
A_23_P146554	4,869	*PTGDS*	NM_000954	prostaglandin biosynthesis; regulation of circadian sleep/wake cycle, sleep; transport
A_23_P162668	4,766	*CPM*	NM_001874	aromatic compound metabolism; morphogenesis; proteolysis and peptidolysis
A_23_P143365	4,765	*CST9L*	NM_080610	sex differentiation
A_23_P146294	4,704	*FLJ11767*	NM_024593	unknown
A_23_P166823	4,676	*TNNC1*	NM_003280	muscle development
A_23_P42127	4,66	*KCNK17*	NM_031460	ion transport; potassium ion transport
A_23_P98200	4,65	*CHST1*	NM_003654	polysaccharide metabolism
A_23_P147485	4,635	*IGHD*	BC021276	unknown
A_23_P44474	4,625	*FLJ13842*	NM_024645	unknown
A_23_P154379	4,581	*NAT8*	NM_003960	response to drug
A_23_P137856	4,566	*MUC1*	NM_182741	cell adhesion
A_23_P2831	4,506	*EDNRB*	NM_003991	G-protein signaling; negative regulation of adenylate cyclase activity; neurogenesis
A_23_P209987	4,465	*POLR1B*	NM_019014	transcription
A_23_P115261	4,452	*AGT*	NM_000029	cell surface receptor linked signal transduction; cell-cell signaling; pregnancy; regulation of blood pressure
A_23_P360949	4,434	*SLC3A1*	NM_000341	L-cystine transport; amino acid metabolism; basic amino acid transport; carbohydrate metabolism
A_23_P75430	4,398	*FN5*	NM_020179	unknown
A_23_P4074	4,388	*LOC146845*	NM_145054	unknown
A_23_P436284	4,347	*OSTbeta*	NM_178859	transport
A_23_P131990	4,272	*VSX1*	NM_014588	development; transcription; visual perception
A_23_P213678	4,26	*PAM*	NM_000919	peptide amidation; peptide metabolism; protein modification
A_23_P129425	4,257	*TSNAXIP1*	NM_018430	Cell proliferation
A_23_P62642	4,247	*NESG1*	NM_012337	unknown
A_23_P203299	4,211	*RCN1*	NM_002901	calcineurin signaling
A_23_P253217	4,205	*OR52E8*	NM_001005168	G-protein coupled receptor protein signaling pathway
A_23_P52121	4,151	*PDZK1*	NM_002614	cell proliferation; transport
A_23_P410653	4,144	*MLLT6*	NM_005937	regulation of transcription
A_23_P14346	4,115	*AKAP6*	NM_004274	protein targeting
A_23_P251293	4,084	*SNCG*	NM_003087	unknown
A_23_P37205	4,024	*NDRG2*	NM_201535	cell differentiation
A_23_P385126	4	*LOC91614*	NM_139160	intracellular signaling cascade

Supplementary Table 3: Highly expressed genes in the cauda epididymidis. Gene listed have an average ratio of the medians CA: CS of 4 or higher. For each gene, the GenBank accession number is shown along with the function. CA, cauda; CS, corpus.

Gene code	Ratio	Gene name	GenBank	Biological function
A_23_P113148	265,1	*ULK1*	NM_003565	protein amino acid phosphorylation; signal transduction
A_23_P29103	47,31	*CESK1*	NM_014406	unknown
A_23_P32605	39,97	*IGLC1*	J00252	unknown
A_23_P203231	17,24	*ABCG4*	NM_022169	transport
A_23_P98805	13,95	*PTD015*	AF092136	unknown
A_23_P431284	12,68	*FLJ32658*	NM_144688	unknown
A_23_P43095	10,46	*ZFHX4*	NM_024721	regulation of transcription
A_23_P36521	10,1	*ARF3*	NM_001659	intracellular protein transport; small GTPase mediated signal transduction
A_23_P417821	9,426	*DMRTB1*	NM_033067	regulation of transcription, sex differentiation
A_23_P52362	9,159	*SLC18A3*	NM_003055	acetylcholine transport
A_23_P42855	8,832	*MGC26647*	NM_152706	unknown
A_23_P155711	7,798	*NEIL3*	NM_018248	DNA repair
A_23_P169017	6,273	*DEFB103A*	NM_018661	immune response
A_23_P397910	5,738	*CBLC*	NM_012116	unknown
A_23_P38732	5,606	*CDH2*	NM_001792	cell adhesion
A_23_P74701	5,526	*COL24A1*	NM_152890	cell adhesion; phosphate transport; transcription
A_23_P101699	4,645	*HDGF2*	NM_032631	unknown
A_23_P116980	4,47	*TULP3*	BC032587	vesicular trafficking; insulin signaling; gene transcription
A_23_P123276	4,233	*FZD6*	NM_003506	G-protein coupled receptor protein signaling pathway; cell surface receptor linked signal transduction; development; establishment of tissue polarity; frizzled signaling pathway
A_23_P435546	4,209	*RUFY2*	NM_017987	unknown
A_23_P64898	4,141	*KLRG1*	NM_005810	cell surface receptor linked signal transduction; cellular defense response; inflammatory response
A_23_P158708	4,125	*DCC*	NM_005215	apoptosis; axonogenesis; development; induction of apoptosis; negative regulation of cell cycle
A_23_P153932	4,098	*ACVR2*	NM_001616	protein amino acid phosphorylation; transmembrane receptor protein serine/threonine kinase signaling pathway
A_23_P136837	4,088	*PTPRC*	NM_002838	cell surface receptor linked signal transduction; protein amino acid dephosphorylation
A_23_P137957	4,027	*RSBN1*	NM_018364	transcriptional regulation in haploid germ cells
A_23_P26994	4,011	*GNGT2*	NM_031498	G-protein coupled receptor protein signaling pathway; phototransduction; signal transduction
A_23_P124773	4,005	*CAPS2*	NM_032606	muscle development

SECTION 2: ALTERATIONS IN GENE EXPRESSION IN THE CAPUT EPIDIDYMIDES OF NON-OBSTRUCTIVE AZOOSPERMIC MEN

Dubé E, Hermo L, Chan PTK, Cyr DG
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2.1 Résumé de l'article en français

La maturation post-testiculaire des spermatozoïdes dans l'épididyme dépend de protéines sécrétées par l'épithélium, de celles responsables de la composition ionique appropriée et du pH luminal ainsi que de la barrière hémato-épididymaire. Chez l'humain, il existe très peu d'informations sur la régulation de ces protéines, et, en particulier, en cas d'infertilité masculine. Nos objectifs étaient de comparer les profils d'expression de gènes dans la tête d'épididymes d'hommes ayant une spermatogenèse normale et d'hommes souffrant d'azoospermie non-obstructive. En utilisant la technique des microréseaux, nous avons mis en évidence 414 gènes qui sont différentiellement exprimés chez les hommes infertiles par un ratio minimal de 2. Plusieurs de ces gènes sont impliqués dans des processus de transcription, de signalisation intracellulaire, d'immunité et de fertilité. Les gènes différentiellement exprimés codent notamment pour des protéines impliquées dans la maturation des spermatozoïdes, pour des canaux ioniques et d'eau ainsi que pour des β-défensines. On peut entre autres citer *CRISP1, SPINLW1, FAM12B, DEFB129,* qui ont un taux d'expression plus élevé dans la tête épididymaire des hommes infertiles que dans la même région de l'épididyme d'hommes fertiles alors que *CFTR, AQP5, KCNK7, KCNK17, SLC6A20, SLC13A3, DEFB126* et *DEFB106A* ont un taux d'expression plus faible. Même si l'expression des gènes codant pour les protéines jonctionnelles ne varient pas, la localisation de CLDN10 et de TJP1 change chez ces patients infertiles contrairement à celle des CLDN1, 3 et 8. Ces données suggèrent que les fonctions paracellulaires de la barrière hémato-épididymaire sont altérées chez les patients azoospermiques non-obstructifs. De plus, l'immunolocalisation d'AQP5, et non de CFTR ou de CRISP1, est différente chez les patients fertiles et infertiles. Le fait que l'expression de plusieurs gènes impliqués dans le transport de l'eau et des ions diminue chez les

patients infertiles suggère que ces gènes sont régulés par la présence de produits testiculaires ou des spermatozoïdes ou qu'ils font partie d'un syndrome plus complexe associé à l'azoospermie non obstructive.

2.2 Contribution de l'étudiante

Toutes les expériences présentées dans cet article ont été réalisées par l'étudiante. L'article a également été rédigé par l'étudiante. Finalement, l'étudiante a participé au choix du journal de publication et aux corrections nécessaires à la publication de la version finale de l'article.

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Alterations in Gene Expression in the Caput Epididymides of Nonobstructive Azoospermic Men[1]

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ABSTRACT

Spermatozoal maturation in the epididymis is dependent on proteins secreted by the epithelium and those that create the proper ionic composition and pH of the lumen as well as the blood-epididymal barrier. For the human epididymis, little information exists about the regulation of these proteins in male infertility. Our objectives were to assess gene expression profiles in the caput epididymidis from men with normal spermatogenesis and men with nonobstructive azoospermia. With microarrays, we identified 414 genes in the caput epididymidis that were differentially regulated in infertile men by at least 2-fold compared with the fertile men. They were mostly involved in transcription, intracellular signaling, immunity, and fertility. Although the expression of genes encoding tight junctional proteins was not affected, the localization of CLDN10 and TJP1, but not CLDNs 1, 3, and 8, was altered in infertile patients, suggesting that there are changes in the paracellular functions of the blood-epididymal barrier. Differentially regulated genes included several encoding proteins involved in spermatozoal maturation, water and ion channels, and beta-defensins: CRISP1, SPINLW1, FAM12B, and DEFB129 were upregulated, whereas CFTR, AQP5, KCNK4, KCNK17, SLC6A20, SLC13A3, DEFB126, and DEFB106A were downregulated. Furthermore, the immunolocalization of AQP5, but not of CFTR or CRISP1, varied in infertile and fertile patients. The observation that the expression of genes involved in water and ion transport were repressed in infertile patients suggests that these genes are regulated by the presence of testicular products or spermatozoa in the epididymal lumen or are part of a broader syndrome associated with nonobstructive azoospermia.

beta-defensins, epididymal junctions, epididymis, gene regulation, genomics, infertility, male reproductive tract, male sexual function, water and ion channels

INTRODUCTION

Infertility is defined as the inability to conceive after 1 yr of unprotected intercourse and affects about 13%–18% of consulting couples. About 40%–50% of infertility cases in couples involve male factor infertility. Male infertility can occur either as an isolated disease or as part of a complex syndrome. In more than half of infertile men, the cause of their infertility is unknown [1–3]. Azoospermia, or the absence of spermatozoa in the ejaculate, may be caused by obstruction of the excurrent ductal system (obstructive azoospermia) or testicular failure (nonobstructive azoospermia), with the latter being the most severe form of male infertility. Various conditions, including congenital or developmental defects of the reproductive system, genetic anomalies, acquired testicular insults, and toxin exposures, can cause nonobstructive azoospermia [4–6]. Recent progress in the treatment of male infertility has enabled the recovery of testicular sperm from nonobstructive azoospermic patients for intracytoplasmic sperm injection to allow these men to father children. Unfortunately, the recovery of testicular sperm is not always successful in these patients. Moreover, the fertilization and pregnancy rates are significantly reduced in patients with nonobstructive azoospermia compared with other types of infertility [7–9]. It is therefore important to better understand the molecular genetics of male infertility to find new therapeutic approaches to alleviate human infertility.

Evidence for the involvement of the epididymis in infertility has been known for several years. The epididymis is a long single convoluted tubule that is generally divided into three segments: the caput, corpus, and cauda (Fig. 1). Its main functions are the maturation, transport protection, and storage of mammalian spermatozoa. Spermatozoal protection is dependent on proteins secreted or reabsorbed by the epithelium and those that create the proper ionic composition and pH of the lumen as well as the blood-epididymal barrier. The blood-epididymal barrier is composed of apical tight junctions between principal cells forming an impenetrable seal, forcing movement of molecules across these cells by specific receptors, ion and water channels, and solute carrier proteins [10–13]. Tight junctional proteins include several peripheral membrane proteins, such as tight junction protein (TJP) 1, TJP2, and TJP3, and transmembrane proteins, such as claudins (CLDNs). Although these proteins are well defined in rodents, their identification in the human epididymis and their regulation in male infertility are relatively unknown [11, 14, 15].

In the male reproductive tract, gene profiling studies have been done on spermatozoa [16], on the epididymis from fertile men [17, 18] and rodents [19–21], on testicular germ cell tumorogenesis [22], on FSH-stimulated Sertoli cells [23], and on testicular development [24]. Elucidation of the transcriptional profiles of epididymal segments in infertile men is a crucial step toward understanding the causes of male infertility

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and could help diagnose male infertility [25]. In the present study, given the extensive modifications associated with spermatozoal maturation that occur in the caput epididymidis, we have investigated possible changes in epididymal gene expression in the caput region of men suffering from nonobstructive azoospermia.

MATERIALS AND METHODS

Tissue Preparation

Epididymal tubules from caput epididymides were obtained microsurgically from four patients (29–50 yr old) undergoing radical orchidectomy for localized testicular cancer (confined within testicular tunica albuginea) [18] and from four nonobstructive azoospermic patients (32–42 yr old) undergoing microsurgical sperm extraction surgery. All patients undergoing orchidectomy had active spermatogenesis. Tissues were received in cold culture medium containing antibiotics (Dulbecco modified Eagle and Ham F-12 medium with penicillin-streptomycin) and were processed as described in the following sections within 1 h of surgery. This study was conducted with the approval of the McGill University ethics committee for research on human subjects, and informed consent was obtained from each patient.

Electron Microscopy

Pieces of tissue (1 mm^3) were fixed by immersion in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h and then washed in cacodylate buffer and postfixed in potassium ferrocyanide-reduced osmium tetroxide for 1 h to enhance the staining of membranes, as described by Hermo and Jacks [26]. After fixation, tissues were rinsed in cacodylate buffer, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Thin sections were cut with a diamond knife, mounted on copper grids, counterstained with uranyl acetate and lead citrate, and examined with an FEI Tecnai 12 electron microscope (FEI Company, The Netherlands).

Microarray Processing and Analysis

Total cellular RNA was isolated with an Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The quality of the RNA was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Wilmington, DE). The Low RNA Input Linear Amplification Kit (Agilent) was used to amplify and label 500 ng of total RNA with either cyanine 3 or cyanine 5 (Perkin-Elmer Inc., Woodbridge, ON, Canada). Human oligo microarrays (20 174 human genes; Agilent) were then hybridized according to the manufacturer's instructions with the In Situ Hybridization kit Plus (Agilent). After hybridization, microarrays were scanned with a ScanArray Express scanner (Perkin-Elmer). Fluorescence ratios for array elements were extracted by ScanArray Express Software (Perkin-Elmer) and imported into GeneSpring 6.1 software (Agilent) for further analysis. Expression analysis was done accordingly to MIAME (minimum information about a microarray experiment) standards [27]. Genes were considered enriched if the expression was 2-fold lower or higher in the caput epididymides of infertile patients compared with the same epididymal segment of fertile patients. Statistical analyses were performed by a one-way ANOVA (significance level set at $P < 0.05$).

Real-Time PCR

Real-time PCR was used to confirm microarray data. Five hundred nanograms of total RNA was reverse transcribed with an Oligo $d(T)_{16}$ primer.

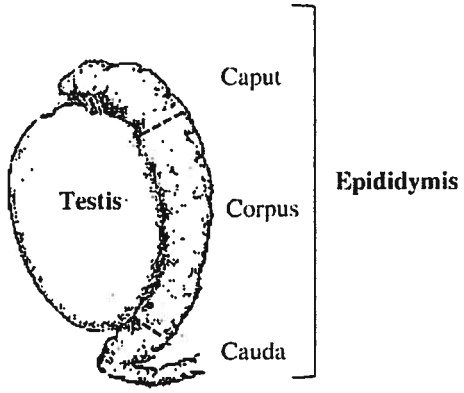


FIG. 1. Schematic representation of the human epididymis. This organ is generally divided into three regions: the caput, the corpus, and the cauda.

Forward and reverse primers for the genes of interest (Table 1) were designed by Oligo Primer Analyses Software (Molecular Biology Insights, Cascade, CO) based on sequences published in GenBank. Real-time PCR was performed with a Rotor-Gene RG3000 (Corbett Research, Cambridgeshire, United Kingdom). A 2-μl aliquot of the RT reaction was amplified in a 15-μl solution containing 1× Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Burlington, ON, Canada) and 0.3 μM (both) reverse and forward primers. The PCR cycling protocols were optimized to maximize the reaction efficiency and ensure that only the target product was contributing to the SYBR Green fluorescence signal. For each quantification, a standard curve was created with suitably appropriate cDNA. Amplification consisted of 40 cycles at 95°C for 15 sec, melting temperature for 30 sec, and 72°C for 30 sec. Primers for the housekeeping gene, *GAPDHS*, were used to normalize values for each sample. Samples were done in duplicate, and identical samples were run in each assay to calibrate for interassay variation. After the PCR amplification, melting curve analysis was performed to ensure the accuracy of quantification.

Immunocytochemistry

Small pieces of epididymal tissue were fixed at the time of surgery by immersion in Bouin fixative (Fisher Scientific, Ottawa, ON, Canada) for 24 h; they were then dehydrated and embedded in paraffin. Thick sections (5 μm) were cut and mounted on glass slides. For immunostaining, the tissue sections were rehydrated through graded ethanol, including 70% alcohol with 1% lithium carbonate for 5 min to remove residual picric acid. The sections were then incubated in 300 mM glycine for 5 min to block free aldehydes and washed in 1 M PBS (pH 7.4). Heat-induced epitope retrieval was done by boiling the slides for 10 min in citrate buffer (1.8 mM citric acid and 8.2 mM sodium citrate) for the immunolocalization of CLDNs 1, 3, 8, and 10, TJP1, and aquaporin-5 (AQP5) and for 20 min for the immunolocalization of the cystic fibrosis transmembrane conductance regulator (CFTR). Immunolocalization was performed according to the manufacturer's instructions (DAKO Catalyzed Signal Amplification System; DAKO, Carpenteria, CA). The primary antibodies used in this study were a rabbit polyclonal antibody against human CLDN1 (5 μg/ml; Zymed Laboratories, San Francisco, CA), a rabbit polyclonal antibody against mouse CLDN3 (2.5 μg/ml; Zymed), a rabbit

TABLE 1. Sequences of primers used in real-time PCR.

Gene	GenBank accession no.	Primer set (5′–3′)[a]	Tm (°C)	Amplicon size (bp)
GAPDHS	AF261085	F: GAA GGT GAA GGT CGG AGT CAA R: GGA AGA TGG TGA TGG GAT TTC	55.1	227
SPINLW1	NM_181502	F: CCT GCA AGA ATA AAC GCT TTC R: TGT TCT GGG AAG GGC TAA G	54.0	161
DEFB126	NM_030931	F: AAA GAA TGG TTG GGC AAT GTG R: GGG GTA GGA GCC ATC GAA G	54.2	183
CRISP1	NM_001131	F: TTG GTT GCT GCT TGC TTA CT R: TTG CTG GCT GGT GGA ACT ACT CTT	55.0	158

[a] F, Forward; R, reverse.

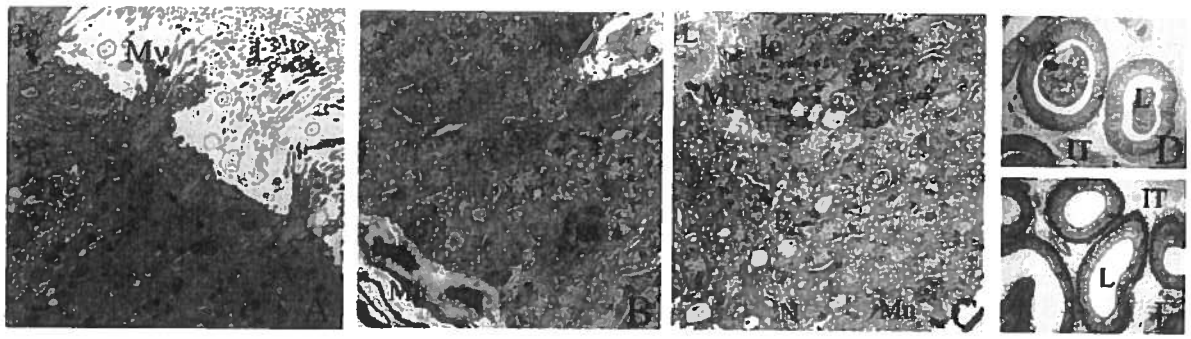


FIG. 2. Electron and light micrographs of the human epididymis. A) Apical region of the caput epididymidis of men with proven fertility. Original magnification ×2550. B) Basal region of the caput epididymidis of men with proven fertility. Original magnification ×2550. C) Epithelium of the caput epididymidis of infertile men. Original magnification ×1250. D) Epididymal tubules of the caput epididymidis of men of proven fertility. Original magnification ×100. E) Epididymal tubules of the caput epididymidis of infertile men. Original magnification ×100. Mu, Muscular coat; P, principal cells; B, basal cells; N, nucleus; L, lumen; Mv, microvilli; Jc, junctional complex; ER, endoplasmic reticulum; M, mitochondria; Ly, lysosomes; V, vesicles; Spz, spermatozoa; IT, interstitial space.

polyclonal antibody against human CLDN8 (3 μg/ml; Genetex Inc., San Antonio, TX), a rabbit polyclonal antibody against human CLDN10 (2 μg/ml; Abcam, Cambridge, MA), a rabbit polyclonal antibody against human TJP1 (0.5 μg/ml; Zymed), a mouse monoclonal antibody against human CFTR (8 μg/ml; Neomarkers, Fremont, CA), a rabbit polyclonal antibody against rat AQP5 (20 μg/ml; Calbiochem, San Diego, CA), and a rabbit polyclonal antibody against human cysteine-rich secretory protein 1 (CRISP1, 20 μg/ml; Santa Cruz, CA). Incubations with the primary antibodies were done overnight at 4°C (CLDN8, CLDN10, and AQP5) for 15–30 min (TJP1, CLDN1, and CLDN3) to 2 h (CFTR) at room temperature or for 2 h (CRISP1) at 37°C. Incubations with the secondary antibodies were done for 15 min at room temperature, except for CFTR, AQP5, and CRISP1, for which the incubations were done for 1–2 h at room temperature. Epididymal sections were counterstained for 2 min with 0.1% methylene blue, dehydrated in ethanol, immersed in Histoclear (Fisher), and mounted in Permount (Fisher). Sections were examined with a Leica DMRE microscope (Leica Microsystems, Inc., Bannockburn, IL).

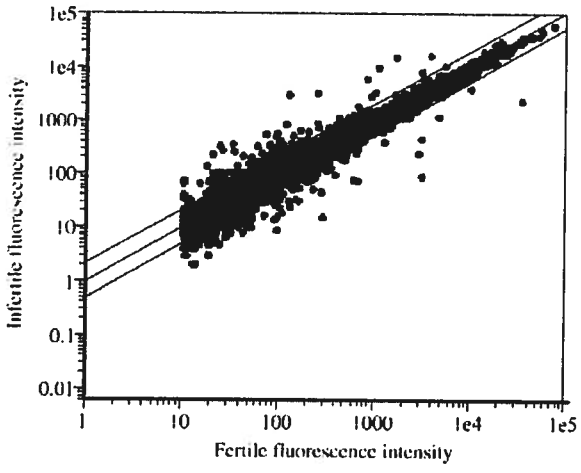


FIG. 3. Scatterplot comparing genes expressed in the caput epididymidis of infertile patients with fertile patients. Axes of the scatterplot represent the log scale of the mean (n = 4) fluorescence intensity value minus the background intensity values for each region. The middle line indicates values that represent a ratio of 1.0 (similar levels of expression in both epididymal regions). The outer lines represent a ratio of 2.0 (upper line; 2-fold greater expression in the caput epididymidis of infertile compared with fertile) and 0.5 (lower line; 2-fold greater expression in the caput epididymidis of fertile compared with infertile).

RESULTS

Ultrastructure of Caput Epididymides of Fertile and Infertile Men

Caput epididymidis of fertile and infertile patients, subsequently used for gene analysis, retained normal ultrastructural features. A pseudostratified epithelium underlying a basement membrane and a muscular coat composed of several layers of smooth muscle cells was observed (Fig. 2, A–C). Columnar principal cells were the main cell type of the epithelium. These cells contained large oval or elongated nuclei located near the base of the epithelium with dispersed chromatin clumps. Principal cells had long microvilli (Fig. 2, A and C). Their cytoplasm contained pale endosomes, dense lysosomes, mitochondria, endoplasmic reticulum, and Golgi apparatus (Fig. 2, A and C). Basal cells were round to ovoid and rested on the basement membrane (Fig. 2, B and C). No apparent changes could be seen in either the epithelial cell height or the distribution of cell types between fertile and infertile patients (Fig. 2, D and E).

Gene Expression Profiling

The human oligonucleotide microarray used in the present study consisted of 20 174 genes. Most genes encoded matrix/structural proteins and proteins dealing with signal transduction, synthesis/translational control, energy/metabolism, and transcription/chromatin. The array was hybridized with probes of the caput epididymidis of fertile and infertile patients to identify genes that were either down- or upregulated in infertile tissues. The data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE9194. In these analyses, 414 genes were differentially expressed in the caput epididymidis of infertile men by a 2-fold change when compared with the caput epididymidis of fertile patients (Fig. 3). There were 251 genes that were downregulated and 163 that were upregulated by a 2-fold change. Most of those genes for which the function was known encoded proteins implicated in transcription, intracellular signaling, fertility, and immunity (Fig. 4). We selected three genes for real-time PCR analysis to authenticate the results from the microarray data. Differential expression

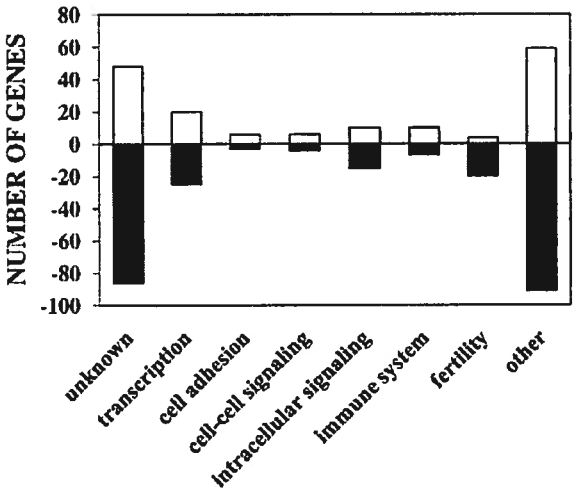


FIG. 4. Functional classification of differentially expressed genes by at least 2-fold in the human caput epididymidis of infertile versus fertile patients. The genes were grouped based on their biological function. The bars in white represent the upregulated genes, whereas the bars in black represent the downregulated genes.

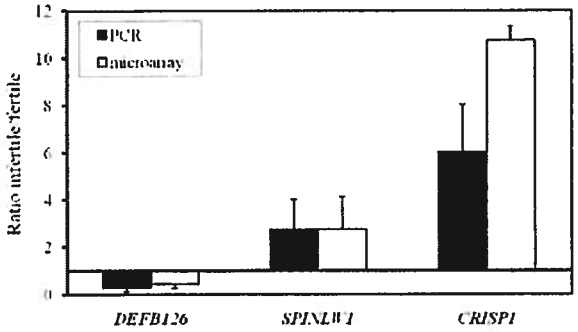


FIG. 5. Confirmation of microarray results by quantitative real-time PCR with selected genes known to be expressed in the epididymis. Messenger RNA expression levels of *DEFB126*, *SPINLW1*, and *CRISP1* in the caput epididymidis of infertile and fertile men were investigated and compared with both microarray results and real-time PCR data. Data are expressed as the ratio of the mRNA levels of infertile in relation to fertile patients. Values represent the mean relative expression ± SEM (n = 4).

patterns of *DEFB126* (beta-defensin 126) and *SPINLW1* (serine protease inhibitor-like, with Kunitz and WAP domains 1, also known as *EPPIN*) and *CRISP1* all showed consistency between microarray and real-time PCR analysis (Fig. 5). For *CRISP1*, the ratio detected in real-time PCR was slightly lower than the ratio detected by microarray analysis. This may be because of the differences in the sensitivity of the assays as well as the specificity of the primers used in real-time PCR. Further analyses were done on genes that were differentially expressed by a 4-fold change (Fig. 6): 7 genes were upregulated, and 10 were downregulated. Most of these genes are implicated in transcriptional regulation (Tables 2 and 3). Otherwise, genes whose expression was altered are involved in immunity, fertilization, sperm motility, DNA packaging, and proteolysis. Genes that are involved in various aspects of fertility were mostly downregulated. Further analysis revealed that several genes encoding for sperm antigens, such as *SPINLW1*, were upregulated by at least 2-fold (Fig. 5).

Genes Encoding for Tight Junctional Proteins

Several genes present on the microarray encoded tight junctional proteins, such as CLDNs 1 to 12, 14 to 19, and 23 and TJP1, TJP2, and TJP3. None of these genes were differentially regulated in the caput epididymidis of infertile patients compared with the same segment of fertile patients (Fig. 7, A and B). Electron microscopy detected intact apical junctional complexes between principal cells in the caput epididymidis of infertile patients (Fig. 7C).

Genes Encoding for Water Channels, Ion Channels, and Solute Carriers

Of the *AQPs* on the array, all, except for *AQP5*, were expressed at similar levels in the caput epididymidis of infertile and fertile patients. *AQP5* was downregulated by at least 2-fold in infertile patients compared with fertile patients (Fig. 8A). Other genes encoding potassium ion channels, *KCNK4* (also known as TRAAK) and *KCNK17* (also known as TALK2 or TASK4), were also downregulated by at least a 2-fold change in infertile patients compared with fertile patients. Genes encoding two solute carriers, *SLC6A20* (amino acid transporter) and *SLC13A3* (anion transporter, also known as NADC3), as well as *CFTR*, which encodes for a cAMP-activated chloride channel, were also downregulated (Fig. 8B).

Genes Encoding for Beta-Defensins

The expression of several genes encoding for beta-defensins was changed by at least 2-fold in infertile patients compared with fertile patients. *DEFB129* was upregulated, whereas *DEFB126* and *DEFB106A* were both downregulated (Fig. 9).

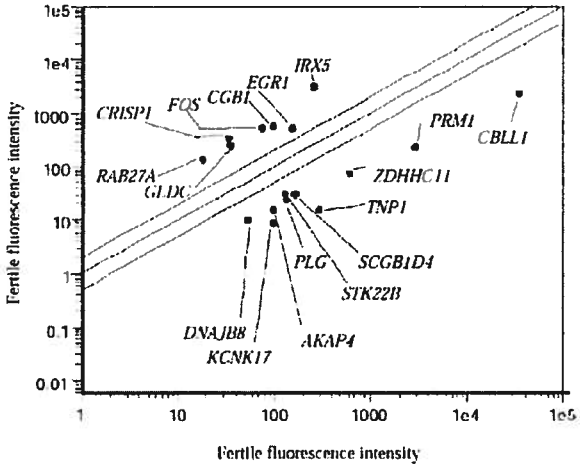


FIG. 6. Scatterplot comparing genes differentially expressed by at least 4-fold in the caput epididymidis of infertile compared with fertile patients. Axes of the scatterplot represent the log scale of the mean (n = 4) fluorescence intensity value minus the background intensity values for each region. The middle line indicates values that represent a ratio of 1.0 (similar levels of expression in both epididymal regions). The outer lines represent a ratio of 2.0 (upper line; 2-fold greater expression in the caput epididymidis of infertile compared with fertile) and 0.5 (lower line; 2-fold greater expression in the caput epididymidis of fertile compared with infertile).

TABLE 2. Upregulated genes in the caput epididymidis of infertile as compared to fertile patients by at least a 4-fold change.

Gene ID	Fold change	Gene symbol	PubMed accession no.	Biological function
A_23_P133722	10.76	*CRISP1*	NM_001131	Fusion of sperm to egg plasma membrane; spermatogenesis
A_23_P106192	9.007	*FOS*	NM_005252	DNA methylation; inflammatory response; regulation of transcription from Pol II promoter
A_23_P123596	7.898	*GLDC*	NM_000170	Glycine catabolism
A_23_P54367	7.522	*RAB27A*	NM_004580	Small GTPase mediated signal transduction
A_23_P9779	4.321	*IRX5*	NM_005853	Regulation of transcription, DNA-dependent
A_23_P39095	4.278	*CGB1*	NM_033377	Unknown
A_23_P214080	4.037	*FGR1*	NM_001964	Regulation of transcription, DNA-dependent

Immunolocalization

Immunocytochemistry performed with anti-CLDN 1, 3, 8, and 10 antibodies showed that they were localized to the area of tight junctions between adjacent principal cells in the caput epididymidis of fertile and infertile patients (Fig. 10). CLDNs 1 and 3 were also localized along the lateral margins of adjacent principal cells as well as between basal and principal cells (Fig. 10, A–F), and CLDN8 was localized to the lateral margins of principal cells in the caput epididymidis of both fertile and infertile patients (Fig. 10, G and H). In contrast with CLDNs 1, 3, and 8, the immunolocalization of CLDN10 in the caput epididymidis of infertile patients was different from fertile patients. In the caput epididymidis of infertile patients, CLDN10, in addition to being localized to the apical tight junctional complex, was localized along the lateral plasma membrane between principal cells (Fig. 10K). The same observation was made for TJP1: in the caput epididymidis of fertile patients, TJP1 was exclusively localized to the apical tight junctional complex, but in infertile patients, the protein was also observed along the lateral margins between adjacent principal cells (Fig. 10, M and N). AQP5 was localized to the apical border of a subpopulation of principal cells in fertile and infertile patients (Fig. 11, A and B), but in infertile patients, AQP5 was also localized along the lateral plasma membrane of principal cells (Fig. 11B). CFTR was localized to the apical membrane of a subpopulation of principal cells in both fertile and infertile patients as well as in the cytoplasm of the narrow cells but with a weaker signal in infertile patients (Fig. 11, D–G). CRISP1 was localized to the apical border of principal cells of the epididymis, and its immunolocalization was the same in both fertile and infertile patients (Fig. 11, H and I).

DISCUSSION

Microarrays constitute a powerful tool to study the molecular basis of male infertility and to highlight genes that may be important in posttesticular spermatozoal maturation, especially because the molecular basis of male infertility is heterogeneous and poorly understood. In the present study, samples from infertile patients were compared with samples originating from four fertile males with histologic confirmation of normal spermatogenesis and intact epididymis [18].

The epididymis displays a complex pattern of gene expression in several species, including mice, rats, and humans, with a larger number of reproductive and somatic genes expressed in the caput epididymidis than in the other epididymal segments [17–19, 28]. Indeed, the caput epididymidis is known to be the most active segment for protein synthesis and secretion in several species and is the region of the epididymis where spermatozoal maturation is initiated [10, 14]. Therefore, it is interesting that 414 genes were differentially expressed in the caput epididymidis of infertile men by at least a 2-fold change when compared with the caput epididymidis of fertile patients (Fig. 3). Although none of the genes encoding tight junctional proteins were changed in nonobstructive azoospermic patients, it is nevertheless interesting that the immunolocalization of CLDN10 and TJP1 is altered. It has been previously shown that *CLDN10* is differentially expressed along the different segments of the human epididymis. Indeed, *CLDN10* is mainly expressed in the cauda epididymidis [18]. Furthermore CLDN10 has been reported to play a role in the paracellular transport of cations across epithelial tight junctions [29]. It has also been suggested that TJP1 is important for the structural integrity of the tight

TABLE 3. Downregulated genes in the caput epididymidis of infertile as compared to fertile patients by at least a 4-fold change.

Gene ID	Fold change	Gene symbol	PubMed accession no.	Biological function
A_23_P98598	0.248	*SCGB1D4*	NM_206998	Immune response
A_23_P121413	0.236	*DNAIB8*	NM_153330	Protein folding
A_23_P40516	0.231	*STK22B*	NM_053006	Protein amino acid phosphorylation; spermatogenesis
A_23_P30693	0.192	*PLG*	NM_000301	Blood coagulation; regulation of cell proliferation; proteolysis and peptidolysis
A_23_P256008	0.179	*ZDHHC11*	NM_024786	Unknown
A_23_P11081	0.162	*AKAP4*	NM_003886	Cell motility; fertilization (sensu Metazoa); signal transduction; sperm motility
A_23_P100189	0.144	*PRM1*	NM_002761	DNA packaging; chromosome organization and biogenesis (sensu Eukaryota); mitotic chromosome condensation; spermatogenesis
A_23_P42127	0.114	*KCNK17*	NM_031460	Ion transport; potassium ion transport
A_23_P165504	0.0703	*TNP1*	NM_003284	Chromatin silencing; chromosome organization and biogenesis (sensu Eukaryota); fertilization, exchange of chromosomal proteins, nucleosome disassembly; sexual reproduction; single strand break repair; sperm motility; spermatid nuclear elongation; spermatogenesis
A_23_P146004	0.0699	*CBLL1*	NM_024814	Protein ubiquitination

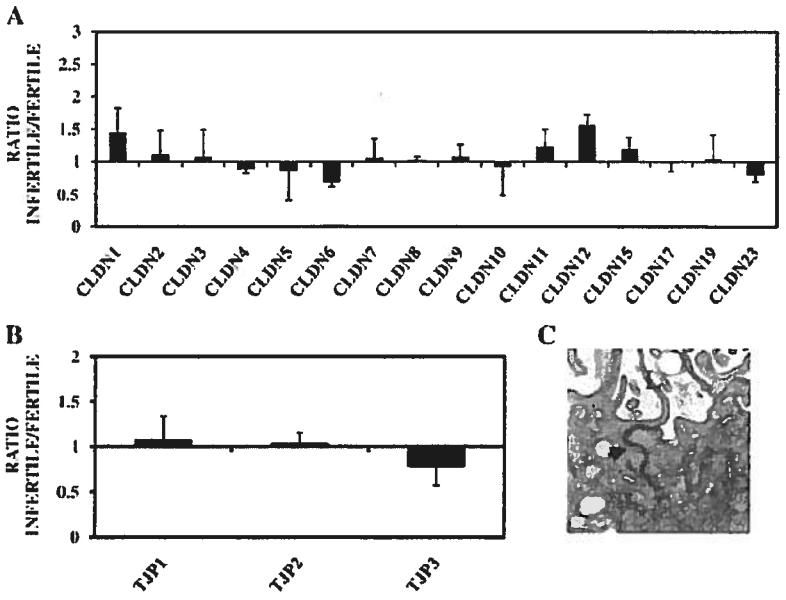


FIG. 7. Ratio of expression of genes encoding for CLDNs (A) and TJPs (B) in the caput epididymidis of infertile versus fertile men (n = 4; ±SEM). C) Electron micrograph of an apical tight junctional complex (arrowhead) in the caput epididymidis of infertile men (×43 000). L, Lumen.

junction [30]. Thus, alterations in the localization of CLDN10 and TJP1 may be suggestive of effects on the permeability of the blood-epididymal barrier and more particularly associated with paracellular cationic transport. Furthermore, several cell adhesion genes are upregulated (Fig. 4). The formation of tight junctions involves different cell adhesion signaling components [31]. Interestingly, one of the upregulated genes in the caput epididymidis of infertile patients is CASK (calcium/calmodulin dependent serine protein kinase), a member of the MAGUK (membrane associated guanylate kinase) family, which is involved in protein targeting and cell polarity in MDCK (Mardin-Darby canine kidney) cells [32]. It is possible that alterations in cell adhesion-mediated intracellular pathways influence, or regulate, the localization of TJPs.

Interestingly, our results also show altered expression of several genes encoding for different water and ion channels, such as CFTR and AQPs, which could result in a major alteration of the specific luminal microenvironment, which is essential for spermatozoal maturation in the human epididymis. Several studies of patients with cystic fibrosis have observed that the male reproductive system is highly dependent on CFTR for normal function. Indeed, more than 95% of men with cystic fibrosis are infertile. Furthermore, CFTR mRNA levels are high in the proximal caput region of the human epididymis. That CFTR expression is reduced could alter the ionic exchange and fluid content within the epididymal lumen, and this could lead to excessive viscosity of the epididymal fluid and the progressive blocking of different segments of the epididymis [33]. CFTR has been demonstrated to be implicated in the transepithelial secretion of electrolytes and water [34, 35] but also to act as a regulator of other membrane transport proteins, such as the epithelial Na^+ channels [36] and AQP3 [37]. Several AQPs, which are involved in the transport of water, are expressed in the rat and human epididymis [17, 38–41]. Therefore, the fluidity of the epididymal lumen appears to be fine-tuned by the presence of both AQPs and CFTR, resulting in a microenvironment that is conductive for spermatozoal maturation. In the human caput epididymidis, it is also possible that CFTR is implicated in the water permeability of AQP5, as suggested for AQP9 [42]. Furthermore, studies have suggested that the lack of expression of several AQPs in the male reproductive system, AQP7 in human sperm [43] and AQP9 in rat epididymal epithelium [42], are important contributors to male infertility. However, the study

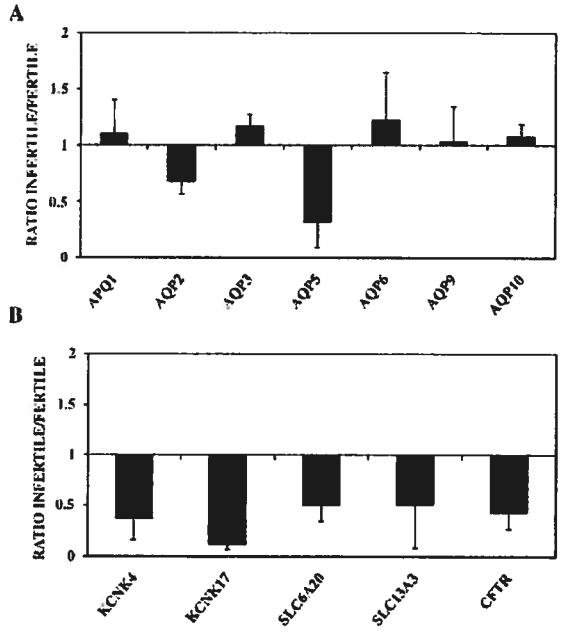


FIG. 8. Ratio of expressed genes encoding for AQPs (A) and other ionic channels (B) in the caput epididymidis of infertile versus fertile patients (n = 4; ±SEM).

FIG. 9. Ratio of expression of genes encoding for beta-defensins. The ratio corresponds to the level of expression of these genes in the caput epididymides of infertile compared with fertile patients ($n = 4$; ±SEM).

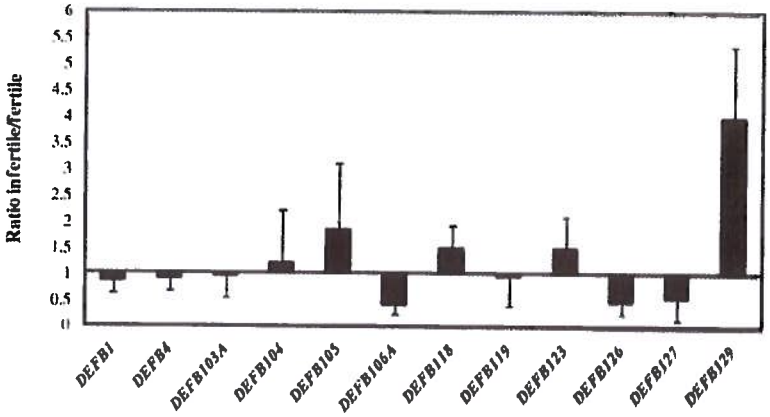
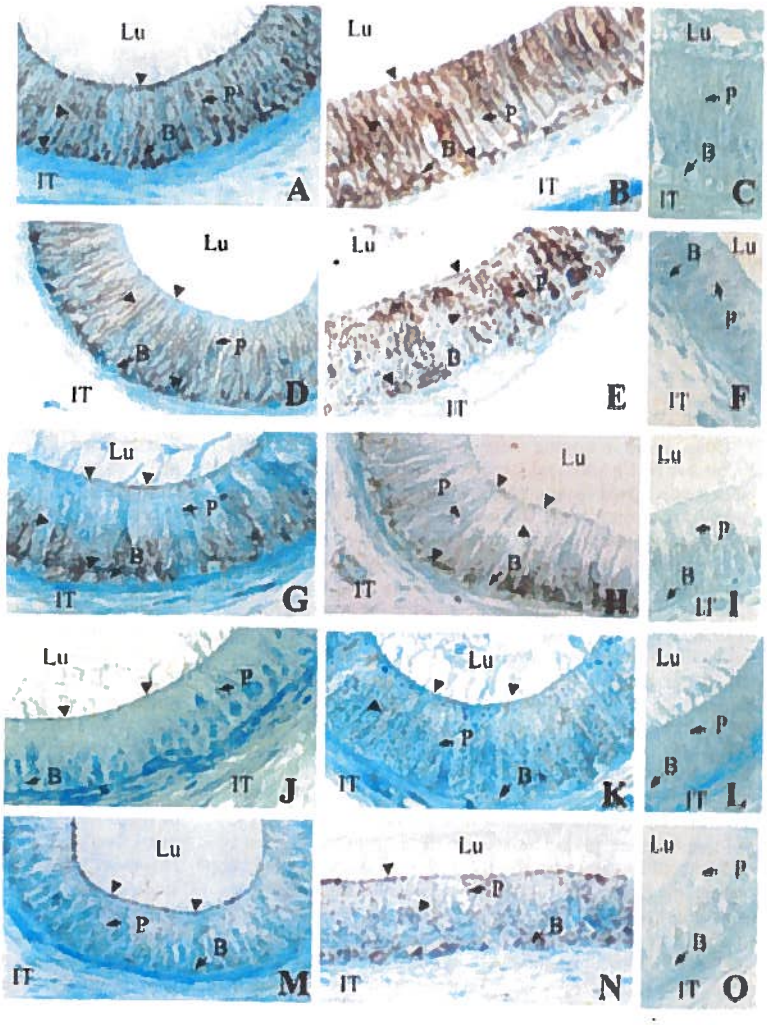


FIG. 10. Immunolocalization of CLDN1 (A–C), CLDN3 (D–F), CLDN8 (G–I), CLDN10 (J–L), and TJP1 (M–O) in the caput epididymides of fertile men (A, D, G, J, M) and infertile men (B, E, H, K, N). Negative controls with no primary antibodies are shown (C, F, I, L, O). Results indicate that CLDNs 1, 3, 8, and 10 and TJP1 were localized (arrowheads) to the area of tight junctions between adjacent principal cells in the caput epididymidis of fertile and infertile patients. In both types of tissues, CLDNs 1 and 3 were localized along the lateral margins of adjacent principal cells as well as between basal and principal cells, whereas CLDN8 was localized to the lateral margins of principal cells. In the caput epididymidis of infertile patients, CLDN10 and TJP1, in addition to being localized to the apical tight junctional complex, were localized along the lateral plasma membrane between principal cells. Original magnification ×640. P, Principal cells; B, basal cells; IT, intertubular space; Lu, lumen.



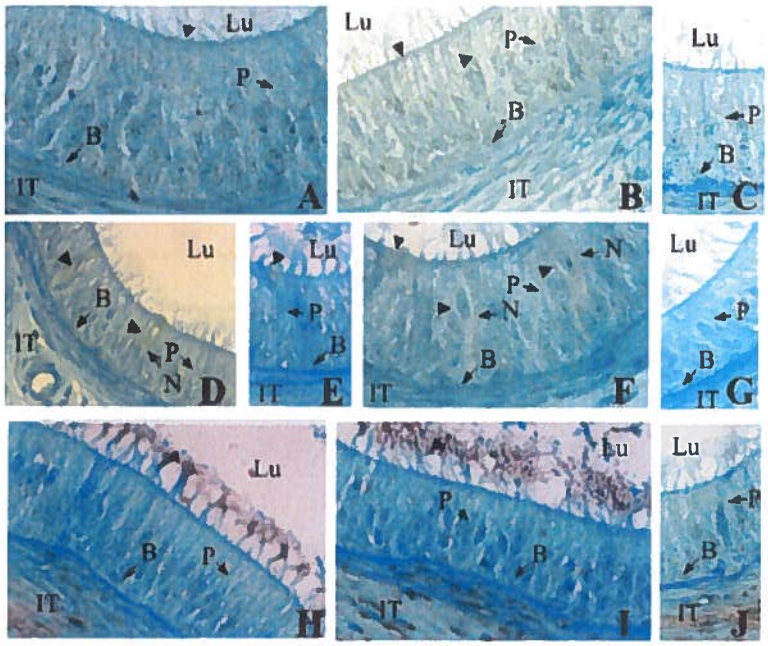


FIG. 11. Immunolocalization of AQP5 (A–C), CFTR (D–G), and CRISP1 (H–J) in the caput epididymides of fertile men (A, D, E, H) and infertile men (B, F, I). Negative controls with no primary antibodies are shown (C, G, J). Results indicate that AQP5 and CFTR were localized (arrowheads) to the apical border of a subpopulation of principal cells in fertile and infertile patients. In addition, CFTR was localized in the cytoplasm of the few narrow cells that could be seen in fertile and infertile patients. In infertile patients, AQP5 was also localized along the lateral plasma membrane of principal cells. CRISP1 was localized (arrowheads) to the apical border of principal cells of the epididymis of fertile and infertile patients. Original magnification in A ×1000; B, C, and F, ×640; D and G, ×400; E, H–J, ×480. P, Principal cells; B, basal cells; N, narrow cells; IT, intertubular space; Lu, lumen.

of various AQP knockout mice (AQP7 and AQP8), which are fertile, indicates that a compensation mechanism exists among the AQPs in rodents [44, 45]. Alteration in the immunostaining pattern of AQP5 suggests that not only the expression but also the targeting and binding of AQP5 to the apical plasma membrane are affected in nonobstructive azoospermic patients. Furthermore, it has been observed that in AQP5−/− mice, there is a decrease in the expression of several TJPs in the salivary glands, suggesting interactions between transcellular and paracellular water transport pathways [46].

Other genes encoding ionic channels, such as KCNK4, KCNK17, SLC6A20, and SLC13A3, are also downregulated by at least a 2-fold change in the caput epididymidis of infertile patients compared with fertile patients. Potassium channels play a role in many cellular processes, including maintenance of the osmotic regulation and ion flow [47]. Furthermore, transport of small hydrophilic substances across cell membranes is mediated by substrate-specific transport proteins. SLC6A20 is a member of the subgroup of transporters with unidentified substrates within the Na^+ and Cl^- coupled transporter family [48], whereas SLC13A3 is implicated in the handling of citrate [49]. Studies have suggested that another solute carrier, SLC26A3, acts in conjunction with CFTR as a ductal $HCO3^-$ secretor and as an absorber of NaCl based on the coimmunolocalization of SLC26A3 and CFTR in the human epididymis [50, 51]. Both of these respective functions have previously been proposed in vitro for the pancreas [52–54] and intestine [55]. The loss or decrease in the number of different solute carriers and water and ionic channels could be responsible for the poor reabsorption of luminal fluid. Whether or not this is related to the absence of spermatozoa in the lumen or is part of a general syndrome associated with infertility is unknown. Although it is tempting to speculate that the presence of spermatozoa alone can regulate gene expression in the epididymis, we cannot discount the possibility that other testicular factors are also missing in nonobstructive patients and that these are also implicated in the regulation of these different water and ion channels.

Interestingly, another group of genes differentially regulated in the caput epididymidis of infertile patients compared with fertile patients encode for proteins that have been known to play a role in spermatozoal maturation, such as CRISP1 [56], SPINLW1 [57], DEFB129, DEFB126, and DEFB106A [58]. CRISP1 has been suggested to play a role in sperm-egg interaction and in the inhibition of the uptake of ions, such as Ca^{2+}, required for capacitation by the spermatozoa [56]. Furthermore, SPINLW1 has been shown to bind to semenogelin in seminal plasma and on human spermatozoa after ejaculation. This complex is believed to be part of a larger network of protein complexes on the sperm surface that provides a protective shield before capacitation in the female reproductive tract [59]. Beta-defensins are also believed to play a role in both fertility and host defense [60–63]. Such sperm-coating proteins function in innate immunity, antimicrobial activity, and inhibition of proteases that may directly attack the sperm plasma membrane. Furthermore, genes downregulated by at least a 4-fold change included several genes that are implicated in spermatogenesis, sperm motility, and fertilization, such as STK22B, AKAP4, PRM1, and TNP1 (Table 3). Several studies have linked AKAP4, PRM1, and TNP1 to different types of male infertility [64–67]. The absence of spermatozoa in the lumen may explain the lack of expression of these genes. Altered expression of the different families of genes could also be due to the absence of testicular factors. However, in contrast with rodents who have been extensively studied [28, 68, 69], it has not been shown in the human epididymis whether or not testicular regulation of gene expression occurs. Some of our results support previous studies of rodents on the testicular regulation of epididymal gene expression. For example, FOS, RAB27A, and EGR1 are upregulated in the caput epididymides of the infertile patients (Fig. 6). This gene has also been shown to be upregulated in

the mouse caput epididymidis after orchidectomy [28]. As well, PRM1 is downregulated in the caput epididymides of the infertile patients (Fig. 6) and has been shown to be downregulated in the mouse caput epididymidis after orchidectomy [28]. This correlation cannot be made for all the differently regulated genes in the infertile versus fertile caput epididymidis. For example, CLDN14 has been demonstrated to be downregulated by orchidectomy in the mouse caput epididymidis [28], whereas in the present study, its expression is not altered. Note, however, that there are important differences between an orchidectomized animal model and nonobstructive azoospermic patients. In nonobstructive azoospermic patients, testicular luminal factors that can act on the epididymis are still present as are factors acting from the testis on the epididymis through circulation, including androgens. This is not the case for an orchidectomized animal, where there are no luminal and no testicular bloodborne factors that can act on the epididymis. It is likely that these differences account for some of the differences between different animal models and observations in the present study.

In conclusion, we have shown that several families of genes are downregulated in the caput epididymidis of infertile nonobstructive azoospermic patients. Many of these genes appear to be implicated in the regulation of spermatozoal maturation and the regulation of ions in the epididymal lumen. Although there are no spermatozoa in the epididymis of these patients, it is unknown whether or not the presence of spermatozoa in the epididymis regulates the expression of these genes or if alterations in their expression are representative of a larger effect, or syndrome, associated with nonobstructive azoospermia. Furthermore, there is no information about the expression of these genes after reconstructive surgery or if these contribute to the lower-quality sperm in patients after vasoepididymostomy.

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REFERENCES

1. Dohle GR, Colpi GM, Hargreave TB, Papp GK, Jungwirth A, Weidner W. EAU guidelines on male infertility. Eur Urol 2005; 48:703–711.
2. Seshagiri PB. Molecular insights into the causes of male infertility. J Biosci 2001; 26:429–435.
3. de Kretser DM. Male infertility. Lancet 1997; 349:787–790.
4. Safran A, Reuhinoff BE, Porat-Katz A, Schenker JG, Lewin A. Assisted reproduction for the treatment of azoospermia. Hum Reprod 1998; 13(suppl 4):47–60.
5. Egozcue S, Blanco J, Vendrell JM, Garcia F, Veiga A, Aran B, Barri PN, Vidal F, Egozcue J. Human male infertility: chromosome anomalies, meiotic disorders, abnormal spermatozoa and recurrent abortion. Hum Reprod Update 2000; 6:93–105.
6. Hargreave TB. Genetic basis of male fertility. Br Med Bull 2000; 56:650–671.
7. Westlander G, Hamberger L, Hanson C, Lundin K, Nilsson L, Soderlund B, Werner C, Bergh C. Diagnostic epididymal and testicular sperm recovery and genetic aspects in azoospermic men. Hum Reprod 1999; 14: 118–122.
8. Fahmy I, Mansour R, Aboulghar M, Serour G, Kamal A, Tawab NA, Ramzy AM, Amin Y. Intracytoplasmic sperm injection using surgically retrieved epididymal and testicular spermatozoa in cases of obstructive and non-obstructive azoospermia. Int J Androl 1997; 20:37–44.
9. Ghazzawi IM, Sarraf MG, Taher MR, Khalifa FA. Comparison of the fertilizing capability of spermatozoa from ejaculates, epididymal aspirates and testicular biopsies using intracytoplasmic sperm injection. Hum Reprod 1998; 13:348–352.
10. Robaire B, Hermo L. Efferent duct, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E, Neill J (eds.), Physiology of Reproduction. New York: Raven Press; 1988:999–1080.
11. Hermo L, Robaire B. Epididymal cell types and their functions. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York: Plenum Press; 2002:81–102.
12. Turner TT. Necessity's potion: inorganic ions and small organic molecules in the epididymal lumen. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York: Plenum Press; 2002:131–150.
13. Dacheux JL, Gatti JL, Dacheux F. Contribution of epididymal secretory proteins for spermatozoa maturation. Microsc Res Tech 2003; 61:7–17.
14. Dacheux JL, Dacheux F. Protein secretion in the epididymis. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecules to Clinical Practice. New York: Plenum Press; 2002:151–168.
15. Robaire B, Hinton B, Orgebin-Crist M-C. The epididymis. In: Neill J (ed.), Knobil and Neill's Physiology of Reproduction, 3rd ed. New York: Elsevier; 2006:1071–1148.
16. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. Lancet 2002; 360:772–777.
17. Zhang JS, Liu Q, Li YM, Hall SH, French FS, Zhang YL. Genome-wide profiling of segmental-regulated transcriptomes in human epididymis using oligo microarray. Mol Cell Endocrinol 2006; 250:169–177.
18. Dube E, Chan PT, Hermo L, Cyr DG. Gene expression profiling and its relevance to the blood-epididymal barrier in the human epididymis. Biol Reprod 2007; 76:1034–1044.
19. Jervis KM, Robaire B. Dynamic changes in gene expression along the rat epididymis. Biol Reprod 2001; 65:696–703.
20. Johnston DS, Jelinsky SA, Bang HJ, DiCandeloro P, Wilson E, Kopf GS, Turner TT. The mouse epididymal transcriptome: transcriptional profiling of segmental gene expression in the epididymis. Biol Reprod 2005; 73: 404–413.
21. Hsia N, Cornwall GA. DNA microarray analysis of region-specific gene expression in the mouse epididymis. Biol Reprod 2004; 70:448–457.
22. Skotheim RI, Monni O, Mousses S, Fossa SD, Kallioniemi OP, Lothe RA, Kallioniemi A. New insights into testicular germ cell tumorigenesis from gene expression profiling. Cancer Res 2002; 62:2359–2364.
23. McLean DJ, Friel PJ, Pouchnik D, Griswold MD. Oligonucleotide microarray analysis of gene expression in follicle-stimulating hormone-treated rat Sertoli cells. Mol Endocrinol 2002; 16:2780–2792.
24. Rodriguez S, Jafer O, Goker H, Summersgill BM, Zafarana G, Gillis AJ, van Gurp RJ, Oosterhuis JW, Lu YJ, Huddart R, Cooper CS, Clark J, et al. Expression profile of genes from 12p in testicular germ cell tumors of adolescents and adults associated with i(12p) and amplification at 12p11.2-p12.1. Oncogene 2003; 22:1880–1891.
25. Moldenhauer JS, Ostermeier GC, Johnson A, Diamond MP, Krawetz SA. Diagnosing male factor infertility using microarrays. J Androl 2003; 24: 783–789.
26. Hermo L, Jacks D. Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. Mol Reprod Dev 2002; 63:394–410.
27. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, et al. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. Nat Genet 2001; 29: 365–371.
28. Sipila P, Pujianto DA, Shariatmadari R, Nikkila J, Lehtoranta M, Huhtaniemi IT, Poutanen M. Differential endocrine regulation of genes enriched in initial segment and distal caput of the mouse epididymis as revealed by genome-wide expression profiling. Biol Reprod 2006; 75: 240–251.
29. Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. Annu Rev Physiol 2006; 68:403–429.
30. Umeda K, Matsui T, Nakayama M, Furuse K, Sasaki H, Furuse M, Tsukita S. Establishment and characterization of cultured epithelial cells lacking expression of ZO-1. J Biol Chem 2004; 279:44785–44794.
31. Cyr DG, Gregory M, Dube E, Dufresne J, Chan PT, Hermo L. Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood-epididymal barrier in animals and humans. Asian J Androl 2007; 9:463–475.
32. Lee S, Fan S, Makarova O, Straight S, Margolis B. A novel and conserved protein-protein interaction domain of mammalian Lin-2/CASK binds and recruits SAP97 to the lateral surface of epithelia. Mol Cell Biol 2002; 22: 1778–1791.
33. Patrizio P, Salameh WA. Expression of the cystic fibrosis transmembrane conductance regulator (CFTR) mRNA in normal and pathological adult human epididymis. J Reprod Fertil Suppl 1998; 53:261–270.
34. Tizzano EF, Silver MM, Chitayat D, Benichou JC, Buchwald M. Differential cellular expression of cystic fibrosis transmembrane regulator in human reproductive tissues: clues for the infertility in patients with cystic fibrosis. Am J Pathol 1994; 144:906–914.

35. Gong X, Burbridge SM, Lewis AC, Wong PY, Linsdell P. Mechanism of lonidamine inhibition of the CFTR chloride channel. Br J Pharmacol 2002; 137:928–936.
36. Schwiebert EM, Egan ME, Hwang TH, Fulmer SB, Allen SS, Cutting GR, Guggino WB. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. Cell 1995; 81:1063–1073.
37. Schreiber R, Greger R, Nitschke R, Kunzelmann K. Cystic fibrosis transmembrane conductance regulator activates water conductance in Xenopus oocytes. Pflugers Arch 1997; 434:841–847.
38. Pastor-Soler N, Bagnis C, Sabolic I, Tyszkowski R, McKee M, Van Hoek A, Breton S, Brown D. Aquaporin 9 expression along the male reproductive tract. Biol Reprod 2001; 65:384–393.
39. Badran HH, Hermo LS. Expression and regulation of aquaporins 1, 8, and 9 in the testis, efferent ducts, and epididymis of adult rats and during postnatal development. J Androl 2002; 23:358–373.
40. Hermo L, Krzeczunowicz D, Ruz R. Cell specificity of aquaporins 0, 3, and 10 expressed in the testis, efferent ducts, and epididymis of adult rats. J Androl 2004; 25:494–505.
41. Da Silva N, Silberstein C, Beaulieu V, Pietrement C, Van Hoek AN, Brown D, Breton S. Postnatal expression of aquaporins in epithelial cells of the rat epididymis. Biol Reprod 2006; 74:427–438.
42. Cheung KH, Leung CT, Leung GP, Wong PY. Synergistic effects of cystic fibrosis transmembrane conductance regulator and aquaporin-9 in the rat epididymis. Biol Reprod 2003; 68:1505–1510.
43. Saito K, Kageyama Y, Okada Y, Kawakami S, Kihara K, Ishibashi K, Sasaki S. Localization of aquaporin-7 in human testis and ejaculated sperm: possible involvement in maintenance of sperm quality. J Urol 2004; 172:2073–2076.
44. Sohara E, Ueda O, Tachibe T, Hani T, Jishage K, Rai T, Sasaki S, Uchida S. Morphologic and functional analysis of sperm and testes in Aquaporin 7 knockout mice. Fertil Steril 2007; 87:671–676.
45. Yang B, Song Y, Zhao D, Verkman AS. Phenotype analysis of aquaporin-8 null mice. Am J Physiol Cell Physiol 2005; 288:C1161–1170.
46. Kawedia JD, Nieman ML, Boivin GP, Melvin JE, Kikuchi K, Hand AR, Lorenz JN, Menon AG. Interaction between transcellular and paracellular water transport pathways through Aquaporin 5 and the tight junction complex. Proc Natl Acad Sci U S A 2007; 104:3621–3626.
47. Miller C. An overview of the potassium channel family. Genome Biol 2000; 1:REVIEWS0004.
48. Broer A, Cavanaugh JA, Rasko JE, Broer S. The molecular basis of neutral aminoaciduria. Pflugers Arch 2006; 451:511–517.
49. Chen X, Tsukaguchi H, Chen XZ, Berger UV, Hediger MA. Molecular and functional analysis of SDCT2, a novel rat sodium-dependent dicarboxylate transporter. J Clin Invest 1999; 103:1159–1168.
50. Clulow J, Jones RC, Hansen LA, Man SY. Fluid and electrolyte reabsorption in the ductuli efferentes testis. J Reprod Fertil Suppl 1998; 53:1–14.
51. Hihnala S, Kujala M, Toppari J, Kere J, Holmberg C, Hoglund P. Expression of SLC26A3, CFTR and NHE3 in the human male reproductive tract: role in male subfertility caused by congenital chloride diarrhoea. Mol Hum Reprod 2006; 12:107–111.
52. Greeley T, Shumaker H, Wang Z, Schweinfest CW, Soleimani M. Downregulated in adenoma and putative anion transporter are regulated by CFTR in cultured pancreatic duct cells. Am J Physiol Gastrointest Liver Physiol 2001; 281:G1301–1308.
53. Ko SB, Shcheynikov N, Choi JY, Luo X, Ishibashi K, Thomas PJ, Kim JY, Kim KH, Lee MG, Naruse S, Muallem S. A molecular mechanism for aberrant CFTR-dependent HCO(3)(-) transport in cystic fibrosis. EMBO J 2002; 21:5662–5672.
54. Ko SB, Zeng W, Dorwart MR, Luo X, Kim KH, Millen L, Goto H, Naruse S, Soyombo A, Thomas PJ, Muallem S. Gating of CFTR by the STAS domain of SLC26 transporters. Nat Cell Biol 2004; 6:343–350.
55. Melvin JE, Park K, Richardson L, Schultheis PJ, Shull GE. Mouse down-regulated in adenoma (DRA) is an intestinal Cl(-)/HCO(3)(-) exchanger and is up-regulated in colon of mice lacking the NHE3 Na(+)/H(+) exchanger. J Biol Chem 1999; 274:22855–22861.
56. Roberts KP, Ensrud KM, Wooters JL, Nolan MA, Johnston DS, Hamilton DW. Epididymal secreted protein Crisp-1 and sperm function. Mol Cell Endocrinol 2006; 250:122–127.
57. Richardson RT, Sivashanmugam P, Hall SH, Hamil KG, Moore PA, Ruben SM, French FS, O'Rand M. Cloning and sequencing of human Eppin: a novel family of protease inhibitors expressed in the epididymis and testis. Gene 2001; 270:93–102.
58. Yudin AI, Treece CA, Tollner TL, Overstreet JW, Cherr GN. The carbohydrate structure of DEFB126, the major component of the cynomolgus Macaque sperm plasma membrane glycocalyx. J Membr Biol 2005; 207:119–129.
59. Wang Z, Widgren EE, Sivashanmugam P, O'Rand MG, Richardson RT. Association of eppin with semenogelin on human spermatozoa. Biol Reprod 2005; 72:1064–1070.
60. Rodriguez-Jimenez FJ, Krause A, Schulz S, Forssmann WG, Conejo-Garcia JR, Schreeb R, Motzkus D. Distribution of new human beta-defensin genes clustered on chromosome 20 in functionally different segments of epididymis. Genomics 2003; 81:175–183.
61. Yenugu S, Hamil KG, Birse CE, Ruben SM, French FS, Hall SH. Antibacterial properties of the sperm-binding proteins and peptides of human epididymis 2 (HE2) family; salt sensitivity, structural dependence and their interaction with outer and cytoplasmic membranes of *Escherichia coli*. Biochem J 2003; 372:473–483.
62. Zanich A, Pascall JC, Jones R. Secreted epididymal glycoprotein 2D6 that binds to the sperm's plasma membrane is a member of the beta-defensin superfamily of pore-forming glycopeptides. Biol Reprod 2003; 69:1831–1842.
63. Zhou CX, Zhang YL, Xiao L, Zheng M, Leung KM, Chan MY, Lo PS, Tsang LL, Wong HY, Ho LS, Chung YW, Chan HC. An epididymis-specific beta-defensin is important for the initiation of sperm maturation. Nat Cell Biol 2004; 6:458–464.
64. Baccetti B, Collodel G, Gambera L, Moretti E, Serafini F, Piomboni P. Fluorescence in situ hybridization and molecular studies in infertile men with dysplasia of the fibrous sheath. Fertil Steril 2005; 84:123–129.
65. Moretti E, Baccetti B, Scapigliati G, Collodel G. Transmission electron microscopy, immunocytochemical and fluorescence in situ hybridisation studies in a case of 100% necrozoospermia: case report. Andrologia 2006; 38:233–238.
66. Ravel C, Chantot-Bastaraud S, El Houate B, Berthaut I, Verstraete L, De Larouziere V, Lourenco D, Dumaine A, Antoine JM, Mandelbaum J, Siffroi JP, McElreavey K. Mutations in the protamine 1 gene associated with male infertility. Mol Hum Reprod 2007; 13:461–464.
67. Miyagawa Y, Nishimura H, Tsujimura A, Matsuoka Y, Matsumiya K, Okuyama A, Nishimune Y, Tanaka H. Single-nucleotide polymorphisms and mutation analyses of the TNP1 and TNP2 genes of fertile and infertile human male populations. J Androl 2005; 26:779–786.
68. Ezer N, Robaire B. Gene expression is differentially regulated in the epididymis after orchidectomy. Endocrinology 2003; 144:975–988.
69. Turner TT, Johnston DS, Jelinsky SA, Tomsig JL, Finger JN. Segment boundaries of the adult rat epididymis limit interstitial signaling by potential paracrine factors and segments lose differential gene expression after efferent duct ligation. Asian J Androl 2007; 9:565–573.

Supplementary table 1: Differentially regulated genes in the caput epididymidis of infertile as compared to fertile patients by at least 2-fold change, that are implicated in fertility.

Gene ID	Fold change	Gene symbol	Pubmed accession no.	Biological function(s)
_23_P133722	10.76	*CRISP1*	NM_001131	Fusion of sperm to egg plasma membrane; spermatogenesis
A_23_P169007	2.3	*NPM2*	NM_182795	chromatin remodeling; embryonic development; fertilization (sensu Metazoa); oocyte differentiation; positive regulation of meiosis; regulation of exit from mitosis; regulation of translation
A_23_P14414	2.131	*FAM12B*	NM_022360	spermatid cell development
A_23_P210503	2.069	*SPINLW1*	NM_181502	fertilization
A_23_P21713	2.013	*SPAG16*	NM_024532	sperm motility
A_23_P25106	0.499	*WNT1*	NM_005430	central nervous system development; frizzled-2 signaling pathway; morphogenesis; spermatogenesis
A_23_P250231	0.45	*ANKRD7*	NM_019644	male gonad development
A_23_P25106	0.499	*WNT1*	NM_005430	central nervous system development; frizzled-2 signaling pathway; morphogenesis; spermatogenesis
A_23_P6041	0.471	*DEFB126*	NM_030931	defense response, fertility
A_23_P71889	0.453	*ODF2*	NM_153437	structural molecule activity (outer dense fiber of sperm tail)
A_23_P250231	0.45	*ANKRD7*	NM_019644	male gonad development
A_23_P123108	0.398	*AKAP9*	NM_147166	spermatogenesis
A_23_P82859	0.359	*C8orf1*	NM_004337	germ cell development; meiosis
A_23_P106600	0.349	*PRM2*	NM_002762	DNA packaging; chromosome organization and biogenesis (sensu Eukaryota); mitotic chromosome condensation; spermatogenesis
A_23_P425815	0.316	*DEFB106*	NM_152251	defense response, fertility
A_23_P40516	0.231	*STK22B*	NM_053006	protein amino acid phosphorylation; spermatogenesis
A_23_P11081	0.162	*AKAP4*	NM_003886	cell motility; fertilization (sensu Metazoa); signal transduction; sperm motility
A_23_100189	0.144	*PRM1*	NM_002761	DNA packaging; chromosome organization and biogenesis (sensu Eukaryota); mitotic chromosome condensation; spermatogenesis
A_23_P393749	0.11	*CATSPER3*	NM_178019	cation transport (cation channel, sperm associated 3)
A_23_P17673	0.106	*DNMT3L*	NM_013369	DNA methylation; imprinting; spermatogenesis
A_23_P165504	0.0703	*TNP1*	NM_024814	chromatin silencing; chromosome organization and biogenesis (sensu Eukaryota); fertilization, exchange of chromosomal proteins; nucleosome disassembly; sexual reproduction; single strand break repair; sperm motility; spermatid nuclear elongation; spermatogenesis
A_23_P390576	0.01	*SPATA9*	NM_031952	protein biosynthesis, spermatogenesis

SECTION 3: ASSESSING THE ROLE OF CLAUDINS IN MAINTAINING THE INTEGRITY OF EPIDIDYMAL TIGHT JUNCTIONS USING NOVEL HUMAN EPIDIDYMAL CELL LINES

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Article sous presse dans *Biology of Reproduction*

3.1 Résumé de l'article en français

L'épididyme est responsable de la maturation post-testiculaire des spermatozoïdes. Cette maturation est possible grâce à l'existence d'un milieu spécifique dans la lumière de l'épididyme. Même si le rôle de l'épididyme dans la maturation des spermatozoïdes est bien caractérisé, les processus moléculaires et cellulaires en arrière de ce phénomène ne sont pas très bien connus, en particulier chez l'humain à cause du manque d'outils biologiques. Nous avons établi les premières lignées cellulaires stables dérivées de deux régions de l'épididyme humain adulte qui ont été transformées avec l'antigène T du virus SV40 (LTAg). Ces lignées cellulaires sont composées de populations homogènes de cellules principales dont les caractéristiques sont similaires à celles des cellules qu'on retrouve *in vivo* (ultrastructure, expression de marqueurs épithéliaux et épididymaires). Les cellules expriment aussi différents gènes codant pour des protéines de jonctions adhérentes (*CDH1, CDH2*), de jonctions serrées (*CLDN1, 2, 3, 4, 7, 8*) et de desmosomes (*DSP*). Les cellules FHCE1 (*Fertile Human Caput Epididymal cell line 1)* forment des jonctions serrées fonctionnelles tel que démontré par la mesure de la résistance transépithéliale (TER) et par la localisation de TJP1, de l'occludine et de CLDN1 le long des membranes plasmiques. De plus, la baisse d'expression des CLDN1, 3, 4 et 7, à l'aide de petits ARNs interférants, entraîne une baisse de TER. Ces CLDNs sont donc importantes pour la fonction de barrière des jonctions serrées. Une altération de leur expression et/ou de leur localisation pourrait être impliquée dans le syndrome de l'infertilité masculine humaine.

3.2 Contribution de l'étudiante

Les expériences présentées dans cet article ont été réalisées par l'étudiante à l'exception du développement de certaines lignées qui a été fait par le deuxième auteur de l'article. L'article a également été rédigé par l'étudiante. Finalement, l'étudiante a participé au choix du journal de publication.

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Assessing the Role of Claudins in Maintaining the Integrity of Epididymal Tight Junctions Using Novel Human Epididymal Cell Lines[1]

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Short title: Claudins in the human epididymis

Summary sentence: Novel human epididymal cell lines were developed and used to demonstrate that CLDN1, CLDN3, CLDN4, CLDN7 are necessary for maintaining the integrity of human tight junctions.

Keywords: male infertility, blood-epididymis barrier, cell line, male reproductive tract, principal cells

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Abstract

The epididymis is responsible for post-testicular sperm maturation. Sperm maturation is dependent on the luminal microenvironments along the epididymis. While the role of the epididymis is well established, the molecular and cellular mechanisms responsible for sperm maturation remain to be elucidated, particularly in the human as there exist limited biological tools. We have established the first stable epithelial cell lines transformed with SV40 large T antigen (LTAg) from two regions of the human adult epididymis. The cell lines are comprised of homogenous populations of diploid principal cells that possess ultrastructural characteristics similar to those of human principal cells *in vivo*. These cells express transcripts for adherens (cadherins *CDH1, CDH2*) and tight (claudins *CLDN1, CLDN2, CLDN3, CLDN4, CLDN7, CLDN8*) junctions as well as desmosomes (desmoplakin, *DSP*). Transepithelial resistance (TER) measurements in Fertile Human Caput Epididymal cell line 1 (FHCE1) as well as the immunolocalization of tight junctional protein 1 (TJP1), occludin and CLDN1 indicate that these cells form functional tight junctions. Furthermore, knockdown of CLDN1, CLDN3, CLDN4 or CLDN7 using specific siRNAs resulted in significant decreases in TER, suggesting that these CLDNs are essential for the barrier function of the blood-epididymis barrier. Disruption of CLDN1, CLDN3, CLDN4 and CLDN7 could, therefore, lead to epididymal dysfunction resulting in male infertility.

Introduction

In mammals, the epididymis plays a major role in sperm maturation, transport, protection and storage. Testicular spermatozoa are immature and need to transit through the epididymis to acquire motility and the ability to fertilize [1, 2]. Specific microenvironments in the lumen of the epididymis are essential for sperm maturation. The creation and maintenance of this luminal environment is due in part to the blood-epididymis barrier [3], which consists of apical tight junctions localized between adjacent principal cells that regulate paracellular transport between the lumen and the blood [4]. Epididymal tight junctions were first described by Friend and Gilula [5] in the rat as a highly developed junctional complex. Suzuki and Nagano [6, 7] showed that the strands of the tight junctions increased in complexity during development until postnatal day 37. Hinton and Howard [8] first showed that the caput epididymidis was relatively impermeable to inulin, thereby demonstrating the presence of a tissue barrier. However, it is only recently that molecular components of the human blood-epididymis barrier have been described. Dubé et al. [9] demonstrated that in human the composition of this barrier is complex and varies along the duct.

As in the rat and mouse, human epididymal tight junctions are comprised of several peripheral (TJP, also known as zonula occludens) and transmembrane proteins (occludin, claudins) [9-14]. Claudins (CLDN) are thought to be the molecular basis of tight junctions [15]. Several CLDNs can be expressed in a specific tissue, defining its permeability characteristics [16], but it is unusual to find as many as 19 CLDNs expressed in one particular tissue [17], as is the case for the human epididymis [9]. In addition, the differential expression of several CLDNs along the human epididymal duct may represent a key element in regulating the changing intraluminal environment necessary for epididymal functions.

Male factor infertility affects almost half of infertile couples [18, 19], and in 25% of cases the causes are unknown [20]. Several lines of evidence have suggested that the epididymis is implicated in male infertility. A recent study demonstrated that a larger number of genes exhibit differential expression in the caput epididymidis of non obstructive azoospermic patients as compared to that of patients with proven fertility [21]. Among these genes, several encoded water and ion channels that can directly influence the luminal environment of the epididymis, while others are known to regulate components of the tight junction [22]. In addition, cytoplasmic immunolocalization of the tight junctional proteins TJP1 and CLDN10, normally localized exclusively to the tight junctions, suggested that the blood-epididymis barrier was altered in the epididymis of infertile patients [21]. However, in a tissue where such a large number of CLDNs are expressed, the significance of a loss, or mistargeting, of a single CLDN is difficult to evaluate. Thus it is important to identify the role of specific CLDNs in order to understand the impact that altered CLDN expression may have on male fertility.

CLDNs form ion-selective channels whose permeability properties depend on the combination and co-polymerization of CLDNs [23]. In order to assess the role of specific CLDNs in the human epididymis, we need to develop biological tools that will allow the study of cell-cell interactions. The difficulty in obtaining quality human epididymal tissues and the limited life-span of primary cell cultures remain a significant obstacle for understanding human epididymal functions [24-27]. Several cell lines have been developed in mouse, rat and dog but their applicability to humans has not been established and may be limited, given the morphological

differences between the human epididymis and that of certain laboratory species [28-32]. Thus, even if the composition of the blood-epididymis barrier is similar between rodents and humans, novel human *in vitro* models would be necessary to study the role of specific junctional proteins, such as CLDNs, in human epididymal pathologies.

The objective of the present study was to develop and characterize human epididymal cell lines in order to assess the role of CLDN1, CLDN3, CLDN4 and CLDN7, that have been shown to be important for barrier function [23, 33], in epididymal tight junctions.

Material and Methods

Tissue Preparation

Epididymides were obtained from a 23-year old patient with active spermatogenesis undergoing radical orchidectomy for localized testicular cancer (confined within testicular tunica albuginea with no sign of epididymal lesion or obstruction). Tissues were surgically removed and placed in cold culture medium (DMEM/HAM's F12 culture medium containing glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 µg/ml), Sigma-Aldrich, Mississauga, Ontario, Canada) and were processed under aseptic conditions within 1 hr of surgery. Epididymides were divided into three regions: the caput, corpus and cauda epididymidis (see Dubé et al. [21] for a schematic representation of human epididymal regions). The study was conducted with the approval of the McGill University ethics committee for research on human subjects after informed consent was obtained from the patient.

Primary Cell Culture

Cells from the different epididymal regions were isolated according to the methods of Dufresne et al. [32]. Briefly, tissue fragments (2-3 mm^3) were placed in DMEM/HAM's F12 culture medium containing glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 µg/ml), collagenase (2 mg/ml; Life Technologies, Inc., Burlington, Ontario, Canada) and DNase (20 U/ml; Promega, Ottawa, Ontario, Canada). The fragments were digested twice for 50 min each time in a shaking water bath at 37°C. Tissues were dissociated between digestions by gently pipetting the small tissue fragments, and these were left to sediment to the bottom of the flasks and the supernatant was replaced with fresh medium. At the end of the final digestion, the cells were centrifuged (34 x g) for 3 min and the pellet resuspended in DMEM/HAM's F12 culture medium containing antibiotics and nutrients (50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 5 nM testosterone, 10 µg/ml insulin, 10 µg/ml transferrin, 80 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor, 10 ng/ml cAMP, 2 ng/ml sodium selenium, 200 ng/ml tocopherol, 200 ng/ml retinol and 1% fetal bovine serum (FBS); Sigma-Aldrich). Small tissue fragments were then placed in 6- or 24-well plates coated with collagen IV (BD Biosciences, Mississauga, Ontario, Canada) and incubated in a humidified chamber at 32° C with 5% CO_2. Once the cells adhered to the bottom of the wells, the culture medium was changed every 48h for 12-18 days until 40-80% confluence was reached. The cells were subsequently used for immortalization.

Immortalization of Epididymal Cells

Epididymal cells were transfected with a pBK-CMV plasmid containing the SV40 LTAg and neomycin resistance genes (a kind gift of Dr. D. W. Silversides, Animal Reproduction Research Center, University of Montreal) using Fugene 6 (70-80% confluent) according to the

manufacturer's instructions (3:2 ratio of Fugene 6:DNA ; Roche Diagnostics, Laval, Quebec, Canada). Stable transfectants were selected by incubating the cells in media containing neomycin (200 µg/ml G418; GIBCO BRL, Burlington, Ontario, Canada) for 14 days. Cell lines were generated from subpopulations of resistant cells (Table 1). The cells were then selected by isolating clusters from the culture wells with cloning cylinders followed by serial dilution and passaging. The homogeneity of the cell lines obtained after selection was verified by electron microscopy and by FACS based on the complexity and size of the cells (data not shown). It should be noted that the selection of a single cell with the cloning cylinder resulted in non-proliferating cells, suggesting a need for cell-cell interaction in the proliferation of human epididymal principal cells.

Cells were cultured in DMEM/HAM's F12 medium containing antibiotics and nutrients, as previously described, as well as 10% FBS. The culture medium was changed every 48h.

Electron Microscopy

Cells were trypsinized (0.05% trypsin, 0.53 mM EDTA), gently aspirated and centrifuged (300 x g) into a 1.5-ml centrifuge tube or grown on plastic chamber slides (Nalge Nunc International, Naperville, IL) before fixation with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24h. The cells were then washed in 0.1M sodium cacodylate buffer and postfixed in 1% ferrocyanide-reduced osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in EPON. Thin sections were cut, mounted on copper grids, counterstained with uranyl acetate and lead citrate and examined with a FEI Tecnai 12 electron microscope.

Cell Cycle Analysis

Cells were trypsinized, recovered by centrifugation (1000g x 7 min), and washed twice in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Cells were fixed by resuspending the pellet in ice-cold ethanol and kept on ice for 30 min. The cells were then washed three times in PBS containing 2% FBS. The pellet was finally resuspended in a solution containing 0.1% sodium citrate, 0.3% NP-40, 100 ug/ml RNase A, 50 ug/ml propidium iodide. DNA content of $2x10^6$ cells per sample was analyzed using a FACScan apparatus (Becton Dickinson, Oakville, Ontario, Canada). THP1 cells (a kind gift of Dr. D. Girard, INRS-Institut Armand-Frappier) were used as control diploid cells.

Cell Growth

Aliquots of 2500 cells per well were plated in 96-well culture plates coated with collagen IV. The number of cells was determined using Trypan blue staining and a hemocytometer (Invitrogen Inc., Burlington, ON). The next day, once the cells had adhered, medium was changed, and this first time point was assigned as time zero. Each time point was done in triplicate. Medium was changed every 24h. At different time points, the culture medium was removed from three of the wells and replaced with 20 µl methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution (Sigma-Aldrich; 0.5 mg/ml in culture medium) to measure cellular proliferation [32]. After 2h, the MTT solution was removed and the formazan crystals were solubilized in 200 µl dimethylsulfoxide. Absorbance (570nm) was measured using a microtiter plate reader (Power Wave X; Bio-Tek Instruments Inc., Winooski, VT). The doubling time was calculated as the inverse of the slope of a semilog plot of the absorbance in function of time, in

the linear portion of the graph (the logarithmic growth phase). Data was presented as mean ± SEM.

Immunofluorescence

Indirect immunofluorescence experiments were done using several antibodies including a rabbit polyclonal anti-cytokeratin (cat#18-0059, Zymed Lab. Inc., San Francisco, US), a mouse monoclonal anti-vimentin (VIM, cat#ab20346, Abcam Inc., Cambridge, MA), a rabbit polyclonal anti-TJP1 (cat#61-7300, Zymed Lab. Inc.), a rabbit polyclonal anti-occludin (OCLN, cat#71-1500, Zymed Lab. Inc.) and a rabbit polyclonal anti-CLDN1 (cat#51-7900, Zymed Lab. Inc.). Cells were grown on plastic chamber slides (Nalge Nunc International). Cells were fixed in methanol at –20°C for 10 min. For cytokeratin and VIM localization, cells were permeabilized in a solution of 0.3% Triton X-100 in PBS for 15 min at room temperature. Cells were then washed in PBS and blocked in the same buffer containing 5% BSA for 30 min at 37 °C (cytokeratin, VIM) or overnight at 4°C (TJP1, OCLN), followed by three 5-min washes in PBS. Samples were then incubated with primary antibodies (cytokeratin, 13.6 μg/ml; VIM, 1 μg/ml; TJP1, 2.5 μg/ml; OCLN, 25 μg/ml) for 90 min at room temperature, and washed three times for 5 min with PBS. For CLDN1 localization, cells were blocked in PBS containing 5% BSA for 60 min at 37 °C, incubated overnight at 4°C with the primary antibody (12.5 μg/ml) and washed three times for 5 min with PBS. Cells were subsequently incubated with Alexa Fluor 488-conjugated anti-rabbit or anti-mouse (2 μg/ml; Molecular Probes, Leiden, The Netherlands) secondary antibody for 45 min at 37°C. Cells were then washed three times in PBS and mounted with Vectashield mounting medium containing propidium iodide (Vector Laboratories, Burlington, Ontario, Canada). Sections were examined with a Leica DMRE microscope (Leica Microsystems, Inc., Bannockburn, IL) or with a Nikon Eclipse E800 microscope equipped with a BioRad Radiance 2000 confocal imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

RT-PCR

Total cellular RNA was isolated using the Illustra RNAspin Mini Isolation kit (GE Healthcare, Baie D'Urfe, QC, Canada) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed using an oligo $(dT)_{16}$ primer (R&D Systems Inc., Minneapolis, MN). Gene specific PCR was subsequently done to identify the presence of different transcripts (see Table 2) using the following program: 94°C for 5 min, 30–40 cycles of 94 °C for 30 sec; melting temperature (Tm) for 30 sec, 72°C for 1 min; and cooled to 4°C. Primer sequences for CLDNs are identical to those described in Dubé et al. [9]. PCR products were separated on an agarose gel (1-2%) and visualized with ethidium bromide using a Fluor-S Multi-Imager densitometer (Bio-Rad Laboratories, Mississauga, ON, Canada). PCR amplification was also done on RNA to confirm that the sample was free of contaminating genomic DNA. RNA extracted from human epididymis and kidney (Biochain, Hayward, CA, USA) were used as positive controls.

Transepithelial Resistance (TER)

Cells were seeded at a density of 1 x 10^5 cells/ml on Costar Transwell 6.5-mm cell culture inserts (pore size 0.4 μm; Corning). Cells were grown in DMEM/HAM's F12 culture medium containing antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin), 2 mM L-glutamine and 5% FBS. Once total confluence was reached, cells were cultured in medium containing 1.8mM $CaCl_2$ for 48h and TER was measured at regular intervals using an EVOM epithelial voltohmmeter with a STX2 electrode (WPI Inc., Sarasota, FL). TER was normalized to the area

of the filter after removal of background resistance of a blank filter that contained only medium. TER was measured as ohms x cm^2. All TER measurements were done in triplicate wells at all of the time points.

RNA Interference

Small interfering RNA (siRNA; final concentration of 5nM) against CLDN1, CLDN3, CLDN4, CLDN7 and a nonsense siRNA (5nM; Qiagen, Mississauga, Ontario, Canada) were transfected into FHCE1 using HiPerfect Transfection Reagent according to the manufacturer's instructions (Qiagen). Cells were seeded on inserts at the time of tranfection and, 24h after transfection, were exposed to $CaCl_2$ as previously described. TER was measured in triplicate wells at regular intervals for 48h. At various time intervals post-transfection, cells were lysed and levels of *CLDNs* were monitored by RT-PCR, as previously described, and by Western blot. Untreated cells, cells treated with transfection reagent and cells transfected with the nonsense siRNA were used as controls. This experiment was repeated at least three times.

Western Blot

Cells were lysed in cold RIPA buffer (PBS, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 10 µg/ml phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Sigma, Missouri, USA) and then centrifuged at 10,000 x g for 10 min at 4°C to remove cellular debris. Protein concentrations in the cell extracts were determined using the Bradford method (Bradford reagent; Sigma). Samples were stored at –80°C until electrophoresis. 25µg of proteins were diluted in Laemmli loading buffer, heated at 94°C for 7 min, separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The resulting blots were stained with 0.6% Ponceau red S to evaluate transfer efficiency. Membranes were blocked 2h at room temperature with 5% non-fat dry milk diluted in Tris-buffered saline (TBS; 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) and subsequently incubated overnight at 4°C or 2h at room temperature with either a polyclonal anti-CLDN1 antibody (Zymed, 0.5 ug/ml), a polyclonal anti-CLDN3 antibody (Zymed, 0.8 ug/ml), a polyclonal anti-CLDN4 antibody (Santa Cruz, 2 ug/ml) or a polyclonal anti-CLDN7 antibody (Zymed, 1 ug/ml) diluted in the blocking buffer. Membranes were then washed three times in TBS with 0.1 % Tween-20 (Fisher scientific) during 10 min and were probed with a HRP-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibody (Santa Cruz, 0.2 ug/ml). Once again, membranes were washed three times 10 min in TBST. Detection was done using the Lumilight Western Blotting Substrate (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. A monoclonal anti-actin antibody (Sigma, 6.4 ug/ml) was used to normalize the protein amounts on the blots.

Results

Establishment of Human Epididymal Cell Lines

Primary cell cultures were derived from the epididymis of a 23-year old man with active spermatogenesis and immortalized with a plasmid containing the coding region of the SV40 LTAg. We obtained five cell lines, four from the caput (FHCE1-4) and one from the corpus epididymidis (FHSE1) as described in Table 1. While cells from the cauda epididymidis from several patients were transfected with the pBK-CMV plasmid, we were unable to maintain these cells in culture over a prolonged period of time. Ultrastructural analyses of the different cell lines indicated that they were comprised of cells which shared similar morphological characteristics

with epithelial principal cells *in vivo [9]*. These characteristics included the presence of microvilli, a large and irregular nucleus, endosomal elements, secreted vesicles resembling epididymosomes, an extensive Golgi network and junctional complexes (FIG.1). Furthermore, the cell lines expressed cytokeratin, an epithelial cell marker, but not vimentin, a mesenchymal cell marker, at both the mRNA and protein levels (FIG.2). These results support the notion that the immortalized epididymal cell lines are of epithelial origin. Furthermore these cells, which have been passaged at least 20 times, are diploid (FIG.3) and have a doubling time of approximately 13 to 20 days (FIG.4).

Characterization of the Human Epididymal Cell Lines

RT-PCR was used to verify the presence of transcripts for several epididymal principal cell markers. *NPC2* (also known as *HE1*), *SPAG11B* (also known as *HE2*), *CD52* (also known as *HE5*) and *DCXR* (also known as *P34H)*, but not *CRISP1* and *SPINLW1* (also known as *EPPIN2*), were expressed in the cell lines (FIG.5). The expression of *SLC9A3*, a Na+/H exchanger also known as *NHE3*, which has been shown to be expressed in the epithelium of human efferent ducts but not in the human epididymis [34], was not expressed in any of the cell lines (FIG.5). Human kidney was used as a positive control for *SLC9A3* [35]. Transcripts for the androgen (*AR*) and estrogen receptors (*ESR1* and *ESR2*) as well as for the two isoforms of 4-ene-steroid-5-alpha-reductase (*SRD5A1* and *SRD5A2*) were also assessed (FIG. 6). The *AR* was expressed in FHCE1, FHCE2 and FHCE3 cell lines, whereas *ESR1*, *ESR2*, *SRD5A1* and *SRD5A2* were expressed in all of the cell lines. Overall our cell lines expressed many of the genes characteristic of differentiated principal cells of the adult human epididymis *in vivo*.

Expression of genes encoding junctional proteins by the different cell lines

The expression of genes encoding different junctional proteins implicated in adherens (cadherins *CDH1* and *CDH2*) and tight junctions (*CLDN1*, *CLDN2*, *CLDN3*, *CLDN4*, *CLDN7* and *CLDN8*) as well as desmosomes (desmoplakin, *DSP*) was verified by RT-PCR (FIG.7). *CDH1, CDH2*, *CLDN1*, *CLDN4* and *CLDN8* and *DSP* were expressed in all cell lines. *CLDN3* was expressed in all cell lines derived from the caput epididymidis but not in the cell line derived from the corpus epididymidis. *CLDN7* was expressed in all cell lines except in FHCE2 cells.

Formation of Tight Junctions

To assess whether or not epididymal epithelial cells were able to form functional tight junctions, we monitored transepithelial resistance (TER), a measurement for the paracellular barrier function and integrity of tight junctions. The formation of tight junctions was induced using a calcium-switch model (FIG. 8A). Several studies have shown that calcium can induce the assembly of tight junctions *in vitro* [36]. Results were obtained using FHCE1 cells which resembled *in vivo* principal cells of the caput epididymidis, the main region implicated in sperm maturation, and expressed the highest TER among the different cell lines (data not shown). In FHCE1 cells exposed to calcium (1.8 mM), TER peaked at 24h after calcium switch (approximately 150 ohms.cm^2) and returned to a lower steady-state level (approximately 20 ohms.cm^2) by 30h (FIG. 8A). This peak was not observed when TER was measured in FHCE1 cells that were not exposed to calcium. Immunocytochemical staining was undertaken to assess the presence and localization of TJP1, OCLN and CLDN1 in FHCE1 cells exposed 24h to calcium. TJP1, OCLN and CLDN1 were localized to the membrane of FHCE1 cells (FIG.8B).

Some cytoplasmic staining was also noted. These results indicate that FHCE1 cells can be stimulated to form functional tight junctions.

Role of CLDN1, CLDN3, CLDN4 and CLDN7 in Tight Junction Integrity

To assess the physiological role of CLDN1, CLDN3, CLDN4, and CLDN7 in the barrier function of epididymal tight junctions, we knocked down each of these CLDNs in FHCE1 cells by using siRNA. As shown in FIG.9A-B, *CLDN1, 3, 4* and *7* mRNA and protein levels were reduced 24h and 48h after the transfection of cells with either *CLDN1, CLDN3, CLDN4* or *CLDN7* siRNA, respectively. Different controls were used to ensure that the effect was specific to the siRNA. These controls included: untreated cells, cells treated with the transfection reagent, and cells transfected with a negative siRNA. *CLDN* mRNA expression levels remained constant in all of the different control conditions. Furthermore, the effect of the siRNA was specific to each CLDN.

The TER of the FHCE1 cells was assessed in cells where the expression of the different CLDNs was knocked-down (FIG. 9B). The results indicate that the peak in TER normally observed in FHCE1 cells at 24h was not achieved when CLDN1, CLDN3, CLDN4 or CLDN7 were only partially knocked-down. These results suggest that CLDN1, CLDN3, CLDN4, and CLDN7 play an important role in the formation of functional human epididymal tight junctions.

Discussion

We have previously shown, using gene expression profiles, that epididymal gene expression in non-obstructive azoospermic infertile men is altered [21]. However, the lack of biological tools and the difficulty in obtaining human epididymis is a limiting factor in understanding the regulation of human epididymal gene expression and their function. In the present study, we developed a series of human epididymal cell lines derived from adult caput and corpus epididymidis. These are the first human epididymal cell lines derived from adult tissue. While we were able to develop cell lines from the caput and corpus epididymidis, we were unsuccessful in developing cell lines from the cauda region. This could be due to the microenvironment in which the cells were maintained *in vitro*. The cauda epididymidis is responsible for sperm storage in a quiescent state. In order to fulfill its function, the luminal microenvironment of the cauda has specific characteristics such as a low pH and high osmolarity as compared to the other epididymal regions [2, 37]. It is possible that the principal cells from this region also require these conditions for long-term survival.

The cell lines developed in this study retained some of the characteristics of *in vivo* principal cells. They exhibited similar ultrastructure, were diploid, and expressed a variety of epididymal markers, at least at the RNA level, which are specifically expressed or highly expressed by human epididymal principal cells (*NPC2, SPAG11B, CD52, DCXR*). The lack of expression for vimentin and *SLC9A3* confirmed that the cells are of epithelial origin and are from the epididymis rather than from the efferent ducts, which are in close proximity to the epididymal tubules in the caput region of the human epididymis [34, 38]. Interestingly, the cells did not express either *CRISP1* or *SPINLW1*. Both of these proteins are implicated in human fertility and the expression of both genes is altered in non-obstructive azoospermic men [21]. CRISP1 is regulated by androgens and other testicular factors [39, 40] while SPINLW1 is regulated by androgens [41]. It is possible that one of the reasons for the low level or lack of expression in our

epididymal cells is related to a requirement for specific testicular factors which regulate their expression. A second possibility is that the immortalization process may alter the expression of these genes. Further studies will be required to address this. The fact that the cells were not completely polarized also suggests that these cells may need an additional nutrient absent from our culture medium or that they need to interact with other cell types such as basal cells.

It is well-established that many epididymal functions are regulated by hormones, especially testosterone [42]. Upon its entry in the epididymis, testosterone can be converted into dihydrotestosterone (DHT) by 5α-reductase (SRD5A). Both isoforms of *SRD5A* that are expressed in our cell lines have also been detected along the human epididymis [43]. Testosterone and DHT bind to the AR, which is expressed along the human epididymis in both principal and basal cells [44]. Most of our human epididymal cell lines expressed the *AR*. While the expression of the *AR* and *SRD5A* isoforms suggest that the cells have the capacity to respond to androgens, it should be noted that in a rat caput epididymal cell line developed in a similar fashion as the present cell lines, the cells had limited responsiveness to androgens. This was most likely due to the immortalization process with LTAg, which inhibits the interaction between the AR and its co-factor, pRb [32]. Our human cells also expressed both isoforms of the estrogen receptor (*ESR1* and *ESR2*) which are present in the human epididymis [45]. Several lines of evidence suggest an important role for estrogens in the mammalian epididymis during development as well as in the adult [46]. Whether or not these cells are responsive to androgens and estrogens remains to be established.

Human epididymal tight junctions, which form the blood-epididymis barrier, are comprised of a multitude of CLDNs [9]. Studies in rodents have also shown the presence of *CLDN1* to *9* in the epididymis [9-11, 13]. CLDN1, CLDN3, CLDN4 and CLDN7, present in our human cell lines, are localized at tight junctions as well as along the lateral plasma membranes between adjacent principal cells and between principal and basal cells, suggesting additional roles in cell-cell interactions [9-11, 13]. The widespread distribution of CLDN1, CLDN3, CLDN4, and CLDN7 in rodents and humans, as well as similarities in their localization, suggest an important role for these CLDNs in the epididymis. In addition, the regulation of epididymal tight junctions is complex and multifactorial [4]. For example, in the rat epididymis, segment-specific regulation of CLDN1 involves androgens [10] and the transcription factors SP1 and SP3 [47]. It is therefore important to identify key components of the tight junctional complex to study their regulation and involvement in epididymal functions.
A study in MDCK cells reported that a loss in expression of CLDN1, CLDN3, CLDN4 or CLDN7 significantly decreased TER [33]. These CLDNs are considered to be particularly important in maintaining the barrier function, or impermeability, of tight junctions [23]. Our results demonstrated that a knockdown of CLDN1, CLDN3, CLDN4 or CLDN7 can totally inhibit TER and thus the formation or the barrier function of human epididymal tight junctions. Therefore, CLDN1, CLDN3, CLDN4 and CLDN7 could play a major role in the proper functioning of the human blood-epididymis barrier. While these CLDNs are expressed at relatively similar levels throughout the epididymis [9], alterations in their regulation in different regions could have varying effects on male fertility. Several studies in the rat epididymis suggest that the permeability of the barrier is not similar along the duct [6, 8, 48]. Even if tight junctions in the caput epididymidis of the rat and mouse are numerous and well-developed, the degree of permeability of the epithelium is not necessarily correlated with the number of tight junctions. It

has been reported that the tight junctions of the caput epididymidis are more permeable than those of the cauda epididymidis [6, 8, 48]. Our FHCE1 cell line can form functional tight junctions, as shown by the expression of different junctional markers (*CDH1, CDH2, TJP1, CLDN1, CLDN2, CLDN3, CLDN4, CLDN7* and *CLDN8*, *DSP*) and the localization of tight junctional proteins TJP1, OCLN and CLDN1 to the plasma membrane. The transformed epididymal cells had a TER of approximately 150 ohms.cm^2, which is similar to previous reports in both tissue and primary cell cultures derived from the human caput and corpus epididymidis [49, 50] and in primary cell cultures derived from the rat caput epididymidis [51].

The complex and multifactorial regulation of tight junctional components could however explain the rapid reduction in resistance in our cell line 24h after the calcium switch. It is possible that the cells need additional factors to maintain tight junctions once they are formed. Nonetheless, the level of resistance suggests that epididymal tight junctions in the human caput and corpus epididymidis may form a leaky barrier as compared to the cauda epididymidis, such as has been reported for the rat epididymis [50, 51], or as compared to the vas deferens [52]. The leakiness of the blood-epididymis barrier in the caput and corpus epididymidis is reflected by the CLDNs expressed and is likely to be important for specific ion flow in the epididymis as has been reported in other leaky epithelia [53]. Dysregulation of CLDNs could result in altered barrier function of the tight junctions, resulting in abnormal sperm maturation or in exposure of sperm to the immune system. Hermo et al., [54] showed that, in cathepsin A deficient mice, reduced expression and mislocalization of CLDN1, CLDN3, CLDN8 and CLDN10 were associated with altered sperm motility. Dysregulation of CLDN1, CLDN3, CLDN4 and CLDN7 may also affect the homeostasis of the luminal microenvironment by changing paracellular permeability. Electrolytes and fluid secretion are important for proper sperm maturation. In LLC-PK1and MDCK cells, CLDN4 and CLDN7 have been shown to be involved in the formation of paracellular channels for Cl^- and Na^+ [33, 55]. Yeung and Cooper [56] reported that changes in luminal Na^+ concentrations has dramatic effects on the ultrastructure of the rat epididymal epithelium. In addition, defective Cl^- secretion in cystic fibrosis patients results in male infertility due to epididymal obstruction [57]. Male infertility could thus involve multiple epididymal dysfunctions associated with different CLDNS leading to the disruption of the blood-epididymis barrier, and /or the paracellular and transcellular transport pathways.

In conclusion, our studies have created a novel approach for examining the role of specific CLDNs in human epididymal tight junctions, as well as other genes involved in epididymal function and sperm maturation. Because CLDNs function as effectors of barrier integrity and paracellular transport, they likely play a critical role in the maintenance of male fertility. The critical functions of CLDN1, CLDN3, CLDN4, and CLDN7 in maintaining the integrity of epididymal tight junctions provide a target that can be assessed to examine the integrity of the blood-epididymis barrier. Future studies on the role of other CLDNs may provide new insights towards understanding the necessity of expression of a large number of CLDNs in the epididymis and how these regulate sperm maturation. Furthermore, the use of human cell lines developed in this study provides an essential tool to examine the regulation of junctional proteins in the epididymis for the development of novel strategies which could eventually target the blood-epididymis barrier in infertile patients with altered barrier function.

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References

1. Robaire B, Hermo L. Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E, Neill J (eds.), The Physiology of Reproduction. New York: Raven Press; 1988: 999-1080.
2. Robaire B, Hinton B, Orgebin-Crist M-C. The epididymis. In: Neill JD (ed.) Knobil and Neill's Physiology of Reproduction, 3th ed. New York: Elsevier; 2006: 1071-1148.
3. Hinton BT, Palladino MA. Epididymal epithelium: its contribution to the formation of a luminal fluid microenvironment. Microsc Res Tech 1995; 30: 67-81.
4. Cyr DG, Gregory M, Dube E, Dufresne J, Chan PT, Hermo L. Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood-epididymal barrier in animals and humans. Asian J Androl 2007; 9: 463-475.
5. Friend DS, Gilula NB. Variations in tight and gap junctions in mammalian tissues. J Cell Biol 1972; 53: 758-776.
6. Suzuki F, Nagano T. Development of tight junctions in the caput epididymal epithelium of the mouse. Dev Biol 1978; 63: 321-334.
7. Suzuki F, Nagano T. Regional differentiation of cell junctions in the excurrent duct epithelium of the rat testis as revealed by freeze-fracture. Anat Rec 1978; 191: 503-519.
8. Hinton BT, Howards SS. Permeability characteristics of the epithelium in the rat caput epididymidis. J Reprod Fertil 1981; 63: 95-99.
9. Dube E, Chan PT, Hermo L, Cyr DG. Gene expression profiling and its relevance to the blood-epididymal barrier in the human epididymis. Biol Reprod 2007; 76: 1034-1044.
10. Gregory M, Dufresne J, Hermo L, Cyr D. Claudin-1 is not restricted to tight junctions in the rat epididymis. Endocrinology 2001; 142: 854-863.
11. Gregory M, Cyr DG. Identification of multiple claudins in the rat epididymis. Mol Reprod Dev 2006; 73: 580-588.
12. Guan X, Inai T, Shibata Y. Segment-specific expression of tight junction proteins, claudin-2 and -10, in the rat epididymal epithelium. Arch Histol Cytol 2005; 68: 213-225.
13. Inai T, Sengoku A, Hirose E, Iida H, Shibata Y. Claudin-7 expressed on lateral membrane of rat epididymal epithelium does not form aberrant tight junction strands. Anat Rec (Hoboken) 2007; 290: 1431-1438.
14. Johnston DS, Jelinsky SA, Bang HJ, DiCandeloro P, Wilson E, Kopf GS, Turner TT. The mouse epididymal transcriptome: transcriptional profiling of segmental gene expression in the epididymis. Biol Reprod 2005; 73: 404-413.
15. Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol 1998; 143: 391-401.
16. Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. Annu Rev Physiol 2006; 68: 403-429.
17. Turksen K, Troy TC. Barriers built on claudins. J Cell Sci 2004; 117: 2435-2447.
18. Mosher WD, Pratt WF. Fecundity and infertility in the United States: incidence and trends. Fertil Steril 1991; 56: 192-193.

19. Thonneau P, Spira A. Prevalence of infertility: international data and problems of measurement. Eur J Obstet Gynecol Reprod Biol 1991; 38: 43-52.
20. Sigman M, Jarow JP. Male Infertility. In: Walsh PC, Retik AB, Vaughan ED, Wein AJ (eds.), Campbell's Urology, 8th ed. Philadelphia: Saunders; 2006: 609-653.
21. Dube E, Hermo L, Chan PT, Cyr DG. Alterations in gene expression in the caput epididymides of nonobstructive azoospermic men. Biol Reprod 2008; 78: 342-351.
22. Rajasekaran SA, Beyenbach KW, Rajasekaran AK. Interactions of tight junctions with membrane channels and transporters. Biochim Biophys Acta 2008; 1778: 757-769.
23. Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, Blasig IE. Structure and function of claudins. Biochim Biophys Acta 2008; 1778: 631-645.
24. Cooper TG, Yeung CH, Meyer R, Schulze H. Maintenance of human epididymal epithelial cell function in monolayer culture. J Reprod Fertil 1990; 90: 81-91.
25. Akhondi MA, Chapple C, Moore HD. Prolonged survival of human spermatozoa when co-incubated with epididymal cell cultures. Hum Reprod 1997; 12: 514-522.
26. Moore HD, Curry MR, Penfold LM, Pryor JP. The culture of human epididymal epithelium and in vitro maturation of epididymal spermatozoa. Fertil Steril 1992; 58: 776-783.
27. Harris A, Coleman L. Ductal epithelial cells cultured from human foetal epididymis and vas deferens: relevance to sterility in cystic fibrosis. J Cell Sci 1989; 92 (Pt 4): 687-690.
28. Kirchhoff C. The dog as a model to study human epididymal function at a molecular level. Mol Hum Reprod 2002; 8: 695-701.
29. Turner TT. De Graaf's thread: the human epididymis. J Androl 2008; 29: 237-250.
30. Araki Y, Suzuki K, Matusik RJ, Obinata M, Orgebin-Crist MC. Immortalized epididymal cell lines from transgenic mice overexpressing temperature-sensitive simian virus 40 large T-antigen gene. J Androl 2002; 23: 854-869.
31. Britan A, Lareyre JJ, Lefrancois-Martinez AM, Manin M, Schwaab V, Greiffeuille V, Vernet P, Drevet JR. Spontaneously immortalized epithelial cells from mouse caput epididymidis. Mol Cell Endocrinol 2004; 224: 41-53.
32. Dufresne J, St-Pierre N, Viger RS, Hermo L, Cyr DG. Characterization of a novel rat epididymal cell line to study epididymal function. Endocrinology 2005; 146: 4710-4720.
33. Hou J, Gomes AS, Paul DL, Goodenough DA. Study of claudin function by RNA interference. J Biol Chem 2006; 281: 36117-36123.
34. Kujala M, Hihnala S, Tienari J, Kaunisto K, Hastbacka J, Holmberg C, Kere J, Hoglund P. Expression of ion transport-associated proteins in human efferent and epididymal ducts. Reproduction 2007; 133: 775-784.
35. Brant SR, Yun CH, Donowitz M, Tse CM. Cloning, tissue distribution, and functional analysis of the human Na+/N+ exchanger isoform, NHE3. Am J Physiol 1995; 269: C198-206.
36. Denker BM, Nigam SK. Molecular structure and assembly of the tight junction. Am J Physiol 1998; 274: F1-9.
37. Jones RC, Murdoch RN. Regulation of the motility and metabolism of spermatozoa for storage in the epididymis of eutherian and marsupial mammals. Reprod Fertil Dev 1996; 8: 553-568.
38. Kasper M, Stosiek P. Immunohistochemical investigation of different cytokeratins and vimentin in the human epididymis from the fetal period up to adulthood. Cell Tissue Res 1989; 257: 661-664.

39. Haendler B, Habenicht UF, Schwidetzky U, Schuttke I, Schleuning WD. Differential androgen regulation of the murine genes for cysteine-rich secretory proteins (CRISP). Eur J Biochem 1997; 250: 440-446.
40. Turner TT, Bomgardner D. On the regulation of Crisp-1 mRNA expression and protein secretion by luminal factors presented in vivo by microperfusion of the rat proximal caput epididymidis. Mol Reprod Dev 2002; 61: 437-444.
41. Sivashanmugam P, Hall SH, Hamil KG, French FS, O'Rand MG, Richardson RT. Characterization of mouse Eppin and a gene cluster of similar protease inhibitors on mouse chromosome 2. Gene 2003; 312: 125-134.
42. Robaire B, Seenundun S, Hamzeh M, Lamour SA. Androgenic regulation of novel genes in the epididymis. Asian J Androl 2007; 9: 545-553.
43. Mahony MC, Swanlund DJ, Billeter M, Roberts KP, Pryor JL. Regional distribution of 5alpha-reductase type 1 and type 2 mRNA along the human epididymis. Fertil Steril 1998; 69: 1116-1121.
44. Ungefroren H, Ivell R, Ergun S. Region-specific expression of the androgen receptor in the human epididymis. Mol Hum Reprod 1997; 3: 933-940.
45. Saunders PT, Sharpe RM, Williams K, Macpherson S, Urquart H, Irvine DS, Millar MR. Differential expression of oestrogen receptor alpha and beta proteins in the testes and male reproductive system of human and non-human primates. Mol Hum Reprod 2001; 7: 227-236.
46. Hess RA, Zhou Q, Nie R. The role of estrogens in the endocrine and paracrine regulation of efferent ductules, epididymis and vas deferens. In: Robaire B, Hinton B (eds.), The epididymis: from molecules to clinical practice. New York: Plenum Press; 2002: 317-337.
47. Dufresne J, Cyr DG. Activation of an SP binding site is crucial for the expression of claudin 1 in rat epididymal principal cells. Biol Reprod 2007; 76: 825-832.
48. Martinez-Palomo A, Erlij D. Structure of tight junctions in epithelia with different permeability. Proc Natl Acad Sci U S A 1975; 72: 4487-4491.
49. Huang SJ, Leung AY, Fu WO, Chung YW, Zhou TS, Chan PS, Wong PY. Electrophysiological studies of anion secretion in cultured human epididymal cells. J Physiol 1992; 455: 455-469.
50. Chan HC, Lai KB, Fu WO, Chung YW, Chan PS, Wong PY. Regional differences in bioelectrical properties and anion secretion in cultured epithelia from rat and human male excurrent ducts. Biol Reprod 1995; 52: 192-198.
51. Byers SW, Citi S, Anderson JM, Hoxter B. Polarized functions and permeability properties of rat epididymal epithelial cells in vitro. J Reprod Fertil 1992; 95: 385-396.
52. Carlin RW, Lee JH, Marcus DC, Schultz BD. Adenosine stimulates anion secretion across cultured and native adult human vas deferens epithelia. Biol Reprod 2003; 68: 1027-1034.
53. Anderson JM, Van Itallie C. Physiology and Function of the Tight Junction. Cold Spring Harbor Perspectives in Biology 2009; 1: a002584.
54. Hermo L, Korah N, Gregory M, Liu LY, Cyr DG, D'Azzo A, Smith CE. Structural alterations of epididymal epithelial cells in cathepsin A-deficient mice affect the blood-epididymal barrier and lead to altered sperm motility. J Androl 2007; 28: 784-797.
55. Alexandre MD, Lu Q, Chen YH. Overexpression of claudin-7 decreases the paracellular Cl- conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. J Cell Sci 2005; 118: 2683-2693.
56. Yeung CH, Cooper TG. Ultrastructure of the perfused rat epididymis: Effect of luminal sodium ion concentration. Cell and Tissue Research 1982; 226: 407-425.

57. Leung AYH, Wong PYD. The Epididymis as a Chloride-Secreting Organ. News Physiol Sci 1994; 9: 31-35.

Figure legends

Figure 1: Ultrastructure analysis by electron microscopy of the cell lines. The images are representative of all cell lines that were either trypsinized (A-E) or grown on plastic chambers coated with collagen IV (F-H). Cells have an irregular nucleus (n), extensive microvilli (mv), numerous mitochondria (m), cytoplasmic vesicles (v), endoplasmic reticulum (ER), lysosomes (L) and junctional complexes (jc). Vesicular structures, which are possibly epididymosomes (ep), are present around the cells. Magnification x4200- 26500.

Figure 2: The expression of cytokeratin, an epithelial marker, and vimentin, a mesenchymal marker, was verified by RT-PCR and immunofluorescence to assess the epithelial morphology of the cells. (A) Representative photomicrograph of the immortalized cell lines grown at confluence on collagen IV coated plates. (B) Cytokeratin 8 (*KRT8*), and not vimentin (*VIM*), mRNA is expressed in all five cell lines. (C) Cytokeratin (green), and not vimentin, is expressed and localized to the cytoplasm in all the cell lines. Nuclei (red) are stained with propidium iodide. Magnification x100-400.

Figure 3: Flow cytometric analysis of FHCE1 cells. DNA was stained with propidium iodide for analysis by flow cytometry. (A) THP1 cells were used as a control diploid population. (B) FHCE1 cells showed two peaks of fluorescence corresponding to resting and dividing cells. (C) The diploid state of the cells is clearly shown when simultanously analyzed with THP1 cells.

Figure 4: Cell growth of the epididymal cell lines at 32°C. Cell growth was evaluated by incubating the cells with MTT solution for 2h at different time points in triplicate. Absorbance was measured at 570 nm (n=3, ± SEM).

Figure 5: Expression of epididymal and efferent ducts markers by RT-PCR. Expression of the different transcripts was verified using specific primers. Human epididymis or kidney was used as a positive control. *NPC2, SPAG11B, CD52* and *DCXR* were expressed in the different cell lines while no band was detected for *CRISP1, SPINLW1* or *SLC9A3*. Neg., negative control; Pos, positive control.

Figure 6: Expression of different hormone receptors and enzymes by RT-PCR. Expression of the different transcripts was verified using specific primers. Human epididymis was used as a positive control. *ESR1, ESR2, SRD5A1, SRD5A2* were expressed in the different cell lines, while *AR* was only expressed in FHCE1, FHCE2 and FHCE3 cell lines.

Figure 7: Expression of genes encoding tight junctional proteins by RT-PCR. Expression of the different transcripts was verified using specific primers. Human epididymis was used as a positive control. *CDH1, CDH2, CLDN1, CLDN2, CLDN3, CLDN4, CLDN8* and *DSP* were expressed in the different cell lines while *CLDN3* was only expressed in the cell lines derived from the caput and not the corpus and *CLDN7* was expressed in all cell lines except FHCE2.

Figure 8: Formation of functional tight junctions by FHCE1 cells. (A) Transepithelial resistance (TER) was measured in FHCE1 cells at different time points after switching from low- to normal calcium- containing medium with a final concentration of 1.8 mM ($n \geq 4$, ± SD). A peak in TER was seen 24 hrs after calcium- switch. (B) Immunolocalization of TJP1, OCLN and CLDN1 in FHCE1 cells exposed 24h to calcium. Tight junctional proteins (green) were mostly localized to the plasma membrane. Nuclei (red) are stained with propidium iodide. Magnification x640.

Figure 9: Effect of knockdown of claudin expression in FHCE1 cells. (A) Western blot of FHCE1 cells expressing siRNA against *CLDN1, CLDN3, CLDN4* and *CLDN7*. (B) RT-PCR of FHCE1 cells expressing siRNA against *CLDN1, CLDN3, CLDN4* and *CLDN7*. (C) Effect of CLDN knockdown on transepithelial resistance ($n \geq 4$, ± SD). Knockdown of each CLDN resulted in a significant decrease in TER. NT, FHCE1 cells not transfected.

Table 1: Description of the different human epididymal cell lines developed in this study. FHCE, Fertile Human Caput Epididymal cell line; FHSE, Fertile Human corpuS Epididymal cell line.

Name of the cell line	Type of patient	Epididymal region
FHCE 1	Fertile	Caput
FHCE 2	Fertile	Caput
FHCE 3	Fertile	Caput
FHCE 4	Fertile	Caput
FHSE 1	Fertile	Corpus

Table 2: Sequences of the primers used for RT-PCR.

Gene	Primer set (5'-3')	Tm (°C)	Amplicon size (bp)
LTAg	F : AATAGCAAAGCAAGCAAGAGT R : GAAAATGGAAGATGGAGTAAA	51.2	304
CRISP1	F : GGGCCACATCTTACC R : TGTTAAATTAATGGCATACGA	50.5	904
SPINLW1	F: GACCGCATTGGCTTACAT R: GGAGGTAGATGCAAGCGTTCT	55.3	919
DCXR	F: ATAGAACCCGTGTGCGTGGAC R: CAAAGTGGAACCCGTGGT	60.8	546
NPC2	F: GCGTTTCCTGGCAGCTACATT R: AGAAAGAGGCCACAAGTTAAT	54.5	734
SPAG11B	F: TTGGCAGACATGAGGCAACGA R: TTAAGCCCTTGGGATACTTCA	56.4	460, 536
CD52	F: GACAGCCACGAAGATCCTACC R: GGATTCTCTTGCGAGTGATG	56.1	278
SLC9A3	F: CCTGGCTTCTGAACCGCTAC R: GGGCCGACCGTCTCAT	59.3	405
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCT	60	450
KRT8	F: AACAAGTTTGCCTCCTTCATAGAC R: GAAGTTGATCTCGTCGGTCAG	56	372
VIM	F : GAATGACCGCTTCGCCAACTACAT R : CCCGCATCTCCTCCTCGTAGAG	60	137
CDH1	F : GAACAGCACGTACACAGCCCTAAT R : GATAGATTCTTGGGTTGGGTCGTT	56.6	280
CDH2	F : ACAGCACTTGATTATGGCATTGAT R :TATTTGATTTGGGAGGATTACC	50.7	208
DSP	F :ACAGTGAAATATCTGGCAAACGAG R :CTCATAAGTCAGTCGGGTGAT	54	551
ESR1	F : CGCCTCAGAAAGTGTTATGAA R : CCACAATACCGCTTATCCT	56	536
ESR2	F : TAGGGTCCATGGCCAGTTAT R : GGGAGCCACACTTCACCAT	56	323
SRD5A1	F : ACCCATTTCTGATGCGAGGAGGAA R : GCATAGCCACACCACTCCATGATT	56	350
SRD5A2	F : AGCTGCCTTCCTTCGCGGTG R : GTGGCCAGGGCATAGCCGAT	56	450
AR	F : GAGCGTGCGCGAAGCGATCCAGAA R : GCTGCCTTCGGATATTACCTCCTGCT	56	524

Figure 1

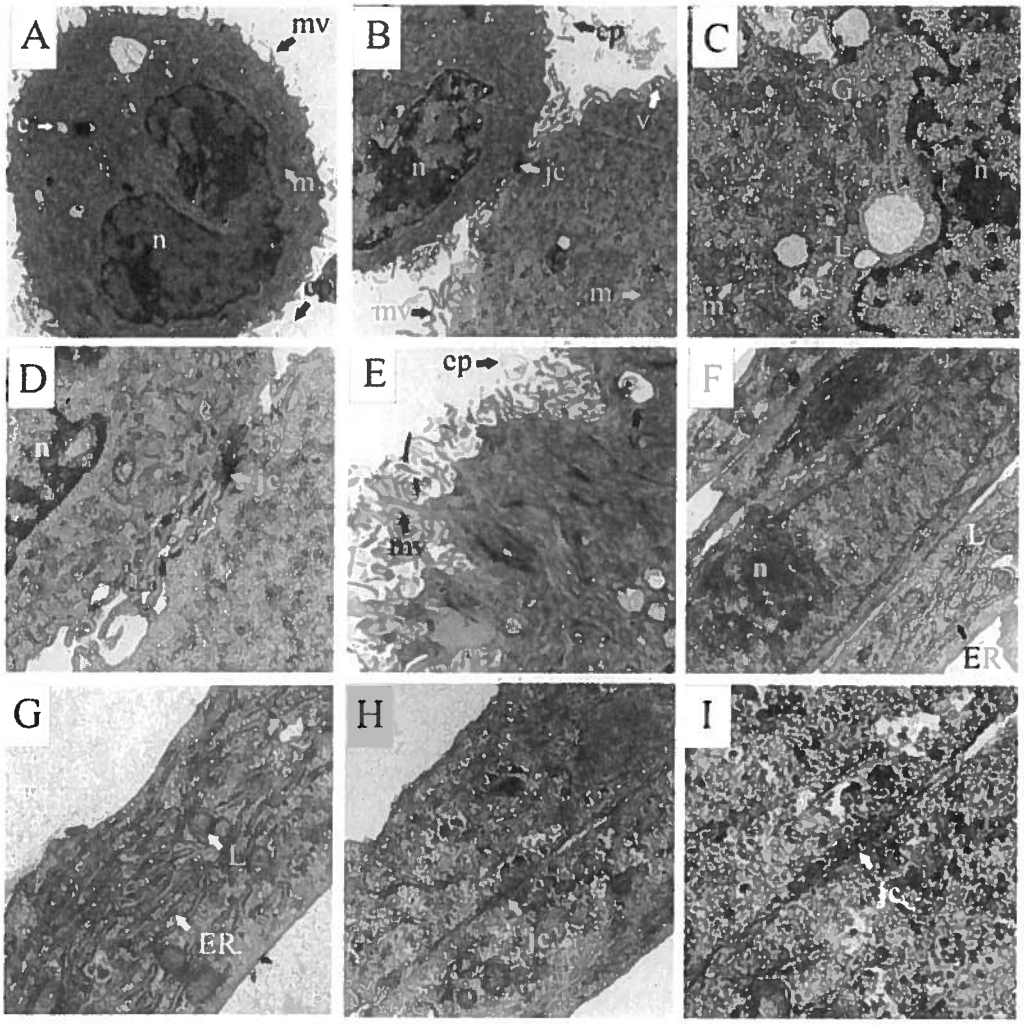
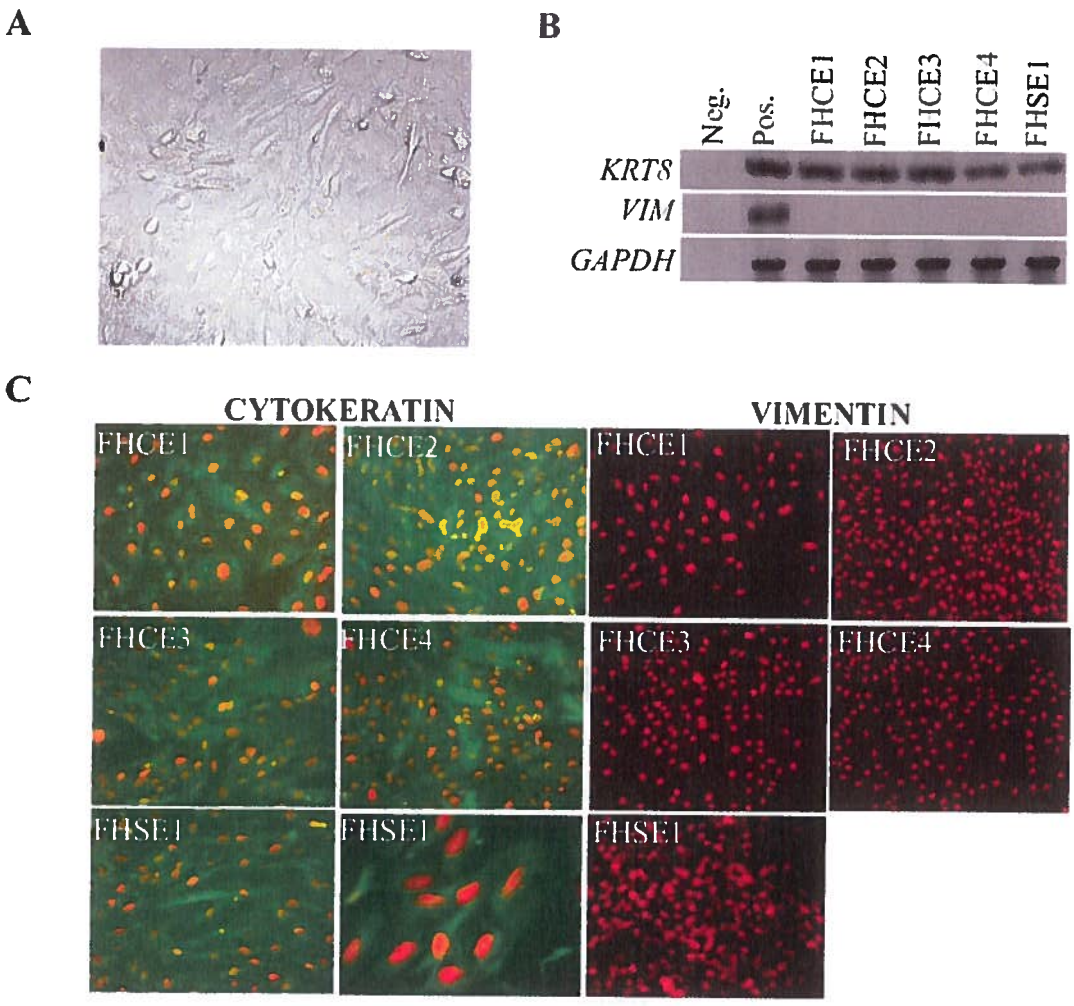


Figure 2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B
Neg.
Pos.
FHCE1
FHCE2
FHCE3
FHCE4
FHSE1
KRT8
VIM
GAPDH
C
CYTOKERATIN
VIMENTIN
FHCE1
FHCE2
FHCE1
FHCE2
FHCE3
FHCE4
FHCE3
FHCE4
FHSE1
FHSE1
FHSE1

Figure 3

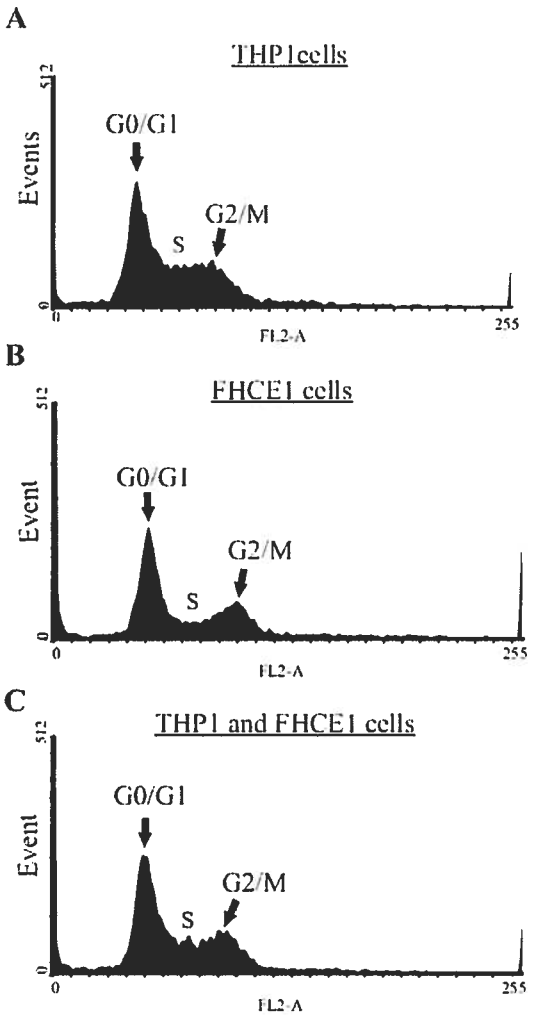

A
THP1cells
512
Events
G0/G1
S
G2/M
0
0
255
FL2-A
B
FHCE1 cells
512
Event
G0/G1
G2/M
S
0
0
255
FL2-A
C
THP1 and FHCE1 cells
512
Event
G0/G1
G2/M
S
0
0
255
FL2-A

Figure 4

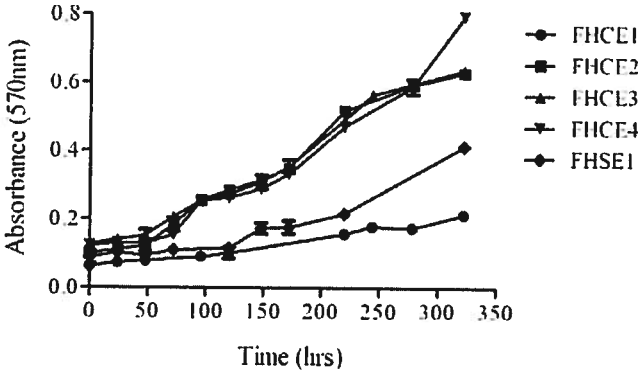
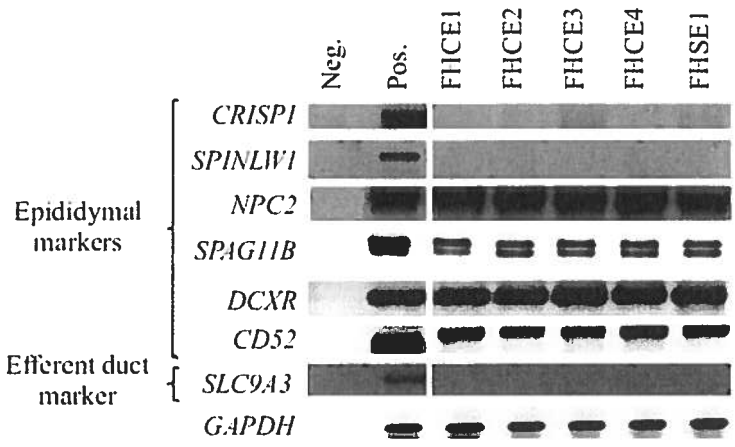

0.8
0.6
0.4
0.2
0.0
Absorbance (570nm)
0
50
100
150
200
250
300
350
Time (hrs)
FHCE1
FHCE2
FHCE3
FHCE4
FHSE1

Figure 5


Neg.
Pos.
FHCE1
FHCE2
FHCE3
FHCE4
FHSE1
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CD52
SLC9A3
GAPDH
Epididymal markers
Efferent duct marker

Figure 6

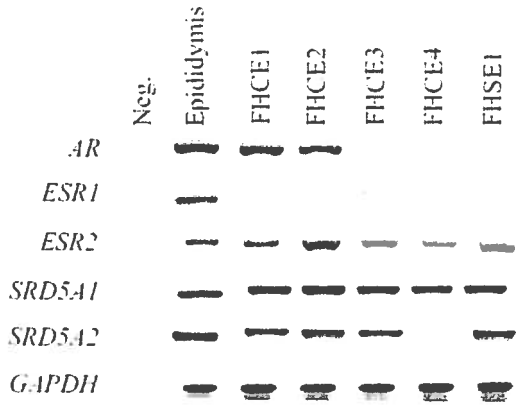

Neg.
Epididymis
FHCE1
FHCE2
FHCE3
FHCE4
FHSE1
AR
ESR1
ESR2
SRD5A1
SRD5A2
GAPDH

Figure 7

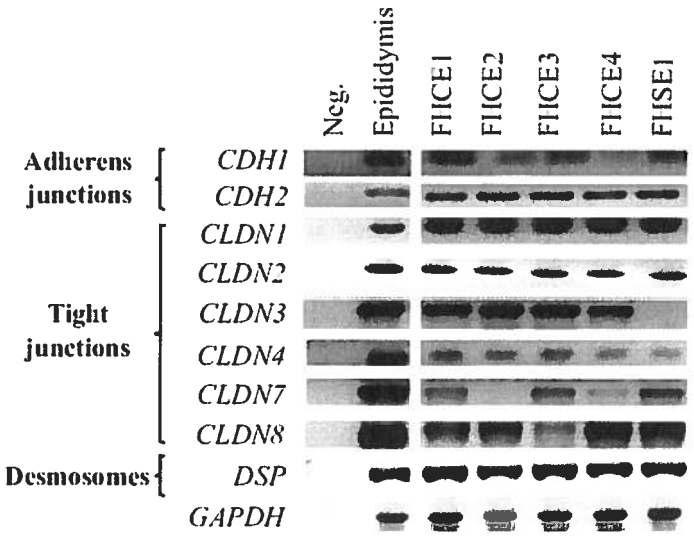
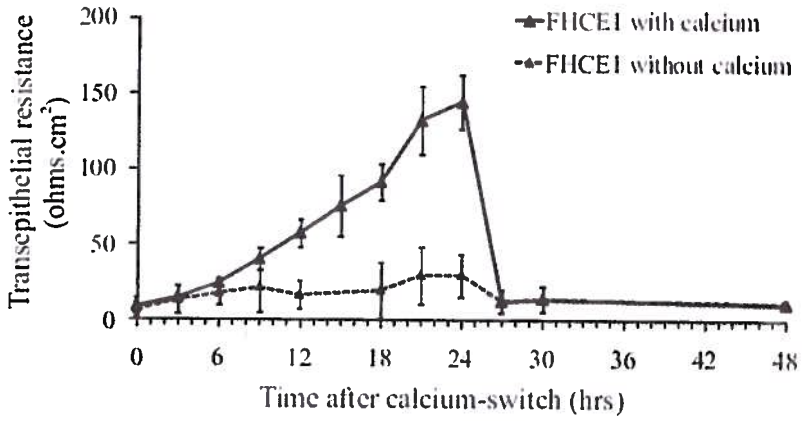

Neg.
Epididymis
FIICE1
FIICE2
FIICE3
FIICE4
FIISE1
Adherens junctions
CDH1
CDH2
Tight junctions
CLDN1
CLDN2
CLDN3
CLDN4
CLDN7
CLDN8
Desmosomes
DSP
GAPDH

Figure 8

A


FHCE1 with calcium
FHCE1 without calcium
Transepithelial resistance (ohms.cm²)
200
150
100
50
0
0
6
12
18
24
30
36
42
48
Time after calcium-switch (hrs)

B

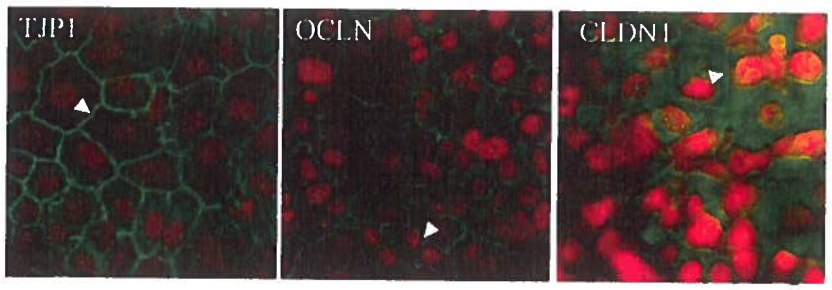
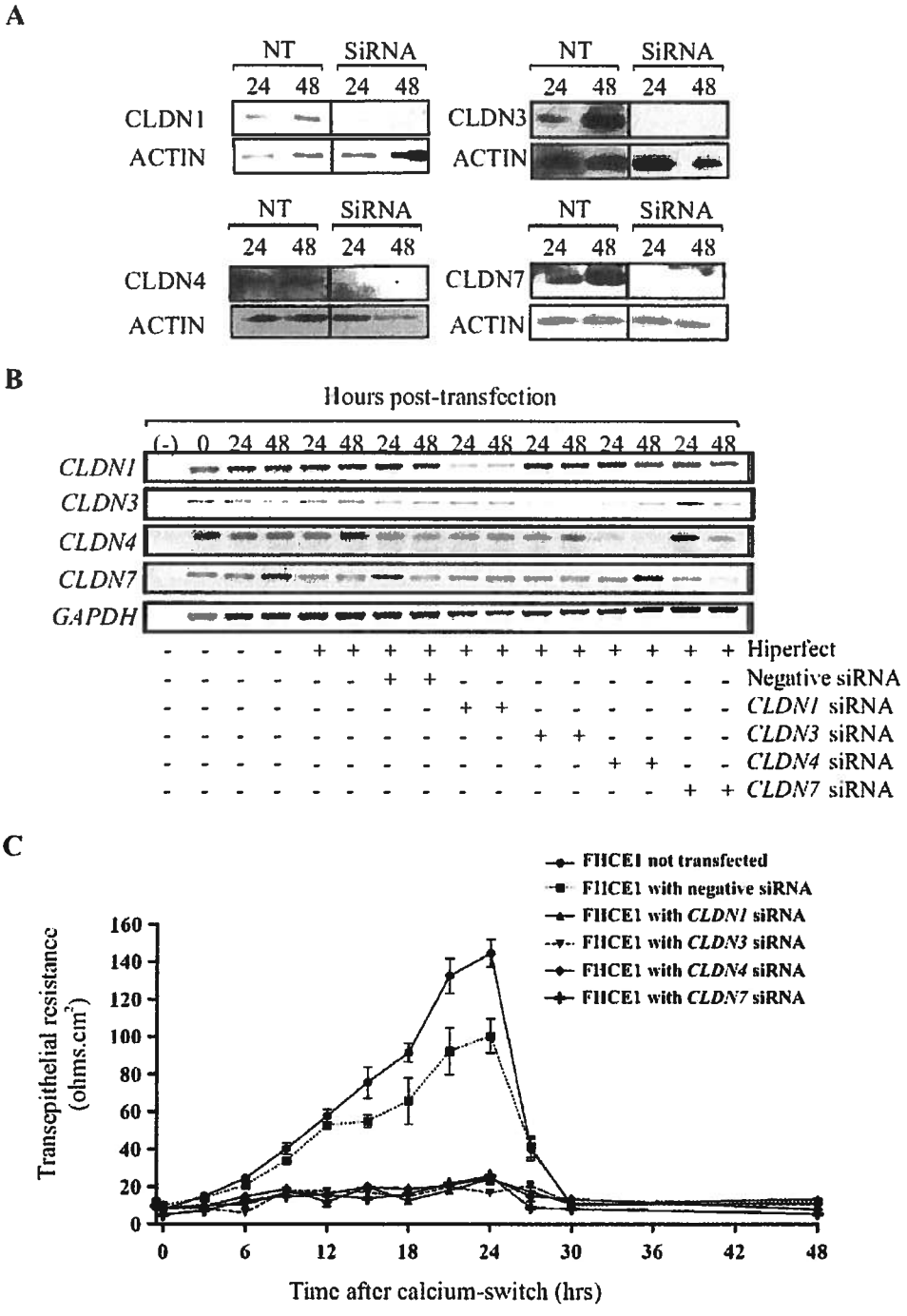

TJP1
OCLN
CLDN1

Figure 9


A
NT
SiRNA
24 48 24 48
CLDN1
ACTIN
CLDN3
ACTIN
CLDN4
ACTIN
CLDN7
ACTIN
B
Hours post-transfection
(-) 0 24 48 24 48 24 48 24 48 24 48 24 48 24 48
CLDN1
CLDN3
CLDN4
CLDN7
GAPDH
Hiperfect
Negative siRNA
CLDN1 siRNA
CLDN3 siRNA
CLDN4 siRNA
CLDN7 siRNA
C
FHCE1 not transfected
FHCE1 with negative siRNA
FHCE1 with CLDN1 siRNA
FHCE1 with CLDN3 siRNA
FHCE1 with CLDN4 siRNA
FHCE1 with CLDN7 siRNA
Transepithelial resistance (ohms.cm²)
160
140
120
100
80
60
40
20
0
0 6 12 18 24 30 36 42 48
Time after calcium-switch (hrs)

SECTION 4: ALTERATIONS OF THE HUMAN BLOOD-EPIDIDYMIS BARRIER IN OBSTRUCTIVE AZOOSPERMIA AND THE DEVELOPMENT OF NOVEL EPIDIDYMAL CELL LINES FROM INFERTILE MEN

Dubé E, Hermo L, Chan PTK, Cyr DG

Article accepté dans *Biology of Reproduction*

4.1 Résumé de l'article en français

L'azoospermie peut être causée par un problème de spermatogenèse ou par une obstruction au niveau de l'épididyme. L'épididyme est responsable de la maturation post-testiculaire des spermatozoïdes et ce processus dépend de la création d'un microenvironnement intraluminal spécifique. Ce microenvironnement est en partie créé par la barrière hémato-épididymaire, qui est composée de jonctions serrées à l'apex des cellules principales adjacentes. Il existe très peu d'informations sur l'expression des gènes chez les patients azoospermiques obstructifs à cause de la difficulté d'obtenir des tissus. Le but de cette étude était de déterminer si les protéines jonctionnelles étaient affectées dans l'épididyme de patients souffrant d'azoospermie obstructive. Nous avons montré que l'expression et/ou la localisation de plusieurs gènes codant pour des protéines jonctionnelles (CDH1, TJP1, CLDNs 1, 4 et 10) sont affectées dans l'épididyme de patients azoospermiques obstructifs. À l'aide de nouvelles lignées épithéliales développées à partir de la tête de l'épididyme d'un patient souffrant d'azoospermie obstructive, nous avons ensuite étudié les mécanismes moléculaires impliqués dans la formation des jonctions épididymaires humaines. Les lignées cellulaires sont composées de populations homogènes de cellules diploïdes qui ressemblent, par leur ultrastructure, aux cellules principales présentes *in vivo*. Les cellules expriment la cytokératine, un marqueur épithélial, HE2, un marqueur épididymaire, les CLDNs 2 et 3, la desmoplakine (DSP) ainsi que la vimentine, un marqueur mésenchymateux. Cependant, les cellules n'expriment pas les marqueurs épididymaires *CRISP1, EPPIN2, HE1, HE5* et *P34H* et les protéines jonctionnelles *CDH1, 2, CLDN1, 4, 7* et *8*. De plus, la lignée cellulaire IHCE1

(*Infertile Human Caput Epididymal cell line 1*) est incapable de former des jonctions serrées tel que démontré par la mesure de la résistance transépithéliale (TER). L'analyse de l'expression des gènes, à l'aide de microréseaux, des cellules IHCE1 et FHCE1 (une lignée cellulaire dérivée de la tête de l'épididyme d'un patient fertile) a mis en évidence un patron d'expression différentiel pour plusieurs gènes codant pour des protéines jonctionnelles, des protéines impliquées dans la régulation des jonctions et des protéines épididymaires. L'azoospermie obstructive impliquerait donc une altération des jonctions adhérentes, serrées et communicantes.

4.2 Contribution de l'étudiante

Les expériences présentées dans cet article ont été réalisées par l'étudiante. L'article a également été rédigé par l'étudiante.

ALTERATIONS IN THE HUMAN BLOOD-EPIDIDYMIS BARRIER IN OBSTRUCTIVE AZOOSPERMIA AND THE DEVELOPMENT OF NOVEL EPIDIDYMAL CELL LINES FROM INFERTILE MEN[1]

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Short title: Epididymal Tight Junctions in Obstructive Azoospermia

Summary sentence: Novel human epididymal cell lines were developed from obstructive azoospermic patients and used to demonstrate dedifferentiation of cells downstream from the blockage and inability of the cells to make tight junctions.

Keywords: epididymis, tight junctions, obstructive azoospermia, human cell lines

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ABSTRACT

Post-testicular sperm maturation requires a specific luminal environment in the epididymis that is created, in part, by the blood-epididymis barrier. There is limited information on gene expression in the epididymis of infertile obstructive azoospermic (OA) patients due to the difficulty in obtaining tissues. The objective of this study was to determine if epididymal tight junction proteins are altered in OA, and to develop cell lines that could serve to understand alterations in the epididymis of infertile men. Epididymal claudins (CLDNs) 1, 4, and 10 mRNA levels were altered in OA downstream from the obstruction site. Epithelial cell lines derived from the caput epididymidis of one OA patient were developed (Infertile Human Caput Epididymal cell line (IHCE)). IHCEs were comprised of homogenous populations of diploid cells that resembled, ultrastructurally, *in vivo* principal cells. The cells expressed cytokeratin, *SPAG11B*, *CLDN2, 3,* desmoplakin and vimentin. However, the cells did not express several other epididymal markers (*CRISP1, SPINLW1, NPC2, CD52, DCXR*) and junctional proteins (*CDH1, 2, CLDN1, 4, 7* and *8*). Further studies using IHCE1 and transepithelial resistance indicated that the cells were unable to form tight junctions. Microarray analyses comparing gene expression in IHCE1 and a recently developed fertile human caput epididymal cell line, revealed differential expression of genes encoding junctional proteins, cell junction regulators and epididymal proteins. Together these data indicate that epididymal cellular junctions appear to be altered in OA.

INTRODUCTION

Between 40 and 50% of infertile couples are unable to conceive after 1 yr of unprotected intercourse, due to male factor infertility [1, 2]. In approximately 25% of these cases, the cause of infertility is unknown and is likely associated with multiple causes [3]. Azoospermia, defined as the absence of spermatozoa in the ejaculate, occurs in 10 to 15% of infertile men. In approximately 40% of cases, azoospermia is due to an obstruction of the ductal system. These can occur as a result of congenital defects or they may be acquired [4, 5]. Advances in the treatment of male infertility allows OA patients to father children using microsurgical reconstruction or surgical retrieval of sperm for intracytoplasmic sperm injection (ICSI) if reconstruction has failed or is impossible [6]. For epididymal obstruction, even if microsurgical reconstruction with epididymovasostomy is successful (patency rates range from 46 to 81%), natural pregnancy rates are low (13 to 44%) [7], especially in cases of primary epididymal obstruction not related to vasectomy [8]. Higher pregnancy rates ranging from 24 to 64% have been observed for OA patients using ICSI [5, 9, 10]. ICSI can be achieved with epididymal sperm or, if the epididymis is inaccessible or no motile sperm can be retrieved, with testicular sperm [11]. However, higher miscarriage rates and lower pregnancy rates occur when testicular sperm are used instead of epididymal sperm [12, 13]. It has also been observed that the use of epipidymal sperm, in cases of acquired obstruction as compared to congenital bilateral absence of vas deferens, results in lower embryo quality and pregnancy rates [12]. Furthermore, the production of antisperm antibodies has been associated with obstructive azoospermia [14]. Antisperm antibodies may be produced when the blood-testis or the blood-epididymis barrier is compromised. Previous studies have demonstrated that defects in the blood-epididymis barrier may be associated with altered sperm motility or non obstructive azoospermia [15].

Epididymal sperm maturation, which is essential for the acquisition of progressive motility and the ability to fertilize [16, 17], depends on the unique luminal environment created by the secretion and absorption of proteins and ions by the epithelium and the selective transport of molecules across the blood-epididymis barrier [18-20]. The blood-epididymis barrier also contributes to the protection of sperm from the immune system [21].

This barrier is comprised of apical tight junctions between principal cells that create a seal between the cells and forces the selective transport of molecules across the epithelium.

Tight junctions are composed of integral proteins such as occludin (OCLN), tricellulin and a larger family of proteins, the CLDNs [22, 23]. It has previously been reported that the rat and human epididymis contain a large number CLDNs [24-28]. The combination of CLDNs determines the selective permeability of the barrier [29]. Tight junctions also include peripheral membrane proteins, such as TJP1, TJP2, and TJP3. The formation of tight junctions involves adherens junctions, which initiate and maintain intercellular adhesion to permit the formation of other types of junctions [30]. Adherens junctions consist of two multiprotein complexes, the nectin-afadin complex and the cadherin-catenin complex [31]. Several cadherins and catenins are expressed in the rat and human epididymis [24, 32, 33].

The reduced fertility in patients following epididymovasostomy may result from antisperm antibodies or epididymal dysfunction. It is therefore important to understand these pathologies and their consequences for the epididymis in order to develop new strategies for the treatment of infertility resulting from OA. The creation of new biological tools is a crucial step toward understanding the causes of male infertility. In the present study, we have identified several defects in the blood-epididymis barrier of OA patients by using novel human epididymal cell lines to study the molecular and cellular mechanisms responsible for these defects.

MATERIAL AND METHODS

Patients

Epididymal tissues were obtained from patients with active spermatogenesis (n=5) undergoing radical orchidectomy for localized testicular cancer (confined within the testicular tunica albuginea with no sign of epididymal lesion or obstruction) or OA patients (n=5) undergoing vasoepididymostomy after informed consent was given. Mean patient age

was 29.6 years (range 20 to 43). Serum FSH (IU/L), LH (IU/L), testosterone (nmol/L) and estradiol (pmol/L) levels, when available, were reviewed using medical records of the patients to demonstrate that patients had comparable physiological parameters (Table 1). The study was conducted with the approval of the McGill University ethics committee for research on human subjects.

Tissue Preparation

For OA patients, at the time of microsurgical epididymovasostomy, a small piece of epididymis was taken distally adjacent to the obstruction site. Epididymal tissue was placed in cold culture medium (DMEM/HAM's F12 culture medium containing glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 µg/ml), Sigma-Aldrich, Mississauga, ON, Canada) and processed under aseptic conditions within 1 hr of surgery. Epididymides were divided into three segments, the caput, corpus and cauda epididymidis (see Dubé et al. [15] for a schematic representation of the different human epididymal segments). Obstructions were situated at the level of the caput or the corpus epididymidis (Table 1).

Real-Time RT-PCR

Real-Time RT-PCR was used to assess the expression of several genes encoding junctional proteins in the epididymis of obstructive azoospermic patients (n=3; patients 3 to 5). Total RNA (500 ng) was reverse-transcribed using an oligo(dT)16 primer (R&D Systems Inc., Minneapolis, MN). Primersare listed in Table 3. Real-Time RT-PCR was performed with a Rotor-Gene RG3000 in duplicate. A 2-µl aliquot of the RT reaction was amplified in a 15-µl solution that contained 1X PerfeCTa™ SYBR Green SuperMix (Quanta Biosciences Inc., Gaithersburg, MD) and 200nM of each of the reverse and forward primers. The PCR cycling protocols were optimized to maximize reaction efficiency and to ensure that only the target product contributed to the SYBR Green fluorescence signal. For each analysis, a standard curve was created using the appropriate cDNA. Amplifications consisted of 40 cycles at 95°C for 15 sec, melting temperature (Tm) for 30 sec, and 72°C for 30 sec. Following PCR amplification, melting curve analysis was performed to ensure the accuracy of the quantification primers for the housekeeping gene, *ACTB* or *GAPDH*, that were used to

normalize the values for each sample. Statistical analyses were performed with one-way ANOVA (significance level set at $P \leq 0.05$).

Immunohistochemistry

Small pieces of epididymal tissue from fertile (n=3) and obstructive patients (n=4) were fixed at the time of surgery by immersion in Bouin's fixative (Fisher Scientific, Ottawa, ON, Canada) for 24 h, dehydrated, and embedded in paraffin. Thick sections (5 μm) were cut and mounted on glass slides. For immunostaining, the tissue sections were rehydrated through graded ethanol, including 70% alcohol with 1% lithium carbonate for 5 min, to remove residual picric acid. The sections were then incubated in 300 mM glycine for 5 min to block free aldehydes, and washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Heat-induced epitope retrieval (HIER) was performed by boiling the slides for 10 min in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate). Immunolocalization was performed with the DAKO Catalyzed Signal Amplification System (DAKO, Carpenteria, CA). The primary antibodies used in this study were: a rabbit polyclonal anti-CDH1 (0.483μg/ml; Cell Signaling, Danvers, MA),a rabbit polyclonal anti-TJP1 (0.25μg/ml; Invitrogen Inc., Burlington, ON, Canada) and a mouse monoclonal anti-vimentin (VIM, 1 μg/ml; Abcam, Cambridge, MA). Primary antibodies were incubated for 60 min at room temperature (CDH1, TJP1) or overnight at 4°C (VIM). Omission of primary antibodies as well as rabbit serum or mouse normal IgG served as negative controls. Epididymal sections were counterstained for 5 min with 0.1% methylene blue, dehydrated in ethanol, immersed in Histoclear (Fisher Scientific), and mounted in Permount (Fisher Scientific). Sections were examined under a Leica DMRE microscope.

Primary Cell Culture

Human epididymal cells were isolated from the caput epididymidis of patient 10 according to the methods of Dufresne et al. [34]. Briefly, tissue fragments (2-3 mm^3) were placed in DMEM/HAM's F12 culture medium containing glutamine (2 mM), penicillin (50

U/ml), streptomycin (50 μg/ml), collagenase (2 mg/ml; Life Technologies, Inc., Burlington, Ontario, Canada) and DNase (20 U/ml; Promega, Ottawa, Ontario, Canada). Tissue fragments were dissociated by successive enzymatic digestions for 50 min in a shaking water bath at 37°C with gentle repetitive pipetting followed by medium replacement between digestions. At the end of the final digestion, the cells were centrifuged (34 x *g*) for 3 min and the pellet resuspended in DMEM/HAM's F12 culture medium containing antibiotics and nutrients (50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10 μg/ml insulin, 10 μg/ml transferrin, 80 ng/ml hydrocortisone, 5 nM testosterone, 10 ng/ml epidermal growth factor, 10 ng/ml cAMP, 2 ng/ml sodium selenium, 200 ng/ml tocopherol, 200 ng/ml retinol and 1% fetal bovine serum (FBS); Sigma-Aldrich). The small tissue fragments were then placed in cell culture plates coated with collagen IV (BD Biosciences, Mississauga, ON, Canada) and incubated in a humidified chamber at 32° C with 5% CO_2. The culture medium was changed every 48 hrs until 40-50% confluence was reached.

Immortalization of Epididymal Cells

Primary culture cells were transfected with a pBK-CMV plasmid containing the SV40 LTAg and neomycin resistance genes (a kind gift of Dr. D. W. Silversides, University of Montreal) by calcium phosphate precipitation as described by Dufresne et al. [34]. Stable transfectants were then selected using neomycin (200 μg/ml G418; GIBCO BRL, Burlington, ON, Canada) in the culture media for 14 days and cells were isolated by serial dilution to generate different stable cell lines (Table 2). Medium containing 10% FBS was changed every 48 hrs.

Electron Microscopy

Immortalized cells were either trypsinized (0.05% trypsin, 0.53 mM EDTA) and collected by centrifugation (300 x *g*) or grown on plastic chamber slides (Nalge Nunc International, Naperville, IL) and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 hrs. The cells were then washed in 0.1M sodium cacodylate buffer and postfixed in 1% ferrocyanide-reduced osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in EPON. Thin sections were cut, mounted on copper grids,

counterstained with uranyl acetate and lead citrate and examined for morphology with FEI Tecnai 12 electron microscope.

Immunofluorescence

Immunofluorescence labeling of the cells was done to ascertain the epithelial origin of the cells. Rabbit polyclonal anti-cytokeratin (cat#18-0059,Invitrogen Inc.) and a mouse monoclonal anti-VIM (cat#ab20346, Abcam Inc., Cambridge, MA) were used. Immortalized cells were grown on plastic chamber slides (Nalge Nunc International) and fixed in methanol at -20°C for 10 min. Cells were permeabilized in a solution of 0.3% Triton X-100 in phosphate buffered saline (PBS, pH 7.4) for 15 min at room temperature. Cells were then washed in PBS and blocked in the same buffer containing 5% BSA for 30 min at 37 °C, followed by three 5-min washes in PBS. Samples were then incubated with primary antibodies (cytokeratin, 13.6 μg/ml; VIM, 1 μg/ml) for 90 min at room temperature, and washed three times for 5 min with PBS. Cells were incubated with Alexa fluor 488 conjugated anti-rabbit or anti-mouse IgG (2μg/ml; Invitrogen Inc.) for 45 min at 37°C. Cells were finally washed three times in PBS and mounted with Vectashield mounting medium containing propidium iodide (Vector Laboratories, Burlington, ON, Canada). Sections were examined under a Leica DMRE microscope.

RT-PCR

RT reactions were prepared as described above. PCR was subsequently done to identify the presence of different transcripts in the cell lines using the following amplification protocol: 94°C for 5 min, 30–40 cycles of 94°C for 30 sec; melting temperature (Tm) for 30 sec, 72°C for 1 min; and cooled to 4°C. The sequence of the primers used for differentiation markers are indicated in Table 3. Other primers have been previously described in Dubé et al. [24]. PCR amplicons were separated on an agarose gel (1-2%) and visualized with ethidium bromide using a Fluor-S Multi-Imager densitometer (Bio-Rad Laboratories, Mississauga, Ontario, Canada). PCR was also done on RNA that was not reverse transcribed to confirm the absence of genomic DNA. RNA extracted from human epididymis and kidney (Biochain, Hayward, CA, USA) were used as positive controls.

Cell Cycle Analysis

Immortalized cells were trypsinized, recovered by centrifugation (1000g x 7 min), and washed twice in PBS (pH 7.4). Cells were fixed by resuspending the pellet in ice-cold ethanol and kept on ice for 30 min. The cells were then washed three times in PBS containing 2% FBS. The pellet was finally resuspended in a solution containing 0.1% sodium citrate, 0.3% NP-40, 100 ug/ml RNase A, 50 ug/ml propidium iodide. DNA content of $2x10^6$ cells per sample was analyzed using a FACScan apparatus (Becton Dickinson, Oakville, Ontario, Canada). THP1 cells (a kind gift of Dr. D. Girard, INRS-Institut Armand-Frappier) were used as control diploid cells as previously shown [37].

Cell Growth

An aliquot of 1250 immortalized cells per well was plated in 96-well culture plates coated with collagen IV. The number of cells was determined using Trypan blue staining (Invitrogen Inc.) and a hemocytometer. The next day, once the cells had adhered, medium was changed, and this first time point was assigned as time zero. Medium was changed every 24 hrs. At different time points done in triplicate (0, 24, 48, 120 and 192 hrs), culture medium was removed from the wells and replaced with 20 µl methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution (Sigma-Aldrich; 0.5 mg/ml in culture medium) to measure cellular proliferation [34]. After 3 hrs, the MTT solution was removed and the formazan crystals were solubilized with 200 µl dimethylsulfoxide (DMSO) per well. The absorbance at 570nm was read using a microtiter plate reader (Power Wave X; Bio-Tek Instruments Inc., Winooski, VT). The doubling time was calculated as the inverse of the slope of a semilog plot of the absorbance in function of time, in the linear portion of the graph (the logarithmic growth phase). Data are presented as the mean ± SEM.

Transepithelial Resistance (TER)

Cells were seeded at a density of 1×10^5 cells/ml on Costar Transwell 6.5-mm cell culture inserts (pore size 0.4 µm; Corning) coated with or without mouse collagen IV (5 $\mu g/cm^2$, BD Biosciences). The medium used in this experiment was DMEM/HAM's F12 culture medium containing antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin), 2 mM L-

glutamine and 5% FBS. Once total confluence was reached, cells were exposed to medium containing 1.8mM $CaCl_2$ during 48 hrs and TER was measured at regular intervals using an EVOM epithelial voltohmmeter with a STX2 electrode (WPI Inc., Sarasota, FL). TER was normalized to the area of the filter after removal of background resistance of a blank filter that contained only medium. TER was measured as ohms x cm^2. TER measurements were done several times in triplicate. FHCE1 cells derived from the caput epididymidis of a fertile man and shown to form functional tight junctions were used as a positive control [37]. Statistical analyses were performed with two-way ANOVA (significance level set at $P \leq 0.05$).

Microarray Processing and Analysis

Microarray analysis was done to compare gene expression in IHCE1 and FHCE1 cells. Total cellular RNA was isolated using the Illustra RNAspin Mini kit (GE Healthcare, Baie D'Urfe, QC, Canada) according to the manufacturer's instructions. The quality of the total RNA was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies). Gene expression profiling was performed with commercially available human oligonucleotide microarrays (20 174 human genes; Agilent Technologies). Analyses were done in triplicate for each cell line. Amplifications and labeling of total RNA (500ng) were performed using the Low RNA Input Linear Amplification Kit (Agilent Technologies). The cRNA was labeled with either cyanine 3 or cyanine 5 (Perkin Elmer, Woodbridge, Canada). Arrays were hybridized according to the manufacturer's instructions using the In Situ Hybridization kit Plus (Agilent Technologies). Following hybridization, microarrays were scanned with a ScanArray Express scanner (Perkin Elmer). Fluorescence ratios for array elements were extracted using the ScanArray Express Software (Perkin Elmer) and imported into the GeneSpring 6.1 software (Agilent Technologies) for further analysis. The data was normalized using a locally weighted regression Lowess method. Statistical analyses were performed with one-way ANOVA (significance level set at $P \leq 0.05$). The data discussed in this publication was deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO, http://www.ncbi.nlm. nih.gov/geo/, GSE21391). Real-Time RT-PCR, as described previously, was used to confirm for two genes, *PRKCA* and *CDC42,* the differences in transcription that were observed in the microarray analysis.

RESULTS

mRNA Levels for Junctional Proteins in Obstructive Azoospermic Patients

Expression of multiple genes encoding junctional proteins (*CDH1, TJP1, CLDN1, 4, 7, 10*) was evaluated in the region of the epididymis downstream of the obstruction in azoospermic patients and compared to approximately the same region in fertile patients (FIG.1). Only a single infertile patient was found to have an obstruction in the region of the caput epididymidis. In the corpus epididymidis of this patient, mRNA levels for *CLDN1* and *4* were almost twice those of fertile patients (FIG. 1c, d) while *CLDN10* mRNA levels were decreased by almost 80% (FIG. 1f). In the cauda epididymidis of OA patients suffering from an obstruction at the level of the corpus, *CLDN10* mRNA levels were significantly lower than those observed in the cauda epididymidis of patients with proven fertility (FIG. 1f). Levels of the other markers were unaltered.

Immunolocalization of Junctional Proteins and Vimentin

Immunolocalization of CDH1 (FIG. 2A) in the corpus and cauda epididymidis revealed that CDH1 was localized along the plasma membrane of adjacent principal cells as well as between basal and principal cells. Some cytoplasmic immunostaining in principal cells was also observed. In the corpus epididymidis of the OA patient, CDH1 was also localized along the lateral margins of adjacent principal cells as well as between basal and principal cells. The immunoreaction, however, appeared to be more intense in the apical region of the cells (FIG. 2Aa, b). In the cauda epididymidis of OA patients, CDH1 was not detected in two of three patients (FIG. 2Ae, f). In the third patient, CDH1 was present throughout the cytoplasm, indicating a mis-targeting of the protein (FIG. 2Ad).

TJP1 in both the corpus and cauda of patients with proven fertility was localized to the area of the tight junction between adjacent principal cells (FIG. 2Ba, c). In the corpus epididymidis of the OA patient, TJP1 was also localized to the area of the tight junctions, although the immunoreaction appeared more intense (FIG. 2Bb). In the cauda epididymidis, the immunoreaction for TJP1 was similar in OA patients to that observed in the fertile patients (FIG. 2Bc, d).

VIM was not expressed in the corpus and cauda epididymidis of fertile patients (FIG. 2Ca, c) but was localized in a small subset of epithelial cells in their cytoplasm and along their lateral margins in the corpus of the obstructive azoospermic patient (FIG. 2Cb) and in the cauda epididymidis of one of the obstructive azoospermic patients (FIG. 2Cd).

Human Epididymal Cell Lines

Cell lines were derived from the caput epididymidis of a 27 year old man with OA (Patient 10, Table 1). The obstruction was localized to the proximal area of the caput epididymidis. Five separate cells lines (IHCE1-5) were derived from the tissue obtained from this patient (Table 2). Ultrastructural analysis of each cell line indicated that these are comprised of epithelial principal cells. The cells have characteristic microvilli or stereocilia on their cell surface, a large and irregular nucleus, endosomal elements and mitochondria (FIG. 3A). The cells derived from the infertile patient appeared more flattened and possessed fewer less developed organelles than the cells previously derived from a fertile patient (FIG. 3B).The cell lines express cytokeratin, an epithelial cell marker, and vimentin (VIM), a mesenchymal marker, at both the mRNA and protein level (FIG. 3C-D). These results indicate that the immortalized epididymal cell lines originate from principal-like cells but that they appear to be somewhat partly dedifferentiated, as suggested by the expression of VIM. Furthermore, the cells, which have been passed at least 20 times, are diploid (FIG. 4) and have a doubling time of approximately 7 to 11 days (FIG. 5). These represent the first stable epididymal cell lines to be derived from the adult epididymis of an infertile patient.

Characterization of Human Epididymal Cell Lines

RT-PCR was used to verify the expression of several differentiation and epididymal markers in the cell lines. Most of the transcripts encoding retinoic acid and thyroid hormone receptors were detected in the cell lines with the exception of *RARB* and *THRB* (FIG. 6A). The expression of these markers was also evaluated in the FHCE1 cell line derived from the caput epididymidis of a fertile patient [37]. Among the epididymal markers, only *SPAG11B* (also known as *HE2*) was expressed in the cell lines derived from the infertile patient (FIG. 6B). The expression of *SLC9A3*, a Na+/H exchanger also known as *NHE3*, which has been

shown to be expressed in the epithelium of the human efferent ducts but not in the epididymis [35], was verified using RNA from human kidney [36] as a positive control. *NHE3* was not expressed in any of the cell lines, thereby confirming that these cells were not of efferent duct origin (FIG.6B). Transcripts for the androgen (*AR*), estrogen receptors (*ESR1* and *ESR2*) and 5-alpha-reductase enzymes (*SRD5A1* and *SRD5A2*) were also determined in each cell line (FIG. 6C). Only the *AR* was expressed in IHCE1 and IHCE2 cell lines. Overall our cell lines from infertile patients retained some of the differentiated functions that characterize *in vivo* principal cells, but not as many as the FHCE1 cell line developed from the caput epididymidis of a fertile patient [37].

mRNA Levels of Genes Encoding for Junctional Proteins

Transcript levels of genes encoding different junctional proteins implicated in adherens (*CDH1* and *CDH2*), tight junctions (*CLDN1, 2, 3, 4, 7* and *8*) and desmosomes (desmoplakin, *DSP*) were assessed (FIG.7A). With the exception of *CLDN2, 3* and *DSP*, these transcripts were not expressed in the cell lines derived from the infertile patient.

Formation of Tight Junctions by IHCE1 Cells

The ability of IHCE1 cells to form functional tight junctions was assessed. Epididymal tight junctions are necessary for the formation of the blood-epididymis barrier and sperm maturation. TER was used to measure the formation of tight junctions in calcium-stimulated cells (FIG7B). Cells derived from the caput epididymidis of a fertile patient were used as a positive control [37]. There was no increase in TER measured in IHCE1 cells, indicating that these cells are not capable of forming tight junctions. Similar results were observed with cells seeded on inserts coated with mouse collagen IV (Data not shown).

Differential gene expression in FHCE1 and IHCE1 cell lines

Microarray analyses were used to compare gene expression profiles in IHCE1 and FHCE1 cells in order to identify potential key elements implicated in the formation, maintenance and/or regulation of human epididymal tight junctions. Arrays were hybridized with probes of each cell line to identify genes that were either down- or upregulated in

IHCE1 cells compared to FHCE1 cells. In these analyses, 2958 genes were differentially expressed in IHCE1 cells by at least a 2-fold change when compared with FHCE1 cells: 1567 genes were downregulated and 1391 were upregulated. Among them, 705 genes were downregulated and 312 were upregulated by at least a 4-fold change (FIG. 8A). Two of the genes, *CDC42* and *PRKCA*, were selected for real-time RT-PCR analysis to validate the microarray data. Expression patterns showed consistency between microarrays and real-time RT-PCR analysis (FIG. 8B). Most of the genes that were differentially regulated, and for which a function was known, encoded proteins implicated in transport (electrons, ions, water and other), signal transduction (including G-proteins and MAPK pathways), protein metabolism, transport, targeting and transcription. Several genes also encoded proteins implicated in apoptosis, cell adhesion and junctions, cell morphogenesis and differentiation, cell growth and proliferation, cell motility, cytoskeleton organization and biogenesis and immunity (FIG.8C). Further analyses were done on genes known to be implicated in the formation of cellular junctions (Table 4). Several genes encoding cadherins (*CDH24*), protocadherins (*PCDHGA8, PCDH18, CDHR2, PCDHB11, CDHR5, PCDHB13*), and catenins (*CTNNB1, CTNNBIP1*) were upregulated in IHCE1 cells as compared to FHCE1 cells by at least a 1.5 fold change. Several genes encoding connexins (*GJC1, GJB4, GJA12*), pannexin-3 (*PANX3*) and claudins (*CLDN9, CLDN11, CLDN18, CLDN17*) were also upregulated by at least a 1.5 fold change in IHCE1 cells as compared to FHCE1 cells. Genes that were downregulated by at least a 1.5 fold change in IHCE1 cells were compared to FHCE1 cells and included N-cadherin (*CDH2*) and two catenins (*CTNNA1, CTNNAL1*).

Further analyses were done on genes that have been shown in other cell types to be implicated in the regulation of cellular junctions (Tables 5-6). Genes that were upregulated by at least a 1.5 fold change in IHCE1 cells compared to FHCE1 cells (Table 5) included genes that encoded several transcription factors, such as the claudin repressor Slug (*SNAI2*), members of the Ras protein family (*RAB36, RAB2, RAB10, RAB21, RAP1A, RAP1B, HRAS, RRAS2, RHOA, RHOG*), oncogenes (*MOS, CSK, NOTCH3, NOTCH2, JUNB, JUND*), protein kinases and their binding proteins (*PRKCA, PRKCD, PRKCDBP*), members of the MAPK family (*MAP2K3*), members of the integrin- mediated signaling pathway (*ILK*), the APC/Wnt signaling pathway (*APC, APC2, WNT2, WIF1*), the JAK-STAT signaling pathway

(*JAK1, JAK3*), and the frizzled gene family (*FZD5, FZD1, FZD2*). Genes that were downregulated by at least a 1.5 fold change in IHCE1 cells compared to FHCE1 cells (Table 6) included genes that encoded oncogenes (*JUN, MYC*), protein kinases (*PRKAG2, PRKCZ, PRKAR1A*), members of the MAPK family (*MAP2K2*), members of the Ras family (*RAB34, RAB18, RAB22A, RAB32, RAB13, RHOC, CDC42, RAC1*), and the Wnt family member, *DVL1*.

Several other genes that have been shown to be expressed in the epididymis were differentially regulated in IHCE1 cells as compared to FHCE1 cells by at least a 1.5 fold change (Table 7). Upregulated genes included WFDC2 (also known as *HE4*), *AKAP1, AQP5, ADAMTS10, ADAM19, ADAM15* while downregulated genes comprised several cathepsins (*CTSZ, CTSB*), *DCXR* (also known as *P34H*) and *MIF*.

DISCUSSION

We have previously shown that epididymal gene expression and the blood-epididymis barrier are altered in non-obstructive azoospermic patients [15]. In the present study, we report that in patients with OA and in epididymal cell lines derived from OA patients, components of the adherens and tight junctions of the epididymis are altered, thereby supporting the notion that the blood-epididymis barrier is altered in infertile patients. Given the low fertility rates of patients following epididymovasostomy to circumvent epididymal obstruction [7, 8, 38], these data suggest that epididymal function also needs to be considered in these infertile patients.

The expression of several genes encoding tight junctional proteins (*CLDN1, 4, 10*) was altered in the corpus epididymidis of an OA patient, while only *CLDN10* expression was altered in the cauda epididymidis of OA patients. These results suggest that the site of the obstruction has an impact on the level of alteration of the blood-epididymis barrier in OA patients. Rajalakshmi et al. [39] have previously reported the occurrence of degenerative changes in the epididymis of obstructive patients, and that these were less pronounced when the site of the obstruction was situated more distally in the epididymis. In addition, our

immunolocalization of CDH1, TJP1 and VIM revealed some differences between obstructive and fertile patients. These results suggest that both the barrier and the paracellular transport as well as adherens junctions are affected in the epididymis of OA patients. Moreover, it is interesting that both TJP1 and CLDN10 are affected in obstructive and non-obstructive azoospermic patients [15]. This suggests that the expression of these two genes may be regulated by testicular factors that are released into the lumen of the epididymis. Previous studies have also reported that CLDN10 mRNA levels in the corpus epididymidis are decreased in patients with vasectomy [40]. Whether or not this is also associated with movement of factors in the luminal fluid from the testis towards the corpus epididymidis is not known. However, it would appear that CLDN10 is particularly sensitive to changes in the lumen environment of the epididymis.

The limited ability to obtain epididymal tissues from infertile patients has greatly hindered the ability to assess the role of the epididymis in male infertility. The development of cell lines that maintain characteristics similar to those found in infertile patients represents a tremendous potential to address cellular and molecular mechanisms of epididymal function in infertile patients. The different cell lines developed in this study retain some of the characteristics of *in vivo* principal cells. These cells have a similar ultrastructure to epididymal principal cells and similar to cell lines derived from the epididymidis of a fertile patient [37], are diploid, and are not from the efferent ducts, as confirmed with absence of *SLC9A3* expression [35].

The cell lines lack the expression of several epididymal (*SPINLW1, CRISP1, NPC2, CD52, DCXR*) and differentiation (*RARB, THRB*) markers, suggesting that these cells may be undergoing epithelial-mesenchymal transition (EMT) [41]. This was not the case in FHCE1 epididymal cell line derived from the caput epididymidis of a fertile patient that express *NPC2*, *SPAG11B*, *CD52* and *DCXR* [37]. The expression of *RARB* and *THRB* is frequently reduced in cancerous cells that undergo an EMT [42, 43]. This is also suggested by the flattened aspect of the cells and the paucity of organelles, which has also been previously observed in the vas deferens epithelium of patients following vasectomy [44]. Furthermore, the epididymal cell lines also express both cytokeratin and VIM, a mesenchymal marker. VIM was also expressed by a small subset of epithelial cells in the

epididymis of obstructive azoospermic patients suggesting that a small percentage of epithelial cells could be less differentiated in these tissues and may represent the origin of the cell lines developed in this study. Previous studies have shown that obstruction in the caput epididymidis can lead to extensive degenerative ultrastructural changes in the principal cells of the caput epididymidis, due to abnormal fluid reabsorption [39], as well as leucocytic infiltration [45] and high levels of antisperm antibodies [14]. Obstruction as a consequence of vasectomy has also been shown to affect the osmolarity and the composition of epididymal intraluminal fluid [46]. All of these changes are consistent with alterations to the blood-epididymis barrier.

IHCE1 cells lack the ability to form tight junctions due to the absence or low expression levels of several genes encoding junctional proteins (*CDH1, CDH2, CLDN1, 3, 4, 7, 8*). Indeed, CLDN1, 3, 4 and 7 have been reported to be important for the integrity of human epididymal tight junctions [37]. Thus, our present results are not surprising, since EMT is characterized by the dissassembly of junctional structures [41]. In addition, many functions in the proximal epididymis are regulated by lumicrine factors, including the expression and localization of several junctional proteins, such as CLDN1 and 8, in rodents [26, 47]. This could explain why the effects are greater following an obstruction in the proximal caput epididymidis as compared to other epididymal regions. In addition, the absence, or low level of expression, of the *AR*, *SRD5A* isoforms and the *ER* in the majority of our cell lines derived from the infertile patient suggests that the cells have limited ability to respond to androgens and estrogens.

There is surprisingly limited information on the hormones and intracellular signaling pathways implicated in the formation and maintenance of the blood-epididymis barrier [23]. Since the IHCE1 cells do not form functional tight junctions, we compared gene expression profiles with human epididymal principal cells, FHCE1, that were derived from a fertile patient and shown to form functional tight junctions, in order to identify regulatory pathways that may be implicated in the regulation of epididymal tight junctions [37]. Several differentially expressed genes which encode junctional proteins such as cadherins, protocadherins, catenins, connexins and pannexin-3 were all shown to have different expression levels between IHCE1 and FHCE1 cells, suggesting that cell adhesion and cell

communication may be impaired in OA. The EMT process implicates signaling pathways such as growth factors (Wnt, Notch and others) that can induce EMT and act in a sequential manner [41]. In addition, several transcription factors, such as SNAI1 (Snail), SNAI2 (Slug) and TWIST, have been shown to be responsible for the downregulation of genes (CDHs, CLDNs, connexins, OCLN and TJPs) which contribute to the assembly of junctional complexes [48]. Many of these effectors were upregulated in IHCE1 cells. In addition, other players important for the assembly of cell junctions [49], such as *RAB13*, *CDC42* and *PKC* isoforms, are downregulated in IHCE1 cells. These results suggest that the regulation of cellular junctions that participate in the creation and maintenance of the human blood-epididymis barrier may be altered in OA. Furthermore, these data suggest that this regulation is accomplished via a variety of signaling pathways as opposed to a single pathway (FIG. 9).

The study of signaling pathways in the epididymis of fertile and infertile patients is necessary to better understand the importance of each pathway in the regulation of human epididymal junctions. The role of these effectors can vary according to the cell type [49] and several of these may be involved in sperm maturation. For example, haploinsufficiency of the PRKAR1A gene is associated with reduced male fertility [50]. In addition, previous studies have demonstrated that epididymal obstruction can disrupt the secretory activities of the epididymal epithelium [46]. For exa nple, reduced expression of DCXR (also known as P34H), which has been associated with male infertility, is observed as a consequence of vasectomy [51, 52] and its expression is not always restored after vasectomy reversal [53]. Several cathepsins are also downregulated and it has been shown in rodents that absence or low expression of specific cathepsins is associated with reduced male fertility [54, 55].

Our study has identified alterations in the expression of epididymal markers and components of cellular junctions in the epididymis of infertile OA patients. Azoospermia due to epididymal obstruction could involve multiples epididymal defects, including an altered blood-epididymis barrier, impaired cell adhesion and communication and differential expression of epididymal markers. However the impact of the obstruction on epididymal structure and/or function probably varies according to the cause, location and duration of obstruction. Overall these alterations could also explain why fertility is not restored in all patients after epididymovasostomy [38]. Clearly, our data suggest that as a result of altered

gene expression to critical transport and junctional proteins, the luminal environment of the epididymis is unlikely to be optimal for sperm maturation and survival after surgical reconstruction. These results support the notion that the epididymis is affected in male infertility and that epididymal function needs to be considered in surgical procedures aimed at improving fertility in azoospermic patients.

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REFERENCES

1. Mosher WD, Pratt WF. Fecundity and infertility in the United States: incidence and trends. Fertil Steril 1991; 56: 192-193.
2. Thonneau P, Spira A. Prevalence of infertility: international data and problems of measurement. Eur J Obstet Gynecol Reprod Biol 1991; 38: 43-52.
3. Sigman M, Jarow JP. Male Infertility. In: Walsh PC, Retik AB, Vaughan ED, Wein AJ (eds.), Campbell's Urology, 8th ed. Philadelphia: Saunders; 2006: 609-653.
4. Schlegel PN. Causes of azoospermia and their management. Reprod Fertil Dev 2004; 16: 561-572.
5. Practice Committee of American Society for Reproductive Medicine in collaboration with Society for Male Reproduction and Urology. The management of infertility due to obstructive azoospermia. Fertil Steril 2008; 90: S121-124.
6. Practice Committee of American Society for Reproductive Medicine in collaboration with Society for Male Reproduction and Urology. Evaluation of the azoospermic male. Fertil Steril 2008; 90: S74-77.
7. Kim ED, Winkel E, Orejuela F, Lipshultz LI. Pathological epididymal obstruction unrelated to vasectomy: results with microsurgical reconstruction. J Urol 1998; 160: 2078-2080.
8. Berardinucci D, Zini A, Jarvi K. Outcome of microsurgical reconstruction in men with suspected epididymal obstruction. J Urol 1998; 159: 831-834.
9. Dohle GR, Ramos L, Pieters MH, Braat DD, Weber RF. Surgical sperm retrieval and intracytoplasmic sperm injection as treatment of obstructive azoospermia. Hum Reprod 1998; 13: 620-623.
10. Palermo GD, Schlegel PN, Hariprashad JJ, Ergun B, Mielnik A, Zaninovic N, Veeck LL, Rosenwaks Z. Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men. Hum Reprod 1999; 14: 741-748.
11. Practice Committee of American Society for Reproductive Medicine. Intracytoplasmic sperm injection (ICSI). Fertil Steril 2006; 86: S122.

12. Buffat C, Patrat C, Merlet F, Guibert J, Epelboin S, Thiounn N, Vieillefond A, Adda-Lievin A, Lebon C, Jouannet P. ICSI outcomes in obstructive azoospermia: influence of the origin of surgically retrieved spermatozoa and the cause of obstruction. Hum Reprod 2006; 21: 1018-1024.
13. Pasqualotto FF, Rossi-Ferragut LM, Rocha CC, Iaconelli A, Jr., Borges E, Jr. Outcome of in vitro fertilization and intracytoplasmic injection of epididymal and testicular sperm obtained from patients with obstructive and nonobstructive azoospermia. J Urol 2002; 167: 1753-1756.
14. Lee R, Goldstein M, Ullery BW, Ehrlich J, Soares M, Razzano RA, Herman MP, Callahan MA, Li PS, Schlegel PN, Witkin SS. Value of serum antisperm antibodies in diagnosing obstructive azoospermia. J Urol 2009; 181: 264-269.
15. Dube E, Hermo L, Chan PT, Cyr DG. Alterations in gene expression in the caput epididymides of nonobstructive azoospermic men. Biol Reprod 2008; 78: 342-351.
16. Robaire B, Hermo L. Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E, Neill J (eds.), The Physiology of Reproduction. New York: Raven Press; 1988: 999-1080.
17. Robaire B, Hinton B, Orgebin-Crist M-C. The epididymis. In: Neill JD (ed.) Knobil and Neill's Physiology of Reproduction, 3th ed. New York: Elsevier; 2006: 1071-1148.
18. Hinton BT, Palladino MA. Epididymal epithelium: its contribution to the formation of a luminal fluid microenvironment. Microsc Res Tech 1995; 30: 67-81.
19. Dacheux JL, Gatti JL, Dacheux F. Contribution of epididymal secretory proteins for spermatozoa maturation. Microsc Res Tech 2003; 61: 7-17.
20. Dacheux JL, Belghazi M, Lanson Y, Dacheux F. Human epididymal secretome and proteome. Mol Cell Endocrinol 2006; 250: 36-42.
21. Itoh M, Terayama H, Naito M, Ogawa Y, Tainosho S. Tissue microcircumstances for leukocytic infiltration into the testis and epididymis in mice. J Reprod Immunol 2005; 67: 57-67.
22. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 2004; 286: C1213-1228.
23. Cyr DG, Gregory M, Dube E, Dufresne J, Chan PT, Hermo L. Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood-epididymal barrier in animals and humans. Asian J Androl 2007; 9: 463-475.
24. Dube E, Chan PT, Hermo L, Cyr DG. Gene expression profiling and its relevance to the blood-epididymal barrier in the human epididymis. Biol Reprod 2007; 76: 1034-1044.
25. Gregory M, Cyr DG. Identification of multiple claudins in the rat epididymis. Mol Reprod Dev 2006; 73: 580-588.
26. Gregory M, Dufresne J, Hermo L, Cyr D. Claudin-1 is not restricted to tight junctions in the rat epididymis. Endocrinology 2001; 142: 854-863.
27. Guan X, Inai T, Shibata Y. Segment-specific expression of tight junction proteins, claudin-2 and -10, in the rat epididymal epithelium. Arch Histol Cytol 2005; 68: 213-225.
28. Inai T, Sengoku A, Hirose E, Iida H, Shibata Y. Claudin-7 expressed on lateral membrane of rat epididymal epithelium does not form aberrant tight junction strands. Anat Rec (Hoboken) 2007; 290: 1431-1438.

29. Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. Annu Rev Physiol 2006; 68: 403-429.
30. Yap AS, Brieher WM, Gumbiner BM. Molecular and functional analysis of cadherin-based adherens junctions. Annu Rev Cell Dev Biol 1997; 13: 119-146.
31. Niessen CM. Tight junctions/adherens junctions: basic structure and function. J Invest Dermatol 2007; 127: 2525-2532.
32. Cyr DG, Robaire B. Developmental regulation of epithelial- and placental-cadherin mRNAs in the rat epididymis. Ann N Y Acad Sci 1991; 637: 399-408.
33. DeBellefeuille S, Hermo L, Gregory M, Dufresne J, Cyr DG. Catenins in the rat epididymis: their expression and regulation in adulthood and during postnatal development. Endocrinology 2003; 144: 5040-5049.
34. Dufresne J, St-Pierre N, Viger RS, Hermo L, Cyr DG. Characterization of a novel rat epididymal cell line to study epididymal function. Endocrinology 2005; 146: 4710-4720.
35. Kujala M, Hihnala S, Tienari J, Kaunisto K, Hastbacka J, Holmberg C, Kere J, Hoglund P. Expression of ion transport-associated proteins in human efferent and epididymal ducts. Reproduction 2007; 133: 775-784.
36. Brant SR, Yun CH, Donowitz M, Tse CM. Cloning, tissue distribution, and functional analysis of the human Na+/N+ exchanger isoform, NHE3. Am J Physiol 1995; 269: C198-206.
37. Dube E, Dufresne J, Chan PTK, Hermo L, Cyr D. Assessing the role of claudins in maintaining the integrity of epididymal tight junctions using novel human epididymal cell lines. Biol Reprod 2010, In Press.
38. Takihara H. The treatment of obstructive azoospermia in male infertility--past, present, and future. Urology 1998; 51: 150-155.
39. Rajalakshmi M, Kumar BV, Kapur MM, Pal PC. Ultrastructural changes in the efferent duct and epididymis of men with obstructive infertility. Anat Rec 1993; 237: 199-207.
40. Thimon V, Calvo E, Koukoui O, Legare C, Sullivan R. Effects of vasectomy on gene expression profiling along the human epididymis. Biol Reprod 2008; 79: 262-273.
41. Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci 2007; 98: 1512-1520.
42. Alvarez S, Germain P, Alvarez R, Rodriguez-Barrios F, Gronemeyer H, de Lera AR. Structure, function and modulation of retinoic acid receptor beta, a tumor suppressor. Int J Biochem Cell Biol 2007; 39: 1406-1415.
43. Gonzalez-Sancho JM, Garcia V, Bonilla F, Munoz A. Thyroid hormone receptors/THR genes in human cancer. Cancer Lett 2003; 192: 121-132.
44. Andonian S, Jarvi K, Zini A, Hermo L. Ultrastructural features of the vas deferens from patients undergoing vasectomy and vasectomy reversal. J Androl 2002; 23: 691-701.
45. Rajalakshmi M, Kumar BV, Ramakrishnan PR, Kapur MM. Histology of the epididymis in men with obstructive infertility. Andrologia 1990; 22: 319-326.
46. Flickinger CJ, Howards SS. Consequences of obstruction on the epididymis. In: Robaire B, Hinton B (eds.), The Epididymis: From Molecules to Clinical Practice. New York: Plenum; 2002: 503-522.

47. Turner TT, Johnston DS, Finger JN, Jelinsky SA. Differential gene expression among the proximal segments of the rat epididymis is lost after efferent duct ligation. Biol Reprod 2007; 77: 165-171.
48. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development 2005; 132: 3151-3161.
49. Gonzalez-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. Biochim Biophys Acta 2008; 1778: 729-756.
50. Veugelers M, Wilkes D, Burton K, McDermott DA, Song Y, Goldstein MM, La Perle K, Vaughan CJ, O'Hagan A, Bennett KR, Meyer BJ, Legius E, Karttunen M, Norio R, Kaariainen H, Lavyne M, Neau JP, Richter G, Kirali K, Farnsworth A, Stapleton K, Morelli P, Takanashi Y, Bamforth JS, Eitelberger F, Noszian I, Manfroi W, Powers J, Mochizuki Y, Imai T, Ko GT, Driscoll DA, Goldmuntz E, Edelberg JM, Collins A, Eccles D, Irvine AD, McKnight GS, Basson CT. Comparative PRKAR1A genotype-phenotype analyses in humans with Carney complex and prkar1a haploinsufficient mice. Proc Natl Acad Sci U S A 2004; 101: 14222-14227.
51. Boue F, Sullivan R. Cases of human infertility are associated with the absence of P34H an epididymal sperm antigen. Biol Reprod 1996; 54: 1018-1024.
52. Legare C, Thabet M, Picard S, Sullivan R. Effect of vasectomy on P34H messenger ribonucleic acid expression along the human excurrent duct: a reflection on the function of the human epididymis. Biol Reprod 2001; 64: 720-727.
53. Guillemette C, Thabet M, Dompierre L, Sullivan R. Some vasovasostomized men are characterized by low levels of P34H, an epididymal sperm protein. J Androl 1999; 20: 214-219.
54. Hermo L, Korah N, Gregory M, Liu LY, Cyr DG, D'Azzo A, Smith CE. Structural alterations of epididymal epithelial cells in cathepsin A-deficient mice affect the blood-epididymal barrier and lead to altered sperm motility. J Androl 2007; 28: 784-797.
55. Jervis KM, Robaire B. Changes in gene expression during aging in the Brown Norway rat epididymis. Exp Gerontol 2002; 37: 897-906.

FIGURE LEGENDS

Figure 1: Altered expression of genes encoding junctional proteins in the epididymis of obstructive azoospermic patients. The mRNA expression levels of (A) *CDH1*, (B) *TJP1*, (C) *CLDN1*, (D) *CLDN4*, (E) *CLDN7* and (F) *CLDN10* were compared by real-time RT-PCR in the epididymis of fertile (n=3) and obstructive azospermic patients suffering from an obstruction at the level of the caput (n=1) or the corpus (n=3). Each sample was done in triplicate. Data is expressed as the mean relative expression (±SEM). **, $P < 0.01$ versus control. CS, corpus; CA, cauda.

Figure 2: Immunolocalization of CDH1 and TJP1 in fertile and obstructive zoospermic patients. (A) Immunolocalization of CDH1 in the epididymis of fertile (a, c) and obstructive azoospermic patients (b, d-f). CDH1 (arrowheads) is localized to the apical tight junctional complex and along the lateral margins of epithelial cells, as well as between the basal and principal cells in the corpus epididymidis of (a) fertile patients (n=3), (b) an obstructive azoospermic patient and in the cauda epididymidis of (c) fertile (n=3) and (d-f) obstructive azoospermic (n=3) patients. In the corpus epididymidis of the obstructive patient and in the cauda epididymidis of one of the obstructive patients, the intensity of the signal for CDH1 was higher than that in fertile patients, whereas in the cauda epididymidis of the two other obstructive patients, the intensity of the signal for CDH1 was weaker than in fertile patients. Original magnification 640x. (B) Immunolocalization of TJP1 in the epididymis of fertile (a,c) and obstructive azoospermic (b,d) patients. TJP1 (arrowheads) is localized to the apical tight junctional complex in the corpus epididymidis of (a) fertile (n=3) and (b) obstructive (n=1) patients as well as in the cauda epididymidis of (c) fertile (n=3) and (d) obstructive (n=3) patients. However, in the corpus epididymidis of the obstructive patient, the intensity of the signal for TJP1 was higher than in fertile patients. Original magnification 640x. (C) Immunolocalization of VIM in the epididymis of fertile (a, c) and obstructive azoospermic patients (b, d). VIM is not expressed in the corpus and cauda epididymidis of fertile patients but is localized in a subset of epithelial cells in the cytoplasm and along their lateral margins in the corpus of an obstructive azoospermic patient and in the cauda epididymidis of one of the obstructive azoospermic patients. Lu, lumen; IT, interstitial space; P, principal cell; B, basal cell. Original magnification 1600x.

Figure 3: Epithelial morphology of the different cell lines. (A) Ultrastructural analysis by electron microscopy of the different cell lines after trypsinization: (a) IHCE1 cells, (b) IHCE2 cells, (c) IHCE3 cells, (d) IHCE4 cells and (e) IHCE5 cells. Cells have an irregular nucleus (n), extensive microvilli or stereocilia (mv), numerous mitochondria (m), cytoplasmic vesicles (v). Vesicular structures, possibly epididymosomes (ep), are also present around the cells. Magnification 4200-6000x. (B) Representative electron micrographs of (a) typical cell line derived from OA patient as compared to (b) FHCE1, a typical cell line derived from a fertile patient [37]. Cells were grown on plastic chambers under identical conditions. ER, endoplasmic reticulum; n, nucleus; v, vesicle; G, Golgi apparatus. Magnification 16500-26500x. (C-D) Cytokeratin, an epithelial marker, and VIM, a mesenchymal marker, expression was assessed by RT-PCR (C) and immunofluorescence staining (D) to assess the epithelial morphology of the cells. Cytokeratin and VIM (green) are expressed and localized to the cytoplasm in all cells lines derived from the infertile patient whereas only cytokeratin is expressed in the FHCE1cell line derived from fertile tissue [37]. Nuclei (red) are stained with propidium iodide.

Figure 4: Flow cytometric analysis of IHCE1 cells. DNA was stained with propidium iodide for analysis by flow cytometry. Human THP1 cells were used as a control diploid population. IHCE1 cells showed two peaks of fluorescence corresponding to resting and dividing cells. The diploid state of the cells is clearly shown when simultaneously analyzed with THP1 cells.

Figure 5: Cell growth of the epididymal cell lines at 32°C. Cell growth was evaluated by incubating the cells with a MTT solution for 3 hrs at different time points in triplicate. Absorbance was measured at 570 nm (n=3, ± SEM).

Figure 6: Expression of differentiation, epididymal, efferent ducts markers and hormone receptors and enzymes. Expression of the different transcripts was verified by RT-PCR using specific primers. Human epididymis or kidney was used as a positive control. (A) The expression of *RARB* and *THRB* was reduced in the cell lines derived from the epididymis of the infertile patient compared to FHCE1 cell line derived from fertile tissue [37]. (B) *SPAG11B* was expressed in the different cell lines derived from infertile tissue while no

band was detected for *CRISP1, SPINLW1, NPC2, DCXR* and *SLC9A3*. (C) *AR* was expressed in the IHCE1 and IHCE2 cell lines while *ESR1, ESR2, SRD5A1, SRD5A2* were not detected in any of the cell lines.. Neg, negative control; Pos, positive control; Epid, epididymis.

Figure 7: Tight junctions in the different cell lines. (A) Expression of genes encoding tight junctional proteins by RT-PCR. Expression of the different transcripts was verified using specific primers. Human epididymis was used as a positive control. Only *CLDN2, 3* and *DSP* were expressed in the different cell lines derived from infertile tissue. (B) Transepithelial resistance (TER) was measured in IHCE1 cells at different time points after switching from low to normal calcium containing medium with a final concentration of 1.8 mM (n=3, ± SEM). Results shown are from a representative experiment that was repeated at least three times. No peak in TER was seen after calcium-switch, in contrast to FHCE1 cells [37]. *, $P < 0.05$ versus IHCE1, ***, $P < 0.001$ versus IHCE1. Neg, negative control; Epid, epididymis.

Figure 8: Analysis of differential gene expression in IHCE1 and FHCE1 cells. (A) Scatterplot comparing genes expressed in both cell lines. Axes of the scatterplot represent the log scale of the mean (n=3) fluorescence intensity value minus the background intensity values for each cell line. The middle line indicates values that represent a ratio of 1.0 (similar levels of expression in both type of cells). The outer lines represent a ratio of 2.0 (upper line; 2-fold greater expression in IHCE1 cells compared with FHCE1 cells) and 0.5 (lower line; 2-fold lower expression in IHCE1 cells compared with FHCE1 cells). (B) Confirmation of microarray results by quantitative real-time RT-PCR with selected genes. Messenger RNA expression levels of *CDC42* and *PRKCA* in IHCE1 and FHCE1 cells were investigated and compared with both microarray results and real-time PCR data. Data are expressed as the ratio of the mRNA levels in IHCE1 cells in relation to FHCE1 cells. Values represent the mean relative expression ± SEM (n=3). (C) Functional classification of differentially expressed genes by at least 2-fold in IHCE1 cells compared to FHCE1 cells. The bars in white represent the downregulated genes, whereas the bars with lines represent the upregulated genes.

Figure 9: Schematic diagram indicating various signaling pathways that appear to be involved in the regulation of epididymal cellular junctions and which may be altered in obstructive azoospermia based on the RT-PCR and microarray analyses. TJ, tight junction; AJ, adherens junction; GJ, gap junction; CLDN, claudin; CDH, cadherin; CTNN, catenin; PANX, pannexin; SYMPL, symplekin; PKC, protein kinase C; PKA, protein kinase A; cdc42, cell division control protein 42; ROCK1, Rho-associated, coiled-coil containing protein kinase 1; MAPK, mitogen-activated protein kinase; JNK, Jun N-terminal kinase; CSK, c-src tyrosine kinase; ILK, integrin linked kinase; GSK3β, glycogen synthase kinase 3 beta; LEF-1, lymphoid enhancer-binding factor 1; JAK, Janus kinase; AKT/PKB, protein kinase B.

Table 1: Description of the patients from which portions of the epididymidis were collected and used in this study. OA, obstructive azoospermia; nd, not determined; IU, international units.

Patient	Fertility status	Age (years)	FSH (IU/L)	Testosterone (nmol/L)	LH (IU/L)	Estradiol (pmol/L)
1	Fertile	36	11	Nd	4.4	56
2	Fertile	20	0.2	25.4	0.2	350
3	Fertile	28	nd	Nd	nd	nd
4	Fertile	26	nd	Nd	nd	nd
5	Fertile	41	4.1	8.1	4.5	113
6	OA (corpus blockage)	43	4	24	nd	50
7	OA (corpus blockage)	37	5	16.5	3	77
8	OA (corpus blockage)	37	5.4	16	3.5	147
9	OA (caput blockage)	37	6.8	16.5	4.4	95
10	OA (proximal caput blockage)	27	nd	Nd	nd	nd

Table 2: Description of the different human epididymal cell lines developed in this study. IHCE, Infertile Human Caput Epididymal cell line; OA, obstructive azoospermia.

Name of the cell line	Type of patient	Epididymal region
IHCE1	Infertile (OA)	Proximal caput
IHCE2	Infertile (OA)	Proximal caput
IHCE3	Infertile (OA)	Proximal caput
IHCE4	Infertile (OA)	Proximal caput
IHCE5	Infertile (OA)	Proximal caput

Table 3: Sequences of the primers used for PCR.

Gene	Primer set (5'-3')	Tm (°C)	Amplicon size (bp)
RARA1	F : GACAAGTCCTCAGGCTACCACTAT R : GCGAACTCCACAGTCTTAATGAT	58	452
RARA2	F :CCCCTAATCCCTTCCTAGTGGTG R : CCTTCTTCTTCTTGTTTCGGTCGT	60	456
RARB1	F : TGAAATGACAGCTGAGTTGGACGA R : AGCTGGTTGGCAAAGGTGAACAC	58	414
RARB2	F : TGAAATGACAGCTGAGTTGGACGA R : AGCTGGTTGGCAAAGGTGAACAC	58	414
RARG1	F :AGAAGGGTCACCTGACAGCTATGA R : CTCCACGATCTTGATGATGCACT	58	212
RARG2	F : AGAAGGGTCACCTGACAGCTATGA R : ACTCCACGATCTTGATGATGCAC	58	212
THRA1	F : AGCAAGGTGGAGTGTGGGTCAGAC R : AAGTGATACAGCGGTAGTGATA	54	193
THRA2	F : TGTTCCCTGAAAACCAGCATGTCA R : ACAAGTGATACAGCGGTAGTGATA	53	114
THRB	F : ATAAACTATTCCTTTGCCTACCTT R : GGGCCATACTTCTTGTCTAAT	50.7	375
ACTB	F : TCTGTGTGGATTGGTGGCTCTA R : CTGCTTGCTGATCCACATCTG	54	69
CDC42	F : TCCCCATCTGGTGCTCTTAG R : TGGCAAACAAATGTCCTTGA	60	204
PRKCA	F : AGC AGGAGAGCGTGAAAGAA R : TCTTCATGGCATAGTGGTTCC	60	186
CDH1	F : TGAAGGTGACAGAGCCTCTGGAT R : TGGGTGAATTCGGGCTTGTT	60	151
TJP1	F : GTGGTTCTTCGAGAAGCTGG R : AGCGTCTCGTGGTTCACTCT	60	132
CLDN1	F : CCGTTGGCATGAAGTGTATG R : CCAGTGAAGAGAGCCTGACC	60	208
CLDN4	F: CTTCTACAATCCGCTGGTGG R: TTACACGTAGTTGCTGGCAG	65	193
CLDN7	F: CCCTTTGATCCCTACCAACA R: GGACAGGAACAGGAGAGCAG	60	112
CLDN10	F: CCACGCTGCCCACCGACTA R: TGAGCACAGCCCTGACAGTATGAA	65	326
GAPDH	F: GAA GGT GAA GGT CGG AGT CAA R: GGA AGA TGG TGA TGG GAT TTC	55.1	227

Table 4: Differentially regulated genes encoding junctional proteins. Results are expressed as the ratio of the relative expression of the gene in the IHCE1 cell line compared to the relative expression of the gene in the FHCE1 cell line (n=3; $p<0.05$). nd, not determined.

Gene name	Fold change	GenBank Accession Number
PCDHGA8	4.863	NM_032088
GJC1	4.569	NM_152219
CTNNB1	4.313	NM_001904
PANX3	2.737	NM_052959
CLDN9	2.631	NM_020982
PCDH18	2.221	NM_019035
CLDN11	2.209	NM_005602
GJB4	2.014	NM_153212
CLDN18	2	NM_016369
CDHR2	1.883	NM_017675
CLDN17	1.852	nd
PCDHB11	1.839	NM_018931
GJA12	1.781	NM_020435
CDHR5	1.775	NM_031264
PCDHB13	1.765	NM_018933
CDH24	1.562	NM_022478
CTNNBIP1	1.556	NM_020248
CDH26	1.419	NM_021810
CTNNA3	1.386	NM_013266
SYMPK	0.696	NM_004819
CDH2	0.448	NM_001792
CTNNA1	0.222	NM_001903
CTNNAL1	0.0969	NM_003798

Table 5: Upregulated genes expressed by IHCE1 cells compared to FHCE1 cells that could be implicated in the regulation of cellular junctions. Results are expressed as the ratio of the relative expression of the gene in the IHCE1 cell line compared to the relative expression of the gene in the FHCE1 cell line (n=3; $p<0.05$).

GENE NAME	Fold change	GenBank Accession Number
JUNB	6.965	NM_002229
JUND	5.514	NM_005354
APC	4.536	NM_000038
RAB36	3.848	NM_004914
MOS	3.383	NM_005372
RAB2	3.551	NM_0022865
NOTCH3	3.319	NM_000435
RAP1A	3.059	NM_001010935
HRAS	2.916	NM_005343
SCRIB	2.897	NM_182706
RAB10	2.885	NM_016131
ILK	2.833	NM_001014795
HRAS	2.665	NM_005343
WIF1	2.616	NM_007191
SNAI2	2.447	NM_003068
APC2	2.414	NM_005883
MAP2K3	2.384	NM_145110
RAB21	2.374	NM_014999
NOTCH2	2.352	NM_024408
FZD5	2.283	NM_003468
PRKCA	2.15	NM_002730
RHOA	2.14	NM_001664
CSK	2.064	NM_004383
WNT2	2.015	NM_003391
PRKCDBP	1.957	NM_145040
FZD1	1.954	NM_003505
PKCD	1.896	NM_006254
RHOG	1.762	NM_001665
FZD2	1.752	NM_001466
RRAS2	1.708	NM_012250
RAP1B	1.68	NM_015646
JAK1	1.535	NM_002227
JAK3	1.517	NM_000215
FZD8	1.483	NM_031866
TWIST1	1.415	NM_000474
ROCK1	1.322	NM_005406
RAC3	1.291	NM_005052

Table 6: Downregulated genes expressed by IHCE1 cells compared to FHCE1 cells that could be implicated in the regulation of cellular junctions. Results are expressed as the ratio of the relative expression of the gene in the IHCE1 cell line compared to the relative expression of the gene in the FHCE1 cell line ($n=3$; $p<0.05$).

Gene name	Fold change	GenBank Accession Number
MYC	0.547	NM_002467
MAP2K2	0.539	NM_030662
PRKAG2	0.484	NM_016203
RAC1	0.454	NM_198829
RAB34	0.367	NM_031934
JUN	0.367	NM_002228
PRKCZ	0.366	NM_002744
RAB18	0.333	NM_021252
RAB22A	0.326	NM_020673
DVL1	0.315	NM_181870
RAB32	0.269	NM_006834
RAB13	0.235	NM_002870
RHOC	0.147	NM_175744
CDC42	0.141	NM_001791
PRKAR1A	0.0804	NM_212472

Table 7: Differentially regulated genes expressed by IHCE1 cells compared to FHCE1 cells that are known to be expressed by the epididymis. Results are expressed as the ratio of the relative expression of the gene in the IHCE1 cell line compared to the relative expression of the gene in the FHCE1 cell line (n=3; $p<0.05$).

Gene name	Fold change	GenBank Accession Number
WFDC2/HE4	16.72	NM_080733
AKAP1	5.394	NM_139275
AQP5	2.801	NM_001651
ADAMTS10	2.731	NM_030957
ADAM19	2.659	NM_033274
ADAM15	1.83	NM_207191
CTSL	0.626	NM_001912
CTSZ	0.333	NM_001336
DCXR/P34H	0.252	NM_001757
CTSB	0.11	NM_147780
MIF	0.0993	NM_002415

Figure 1

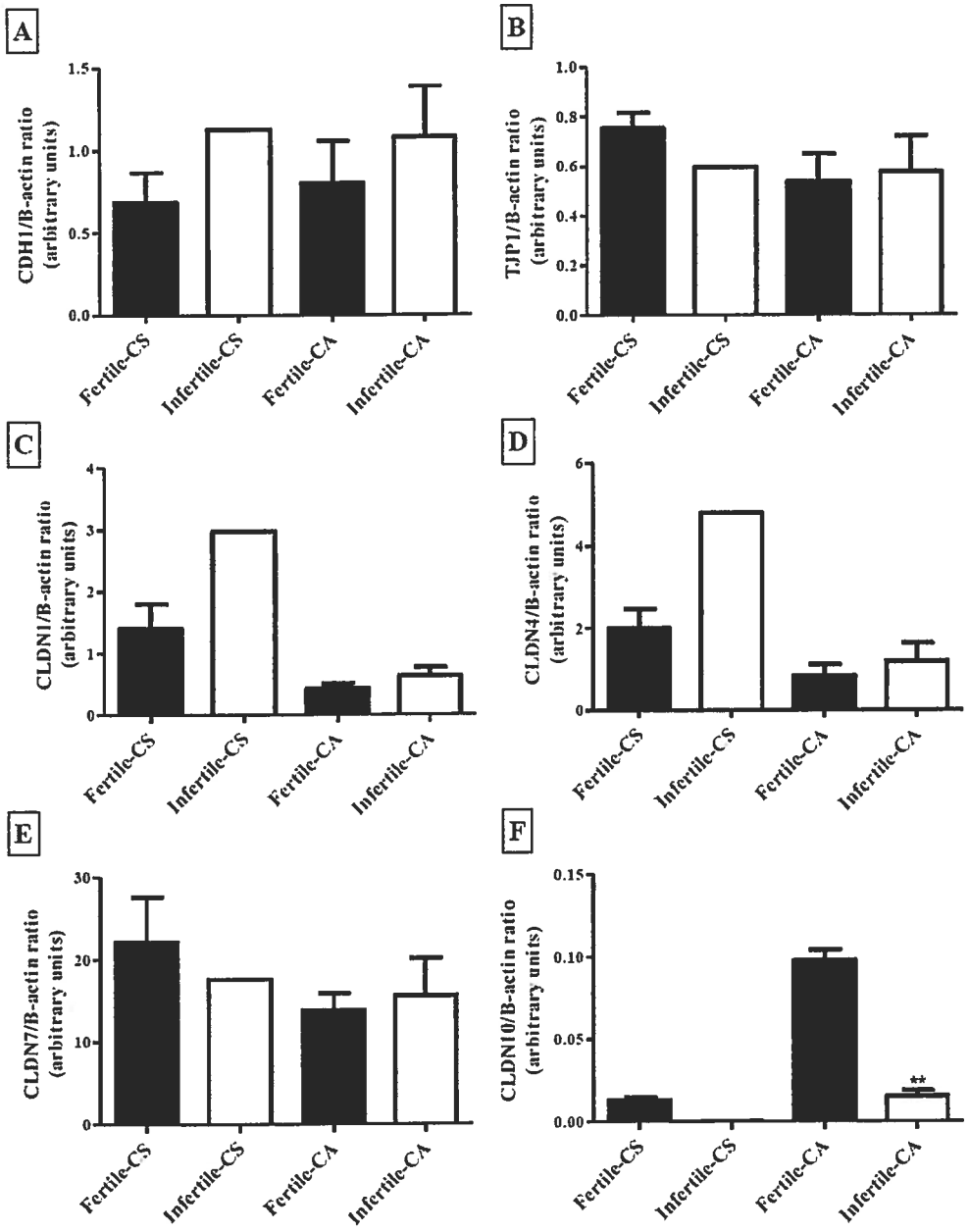

A
CDH1/B-actin ratio (arbitrary units)
1.5
1.0
0.5
0.0
Fertile-CS
Infertile-CS
Fertile-CA
Infertile-CA
B
TJP1/B-actin ratio (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
Fertile-CS
Infertile-CS
Fertile-CA
Infertile-CA
C
CLDN1/B-actin ratio (arbitrary units)
4
3
2
1
0
Fertile-CS
Infertile-CS
Fertile-CA
Infertile-CA
D
CLDN4/B-actin ratio (arbitrary units)
6
4
2
0
Fertile-CS
Infertile-CS
Fertile-CA
Infertile-CA
E
CLDN7/B-actin ratio (arbitrary units)
30
20
10
0
Fertile-CS
Infertile-CS
Fertile-CA
Infertile-CA
F
CLDN10/B-actin ratio (arbitrary units)
0.15
0.10
0.05
0.00
**
Fertile-CS
Infertile-CS
Fertile-CA
Infertile-CA

Figure 2

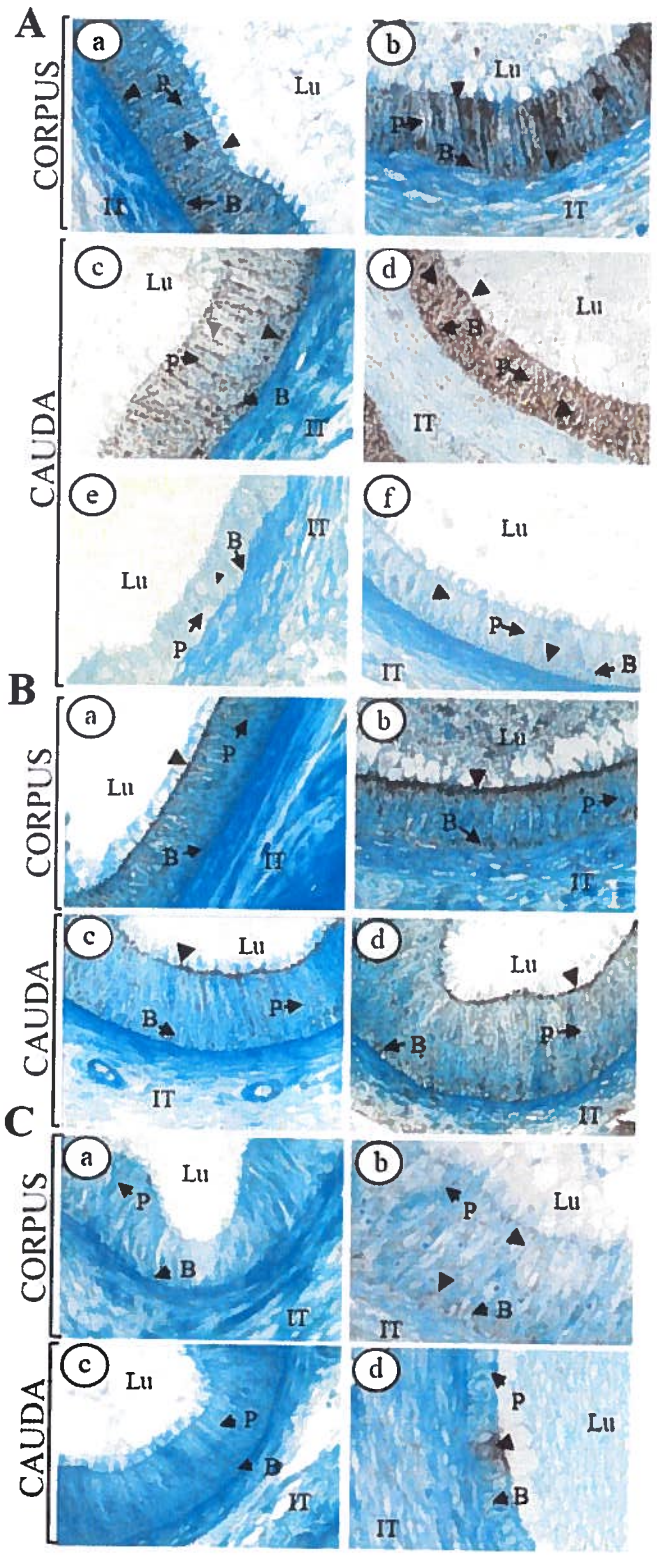

A
CORPUS
a
Lu
P
B
IT
b
Lu
P
B
IT
CAUDA
c
Lu
P
B
IT
d
Lu
B
P
IT
e
B
IT
Lu
P
f
Lu
P
IT
B
B
CORPUS
a
Lu
P
B
IT
b
Lu
P
B
IT
CAUDA
c
Lu
P
B
IT
d
Lu
P
B
IT
C
CORPUS
a
Lu
P
B
IT
b
P
Lu
B
IT
CAUDA
c
Lu
P
B
IT
d
P
Lu
B
IT

Figure 3

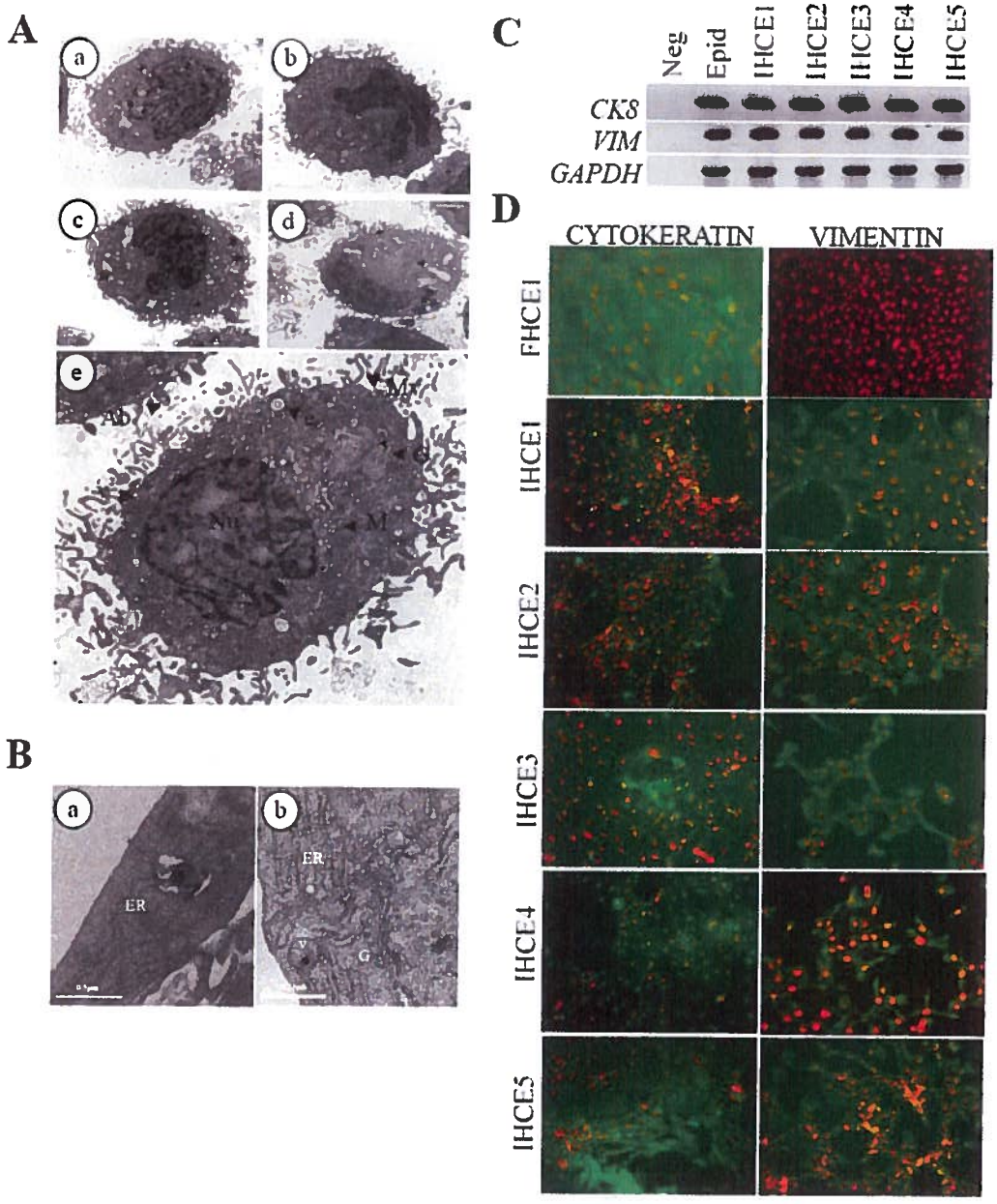

A
a
b
c
d
e
Ab
Mx
Nu
M
B
a
b
ER
ER
v
G
C
Neg
Epid
IHCE1
IHCE2
IHCE3
IHCE4
IHCE5
CK8
VIM
GAPDH
D
CYTOKERATIN
VIMENTIN
FHCE1
IHCE1
IHCE2
IHCE3
IHCE4
IHCE5

Figure 4

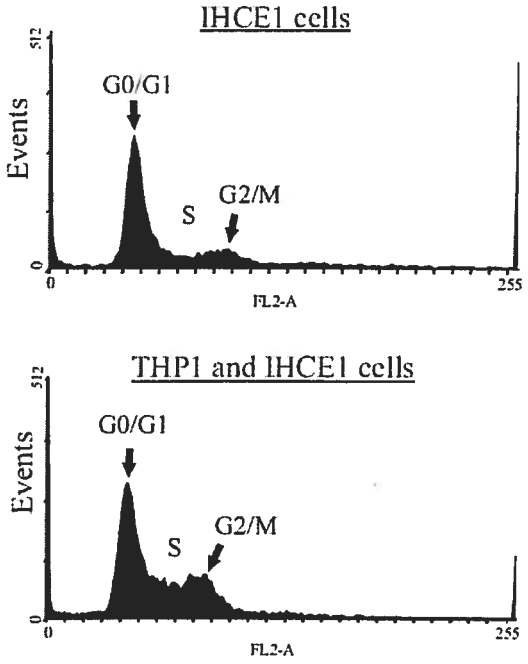

lHCE1 cells
512
Events
0
G0/G1
S
G2/M
0
255
FL2-A
THP1 and lHCE1 cells
512
Events
0
G0/G1
S
G2/M
0
255
FL2-A

Figure 5

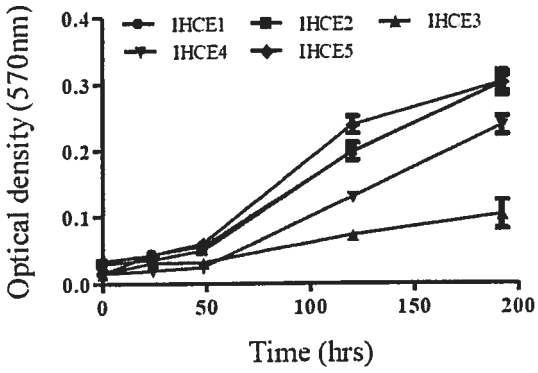

IHCE1
IHCE2
IHCE3
IHCE4
IHCE5
Optical density (570nm)
0.4
0.3
0.2
0.1
0.0
0
50
100
150
200
Time (hrs)

Figure 6

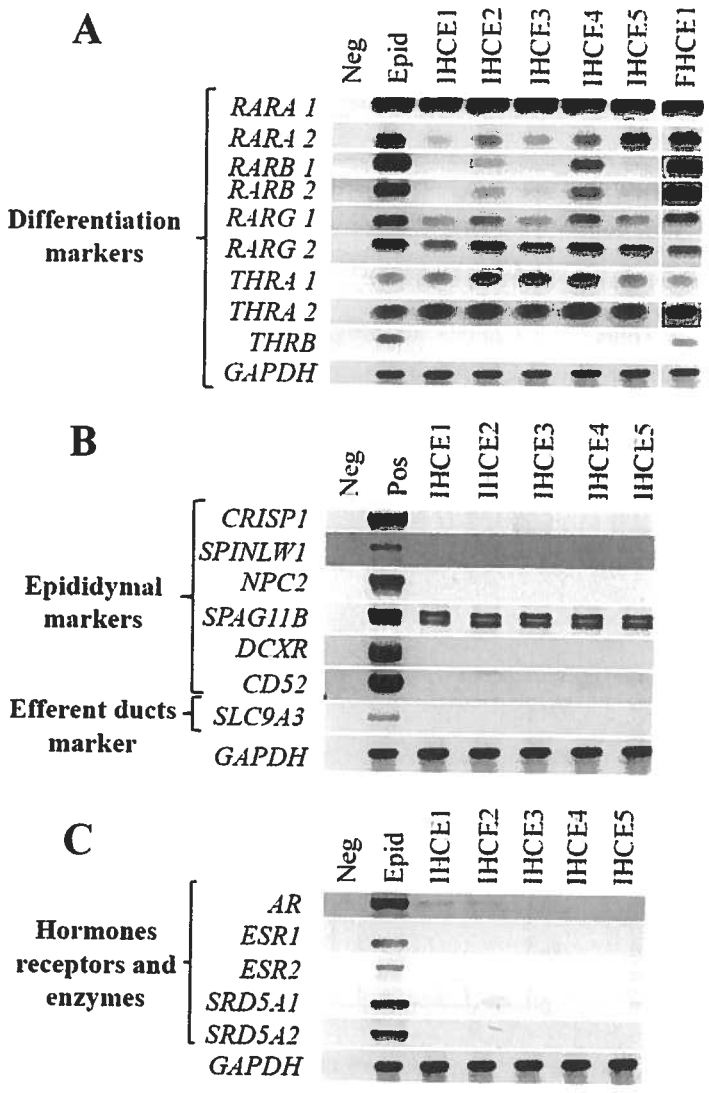

A
Neg
Epid
IHCE1
IHCE2
IHCE3
IHCE4
IHCE5
FHCE1
Differentiation markers
RARA 1
RARA 2
RARB 1
RARB 2
RARG 1
RARG 2
THRA 1
THRA 2
THRB
GAPDH
B
Neg
Pos
IHCE1
IHCE2
IHCE3
IHCE4
IHCE5
Epididymal markers
CRISP1
SPINLW1
NPC2
SPAG11B
DCXR
CD52
Efferent ducts marker
SLC9A3
GAPDH
C
Neg
Epid
IHCE1
IHCE2
IHCE3
IHCE4
IHCE5
Hormones receptors and enzymes
AR
ESR1
ESR2
SRD5A1
SRD5A2
GAPDH

Figure 7

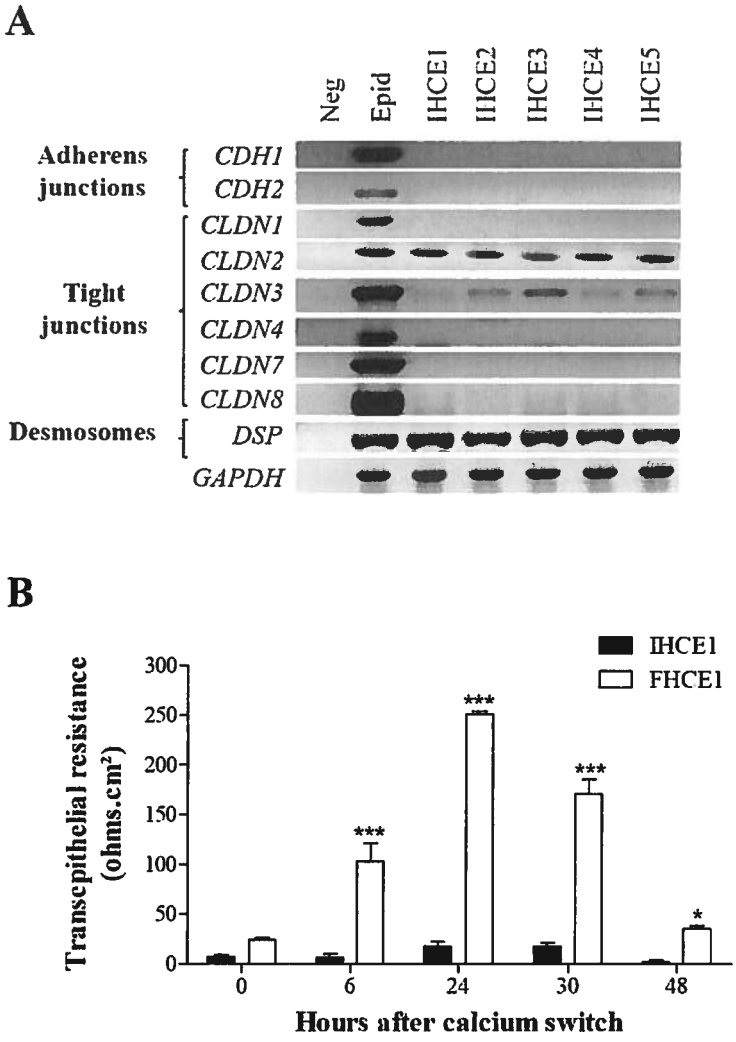

A
Neg
Epid
IHCE1
IHCE2
IHCE3
IHCE4
IHCE5
Adherens junctions
CDH1
CDH2
Tight junctions
CLDN1
CLDN2
CLDN3
CLDN4
CLDN7
CLDN8
Desmosomes
DSP
GAPDH
B
IHCE1
FHCE1
Transcpithelial resistance (ohms.cm²)
300
250
200
150
100
50
0
***
***
***
*
0
6
24
30
48
Hours after calcium switch

Figure 8

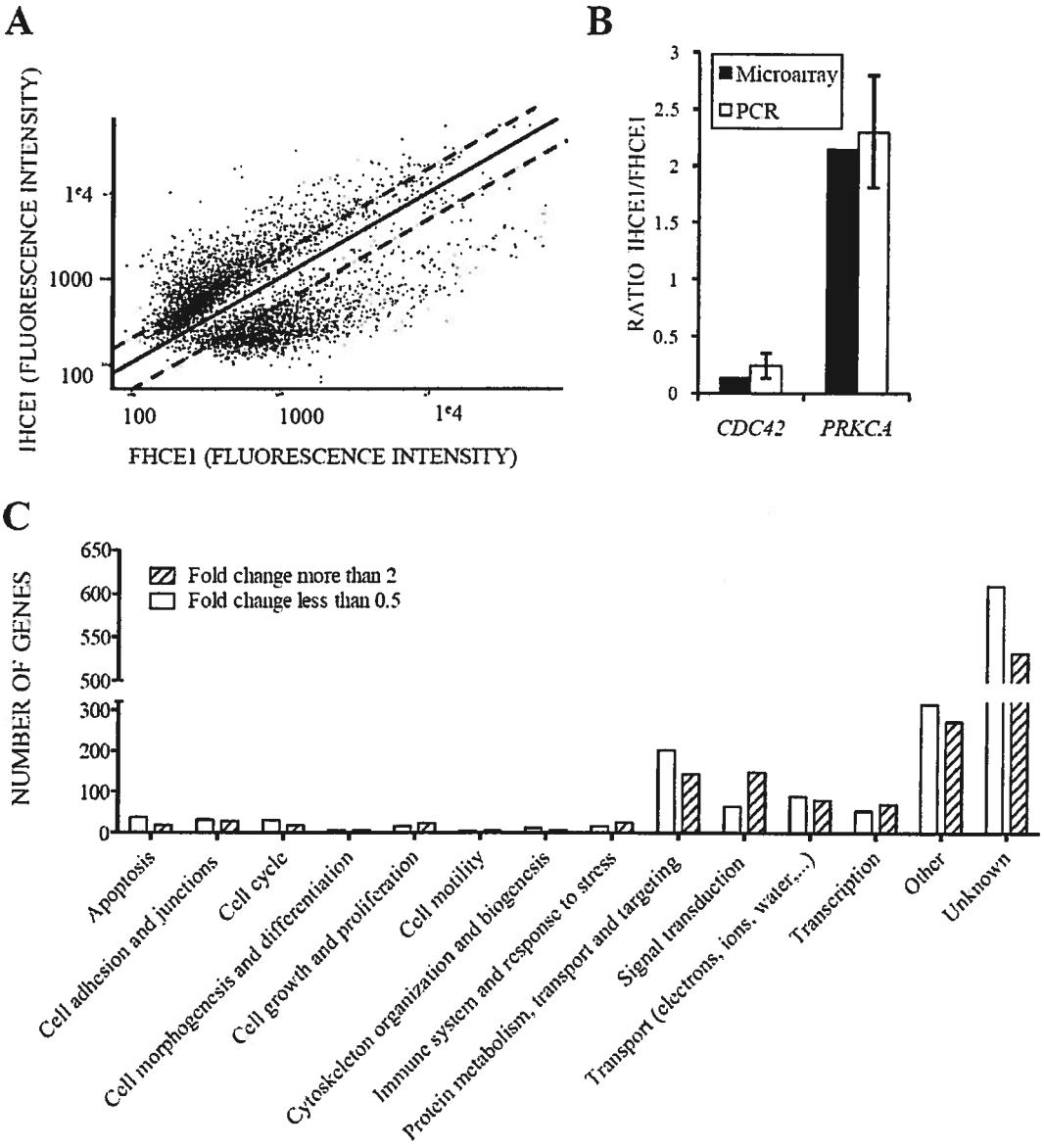
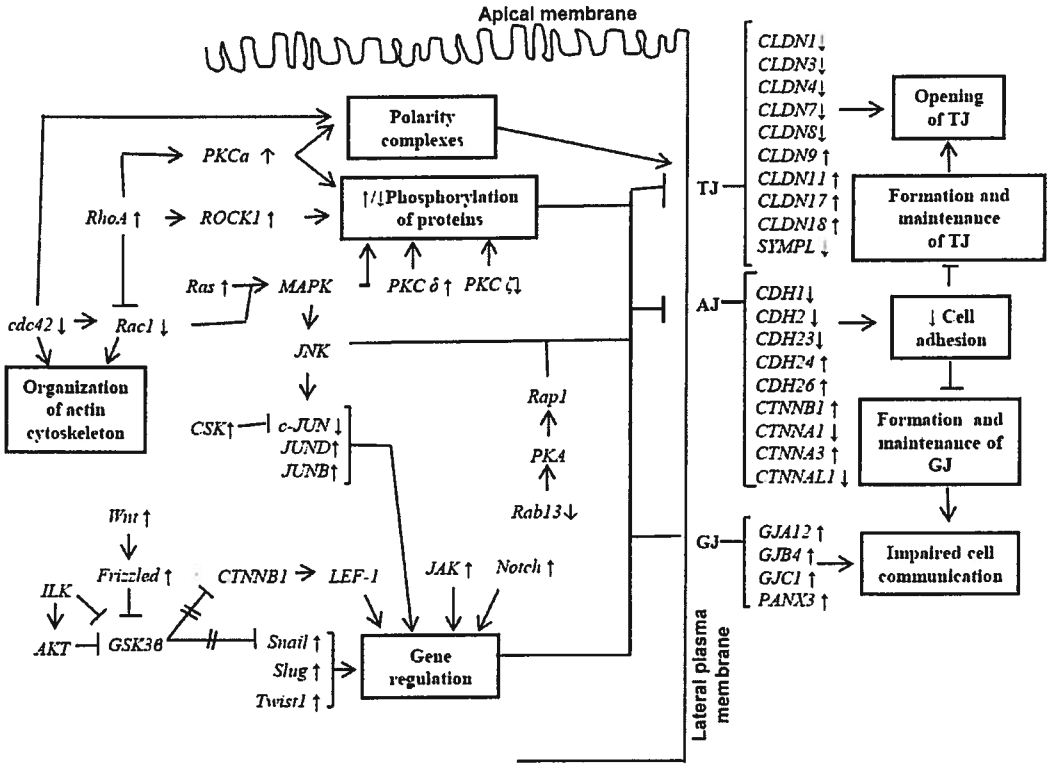

A
IHCE1 (FLUORESCENCE INTENSITY)
1e4
1000
100
100
1000
1e4
FHCE1 (FLUORESCENCE INTENSITY)
B
Microarray
PCR
RATIO IHCE1/FHCE1
3
2.5
2
1.5
1
0.5
0
CDC42
PRKCA
C
NUMBER OF GENES
650
600
550
500
300
200
100
0
Fold change more than 2
Fold change less than 0.5
Apoptosis
Cell adhesion and junctions
Cell cycle
Cell morphogenesis and differentiation
Cell growth and proliferation
Cell motility
Cytoskeleton organization and biogenesis
Immune system and response to stress
Protein metabolism, transport and targeting
Signal transduction
Transport (electrons, ions, water,...)
Transcription
Other
Unknown

Figure 9


Apical membrane
Polarity complexes
PKCa ↑
↑/↓Phosphorylation of proteins
RhoA ↑
ROCK1 ↑
Ras ↑
MAPK
PKC δ ↑
PKC ζ↓
cdc42 ↓
Rac1 ↓
JNK
Organization of actin cytoskeleton
CSK↑
c-JUN ↓
JUND↑
JUNB↑
Rap1
PKA
Rab13↓
Wnt ↑
Frizzled ↑
CTNNB1
LEF-1
JAK ↑
Notch ↑
ILK
AKT
GSK3β
Snail ↑
Slug ↑
Twist1 ↑
Gene regulation
Lateral plasma membrane
TJ
CLDN1↓
CLDN3↓
CLDN4↓
CLDN7↓
CLDN8↓
CLDN9 ↑
CLDN11 ↑
CLDN17 ↑
CLDN18 ↑
SYMPL ↓
Opening of TJ
Formation and maintenance of TJ
AJ
CDH1↓
CDH2 ↓
CDH23↓
CDH24 ↑
CDH26 ↑
CTNNB1 ↑
CTNNA1 ↓
CTNNA3 ↑
CTNNAL1 ↓
↓ Cell adhesion
Formation and maintenance of GJ
GJ
GJA12 ↑
GJB4 ↑
GJC1 ↑
PANX3 ↑
Impaired cell communication

CONCLUSIONS ET DISCUSSION GÉNÉRALES

L'infertilité masculine touche un grand nombre de couples consultant pour des problèmes de fertilité [233]. La baisse de fertilité associée à des altérations des jonctions épididymaires des rats âgés [13] nous a laissé suspecter qu'un dysfonctionnement de la barrière hémato-épididymaire pouvait être impliqué dans l'infertilité masculine humaine. Les jonctions serrées permettent, en effet, de protéger les spermatozoïdes en formant différentes barrières cellulaires le long du tractus reproducteur mâle telles que la barrière hémato-testiculaire [234], la barrière hémato-épididymaire [151] et la barrière hémato-prostatique [235]. Le but de notre étude était de déterminer si la barrière hémato-épididymaire était compromise chez des hommes infertiles souffrant d'azoospermie.

Dans un premier temps, nous avons démontré que de nombreux gènes codant pour des protéines impliquées dans les jonctions adhérentes (Figure 6) et serrées (Figure 7) sont exprimées le long de l'épididyme humain grâce à l'utilisation des microréseaux [236]. On peut, entre autres, citer CDH1, CTNNA1, CTNNB1, TJP1 et les CLDN1, 3, 4, 7. La technique des microréseaux nous a permis d'étudier le patron d'expression de ces gènes dans l'épididyme humain malgré la difficulté d'obtenir du tissu en grande quantité, cependant des études additionnelles au niveau du protéome seraient importantes afin de compléter ces données. En effet, le niveau d'expression des ARNm n'est pas toujours corrélé au niveau d'expression des protéines correspondant à ces ARNm. On peut, par exemple, parler des modifications post-traductionnelles que peuvent subir les CLDNs telle que la phosphorylation [114]. Ces études seraient maintenant possibles grâce au développement de plusieurs anticorps dirigés contre les protéines jonctionnelles et le développement des biopuces à protéines permettant d'étudier plusieurs protéines en parallèle en utilisant une quantité de tissu moins importante que les techniques classiques d'immunobuvardage de type western. De plus, l'utilisation des lignées cellulaires va permettre d'élucider le rôle fonctionnel des différentes protéines jonctionnelles dans le maintien de la barrière et donc, indirectement, dans la composition et le maintien du milieu intraluminal de l'épididyme humain.

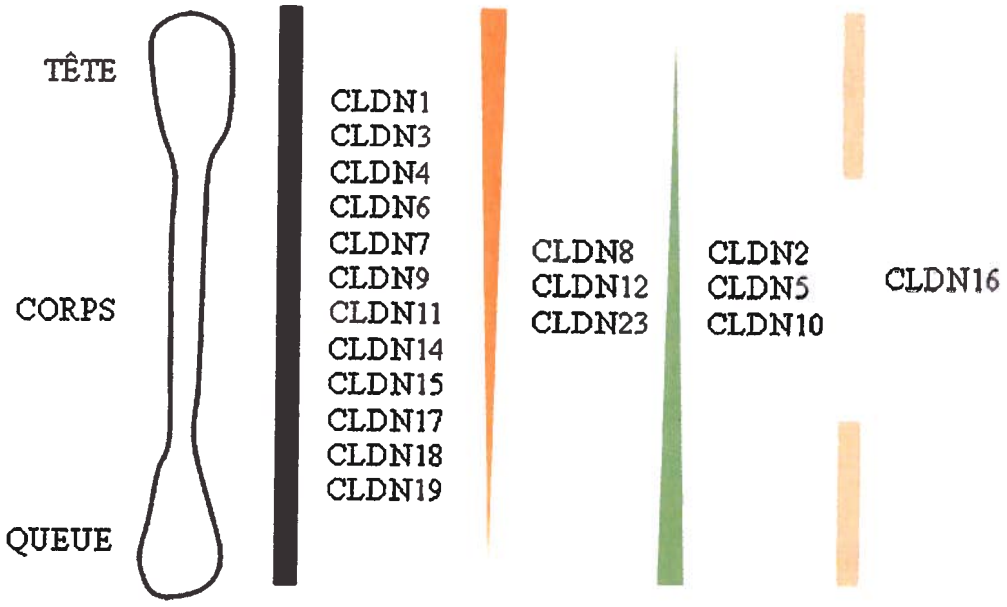


Figure 6 : Patron d'expression des CLDNs dans l'épididyme humain. De nombreuses CLDNs sont exprimées dans l'épididyme humain (CLDNs 1 à 12, 14 à 19, 23). La majorité est exprimée de manière similaire le long de l'épididyme (en noir) alors que d'autres ont un patron d'expression qui varie entre les différentes régions de l'épididyme : CLDNs 8, 12 et 23 sont plus exprimées dans la tête que dans la queue de l'épididyme (en orange) alors que CLDNs 2, 5 et 10 sont plus exprimées dans la queue que dans la tête (en vert). Quant à CLDN16 (en rose saumon), elle est exprimée uniquement dans la tête et la queue de l'épididyme.

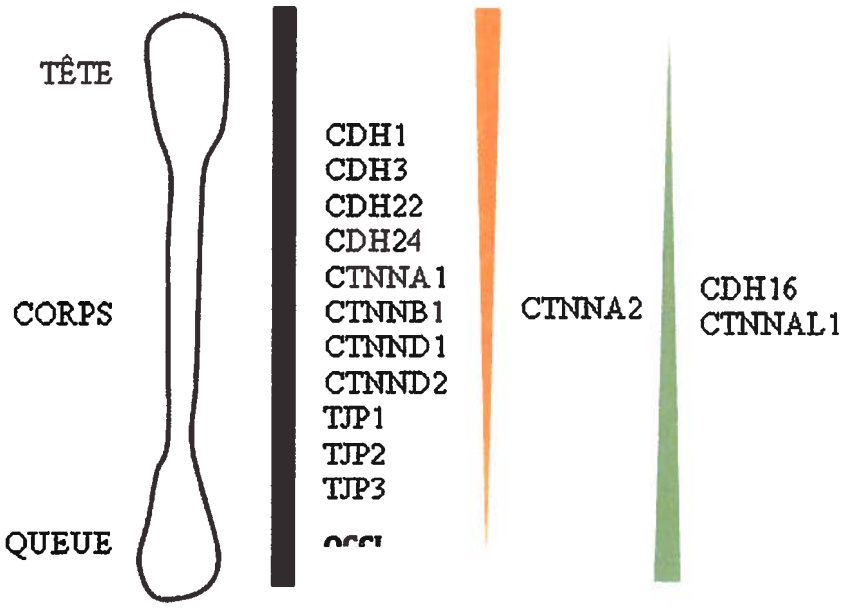


Figure 7 : Patron d'expression des CDHs, des CTNNs, des TJPs et de l'occludine dans l'épididyme humain. De nombreuses CDHs, CTNNs et TJPs ainsi que l'occludine sont exprimées dans l'épididyme humain. La majorité est exprimée de manière similaire le long de l'épididyme (en noir) alors que d'autres ont un patron d'expression qui varie entre les différentes régions de l'épididyme : CTNNA2 est plus exprimée dans la tête que dans la queue de l'épididyme (en orange) alors que CDH16 et CTNNAL1 sont plus exprimées dans la queue que dans la tête (en vert).

Plusieurs études démontrent que les CLDN1 [237, 238], CLDN3 [239, 240], CLDN4 [239, 241-243], CLDN7 [242, 244, 245] , CLDN14 [12, 246] et CLDN18 [247] sont impliquées dans l'étanchéité des jonctions serrées, et ce, dans plusieurs modèles cellulaires en ce qui concerne les CLDN1, 3, 4 et 7. Nous l'avons également observé dans notre lignée cellulaire développée à partir de la tête de l'épididyme d'un patient fertile. L'effet de l'inhibition de chacune de ces CLDNs sur la résistance transépithéliale suggère que des altérations locales dans l'expression de certains gènes ou des protéines correspondantes sont susceptibles d'affecter la composition du milieu luminal. Le rôle de ces CLDNs dans la fonction de barrière pourrait aussi expliquer la nécessité de leur présence le long de l'épididyme. De plus, ces CLDNs ne sont pas exclusivement localisées aux jonctions serrées épididymaires, mais aussi le long des membranes plasmiques des cellules épithéliales [11, 236]. Cette localisation suggère d'autres rôles, comme par exemple au niveau de l'adhésion cellulaire. Plusieurs études semblent indiquer que les CLDNs seraient impliquées dans d'autres processus que la formation et le maintien des jonctions serrées. Par exemple, CLDN7 serait impliquée dans la régulation de CDH1 [199] et de PSA [248] alors que CLDN4 aurait des propriétés angiogéniques [241]. Les CLDN6, 11 et 15 sont également impliquées dans la fonction de barrière des jonctions serrées mais leur rôle précis dépend du modèle cellulaire utilisé [6, 239, 243, 249]. Des différences dans le patron d'expression de plusieurs gènes (CDH16, CTNNA2, CTNNAL1, CLDN2, 5, 8, 10, 12, 16 et 23) ont également été observées entre les régions épididymaires. On peut en conclure que les caractéristiques de la barrière hémato-épididymaire changent le long du tubule. Parmi ces protéines, les CLDN2, 10 et 12 favorisent l'ouverture des jonctions serrées [242, 250-252] alors que la CLDN8 favorise l'étanchéité de la barrière [253, 254]. L'expression segment-spécifique de plusieurs CLDNs suggère que la barrière n'a pas la même perméabilité le long de l'épididyme humain tel que cela a été démontré chez le rat [75]. Il ne faut pas oublier que les caractéristiques d'une jonction dépendent de l'interaction entre les différentes CLDNs présentes au sein du complexe jonctionnel [116, 255]. De plus, certaines CLDNs pourraient être présentes dans l'épididyme afin de compenser les effets d'autres CLDNs. En effet, une étude a observé que l'expression de la CLDN8 pouvait compenser les effets

de la CLDN2 en réduisant son expression et sa localisation aux jonctions serrées, et ainsi améliorer la fonction de barrière [256].

La composition de la barrière hémato-épididymaire joue aussi un rôle dans la composition du microenvironnement intraluminal épididymaire. En effet, les CLDN2, 12 et 15 contribuent à la création de pores permettant le passage des cations (Na^+, Ca^{2+}) alors que les CLDN4, 8, 9, 11, 14 et 18 empêchent l'entrée des cations (Na^+, H^+, $NH4^+$) et que les CLDN4 et 8 permettent l'entrée des anions (Cl^-, $HCO3^-$) [12, 112, 115, 239, 242, 243, 247, 249-251, 254, 257]. Les CLDN7 et 10 permettent également la création de pores sélectifs mais les résultats obtenus varient selon le modèle cellulaire ou l'isoforme pour CLDN10 [105, 244, 245, 258]. Quant à CLDN16, elle joue un rôle dans le transport du magnésium et du calcium [259-261]. Plusieurs de ces ions sont présents dans la lumière épididymaire et leur concentration varie entre les différentes régions de l'épididyme, mais aussi entre la lumière et le sang (Tableau 1). En participant à la création de ces gradients, les CLDNs jouent un rôle important dans la création d'un milieu propice à la survie des spermatozoïdes. En effet, il a été suggéré que ces gradients d'ions contribueraient au développement de la motilité et au maintien des spermatozoïdes dans un état quiescent pour éviter toute activation précoce qui les conduirait à leur mort [17]. Une perturbation de ces gradients pourrait conduire à l'infertilité masculine.

Tableau 1 : Concentrations approximatives de différents ions dans le sang et dans les différents compartiments intraluminaux de l'épididyme de rat. Les données ont été extraites de Robaire and Hermo, 1988 [3]. Les valeurs sont exprimées en mEq/L.

Ions	Sérum	Segment initial	Tête	Corps	Queue
Sodium	135	120	110	87	40
Potassium	5	10	20	27	27
Chlorure	125	125	25	30	25
Phosphore	5	5	50	80	75
Calcium	1	2	1.5	1	1
Magnésium	1	1.5	1.5	2	1.5

Dans un deuxième temps, nous avons montré que l'infertilité masculine due à l'azoospermie non obstructive implique une perturbation de la barrière hémato-épididymaire, du transport paracellulaire et transcellulaire [262]. En effet, plusieurs gènes différentiellement exprimés dans la tête de l'épididyme de patients azoospermiques non obstructifs codent pour des protéines impliquées dans la maturation des spermatozoïdes, pour des canaux ioniques et aqueux ainsi que pour des β-défensines, comme par exemple *CFTR, AQP5, KCNK7, KCNK17, SLC6A20, SLC13A3, DEFB126* et *DEFB106A*. Plusieurs de ces gènes ont déjà été associés à l'infertilité masculine [263, 264]. De plus, la localisation de CLDN10 et de TJP1 change chez ces patients infertiles suggérant que la fonction de barrière et la perméabilité sélective des jonctions serrées sont compromises. En effet, TJP1 est impliqué dans la stabilisation des jonctions serrées [265]. La barrière hémato-épididymaire ainsi compromise pourrait laisser passer des anticorps dirigés contre les spermatozoïdes et affecter la composition du milieu intraluminal via le transport des ions Na^+ et Cl^-. Une baisse de CLDN10 a aussi été observée dans l'épididyme de souris infertiles, suite à une invalidation du gène HE6 [266, 267]. L'altération de l'expression ou de la localisation de CLDNs pourrait également affecter les fonctions des différents types cellulaires de l'épithélium épididymaire via la modification de leur environnement ionique.

Dans un troisième temps, nous avons démontré que la barrière hémato-épididymaire est compromise en cas d'azoospermie obstructive suite à une expression et/ou à une localisation aberrante de CDH1, TJP1, CLDN1, 4 et 10. Il a d'ailleurs été observé qu'après une vasectomie, qui est une cause fréquente d'obstructions de l'épididyme, l'expression des CLDNs 8 et 10 était altérée [230]. De nombreuses études rapportent que les niveaux d'expression de plusieurs CLDNs sont affectés en cas de cancer mais l'expression d'une même CLDN peut être augmentée ou diminuée dépendamment du type de cancer [268]. Les mécanismes moléculaires qui pourraient expliquer ces résultats variables ne sont pas connus. Il est cependant intéressant de constater que CLDN10 est la seule CLDN dont l'expression diminue chez tous les patients azoospermiques obstructifs. En comparant l'expression des gènes dans nos lignées dérivées de la tête de l'épididyme

d'un patient fertile et de celle d'un patient souffrant d'azoospermie obstructive, nous nous sommes rendu compte que les jonctions adhérentes et communicantes sont aussi affectées en cas d'azoospermie obstructive ainsi qu'une multitude de voies de signalisation. Ces données pourraient contribuer à expliquer pourquoi, même après une reconstruction chirurgicale, certains patients démontrent des signes de fertilité réduite [269, 270]. De plus, les différences observées entre les patients azoospermiques obstructifs suggèrent une régulation des jonctions complexe et spécifique à chaque segment. La comparaison de nos modèles cellulaires FHCE1 et IHCE1, qui ne possèdent pas la même capacité de former des jonctions serrées, nous permet d'identifier des candidats qui pourraient être impliqués dans la régulation des jonctions serrées de la tête de l'épididyme (Figure 8) [155, 271]. Des études additionnelles sont essentielles afin de mieux comprendre les mécanismes exacts de régulation tant au niveau transcriptionnel que post-traductionnel. Une meilleure compréhension du rôle de chaque CLDN dans la barrière hémato-épididymaire pourrait également permettre de découvrir de nouvelles stratégies thérapeutiques pour traiter l'infertilité.

De plus, de nombreux cas d'infertilité masculine sont dûs à des infections du système reproducteur mâle. Ces infections, qui sont détectées dans 15% des cas, peuvent affecter les spermatozoïdes au cours de leur transport et de leur maturation [272]. Par exemple, *Chlamydia trachomatis* altère *in vitro* la motilité des spermatozoïdes [273] et une infection avec cette bactérie a été corrélée avec la production d'anticorps anti-spermatiques [274]. D'autres virus (le virus de l'herpès, les papillomavirus humains, le virus de l'immunodéficience humaine) ont également été associés à l'infertilité masculine [272]. La présence de ces agents pathogènes dans l'épididyme pourrait être due à une ouverture de la barrière hémato-épididymaire ou au fait que plusieurs protéines jonctionnelles peuvent servir de co-récepteurs à certains agents pathogènes. En effet, il a été démontré que les CLDN1, 3, 4, 6 et 9 servent de récepteurs pour différents agents pathogènes [275-279]. De plus, la composition de la barrière hémato-épididymaire pourrait expliquer pourquoi certaines régions épididymaires sont plus affectées que d'autres dans certaines infections [280, 281]. Ces interactions ont amené plusieurs

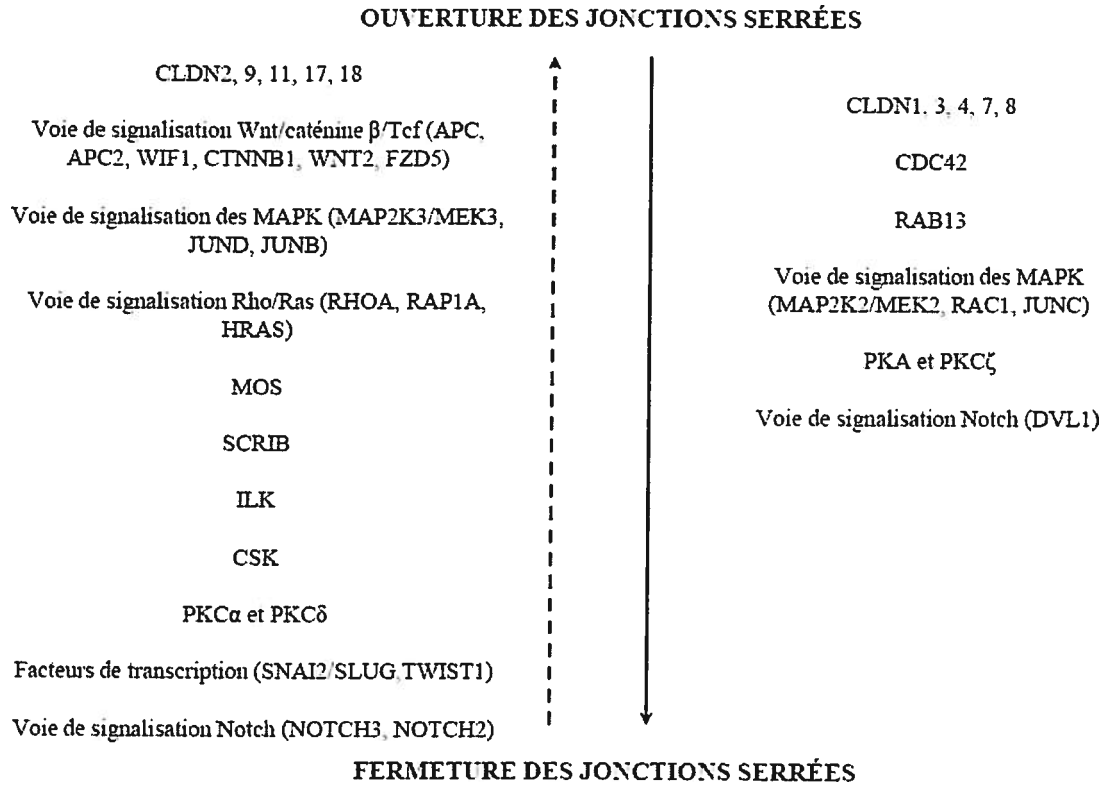


Figure 8 : Modèle proposé pour la régulation des jonctions serrées dans la tête de l'épididyme humain.

groupes de recherche à s'intéresser au développement de drogues qui agissent sur les jonctions serrées de manière réversible [282, 283]. Cette approche pourrait être explorée dans le cas de l'infertilité masculine ou de la contraception masculine. Il serait, par exemple, intéressant de développer des moyens de contraception masculine en couplant soit l'immunisation active contre des protéines épididymaires, soit l'utilisation d'inhibiteurs de protéines épididymaires à une modulation des jonctions serrées afin d'en augmenter l'efficacité. Plusieurs protéines épididymaires ont été identifiées comme cibles potentielles de la contraception masculine. On peut parler entre autres de P34H [284], CRISP1 [285], SPINLW1[286] et CD52 [287]. Il est intéressant de noter que ces mêmes gènes sont affectés chez les patients azoospermiques. Cependant, il serait important de cibler uniquement des protéines jonctionnelles dans l'épididyme afin d'éviter tout effet secondaire.

Dans l'ensemble, ces travaux ont permis de mieux comprendre l'implication de la barrière hémato-épididymaire dans le maintien de la fertilité masculine, de développer de nouveaux outils cellulaires et de considérer de nouvelles pistes pour traiter l'infertilité masculine et améliorer les techniques de contraception masculine.

CONTRIBUTION À L'AVANCEMENT DES CONNAISSANCES

Les résultats obtenus au cours de ce projet de doctorat contribuent à l'avancement des connaissances de plusieurs manières. Tout d'abord ils ont permis d'identifier les composantes de la barrière hémato-épididymaire humaine. Malgré un certain degré de conservation, il existe de nombreuses différences en terme d'expression et de localisation des protéines jonctionnelles entre l'humain et les rongeurs. De plus, la composition de la barrière semble beaucoup plus complexe chez l'humain que chez le rat. Parmi les protéines qui la composent, les CLDN1, 3, 4 et 7 sont essentielles pour sa fonction de barrière. Des études supplémentaires seraient nécessaires afin d'identifier les voies de régulation de la barrière hémato-épididymaire humaine. Une étude plus approfondie des différentes protéines telle que CTNNAL1 pourrait contribué à l'identification de nouvelles voies de signalisation qui régulent les fonctions de l'épididyme humain. Ces résultats ont permis de mettre en évidence de nouvelles causes de l'infertilité masculine humaine qui consistent en des perturbations de la fonction de barrière des jonctions serrées, du transport paracellulaire et du transport transcellulaire. Ces dysfonctionnements peuvent interférer avec le processus de maturation épididymaire des spermatozoïdes en exposant ces derniers aux attaques du système immunitaire, à différents produits toxiques et en changeant la composition du milieu intraluminal si important à leur survie. Finalement, ce projet de doctorat a conduit à la création de nouveaux outils biologiques. Il s'agit d'un important avancement dans l'étude de l'épididyme humain car il est très difficile d'obtenir des tissus en quantité considérable et de plus en plus de données suggèrent que les résultats obtenus à l'aide de modèles animaux ne sont pas toujours applicables à l'humain. De plus, ces lignées provenant de patients fertiles et infertiles ont permis d'identifier différentes voies de signalisation qui réguleraient les jonctions dans l'épididyme humain et qui pourraient être altérées en cas d'infertilité masculine humaine. Ces lignées pourraient servir à étudier un nombre incalculable d'aspects de la physiologie de l'épididyme humain, comme par exemple l'étude *in vitro* de la maturation des spermatozoïdes humains. Il s'agit donc d'un avancement majeur dans la compréhension de la fertilité masculine, qui pourrait conduire au développement de nouvelles stratégies thérapeutiques contre l'infertilité et à l'amélioration des techniques de fécondation *in vitro*.

RÉFÉRENCES

1. Spira, A., *Epidemiology of human reproduction.* Hum Reprod, 1986. **1**(2): p. 111-5.
2. Schoysman, R. and G. Segal-Bertin, *[Physiology and disorders of the epididymis].* Contracept Fertil Sex, 1995. **23**(3): p. 177-87.
3. Robaire, B. and L. Hermo, *Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation*, in *The Physiology of Reproduction*, E. Knobil and J. Neill, Editors. 1988, Raven Press: New York. p. 999-1080.
4. Sawada, N., et al., *Tight junctions and human diseases.* Med Electron Microsc, 2003. **36**(3): p. 147-56.
5. Mitic, L.L., C.M. Van Itallie, and J.M. Anderson, *Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins.* Am J Physiol Gastrointest Liver Physiol, 2000. **279**(2): p. G250-4.
6. Turksen, K. and T.C. Troy, *Permeability barrier dysfunction in transgenic mice overexpressing claudin 6.* Development, 2002. **129**(7): p. 1775-84.
7. Furuse, M., *Knockout animals and natural mutations as experimental and diagnostic tool for studying tight junction functions in vivo.* Biochim Biophys Acta, 2009. **1788**(4): p. 813-9.
8. Gregory, M., et al., *Claudin-1 is not restricted to tight junctions in the rat epididymis.* Endocrinology, 2001. **142**(2): p. 854-63.
9. Gregory, M. and D.G. Cyr, *Identification of multiple claudins in the rat epididymis.* Mol Reprod Dev, 2006. **73**(5): p. 580-8.
10. Guan, X., T. Inai, and Y. Shibata, *Segment-specific expression of tight junction proteins, claudin-2 and -10, in the rat epididymal epithelium.* Arch Histol Cytol, 2005. **68**(3): p. 213-25.
11. Inai, T., et al., *Claudin-7 expressed on lateral membrane of rat epididymal epithelium does not form aberrant tight junction strands.* Anat Rec (Hoboken), 2007. **290**(11): p. 1431-8.
12. Van Itallie, C.M. and J.M. Anderson, *The role of claudins in determining paracellular charge selectivity.* Proc Am Thorac Soc, 2004. **1**(1): p. 38-41.
13. Levy, S. and B. Robaire, *Segment-specific changes with age in the expression of junctional proteins and the permeability of the blood-epididymis barrier in rats.* Biol Reprod, 1999. **60**(6): p. 1392-401.
14. Vermeulen, A., *Clinical review 24: Androgens in the aging male.* J Clin Endocrinol Metab, 1991. **73**(2): p. 221-4.
15. Cooper, T.G., *Epididymis*, in *The Encyclopaedia of Reproduction*, E. Knobil and J.D. Neill, Editors. 1999, Academic Press: New York. p. 1-17.
16. Turner, T.T., *De Graaf's thread: the human epididymis.* J Androl, 2008. **29**(3): p. 237-50.
17. Robaire, B., B. Hinton, and M.-C. Orgebin-Crist, *The epididymis*, in *Knobil and Neill's Physiology of Reproduction*, J.D. Neill, Editor. 2006, Elsevier: New York. p. 1071-1148.

18. Johnston, D.S., et al., *The mouse epididymal transcriptome: transcriptional profiling of segmental gene expression in the epididymis.* Biol Reprod, 2005. **73**(3): p. 404-13.
19. Jelinsky, S.A., et al., *The rat epididymal transcriptome: comparison of segmental gene expression in the rat and mouse epididymides.* Biol Reprod, 2007. **76**(4): p. 561-70.
20. Yeung, C.H., et al., *Organization of tubules in the human caput epididymidis and the ultrastructure of their epithelia.* Am J Anat, 1991. **191**(3): p. 261-79.
21. Vendrely, E. and J.P. Dadoune, *Quantitative ultrastructural analysis of the principal cells in the human epididymis.* Reprod Nutr Dev, 1988. **28**(5): p. 1225-35.
22. Palacios, J., et al., *Apical mitochondria-rich cells in the human epididymis: an ultrastructural, enzymohistochemical, and immunohistochemical study.* Anat Rec, 1991. **231**(1): p. 82-8.
23. Yeung, C.H., et al., *Basal cells of the human epididymis--antigenic and ultrastructural similarities to tissue-fixed macrophages.* Biol Reprod, 1994. **50**(4): p. 917-26.
24. Adamali, H.I. and L. Hermo, *Apical and narrow cells are distinct cell types differing in their structure, distribution, and functions in the adult rat epididymis.* J Androl, 1996. **17**(3): p. 208-22.
25. Hermo, L. and B. Robaire, *Epididymal cell types and their functions*, in *The epididymis: from molecules to clinical practice.*, B. Robaire and B. Hinton, Editors. 2002, Plenum Press: New York. p. 81-102.
26. Shum, W.W., et al., *Transepithelial projections from basal cells are luminal sensors in pseudostratified epithelia.* Cell, 2008. **135**(6): p. 1108-17.
27. Martinez-Garcia, F., et al., *The apical mitochondria-rich cells of the mammalian epididymis.* Andrologia, 1995. **27**(4): p. 195-206.
28. Wang, Y.F. and A.F. Holstein, *Intraepithelial lymphocytes and macrophages in the human epididymis.* Cell Tissue Res, 1983. **233**(3): p. 517-21.
29. Clulow, J., R.C. Jones, and L.A. Hansen, *Micropuncture and cannulation studies of fluid composition and transport in the ductuli efferentes testis of the rat: comparisons with the homologous metanephric proximal tubule.* Exp Physiol, 1994. **79**(6): p. 915-28.
30. Cyr, D., et al., *Cellular interactions and the blood-epididymal barrier*, in *The Epididymis: From Molecules to Clinical Practice*, B. Robaire and B. Hinton, Editors. 2002, Plenum Press: New York. p. 103-118.
31. Dacheux, J.-L. and F. Dacheux, *Protein secretion in the epididymis*, in *The Epididymis: From Molecules to Clinical Practice*, B. Robaire and B. Hinton, Editors. 2002, Plenum Press: New York. p. 151-168.
32. Dacheux, J.L., J.L. Gatti, and F. Dacheux, *Contribution of epididymal secretory proteins for spermatozoa maturation.* Microsc Res Tech, 2003. **61**(1): p. 7-17.
33. Hinton, B.T. and M.A. Palladino, *Epididymal epithelium: its contribution to the formation of a luminal fluid microenvironment.* Microsc Res Tech, 1995. **30**(1): p. 67-81.

34. Cyr, D.G., B. Robaire, and L. Hermo, *Structure and turnover of junctional complexes between principal cells of the rat epididymis.* Microsc Res Tech, 1995. **30**(1): p. 54-66.
35. Yeung, C.H. and T.G. Cooper, *Acquisition and development of sperm motility upon maturation in the epididymis*, in *The Epididymis: From Molecules to Clinical Practice*, B. Robaire and B. Hinton, Editors. 2002, Plenum Press: New York. p. 417-434.
36. Haidl, G., et al., *Disturbances of sperm flagella due to failure of epididymal maturation and their possible relationship to phospholipids.* Hum Reprod, 1993. **8**(7): p. 1070-3.
37. Young, W.C., *A study of the function of the epididymis. I. Is the attainment of full spermatozoan maturity attribiable to some specific action of epididymal secretion ?* J Morphol Physiol, 1929. **47**: p. 479-495.
38. Young, W.C., *A study of the function of the epididymis. II. The importance of an aging process in sperm for the length of the period during which fertilizing capacity is retained by sperm isolated in the epididymis of the guinea-pig.* J Morphol Physiol, 1929. **48**: p. 475-491.
39. Young, W.C., *A study of the function of the epididymis. III. Functional changes undergone by spermatozoa during their passage trough the epididymis and vas deferens in the guinea pig.* J Exp Biol, 1931. **8**: p. 151-162.
40. Cooper, T.G., *Interactions between epididymal secretions and spermatozoa.* J Reprod Fertil Suppl, 1998. **53**: p. 119-36.
41. Cooper, T.G., *Cytoplasmic droplets: the good, the bad or just confusing?* Hum Reprod, 2005. **20**(1): p. 9-11.
42. Misell, L.M., et al., *A stable isotope-mass spectrometric method for measuring human spermatogenesis kinetics in vivo.* J Urol, 2006. **175**(1): p. 242-6; discussion 246.
43. Amann, R.P. and S.S. Howards, *Daily spermatozoal production and epididymal spermatozoal reserves of the human male.* J Urol, 1980. **124**(2): p. 211-5.
44. Johnson, L. and D.D. Varner, *Effect of daily spermatozoan production but not age on transit time of spermatozoa through the human epididymis.* Biol Reprod, 1988. **39**(4): p. 812-7.
45. Silber, S.J., et al., *The use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection: the genetic implications for male infertility.* Hum Reprod, 1995. **10**(8): p. 2031-43.
46. Silber, S.J., *Apparent fertility of human spermatozoa from the caput epididymidis.* J Androl, 1989. **10**(4): p. 263-9.
47. Shulman, A., et al., *In-vitro fertilization treatment for severe male factor: the fertilization potential of immotile spermatozoa obtained by testicular extraction.* Hum Reprod, 1999. **14**(3): p. 749-52.
48. Moghadam, K.K., et al., *The motility of epididymal or testicular spermatozoa does not directly affect IVF/ICSI pregnancy outcomes.* J Androl, 2005. **26**(5): p. 619-23.

49. Qian, Y., et al., *Fertilization of in vitro matured human oocytes by intracytoplasmic sperm injection (ICSI) using ejaculated and testicular spermatozoa.* Asian J Androl, 2005. 7(1): p. 39-43.
50. Patrizio, P., et al., *Correlation between epididymal length and fertilization rate in men with congenital absence of the vas deferens.* Fertil Steril, 1994. **61**(2): p. 265-8.
51. Dacheux, J.L. and M. Paquignon, *Relations between the fertilizing ability, motility and metabolism of epididymal spermatozoa.* Reprod Nutr Dev, 1980. **20**(4A): p. 1085-99.
52. Hinrichsen, M.J. and J.A. Blaquier, *Evidence supporting the existence of sperm maturation in the human epididymis.* J Reprod Fertil, 1980. **60**(2): p. 291-4.
53. Yeung, C.H., et al., *Maturation of human spermatozoa (from selected epididymides of prostatic carcinoma patients) with respect to their morphology and ability to undergo the acrosome reaction.* Hum Reprod Update, 1997. **3**(3): p. 205-13.
54. Yeung, C.H., et al., *Changes in movement characteristics of human spermatozoa along the length of the epididymis.* Biol Reprod, 1993. **49**(2): p. 274-80.
55. Kirchhoff, C., et al., *The molecular biology of the sperm surface. Post-testicular membrane remodelling.* Adv Exp Med Biol, 1997. **424**: p. 221-32.
56. Cooper, T.G., *The human epididymis, sperm maturation and storage.* Official Journal of the hellenic society of andrology, 2007. **9**(1): p. 18-24.
57. Krull, N., et al., *Region-specific variation of gene expression in the human epididymis as revealed by in situ hybridization with tissue-specific cDNAs.* Mol Reprod Dev, 1993. **34**(1): p. 16-24.
58. Boue, F., J. Blais, and R. Sullivan, *Surface localization of P34H an epididymal protein, during maturation, capacitation, and acrosome reaction of human spermatozoa.* Biol Reprod, 1996. **54**(5): p. 1009-17.
59. Hayashi, M., et al., *Characterization of a human glycoprotein with a potential role in sperm-egg fusion: cDNA cloning, immunohistochemical localization, and chromosomal assignment of the gene (AEGL1).* Genomics, 1996. **32**(3): p. 367-74.
60. Kirchhoff, C., et al., *Function of human epididymal proteins in sperm maturation.* Andrologia, 1998. **30**(4-5): p. 225-32.
61. Richardson, R.T., et al., *Cloning and sequencing of human Eppin: a novel family of protease inhibitors expressed in the epididymis and testis.* Gene, 2001. **270**(1-2): p. 93-102.
62. Friend, D.S. and N.B. Gilula, *Variations in tight and gap junctions in mammalian tissues.* J Cell Biol, 1972. **53**(3): p. 758-76.
63. Johnson, L.G., *Applications of imaging techniques to studies of epithelial tight junctions.* Adv Drug Deliv Rev, 2005. **57**(1): p. 111-21.
64. Suzuki, F. and T. Nagano, *Development of tight junctions in the caput epididymal epithelium of the mouse.* Dev Biol, 1978. **63**(2): p. 321-34.
65. Cavicchia, J.C., *Fine structure of the monkey epididymis: a correlated thin-section and freeze-cleave study.* Cell Tissue Res, 1979. **201**(3): p. 451-8.

66. Pelletier, R.M., *Freeze-fracture study of cell junctions in the epididymis and vas deferens of a seasonal breeder: the mink (Mustela vison).* Microsc Res Tech, 1995. **30**(1): p. 37-53.
67. Lopez, M.L., et al., *Regional differentiation of the blood-epididymis barrier in stallion (Equus caballus).* J Submicrosc Cytol Pathol, 1997. **29**(3): p. 353-63.
68. Cambrosio Mann, M., A.E. Friess, and M.H. Stoffel, *Blood-tissue barriers in the male reproductive tract of the dog: a morphological study using lanthanum nitrate as an electron-opaque tracer.* Cells Tissues Organs, 2003. **174**(4): p. 162-9.
69. Agarwal, A. and A.P. Hoffer, *Ultrastructural studies on the development of the blood-epididymis barrier in immature rats.* J Androl, 1989. **10**(6): p. 425-31.
70. Suzuki, F. and T. Nagano, *Regional differentiation of cell junctions in the excurrent duct epithelium of the rat testis as revealed by freeze-fracture.* Anat Rec, 1978. **191**(4): p. 503-19.
71. Gonzalez-Mariscal, L., et al., *Tight junction proteins.* Prog Biophys Mol Biol, 2003. **81**(1): p. 1-44.
72. Hoffer, A.P. and B.T. Hinton, *Morphological evidence for a blood-epididymis barrier and the effects of gossypol on its integrity.* Biol Reprod, 1984. **30**(4): p. 991-1004.
73. Turner, T.T., *Spermatozoa are exposed to a complex microenvironment as they traverse the epididymis.* Ann N Y Acad Sci, 1991. **637**: p. 364-83.
74. Martinez-Palomo, A. and D. Erlij, *Structure of tight junctions in epithelia with different permeability.* Proc Natl Acad Sci U S A, 1975. **72**(11): p. 4487-91.
75. Chan, H.C., et al., *Regional differences in bioelectrical properties and anion secretion in cultured epithelia from rat and human male excurrent ducts.* Biol Reprod, 1995. **52**(1): p. 192-8.
76. Gould, S.F. and M.H. Bernstein, *Fine structure of fetal human testis and epididymis.* Arch Androl, 1979. **2**(2): p. 93-9.
77. Yeung, C.H., et al., *Fluid-phase transcytosis in the primate epididymis in vitro and in vivo.* Int J Androl, 1989. **12**(5): p. 384-94.
78. Cooper, T.G., et al., *Maintenance of human epididymal epithelial cell function in monolayer culture.* J Reprod Fertil, 1990. **90**(1): p. 81-91.
79. Schneeberger, E.E. and R.D. Lynch, *The tight junction: a multifunctional complex.* Am J Physiol Cell Physiol, 2004. **286**(6): p. C1213-28.
80. Furuse, M., et al., *Occludin: a novel integral membrane protein localizing at tight junctions.* J Cell Biol, 1993. **123**(6 Pt 2): p. 1777-88.
81. Cyr, D.G., et al., *Cellular immunolocalization of occludin during embryonic and postnatal development of the mouse testis and epididymis.* Endocrinology, 1999. **140**(8): p. 3815-25.
82. Yoon, S.I., et al., *Expression of Occludin in Testis and Epididymis of Wild Rabbits, Lepus sinensis coreanus.* Reprod Domest Anim, 2008.
83. Gye, M.C., *Expression of occludin in canine testis and epididymis.* Reprod Domest Anim, 2004. **39**(1): p. 43-7.

84. Furuse, M., et al., *Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions.* J Cell Biol, 1994. **127**(6 Pt 1): p. 1617-26.
85. Fanning, A.S., et al., *The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton.* J Biol Chem, 1998. **273**(45): p. 29745-53.
86. Haskins, J., et al., *ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin.* J Cell Biol, 1998. **141**(1): p. 199-208.
87. Itoh, M., K. Morita, and S. Tsukita, *Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin.* J Biol Chem, 1999. **274**(9): p. 5981-6.
88. Cordenonsi, M., et al., *Xenopus laevis occludin. Identification of in vitro phosphorylation sites by protein kinase CK2 and association with cingulin.* Eur J Biochem, 1999. **264**(2): p. 374-84.
89. Clarke, H., et al., *Modification of tight junction function by protein kinase C isoforms.* Adv Drug Deliv Rev, 2000. **41**(3): p. 283-301.
90. Chen, Y.H., et al., *Nonreceptor tyrosine kinase c-Yes interacts with occludin during tight junction formation in canine kidney epithelial cells.* Mol Biol Cell, 2002. **13**(4): p. 1227-37.
91. Nunbhakdi-Craig, V., et al., *Protein phosphatase 2A associates with and regulates atypical PKC and the epithelial tight junction complex.* J Cell Biol, 2002. **158**(5): p. 967-78.
92. Wong, V., *Phosphorylation of occludin correlates with occludin localization and function at the tight junction.* Am J Physiol, 1997. **273**(6 Pt 1): p. C1859-67.
93. Balda, M.S., et al., *Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein.* J Cell Biol, 1996. **134**(4): p. 1031-49.
94. Chen, Y., et al., *COOH terminus of occludin is required for tight junction barrier function in early Xenopus embryos.* J Cell Biol, 1997. **138**(4): p. 891-9.
95. Wong, V. and B.M. Gumbiner, *A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier.* J Cell Biol, 1997. **136**(2): p. 399-409.
96. Saitou, M., et al., *Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions.* J Cell Biol, 1998. **141**(2): p. 397-408.
97. Saitou, M., et al., *Complex phenotype of mice lacking occludin, a component of tight junction strands.* Mol Biol Cell, 2000. **11**(12): p. 4131-42.
98. Matter, K., et al., *Mammalian tight junctions in the regulation of epithelial differentiation and proliferation.* Curr Opin Cell Biol, 2005. **17**(5): p. 453-8.
99. Ikenouchi, J., et al., *Loss of occludin affects tricellular localization of tricellulin.* Mol Biol Cell, 2008. **19**(11): p. 4687-93.
100. Ikenouchi, J., et al., *Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells.* J Cell Biol, 2005. **171**(6): p. 939-45.

101. Riazuddin, S., et al., *Tricellulin is a tight-junction protein necessary for hearing.* Am J Hum Genet, 2006. **79**(6): p. 1040-51.
102. Krug, S.M., et al., *Tricellulin Forms a Barrier to Macromolecules in Tricellular Tight Junctions without Affecting Ion Permeability.* Mol Biol Cell, 2009.
103. Furuse, M., et al., *A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts.* J Cell Biol, 1998. **143**(2): p. 391-401.
104. Chiba, H., et al., *Transmembrane proteins of tight junctions.* Biochim Biophys Acta, 2008. **1778**(3): p. 588-600.
105. Van Itallie, C.M., et al., *Two splice variants of claudin-10 in the kidney create paracellular pores with different ion selectivities.* Am J Physiol Renal Physiol, 2006. **291**(6): p. F1288-99.
106. Niimi, T., et al., *claudin-18, a novel downstream target gene for the T/EBP/NKX2.1 homeodomain transcription factor, encodes lung- and stomach-specific isoforms through alternative splicing.* Mol Cell Biol, 2001. **21**(21): p. 7380-90.
107. Lal-Nag, M. and P.J. Morin, *The claudins.* Genome Biol, 2009. **10**(8): p. 235.
108. Hamazaki, Y., et al., *Multi-PDZ domain protein 1 (MUPP1) is concentrated at tight junctions through its possible interaction with claudin-1 and junctional adhesion molecule.* J Biol Chem, 2002. **277**(1): p. 455-61.
109. Itoh, M., et al., *Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins.* J Cell Biol, 1999. **147**(6): p. 1351-63.
110. Umeda, K., et al., *ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation.* Cell, 2006. **126**(4): p. 741-54.
111. Tsukita, S. and M. Furuse, *Pores in the wall: claudins constitute tight junction strands containing aqueous pores.* J Cell Biol, 2000. **149**(1): p. 13-6.
112. Van Itallie, C.M. and J.M. Anderson, *Claudins and epithelial paracellular transport.* Annu Rev Physiol, 2006. **68**: p. 403-29.
113. Van Itallie, C.M. and J.M. Anderson, *The molecular physiology of tight junction pores.* Physiology (Bethesda), 2004. **19**: p. 331-8.
114. Findley, M.K. and M. Koval, *Regulation and roles for claudin-family tight junction proteins.* IUBMB Life, 2009. **61**(4): p. 431-7.
115. Colegio, O.R., et al., *Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture.* Am J Physiol Cell Physiol, 2003. **284**(6): p. C1346-54.
116. Krause, G., et al., *Structure and function of claudins.* Biochim Biophys Acta, 2008. **1778**(3): p. 631-45.
117. Mandell, K.J. and C.A. Parkos, *The JAM family of proteins.* Adv Drug Deliv Rev, 2005. **57**(6): p. 857-67.
118. Ebnet, K., et al., *The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM).* EMBO J, 2001. **20**(14): p. 3738-48.
119. Ebnet, K., et al., *The junctional adhesion molecule (JAM) family members JAM-2 and JAM-3 associate with the cell polarity protein PAR-3: a possible role for JAMs in endothelial cell polarity.* J Cell Sci, 2003. **116**(Pt 19): p. 3879-91.

120. Ebnet, K., et al., *Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1.* J Biol Chem, 2000. **275**(36): p. 27979-88.
121. Bazzoni, G., et al., *Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin.* J Biol Chem, 2000. **275**(27): p. 20520-6.
122. Liu, Y., et al., *Human junction adhesion molecule regulates tight junction resealing in epithelia.* J Cell Sci, 2000. **113 (Pt 13)**: p. 2363-74.
123. Mandell, K.J., et al., *Expression of JAM-A in the human corneal endothelium and retinal pigment epithelium: localization and evidence for role in barrier function.* Invest Ophthalmol Vis Sci, 2007. **48**(9): p. 3928-36.
124. Stevenson, B.R., et al., *Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia.* J Cell Biol, 1986. **103**(3): p. 755-66.
125. Stevenson, B.R., et al., *ZO-1 and cingulin: tight junction proteins with distinct identities and localizations.* Am J Physiol, 1989. **257**(4 Pt 1): p. C621-8.
126. Anderson, J.M., et al., *Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells.* J Cell Biol, 1988. **106**(4): p. 1141-9.
127. Gumbiner, B., T. Lowenkopf, and D. Apatira, *Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1.* Proc Natl Acad Sci U S A, 1991. **88**(8): p. 3460-4.
128. Wittchen, E.S., J. Haskins, and B.R. Stevenson, *Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3.* J Biol Chem, 1999. **274**(49): p. 35179-85.
129. Adachi, M., et al., *Normal establishment of epithelial tight junctions in mice and cultured cells lacking expression of ZO-3, a tight-junction MAGUK protein.* Mol Cell Biol, 2006. **26**(23): p. 9003-15.
130. Hirabayashi, S., et al., *MAGI-1 is a component of the glomerular slit diaphragm that is tightly associated with nephrin.* Lab Invest, 2005. **85**(12): p. 1528-43.
131. Ide, N., et al., *Localization of membrane-associated guanylate kinase (MAGI)-1/BAI-associated protein (BAP) 1 at tight junctions of epithelial cells.* Oncogene, 1999. **18**(54): p. 7810-5.
132. Dobrosotskaya, I.Y. and G.L. James, *MAGI-1 interacts with beta-catenin and is associated with cell-cell adhesion structures.* Biochem Biophys Res Commun, 2000. **270**(3): p. 903-9.
133. Patrie, K.M., et al., *Interaction of two actin-binding proteins, synaptopodin and alpha-actinin-4, with the tight junction protein MAGI-1.* J Biol Chem, 2002. **277**(33): p. 30183-90.
134. Dobrosotskaya, I., R.K. Guy, and G.L. James, *MAGI-1, a membrane-associated guanylate kinase with a unique arrangement of protein-protein interaction domains.* J Biol Chem, 1997. **272**(50): p. 31589-97.
135. Wu, Y., et al., *Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase.* J Biol Chem, 2000. **275**(28): p. 21477-85.

136. Wu, X., et al., *Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2.* Proc Natl Acad Sci U S A, 2000. **97**(8): p. 4233-8.
137. Ullmer, C., et al., *Cloning and characterization of MUPP1, a novel PDZ domain protein.* FEBS Lett, 1998. **424**(1-2): p. 63-8.
138. Jeansonne, B., et al., *Claudin-8 interacts with multi-PDZ domain protein 1 (MUPP1) and reduces paracellular conductance in epithelial cells.* Cell Mol Biol (Noisy-le-grand), 2003. **49**(1): p. 13-21.
139. Adachi, M., et al., *Similar and distinct properties of MUPP1 and Patj, two homologous PDZ domain-containing tight-junction proteins.* Mol Cell Biol, 2009. **29**(9): p. 2372-89.
140. Lanaspa, M.A., et al., *The tight junction protein, MUPP1, is up-regulated by hypertonicity and is important in the osmotic stress response in kidney cells.* Proc Natl Acad Sci U S A, 2007. **104**(34): p. 13672-7.
141. Byers, S.W., et al., *Polarized functions and permeability properties of rat epididymal epithelial cells in vitro.* J Reprod Fertil, 1992. **95**(2): p. 385-96.
142. Cordenonsi, M., et al., *Cingulin contains globular and coiled-coil domains and interacts with ZO-1, ZO-2, ZO-3, and myosin.* J Cell Biol, 1999. **147**(7): p. 1569-82.
143. D'Atri, F. and S. Citi, *Cingulin interacts with F-actin in vitro.* FEBS Lett, 2001. **507**(1): p. 21-4.
144. Guillemot, L., et al., *Disruption of the cingulin gene does not prevent tight junction formation but alters gene expression.* J Cell Sci, 2004. **117**(Pt 22): p. 5245-56.
145. Guillemot, L. and S. Citi, *Cingulin regulates claudin-2 expression and cell proliferation through the small GTPase RhoA.* Mol Biol Cell, 2006. **17**(8): p. 3569-77.
146. Ohnishi, H., et al., *JACOP, a novel plaque protein localizing at the apical junctional complex with sequence similarity to cingulin.* J Biol Chem, 2004. **279**(44): p. 46014-22.
147. Guillemot, L., et al., *Paracingulin regulates the activity of Rac1 and RhoA GTPases by recruiting Tiam1 and GEF-H1 to epithelial junctions.* Mol Biol Cell, 2008. **19**(10): p. 4442-53.
148. Keon, B.H., et al., *Symplekin, a novel type of tight junction plaque protein.* J Cell Biol, 1996. **134**(4): p. 1003-18.
149. Kavanagh, E., et al., *Functional interaction between the ZO-1-interacting transcription factor ZONAB/DbpA and the RNA processing factor symplekin.* J Cell Sci, 2006. **119**(Pt 24): p. 5098-105.
150. Robaire, B., et al., *Androgenic regulation of novel genes in the epididymis.* Asian J Androl, 2007. **9**(4): p. 545-53.
151. Cyr, D.G., et al., *Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood-epididymal barrier in animals and humans.* Asian J Androl, 2007. **9**(4): p. 463-75.

152. Turner, T.T., et al., *Differential gene expression among the proximal segments of the rat epididymis is lost after efferent duct ligation.* Biol Reprod, 2007. 77(1): p. 165-71.
153. Turner, T.T., R.D. Giles, and S.S. Howards, *Effect of oestradiol valerate on the rat blood--testis and blood--epididymal barriers to [3H]inulin.* J Reprod Fertil, 1981. **63**(2): p. 355-8.
154. Guillemot, L., et al., *The cytoplasmic plaque of tight junctions: a scaffolding and signalling center.* Biochim Biophys Acta, 2008. **1778**(3): p. 601-13.
155. Gonzalez-Mariscal, L., R. Tapia, and D. Chamorro, *Crosstalk of tight junction components with signaling pathways.* Biochim Biophys Acta, 2008. **1778**(3): p. 729-56.
156. Ikenouchi, J., et al., *Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail.* J Cell Sci, 2003. **116**(Pt 10): p. 1959-67.
157. Ohkubo, T. and M. Ozawa, *The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation.* J Cell Sci, 2004. **117**(Pt 9): p. 1675-85.
158. Dufresne, J. and D.G. Cyr, *Activation of an SP binding site is crucial for the expression of claudin 1 in rat epididymal principal cells.* Biol Reprod, 2007. **76**(5): p. 825-32.
159. Li, L., et al., *Gene regulation by Sp1 and Sp3.* Biochem Cell Biol, 2004. **82**(4): p. 460-71.
160. Honda, H., et al., *Crucial roles of Sp1 and epigenetic modifications in the regulation of the CLDN4 promoter in ovarian cancer cells.* J Biol Chem, 2006. **281**(30): p. 21433-44.
161. Honda, H., et al., *Regulation of the CLDN3 gene in ovarian cancer cells.* Cancer Biol Ther, 2007. **6**(11): p. 1733-42.
162. Ikari, A., et al., *Epidermal growth factor increases claudin-4 expression mediated by Sp1 elevation in MDCK cells.* Biochem Biophys Res Commun, 2009.
163. Luk, J.M., et al., *Sp1 site is crucial for the mouse claudin-19 gene expression in the kidney cells.* FEBS Lett, 2004. **578**(3): p. 251-6.
164. Lopardo, T., et al., *Claudin-1 is a p63 target gene with a crucial role in epithelial development.* PLoS ONE, 2008. **3**(7): p. e2715.
165. Hayashi, T., et al., *Expression of the p63 and Notch signaling systems in rat testes during postnatal development: comparison with their expression levels in the epididymis and vas deferens.* J Androl, 2004. **25**(5): p. 692-8.
166. Saito, K., et al., *Spatial and isoform specific p63 expression in the male human urogenital tract.* J Urol, 2006. **176**(5): p. 2268-73.
167. Yap, A.S., W.M. Brieher, and B.M. Gumbiner, *Molecular and functional analysis of cadherin-based adherens junctions.* Annu Rev Cell Dev Biol, 1997. **13**: p. 119-46.
168. Niessen, C.M., *Tight junctions/adherens junctions: basic structure and function.* J Invest Dermatol, 2007. **127**(11): p. 2525-32.
169. Takai, Y. and H. Nakanishi, *Nectin and afadin: novel organizers of intercellular junctions.* J Cell Sci, 2003. **116**(Pt 1): p. 17-27.

170. Watari, Y., et al., *Identification of Ce-AF-6, a novel Caenorhabditis elegans protein, as a putative Ras effector.* Gene, 1998. **224**(1-2): p. 53-8.
171. Takahashi, K., et al., *Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein.* J Cell Biol, 1999. **145**(3): p. 539-49.
172. Okamoto, R., et al., *Recruitment of E-cadherin associated with alpha- and beta-catenins and p120ctn to the nectin-based cell-cell adhesion sites by the action of 12-O-tetradecanoylphorbol-13-acetate in MDCK cells.* Genes Cells, 2005. **10**(5): p. 435-45.
173. Fukuhara, A., et al., *Role of nectin in organization of tight junctions in epithelial cells.* Genes Cells, 2002. **7**(10): p. 1059-72.
174. Fukuhara, A., et al., *Involvement of nectin in the localization of junctional adhesion molecule at tight junctions.* Oncogene, 2002. **21**(50): p. 7642-55.
175. Ogita, H. and Y. Takai, *Activation of Rap1, Cdc42, and rac by nectin adhesion system.* Methods Enzymol, 2006. **406**: p. 415-24.
176. Honda, T., et al., *Regulation by nectin of the velocity of the formation of adherens junctions and tight junctions.* Biochem Biophys Res Commun, 2003. **306**(1): p. 104-9.
177. Hoshino, T., et al., *A novel role of nectins in inhibition of the E-cadherin-induced activation of Rac and formation of cell-cell adherens junctions.* Mol Biol Cell, 2004. **15**(3): p. 1077-88.
178. Sato, T., et al., *Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells.* J Biol Chem, 2006. **281**(8): p. 5288-99.
179. Halbleib, J.M. and W.J. Nelson, *Cadherins in development: cell adhesion, sorting, and tissue morphogenesis.* Genes Dev, 2006. **20**(23): p. 3199-214.
180. Chen, X. and B.M. Gumbiner, *Crosstalk between different adhesion molecules.* Curr Opin Cell Biol, 2006. **18**(5): p. 572-8.
181. Cyr, D.G., et al., *Distribution and regulation of epithelial cadherin messenger ribonucleic acid and immunocytochemical localization of epithelial cadherin in the rat epididymis.* Endocrinology, 1992. **130**(1): p. 353-63.
182. Cyr, D.G. and B. Robaire, *Developmental regulation of epithelial- and placental-cadherin mRNAs in the rat epididymis.* Ann N Y Acad Sci, 1991. **637**: p. 399-408.
183. Andersson, A.M., K. Edvardsen, and N.E. Skakkebaek, *Expression and localization of N- and E-cadherin in the human testis and epididymis.* Int J Androl, 1994. **17**(4): p. 174-80.
184. Smith, S.R., et al., *N- and E-cadherin expression in human ovarian and urogenital duct development.* Fertil Steril, 2009.
185. Hartsock, A. and W.J. Nelson, *Adherens and tight junctions: structure, function and connections to the actin cytoskeleton.* Biochim Biophys Acta, 2008. **1778**(3): p. 660-9.
186. Barth, A.I., I.S. Nathke, and W.J. Nelson, *Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signaling pathways.* Curr Opin Cell Biol, 1997. **9**(5): p. 683-90.

187. Capaldo, C.T. and I.G. Macara, *Depletion of E-cadherin disrupts establishment but not maintenance of cell junctions in Madin-Darby canine kidney epithelial cells.* Mol Biol Cell, 2007. **18**(1): p. 189-200.
188. Davis, M.A., R.C. Ireton, and A.B. Reynolds, *A core function for p120-catenin in cadherin turnover.* J Cell Biol, 2003. **163**(3): p. 525-34.
189. Itoh, M., et al., *Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments.* J Cell Biol, 1997. **138**(1): p. 181-92.
190. DeBellefeuille, S., et al., *Catenins in the rat epididymis: their expression and regulation in adulthood and during postnatal development.* Endocrinology, 2003. **144**(11): p. 5040-9.
191. Merdek, K.D., N.T. Nguyen, and D. Toksoz, *Distinct activities of the alpha-catenin family, alpha-catulin and alpha-catenin, on beta-catenin-mediated signaling.* Mol Cell Biol, 2004. **24**(6): p. 2410-22.
192. Wiesner, C., et al., *Alpha-catulin, a Rho signalling component, can regulate NF-kappaB through binding to IKK-beta, and confers resistance to apoptosis.* Oncogene, 2008. **27**(15): p. 2159-69.
193. Thedieck, C., et al., *alpha B-crystallin is a cytoplasmic interaction partner of the kidney-specific cadherin-16.* J Mol Biol, 2008. **378**(1): p. 145-53.
194. Wertz, K. and B.G. Herrmann, *Kidney-specific cadherin (cdh16) is expressed in embryonic kidney, lung, and sex ducts.* Mech Dev, 1999. **84**(1-2): p. 185-8.
195. Boutet, A., et al., *Snail activation disrupts tissue homeostasis and induces fibrosis in the adult kidney.* EMBO J, 2006. **25**(23): p. 5603-13.
196. Stemmler, M.P., *Cadherins in development and cancer.* Mol Biosyst, 2008. **4**(8): p. 835-50.
197. Hernandez, S., B. Chavez Munguia, and L. Gonzalez-Mariscal, *ZO-2 silencing in epithelial cells perturbs the gate and fence function of tight junctions and leads to an atypical monolayer architecture.* Exp Cell Res, 2007. **313**(8): p. 1533-47.
198. Wittchen, E.S., J. Haskins, and B.R. Stevenson, *Exogenous expression of the amino-terminal half of the tight junction protein ZO-3 perturbs junctional complex assembly.* J Cell Biol, 2000. **151**(4): p. 825-36.
199. Lioni, M., et al., *Dysregulation of claudin-7 leads to loss of E-cadherin expression and the increased invasion of esophageal squamous cell carcinoma cells.* Am J Pathol, 2007. **170**(2): p. 709-21.
200. Wang, Q., X.W. Chen, and B. Margolis, *PALS1 regulates E-cadherin trafficking in mammalian epithelial cells.* Mol Biol Cell, 2007. **18**(3): p. 874-85.
201. Kumar, N.M. and N.B. Gilula, *The gap junction communication channel.* Cell, 1996. **84**(3): p. 381-8.
202. Mese, G., G. Richard, and T.W. White, *Gap junctions: basic structure and function.* J Invest Dermatol, 2007. **127**(11): p. 2516-24.
203. Segretain, D. and M.M. Falk, *Regulation of connexin biosynthesis, assembly, gap junction formation, and removal.* Biochim Biophys Acta, 2004. **1662**(1-2): p. 3-21.

204. Nagano, T., Y. Toyama, and F. Suzuki, *Further observations on the Sertoli cell junctions of the mouse testis after metal contract freeze-fracture, and comparisons with cellular junctions of other epithelial cells.* Am J Anat, 1982. **163**(1): p. 47-58.
205. Cyr, D.G., L. Hermo, and D.W. Laird, *Immunocytochemical localization and regulation of connexin43 in the adult rat epididymis.* Endocrinology, 1996. **137**(4): p. 1474-84.
206. St-Pierre, N., et al., *Neonatal hypothyroidism alters the localization of gap junctional protein connexin 43 in the testis and messenger RNA levels in the epididymis of the rat.* Biol Reprod, 2003. **68**(4): p. 1232-40.
207. Dufresne, J., et al., *Expression of multiple connexins in the rat epididymis indicates a complex regulation of gap junctional communication.* Am J Physiol Cell Physiol, 2003. **284**(1): p. C33-43.
208. Stout, C., D.A. Goodenough, and D.L. Paul, *Connexins: functions without junctions.* Curr Opin Cell Biol, 2004. **16**(5): p. 507-12.
209. Jongen, W.M., et al., *Regulation of connexin 43-mediated gap junctional intercellular communication by Ca2+ in mouse epidermal cells is controlled by E-cadherin.* J Cell Biol, 1991. **114**(3): p. 545-55.
210. Wei, C.J., et al., *Connexin43 associated with an N-cadherin-containing multiprotein complex is required for gap junction formation in NIH3T3 cells.* J Biol Chem, 2005. **280**(20): p. 19925-36.
211. Xu, X., et al., *N-cadherin and Cx43alpha1 gap junctions modulates mouse neural crest cell motility via distinct pathways.* Cell Commun Adhes, 2001. **8**(4-6): p. 321-4.
212. Wu, J.C., R.Y. Tsai, and T.H. Chung, *Role of catenins in the development of gap junctions in rat cardiomyocytes.* J Cell Biochem, 2003. **88**(4): p. 823-35.
213. Meyer, R.A., et al., *Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies.* J Cell Biol, 1992. **119**(1): p. 179-89.
214. Giepmans, B.N. and W.H. Moolenaar, *The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein.* Curr Biol, 1998. **8**(16): p. 931-4.
215. Toyofuku, T., et al., *Direct association of the gap junction protein connexin-43 with ZO-1 in cardiac myocytes.* J Biol Chem, 1998. **273**(21): p. 12725-31.
216. Li, X., et al., *Connexin47, connexin29 and connexin32 co-expression in oligodendrocytes and Cx47 association with zonula occludens-1 (ZO-1) in mouse brain.* Neuroscience, 2004. **126**(3): p. 611-30.
217. Nielsen, P.A., et al., *Molecular cloning, functional expression, and tissue distribution of a novel human gap junction-forming protein, connexin-31.9. Interaction with zona occludens protein-1.* J Biol Chem, 2002. **277**(41): p. 38272-83.
218. Nagasawa, K., et al., *Possible involvement of gap junctions in the barrier function of tight junctions of brain and lung endothelial cells.* J Cell Physiol, 2006. **208**(1): p. 123-32.
219. Singh, D., et al., *Connexin 43 interacts with zona occludens-1 and -2 proteins in a cell cycle stage-specific manner.* J Biol Chem, 2005. **280**(34): p. 30416-21.

220. Kausalya, P.J., M. Reichert, and W. Hunziker, *Connexin45 directly binds to ZO-1 and localizes to the tight junction region in epithelial MDCK cells.* FEBS Lett, 2001. **505**(1): p. 92-6.
221. Li, Q., et al., *Disruption of tight junctions during polymicrobial sepsis in vivo.* J Pathol, 2009.
222. Lisewski, U., et al., *The tight junction protein CAR regulates cardiac conduction and cell-cell communication.* J Exp Med, 2008. **205**(10): p. 2369-79.
223. Kojima, T., et al., *Growth-suppressive function of human connexin32 in a conditional immortalized mouse hepatocyte cell line.* In Vitro Cell Dev Biol Anim, 2001. **37**(9): p. 589-98.
224. Kojima, T., et al., *Induction of tight junctions in human connexin 32 (hCx32)-transfected mouse hepatocytes: connexin 32 interacts with occludin.* Biochem Biophys Res Commun, 1999. **266**(1): p. 222-9.
225. Murata, M., et al., *Tight junction protein MAGI-1 is up-regulated by transfection with connexin 32 in an immortalized mouse hepatic cell line: cDNA microarray analysis.* Cell Tissue Res, 2005. **319**(2): p. 341-7.
226. Go, M., et al., *Connexin 26 expression prevents down-regulation of barrier and fence functions of tight junctions by Na+/K+-ATPase inhibitor ouabain in human airway epithelial cell line Calu-3.* Exp Cell Res, 2006. **312**(19): p. 3847-56.
227. Chamley, L.W. and G.N. Clarke, *Antisperm antibodies and conception.* Semin Immunopathol, 2007. **29**(2): p. 169-84.
228. Ohl, D.A. and R.K. Naz, *Infertility due to antisperm antibodies.* Urology, 1995. **46**(4): p. 591-602.
229. Flickinger, C.J., S.S. Howards, and J.C. Herr, *Effects of vasectomy on the epididymis.* Microsc Res Tech, 1995. **30**(1): p. 82-100.
230. Thimon, V., et al., *Effects of vasectomy on gene expression profiling along the human epididymis.* Biol Reprod, 2008. **79**(2): p. 262-73.
231. Cereijido, M., et al., *New diseases derived or associated with the tight junction.* Arch Med Res, 2007. **38**(5): p. 465-78.
232. Hermo, L., et al., *Structural alterations of epididymal epithelial cells in cathepsin A-deficient mice affect the blood-epididymal barrier and lead to altered sperm motility.* J Androl, 2007. **28**(5): p. 784-97.
233. Sigman, M. and J.P. Jarow, *Male Infertility*, in *Campbell's Urology*, P.C. Walsh, et al., Editors. 2006, Saunders: Philadelphia. p. 609-653.
234. Wong, C.H. and C.Y. Cheng, *The blood-testis barrier: its biology, regulation, and physiological role in spermatogenesis.* Curr Top Dev Biol, 2005. **71**: p. 263-96.
235. Fulmer, B.R. and T.T. Turner, *A blood-prostate barrier restricts cell and molecular movement across the rat ventral prostate epithelium.* J Urol, 2000. **163**(5): p. 1591-4.
236. Dube, E., et al., *Gene expression profiling and its relevance to the blood-epididymal barrier in the human epididymis.* Biol Reprod, 2007. **76**(6): p. 1034-44.
237. Inai, T., J. Kobayashi, and Y. Shibata, *Claudin-1 contributes to the epithelial barrier function in MDCK cells.* Eur J Cell Biol, 1999. **78**(12): p. 849-55.

238. Furuse, M., et al., *Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice.* J Cell Biol, 2002. **156**(6): p. 1099-111.
239. Takehara, M., et al., *Effect of claudin expression on paracellular permeability, migration and invasion of colonic cancer cells.* Biol Pharm Bull, 2009. **32**(5): p. 825-31.
240. Agarwal, R., T. D'Souza, and P.J. Morin, *Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity.* Cancer Res, 2005. **65**(16): p. 7378-85.
241. Li, J., et al., *Possible angiogenic roles for claudin-4 in ovarian cancer.* Cancer Biol Ther, 2009. **8**(19).
242. Hou, J., et al., *Study of claudin function by RNA interference.* J Biol Chem, 2006. **281**(47): p. 36117-23.
243. Van Itallie, C.M., A.S. Fanning, and J.M. Anderson, *Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins.* Am J Physiol Renal Physiol, 2003. **285**(6): p. F1078-84.
244. Alexandre, M.D., Q. Lu, and Y.H. Chen, *Overexpression of claudin-7 decreases the paracellular Cl- conductance and increases the paracellular Na+ conductance in LLC-PK1 cells.* J Cell Sci, 2005. **118**(Pt 12): p. 2683-93.
245. Tatum, R., et al., *Renal Salt Wasting and Chronic Dehydration in Claudin-7-Deficient Mice.* Am J Physiol Renal Physiol, 2009.
246. Ben-Yosef, T., et al., *Claudin 14 knockout mice, a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration.* Hum Mol Genet, 2003. **12**(16): p. 2049-61.
247. Jovov, B., et al., *Claudin-18: a dominant tight junction protein in Barrett's esophagus and likely contributor to its acid resistance.* Am J Physiol Gastrointest Liver Physiol, 2007. **293**(6): p. G1106-13.
248. Zheng, J.Y., et al., *Regulation of the expression of the prostate-specific antigen by claudin-7.* J Membr Biol, 2003. **194**(3): p. 187-97.
249. Van Itallie, C.M., O.R. Colegio, and J.M. Anderson, *The cytoplasmic tails of claudins can influence tight junction barrier properties through effects on protein stability.* J Membr Biol, 2004. **199**(1): p. 29-38.
250. Fujita, H., et al., *Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca2+ absorption between enterocytes.* Mol Biol Cell, 2008. **19**(5): p. 1912-21.
251. Amasheh, S., et al., *Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells.* J Cell Sci, 2002. **115**(Pt 24): p. 4969-76.
252. Gunzel, D., et al., *Claudin-10 exists in six alternatively spliced isoforms that exhibit distinct localization and function.* J Cell Sci, 2009. **122**(Pt 10): p. 1507-17.
253. Yu, A.S., et al., *Claudin-8 expression in Madin-Darby canine kidney cells augments the paracellular barrier to cation permeation.* J Biol Chem, 2003. **278**(19): p. 17350-9.
254. Angelow, S., K.J. Kim, and A.S. Yu, *Claudin-8 modulates paracellular permeability to acidic and basic ions in MDCK II cells.* J Physiol, 2006. **571**(Pt 1): p. 15-26.

255. Furuse, M., H. Sasaki, and S. Tsukita, *Manner of interaction of heterogeneous claudin species within and between tight junction strands.* J Cell Biol, 1999. **147**(4): p. 891-903.
256. Angelow, S., E.E. Schneeberger, and A.S. Yu, *Claudin-8 expression in renal epithelial cells augments the paracellular barrier by replacing endogenous claudin-2.* J Membr Biol, 2007. **215**(2-3): p. 147-59.
257. Nakano, Y., et al., *A claudin-9-based ion permeability barrier is essential for hearing.* PLoS Genet, 2009. **5**(8): p. e1000610.
258. Alexandre, M.D., et al., *The first extracellular domain of claudin-7 affects paracellular Cl- permeability.* Biochem Biophys Res Commun, 2007. **357**(1): p. 87-91.
259. Hou, J., et al., *Claudin-16 and claudin-19 interaction is required for their assembly into tight junctions and for renal reabsorption of magnesium.* Proc Natl Acad Sci U S A, 2009.
260. Hou, J., et al., *Transgenic RNAi depletion of claudin-16 and the renal handling of magnesium.* J Biol Chem, 2007. **282**(23): p. 17114-22.
261. Shan, Q., et al., *Insights into driving forces and paracellular permeability from claudin-16 knockdown mouse.* Ann N Y Acad Sci, 2009. **1165**: p. 148-51.
262. Dube, E., et al., *Alterations in gene expression in the caput epididymides of nonobstructive azoospermic men.* Biol Reprod, 2008. **78**(2): p. 342-51.
263. Hihnala, S., et al., *Expression of SLC26A3, CFTR and NHE3 in the human male reproductive tract: role in male subfertility caused by congenital chloride diarrhoea.* Mol Hum Reprod, 2006. **12**(2): p. 107-11.
264. Wang, X.F., et al., *Involvement of CFTR in uterine bicarbonate secretion and the fertilizing capacity of sperm.* Nat Cell Biol, 2003. **5**(10): p. 902-6.
265. Van Itallie, C.M., et al., *ZO-1 stabilizes the tight junction solute barrier through coupling to the perijunctional cytoskeleton.* Mol Biol Cell, 2009. **20**(17): p. 3930-40.
266. Davies, B., et al., *Novel epididymis-specific mRNAs downregulated by HE6/Gpr64 receptor gene disruption.* Mol Reprod Dev, 2007. **74**(5): p. 539-53.
267. Kirchhoff, C., et al., *Role of epididymal receptor HE6 in the regulation of sperm microenvironment.* Mol Cell Endocrinol, 2006. **250**(1-2): p. 43-8.
268. Morin, P.J., *Claudin proteins in human cancer: promising new targets for diagnosis and therapy.* Cancer Res, 2005. **65**(21): p. 9603-6.
269. Belker, A.M., et al., *Results of 1,469 microsurgical vasectomy reversals by the Vasovasostomy Study Group.* J Urol, 1991. **145**(3): p. 505-11.
270. Abdelmassih, V., et al., *Relationship between time period after vasectomy and the reproductive capacity of sperm obtained by epididymal aspiration.* Hum Reprod, 2002. **17**(3): p. 736-40.
271. Balda, M.S. and K. Matter, *Tight junctions and the regulation of gene expression.* Biochim Biophys Acta, 2009. **1788**(4): p. 761-7.
272. Pellati, D., et al., *Genital tract infections and infertility.* Eur J Obstet Gynecol Reprod Biol, 2008. **140**(1): p. 3-11.
273. Eley, A., et al., *Can Chlamydia trachomatis directly damage your sperm?* Lancet Infect Dis, 2005. **5**(1): p. 53-7.

274. Witkin, S.S., I. Kligman, and A.M. Bongiovanni, *Relationship between an asymptomatic male genital tract exposure to Chlamydia trachomatis and an autoimmune response to spermatozoa.* Hum Reprod, 1995. **10**(11): p. 2952-5.
275. Sousa, S., M. Lecuit, and P. Cossart, *Microbial strategies to target, cross or disrupt epithelia.* Curr Opin Cell Biol, 2005. **17**(5): p. 489-98.
276. Gonzalez-Mariscal, L., E. Garay, and S. Lechuga, *Virus interaction with the apical junctional complex.* Front Biosci, 2009. **14**: p. 731-68.
277. Evans, M.J., et al., *Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry.* Nature, 2007. **446**(7137): p. 801-5.
278. McClane, B.A., *The complex interactions between Clostridium perfringens enterotoxin and epithelial tight junctions.* Toxicon, 2001. **39**(11): p. 1781-91.
279. Zheng, A., et al., *Claudin-6 and claudin-9 function as additional coreceptors for hepatitis C virus.* J Virol, 2007. **81**(22): p. 12465-71.
280. Dohle, G.R., *Inflammatory-associated obstructions of the male reproductive tract.* Andrologia, 2003. **35**(5): p. 321-4.
281. Jantos, C., et al., *Experimental epididymitis due to Chlamydia trachomatis in rats.* Infect Immun, 1992. **60**(6): p. 2324-8.
282. Kondoh, M. and K. Yagi, *Tight junction modulators: promising candidates for drug delivery.* Curr Med Chem, 2007. **14**(23): p. 2482-8.
283. Kominsky, S.L., *Claudins: emerging targets for cancer therapy.* Expert Rev Mol Med, 2006. **8**(18): p. 1-11.
284. Sullivan, R., *Male fertility markers, myth or reality.* Anim Reprod Sci, 2004. **82-83**: p. 341-7.
285. Cohen, D.J., et al., *Participation of epididymal cysteine-rich secretory proteins in sperm-egg fusion and their potential use for male fertility regulation.* Asian J Androl, 2007. **9**(4): p. 528-32.
286. O'Rand M, G., et al., *Reversible immunocontraception in male monkeys immunized with eppin.* Science, 2004. **306**(5699): p. 1189-90.
287. Koyama, K., K. Ito, and A. Hasegawa, *Role of male reproductive tract CD52 (mrt-CD52) in reproduction.* Soc Reprod Fertil Suppl, 2007. **63**: p. 103-10.

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