UNIVERSITÀ DELLA CALABRIA

Dipartimento di Farmacia e Scienze della Salute e della Nutrizione

Dottorato di Ricerca in

Biochimica Cellulare ed Attività dei Farmaci in Oncologia

CICLO XXVIII°

Identificazione del recettore estrogenico GPER (G- protein- coupled estrogen receptor) nella ghiandola prostatica umana: valutazione del GPER nei tessuti prostatici benigni e neoplastici.

Settore Scientifico Disciplinare BIO16

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SOMMARIO

Gli estrogeni sono coinvolti nella crescita, differenziazione e patogenesi della prostata umana attraverso la mediazione dei classici recettori estrogenici (ERα e ERβ). D'altro canto, il meccanismo non genomico degli estrogeni sembra esercitare un ruolo importante su segnalazioni di percorsi che dirigono o indirettamente modulano l'espressione genica. In questo scenario, il G protein-coupled receptor, GPER (precedentemente chiamato GPR30), è stato implicato nella mediazione rapida degli eventi trascrizionali in risposta agli estrogeni. Alcuni studi supportano l'ipotesi che GPER rappresenta un recettore estrogeno-sensibile e la sua iper-espressione sembra essere fondamentale i diverse patologie neoplasiche. L'espressione di GPER è stata recentemente evidenziata in alcuni tessuti riproduttivi umani, ma la sua espressione a livello prostatico è ancora sconosciuta. In questo studio, abbiamo valutato, l'espressione di GPER in 5 pazienti affetti da patologie prostatiche non neoplastiche, e in 50 pazienti affetti da adenocarcinoma, mediante analisi immunoistochimica e Western blot.. Le aree normali della prostata benigna hanno mostrato una forte immunoreattività del GPER nel citoplasma delle cellule epiteliali basali insieme ad una debole colorazione nel citoplasma delle cellule stromali. Nessuna immunocolorazione è stata invece osservata nelle cellule epiteliali luminali secretorie. L'analisi immunoistochimica ha evidenziato l'espressione cellulare di GPER in tutti i campioni di adenocarcinoma esaminati ma con una variabilità correlata alle diverse architetture delle cellule tumorali (Gleason patterns). Le regioni che presentavano lesioni pre-neoplastiche HGPIN (high-grade prostatic intraepithelial neoplasia) hanno evidenziato una intensa immunoreattività per il recettore, mentre nelle aree tumorali la positività al GPER è stata correlata ai "Gleason patterns" e valutata con il metodo di Allred. Una intensa immunopositivtà al GPER è stata evidenziata nelle aree tumorali Gleason pattern 2 e pattern Gleason 3 (leggermente ridotta in queste ultime), mentre debolmente colorate apparivano le aree con Gleason pattern 4.

L'analisi Western blot degli estratti proteici benigni e tumorali ha confermato questo risultato. È stato inoltre osservato un aumento delle forme fosforilate dei livelli di Akt e di CREB nei campioni di pazienti affetti da adenocarcinoma scarsamente differenziato rispetto alle altre categorie.

In conclusione, nel presente lavoro, per la prima volta, abbiamo identificato GPER nelle cellule basali epiteliali della prostata umana non neoplastica, con una diversa localizzazione rispetto ai classici recettori estrogenici. Abbiamo inoltre evidenziato l'espressione di GPER nelle cellule di adenocarcinoma prostatico ma con una modulazione della sua intensità dipendente dall'organizzazione delle cellule neoplastiche. La immunoreattività al GPER appare quindi inversamente correlata al grado di differenziazione tumorale.

INTRODUZIONE

Gli androgeni sono necessari per la crescita e la differenziazione della ghiandola prostatica, anche se diversi autori hanno riportato un'associazione tra elevati livelli sierici e/o intraghiandolari di tali ormoni e un aumentato rischio di cancro alla prostata (Noble,1977; Henderson et al., 1982; Carter et al., 1995; Heracek et al 2007; Roddam et al., 2008, Cooper & Page 2014). In aggiunta agli androgeni, anche gli estrogeni regolano crescita e funzione della prostata in diversi stadi di sviluppo attraverso meccanismi sia diretti che indiretti (Prossnits & Barton, 2011). Studi precedenti hanno evidenziato che l'estrogenizzazione neonatale con estradiolo e DES provoca un'inibizione tempo- e dose-dipendente della crescita e della funzionalità della prostata in topi e ratti (Naslund&Coffey, 1986; Prins, 1992; Prins et al., 1993; Pylkkanen et al., 1991) e inoltre promuove le infiammazioni, l'iperplasia epiteliale e lo sviluppo di lesioni displastiche, che istologicamente assomigliano alle lesioni preneoplastiche PIN (Pylkkanen et al., 1991; Prins&Birch, 1997). Oggi, prove crescenti suggeriscono un ruolo chiave giocato dagli estrogeni nella carcinogenesi prostatica (Bonkhoff and Berges 2009).

Un'azione diretta della segnalazione estrogenica nel tessuto prostatico è stata supportata dall'identificazione nel tessuto ghiandolare dei classici recettori estrogenici ERα e ERβ (McPherson *et al*. 2008; Prins & Korach 2008). L'espressione di ERα e ERβ è stata ampiamente studiata localizzando così ERα principalmente nelle cellule stromali ed ERβ prevalentemente nelle cellule epiteliali (Weihua et al., 2002; Bosland 2005; Ho e Habib, 2011; Kawashima & Nakatani 2012) ERα e ERβ sono stati evidenziati anche nel tessuto prostatico neoplastico nonché in diverse linee cellulari di cancro alla prostata, (Chang and Prins , 1999; Lau et al., 2000; Latil et al., 2001; Rayuela et al., 2001; Tsurusaki et al., 2003; Linja et al.,2003.) Tali studi avevano suggerito il tradizionale paradigma in cui, nella carcinogenesi prostatica, ERβ aveva un ruolo protettivo , attraverso effetti anti tumorali e pro-apoptotici, mentre ERα era pro-cancerogenesi (Risbridger *et al.* 2007; Bonkhoff & Berges 2009; Celhay *et al*. 210; McPherson *et al*. 2010; Nakajima *et al*. 2011; Attia & Ederveen 2012). Di recente, però, questo paradigma è stato messo in discussione dimostrando una variabile espressione di

ERβ in diversi stadi tumorali (Zellweger *et al.* 2013) e un possibile ruolo oncogenico delle splice-variants di ERβ, ERβ2 e ERβ5 (Dey *et al.* 2012; Nelson *et al*. 2014).

Al contrario, nel tessuto prostatico, è poco nota la possibile mediazione dell'azione estrogenica da parte del GPER (G protein-coupled estrogen receptor), un recettore associato alla membrane che media effetti estrogenici rapidi su molteplici cellule targets (Prossnitz *et al.* 2008; Maggiolini & Picard 2010). L'espressione del GPER nelle cellule dell'apparato riproduttivo maschile è stata investigata solo di recente, con l' identificazione del recettore nelle cellule somatiche e germinali testicolari e negli spermatozoi umani (Franco *et al*, 2010; Rago *et al* 2011, 2014). Uno studio ha inoltre dimostrato la presenza di GPER nella prostata di coniglio, suggerendo per tale recettore un ruolo di mediatore dell'azione estrogenica (Comeglio *et al* 2014). Un possibile ruolo di GPER nella carcinogenesi prostatica è stato suggerito da Chan (Chan *et al.* 2010) che ha investigato *in vitro* linee cellulari di carcinoma prostatico sia androgeno dipendenti che androgeno indipendenti, e *in vivo* PC3 xenografts dove i trattamenti con un agonista di GPER, il G-1, provocava un arresto della crescita cellulare in fase G2. Scopo del presente lavoro è stato quello di investigare l'espressione di GPER nel tessuto prostatico normale e neoplastico al fine di evidenziare se tale recettore possa contribuire alla mediazione dell'azione estrogenica nella ghiandola sessuale accessoria.

Capitolo I

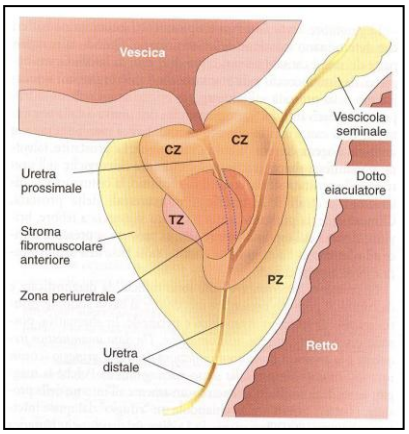
CARCINOMA PROSTATICO

Anatomia della prostata

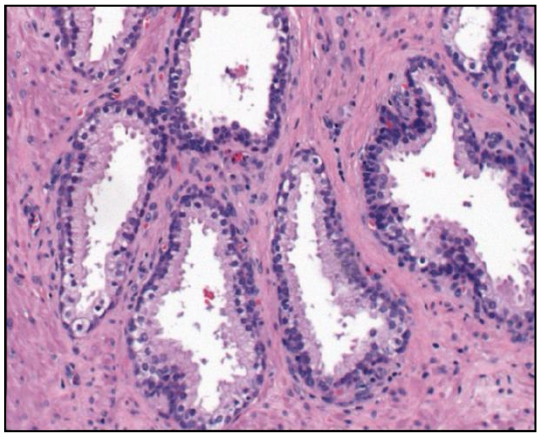
La prostata è un organo ghiandolare e fibromuscolare che, circonda la porzione prossimale dell'uretra, è posta al di sotto del collo vescicale e davanti al l retto da cui è separata dalla fascia di Denonvilliers. L'organo ha la forma di una castagna con la base rivolta verso la vescica e l'apice verso il diaframma uro-genitale. Nell'adulto il peso della ghiandola è di 20-25 grammi, il diametro longitudinale è di 30 mm, il trasverso di 40 mm e quello antero-posteriore di 25 mm. Le porzioni inferiore e posteriore della prostata prendono rapporti con l'ampolla rettale essendo dirette obliquamente in basso ed in avanti, l'esplorazione rettale ne potrà apprezzare quindi la porzione posteriore con l'interposizione della parete intestinale e della aponeurosi di Denonvilliers.

La vascolarizzazione arteriosa e venosa della ghiandola è molto abbondante, la prima è particolarmente sviluppata nel distretto periuretrale, la seconda è raccolta in tortuosi plessi che circondano l'organo ghiandolare. La prostata infine contrae delicati rapporti prossimalmente con lo sfintere uretrale liscio, da cui è rivestita la sua faccia superiore, e distalmente con lo sfintere striato con cui è in continuità tramite il suo apice. I dotti ghiandolari si aprono nell'uretra prostatica nel cui lume, a livello della parete posteriore ed in corrispondenza di una rilevanza definita *veru montanum*, sboccano anche i dotti eiaculatori dopo aver attraversato il parenchima ghiandolare. La ghiandola prostatica entra quindi in intimo rapporto con il sistema vescico-uretrale e va considerata uno degli organi sessuali secondari.

Nella prostata si possono identificare tre distinte regioni anatomiche: la zona periferica (PZ), quella centrale(CZ) e quella di transizione (TZ). La zona periferica comprende circa il 65% della prostata. La zona centrale, situata attorno ai dotti eiaculatori, comprende circa il 25%. La zona di transizione è composta da due lobi posti ai due lati del tratto iniziale dell'uretra e comprende il 5-10% della prostata. La zona di transizione è separata dalle altre due zone da una banda di tessuto fibro-muscolare.

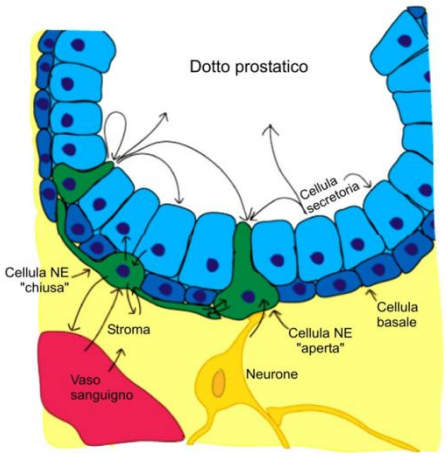


Dal punto di vista istologico il tessuto ghiandolare prostatico è costituito da un numero variabile (30 - 50) di ghiandole tubulo acinose ramificate, spesso chiamate anche ghiandole otricolari, immerse in uno stroma fibromuscolare.La prostata infatti ricorda un grappolo d'uva (componente ghiandolare) immerso in una gelatina organizzata da tralci fibrosi (stroma).



Prostata umana

I dotti ed gli acini sono rivestiti da un epitelio bistratificato. Questo comprende lo strato di *cellule basali,* contenenti le citocheratine CK5 e CK14 e l'antigene nucleare p63, e le cellule luminali secretorie contenenti le citocheratine CK8 e CK18 , il PSA (*prostate-specific-antigen*) , il PSAP (*prostatic-specific acid phosphatase*) e l' AR (*recettore androgenic*o) L'epitelio prostatico comprende anche rare cellule neuroendocrine. Queste producono diverse sostanze ormonali, in particolare la cromogranina A. Le cellule neuroendocrine hanno la funzione di favorire lo sviluppo e



la crescita della prostata , queste cellule non contengono recettori per gli androgeni, pertanto la loro funzione non è modulata dalla stimolazione ormonale.

Oltre queste cellule esiste un subpopolazione di cellule basali, note come cellule intermediarie che esprimono sia i marcatori delle cellule basali che di quelle luminali. Inoltre è noto che la prostata adulta va incontro a cicli di regressione e rigenerazione cellulare , soprattutto inseguito a tecniche di ablazione androginica, il che implica l'esistenza di cellule staminali , contenute nell'epitelio prostatico capaci di arginare i danni dell'ablazione androginica e promuovere una rigenerazione dell'epitelio. Fino ad oggi queste cellule staminali sono state identificate sia nelle cellule basali che in quelle. In particolare, sottopopolazioni di cellule basali, isolate utilizzando marcatori specifici marcatori celllulari , mostravano caratteristiche di multi potenza e di auto-rinnovamento. E' stata inoltre identificata una rara popolazione di cellule luminale, resistente alla castrazione che esprimevano marcatori tipici delle cellule secretorie. Queste cellule staminali sembra siano coinvolte nella tumorogenesi prostatica, soprattutto nel momento in cui dopo un iniziale trattamento del cancro , mediante l'ablazione androginica, si sviluppano forme tumorali molto aggressive ed ormono indipendenti. Questa osservazione porta ad ipotizzare che la sottopopolazione di cellule tumorali residue dopo l'ablazione androginica, contiene queste cellule primitive che possono potenzialmente causare la ricorrenza della malattia Lo stroma prostatico, molto abbondante è costituito principalmente da tessuto connettivo con molte fibre elastiche e da tessuto muscolare liscio. Le zone periferica e centrale hanno una morfologia analoga: spazi acinari , rivestiti da cellule secretorie alte, separati da stroma muscolare liscio. Nella zona di transizione gli acini hanno una struttura lobulare . I dotti convergono verso il verumontanum (*colliculus seminalis*). Lo stroma è più compatto di quello della zona periferica. Il significato clinico delle tre zone

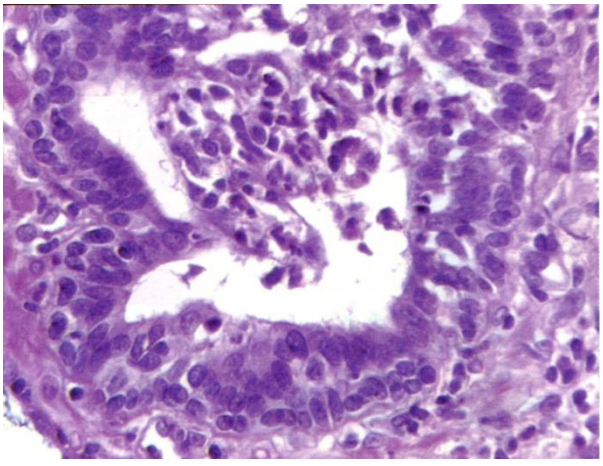
della prostata è connesso al fatto che quella di transizione è la sede dell'iperplasia prostatica benigna (IPB) e del 25% degli adenocarcinomi, mentre le zone periferica e centrale sono la sede di origine di circa il 75% dei carcinomi della ghiandola

Patologie prostatiche non neoplastiche

La prostata è soggetta a diverse alterazioni quali, prostatiti, atrofia, metaplasie, ed iperplasia.

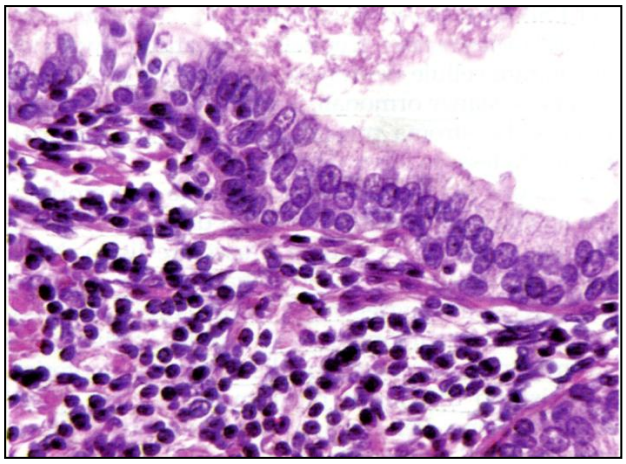
Prostatiti

L'infiammazione della prostata , usualmente indicata come prostatite , è frequente nella pratica urologica, è diagnosticata clinicamente ed in genere è sostenuta da una infezione batterica, quindi trattata con antibiotici. Può essere sia acuta che cronica. L'infiammazione acuta è caratterizzata istologicamente dalla presenza di granulociti neutrofili, sia nell'interstizio che nel citoplasma della cellule epiteliali e nel lume dei dotti e degli acini . Lo stroma, soprattutto peri-ghiandolare, è edematoso e sede di infiltrazione neutrofila.



Infiammazione acuta con granulociti neutrofili nel lume degli acini prostatici.

L'infiammazione cronica è caratterizzata dalla presenza soprattutto di linfociti T e B, talora associati a plasmacellule e istiociti. La flogosi è soprattutto stromale, per-ghiandolare ed anche perineurale o vascolare.



Infiammazione cronica localizzata a livello dello stroma peri-ghiandolare.

Le prostatiti possono essere anche granulomatose.

La prostatite granulomatosa specifica, è generalmente di natura micobatterica e può osservarsi in pazienti con tubercolosi sistemica, mentre la prostatite granulomatosa non specificaè il tipo più frequente di prostatite granulomatosa. L'eziologia di queste lesioni è attribuita ad una reazione a tossine batteriche, a detriti cellulari ed all'infiltrazione nello stroma di secrezioni ghiandolari provenienti da dotti ostruiti. Dal punto di vista istologico il tessuto prostatico è obliterato da un denso infiltrato nodulare misto , talora posto attorno a dotti e acini e costituito da linfociti , plasmacellule, neutrofili, eosinofili e istiociti con cellule giganti plurinucleate.

Le prostatiti possono essere micotiche e si verificano in soggetti immunodepressi e con micosi disseminata. Le forme più comuni sono la blastomicosi, la coccidiosi e la criptococcosi.

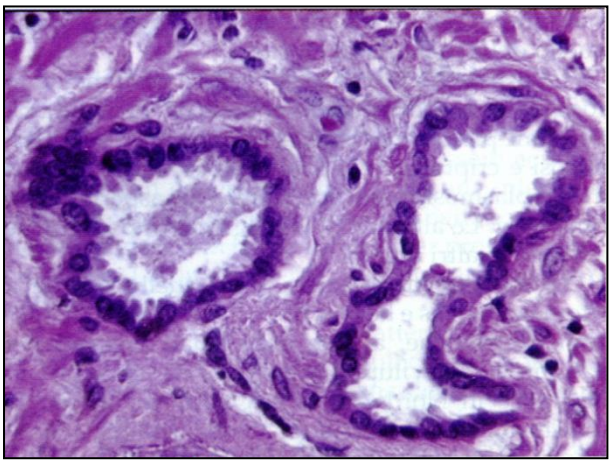
Inoltre appartiene alle prostatiti anche la Malocoplachia prostatica, che può simulare clinicamente un tumore, poiché determina un indurimento della ghiandola all'esplorazione rettale ed una lesione ipoecogena all'ecografia transrettale. Generalmente questa lesione è accompagnata da infezioni urinarie da *E. Coli* .

Atrofia

L'atrofia prostatica è comune in soggetti di età superiore ai 50 anni. Diversi fattori possono indurre l'atrofiadelle strutture acinari e duttali e dell'epitelio di rivestimento:

compressione da parte di noduli iperplastici, infiammazione, ostruzione, ischemia come quella da aterosclerosi, fattori dietetici ed inibizione ormonale.

L'atrofia semplice è usualmente osservata nella zona periferica. Può essere focale e limitata a gruppi di dotti ed acini oppure essere diffusa. Può essere cistica e non cistica. La forma cistica è comune ed è caratterizzata da dotti ed acini con lume ampio e dilatato, talora fino a 2 cm. L'epitelio è appiattito e i nuclei sono piccoli e con cromatina addensata. La forma cistica è osservabile sia nella zona periferica dove assume una configurazione lobulare oppure nella zona di transizione dove dotti ed acini con epitelio atrofico si possono trovare in contiguità con epitelio iperplastico. L'atrofia non cistica è costituita da aggregati di piccoli acini, talora raggruppati alla periferia di un dotto centrale atrofico e dilatato. Gli acini hanno un contorno rotondeggiante e sono rivestiti da epitelio cuboidale con citoplasma basofilo e nuclei rotondi. Nucleoli prominenti sono osservabili nel 10% dei casi in associazione alla flogosi acuta. Nel lume possono osservarsi corpi amilacei mentre lo stroma è spesso sede di fibrosi con associato inspessimento del collagene periacinare.

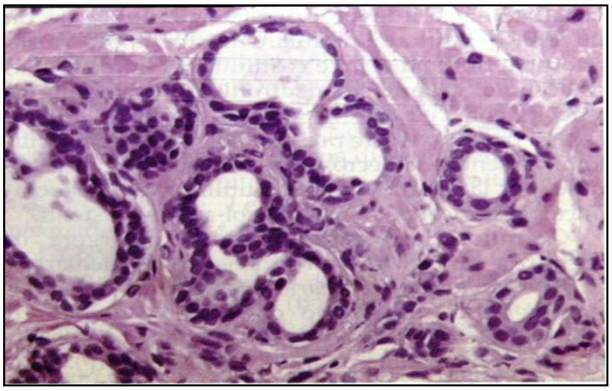


Atrofia semplice.

L'atrofia sclerotica, questa è generalmente localizzata nella zona di transizione. Morfologicamente si presenta con gruppi di acini distorti, a contorno irregolare. La citologia delle cellule è blanda e le cellule basali sono difficilmente evidenziabili nelle sezioni colorate con Ematossilina ed Eosina.

L'atrofia parziale è una variante di atrofia nella quale i dotti e gli acini mostrano profilo variabile da ondulato a stellato e lume modicamente dilatato e contenente materiale amorfo non tingibile. I dotti e gli acini sono rivestiti da cellule cuboidali con citoplasma chiaro. I nuclei sono di forma cilindrica ed evidenziabili solo in un quarto dei casi.

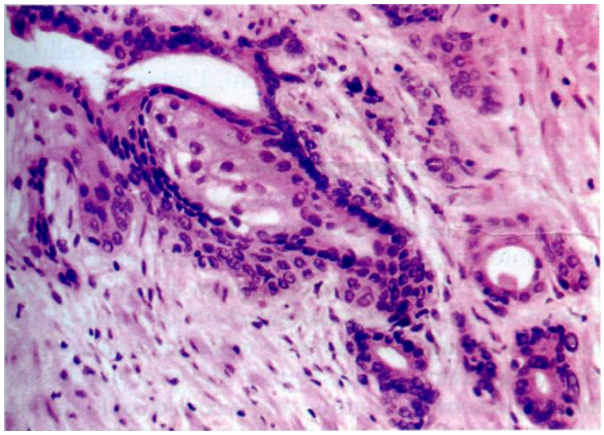
L'iperplasia post-atrofica o atrofia iperplastica si osserva nella zona periferica. Istologicamente è costituita da un dotto centrale dilatato rivestito da epitelio secretorio con citoplasma scarso e chiar, il dotto è circondato da numerosi acini di piccole dimensioni con scarso stroma interposto. Gli acini sono rivestiti da epitelio bi stratificato in cui si identificano lo strato basale e quello secretorio. Nel lume sono presenti corpi amilacei . lo stroma interposto è scarso e mostra atrofia delle fibre muscolari lisce.



Atrofia iperplastica

Metaplasie

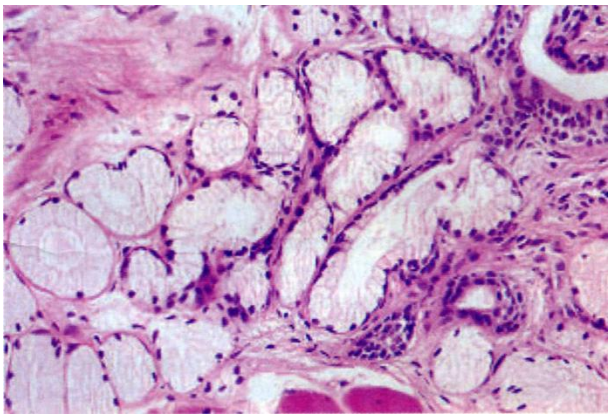
L'epitelio prostatico può mostrare una varietà di lesioni metaplastiche e proliferative che possono simulare l'adenocarcinoma, tra queste la metaplasia squamosa , la uroteliale , la mucinosa e la metaplasia a cellule endocrine con granuli eosinofili. La metaplasia squamosa è una condizione benigna che si associa ad una varietà di condizioni come la prostatite acuta , l'infarto e la terapia ormonale (deprivazione androgenica). Le modificazioni dell'epitelio possono essere focali o diffuse. Dal punto di vista istologico, nella maggior parte dei casi si osserva un epitelio pavimentoso non cheratinizzante.



Metaplasia squamosa

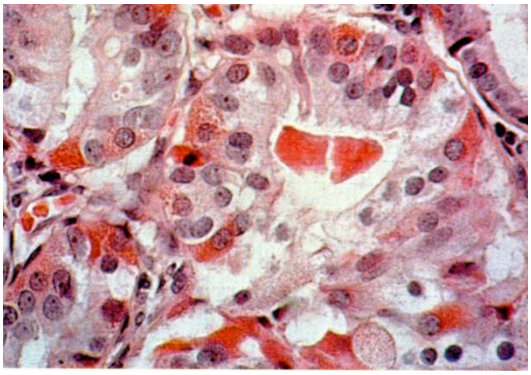
La metaplasia uroteliale (o transazionale) si osserva soprattutto in pazienti sottoposti a manipolazione androgenica. Istologicamente è caratterizzata dalla presenza di urotelio nei dotti ed acini.

La metaplasia mucinosa è di rara osservazione ed è caratterizzata dalla presenza di gruppi di cellule colonnari e caliciformi monostratificate, il lume di questi dotti può contenere mucina.



Metaplasia mucinosa

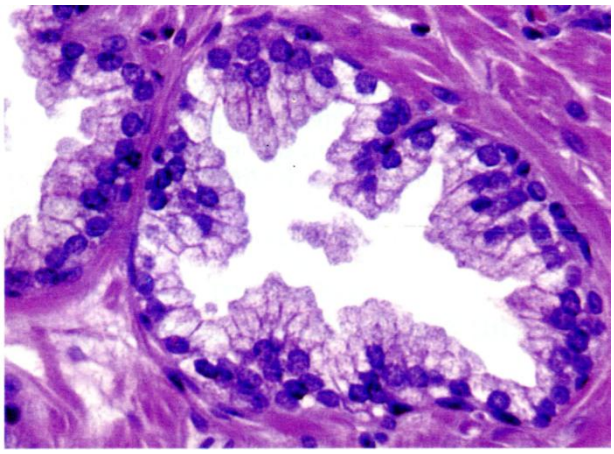
Metaplasia a cellule endocrine con granuli eosinofili rappresenta una forma distinta di differenziazione neuroendocrina dell'epitelio prostatico. E' presente nel 10% delle prostatectomie radicali sotto forma di cellule isolate o in piccoli nidi. Le cellule hanno un citoplasma ampio e ricco di granuli eosinofili.



Metaplasia a cellule neuroendocrine con granuli eosinofili

Iperplasia prostatica benigna (IPB)

Iperplasia Prostatica benigna è il nome comunemente usato per indicare l'accrescimento benigno della zona di transizione della prostata che può determinare vari gradi di ostruzione delle vie urinarie escretrici tale da richiedere talora l'intervento chirurgico. La malattia consiste in un accrescimento nodulare della ghiandola, causato da un'iperplasia di entrambe le componenti, ghiandolare e stromale . Dal punto di vista macroscopico la superficie di taglio mostra noduli di varie dimensioni, con colore che va dal grigio al giallo e di aspetto granulare. In particolare, i noduli sono costituiti da gruppi di dotti ed acini con lume ampio e talora misticamente dilatato, talora con proiezioni papillari endo-luminali. Dal punto di vista microscopico, l'IPB è caratterizzata da un aumento del numero delle cellule epiteliali e stromali Dotti ed acini sono rivestiti da epitelio bi-stratificato identico a quello delle prostate non iperplastiche. In particolare i nuclei sono rotondeggianti con nucleoli non sempre visibili. Nel lume dei dotti ed acini si apprezzano talora, corpi amilacei e calcificazioni. Accanto ai noduli epiteliali si possono osservare noduli stromali costituiti da tessuto muscolare liscio o da connettivo lasso. Lo stroma è frequentemente sede di infiammazione acuta e cronica.



Iperplasia Prostatica Benigna

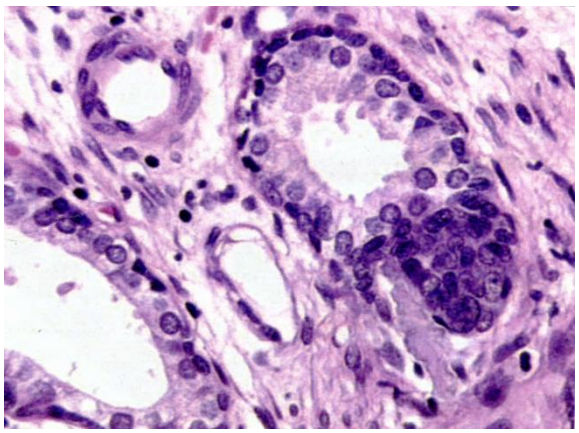
L'etiopatogenesi con l'esatto meccanismo molecolare alla base del processo iperplastico non è del tutto nota. L'aumento del numero delle cellule può essere dovuto o alla proliferazione epiteliale e stromale oppure ad una riduzione della morte cellulare programmata che conduce ad un accumulo cellulare. La teoria "*embryonic reawakening*" considera l'IPB un processo di formazione di nuovi dotti ed acini indotto dalle cellule stromali. Gli androgeni , gli estrogeni, l'interazione stroma – epitelio, i fattori di crescita e i neurotrasmettitori agiscono singolarmente o in combinazione nell'eziologia del processo iperplastico . L'interazione stroma-epitelio osservata nello sviluppo della prostata e della IPB può essere mediata da fattori di crescita solubili o dalla matrice extracellulare, la quale ha capacità simili ai fattori di crescita. Fattori di crescita come FGF (Fibroblast Growth Factor) , EGF (Epidermal Growth Factor), KGF (Keratinocyte Growth Factor) e la IGF (Insulin Growth Factor) hanno un ruolo positive (o di stimolo alla proliferazione) nello sviluppo della IPB; ruolo che è modulato dal DHT (di-idrotestosterone). Il KGF è considerato uno dei fattori più importanti che regolano la proliferazione epiteliale mediata dallo stroma. Un meccanismo opposto a quello stimolatorio è dovuto all'effetto del TGFβ1 (Trasforming Growth Factor). Questo è un potente mitogeno per le cellule stromali e, nello stesso tempo , inibisce la proliferazione epiteliale

Proliferazioni benigne delle cellule basali

Vengono oggi descritte sia forme benigne che maligne della proliferazione delle cellule basali. Le prime comprendono l'iperplasia usuale delle cellule basali, l'iperplasia atipica

e l'adenoma, le seconde invece comprendono il carcinoma basocellulare e le sue varianti morfologiche.

L'iperplasia usuale delle cellule basali è definita anche fetalizzazione o iperplasia embrionale per la sua somiglianza morfologica con la prostata fetale. Si osserva prevalentemente nella zona di transizione molto frequentemente in soggetti sottoposti a manipolazione ormonale. Dal punto di vista istologico questa iperplasia è spesso multifocale ed è caratterizzata dalla presenza di due o più strati di cellule rotondeggianti o allungate. I nuclei sono in genere rotondi e con cromatina finemente dispersa. Rare sono le figure mitotiche. I dotti e gli acini possono mostrare diversi gradi di proliferazione concentrica od eccentrica delle cellule basali , fino alla formazione di nidi solidi con obliterazione del lume e scomparsa delle cellule secretorie.

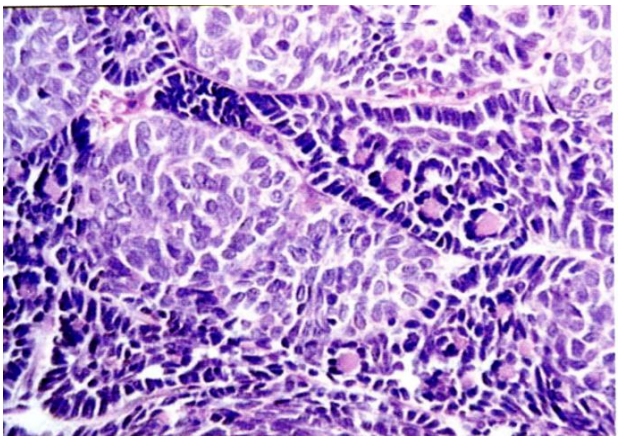


Iperplasia usuale delle cellule basali

L'iperplasia atipica delle cellule basali (o iperplasia con nucleoli prominenti) è morfologicamente simile alla forma usuale , la differenza è rappresentata dalla presenza di nucleoli prominenti con diametro superiore a 1,3 micron.

L'adenoma delle cellule basali è mitologicamente simile all'iperplasia. La differenza è rappresentata dal fatto che nell'adenoma la proliferazione è di tipo nodulare con margini periferici arrotondati.

Il carcinoma basocellulare è un tumore aso potenziale di malignità con crescita ed infiltrazione locale, che solo raramente da metastasi. E' usualmente osservato nella zona di transizione. E' un tumore raro , si tratta di una neoplasia costituita da nidi solidi, tubuli e cordoni con cellule con morfologia basaloide e disposte perifericamente a palizzata. La morfologia è analoga a quella del carcinoma basocellulare della cute. In alcuni casi si osserva la presenza di lumi contenenti mucina o materiale eosinofilo simil-membrana basale.

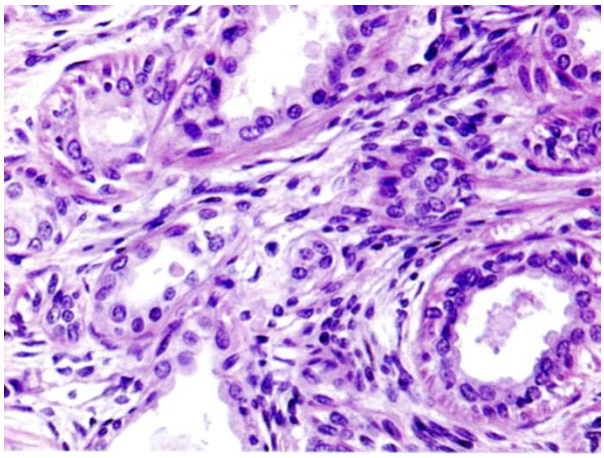


Carcinoma basocellulare

Adenosi sclerosante

L'adenosi sclerosante è una forma di proliferazione microacinare benigna con stroma a cellule fusate. E' considerata una proliferazione metaplastica originante dalle cellule basali, è generalmente presente nella zona di transizione dove si associa all'iperplasia nodulare benigna.Dal punto di vista istologico la lesione mostra piccoli noduli singoli o multipli, di dimensioni variabili da 1,5 a 11 mm. E' costituita da piccoli acini con scarso stroma interposto, gli acini possono essere rotondeggianti, ovalari o ramificati, occasionalmente sono compressi e senza lume. Gli acini sono rivestiti da

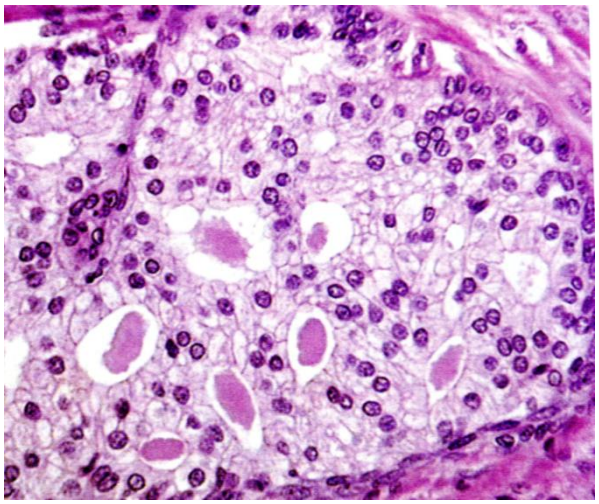
epitelio bi stratificato. Le cellule basali mostrano un fenotipo mio epiteliale. Le cellule secretorie hanno uno scarso citoplasma vacuolato o anfofilo e nuclei in genere rotondeggianti. Nel lume degli acini si osserva la presenza di cristalloidi e di mucina. La membrana basale è ben evidenziabile es eosinofila.Lo stroma è densamente cellulato e costituito da cellule fusate separate da matrice collagena o mixoide, debolmente basofilo.



Adenosi sclerosante

Iperplasie benigne rare

L'iperplasia cribiforme è una variante di iperplasia epiteliale che si osserva nell'8% delle prostate non trattate. E' una lesione benigna e non mostra alcun rapporto con lo sviluppo della neoplasia prostatica. Dal punto di vista istologico i dotti e gli acini coinvolti, localizzati prevalentemente nella zona di transizione, hanno architettura cribiforme dell'epitelio secretorio con obliterazione del lume. Le cellule mostrano spesso citoplasma chiaro e nuclei rotondi con nucleoli non identificabili. Lo strato basale cellulare è intatto ed è facilmente identificabile in ematossilina ed eosina.



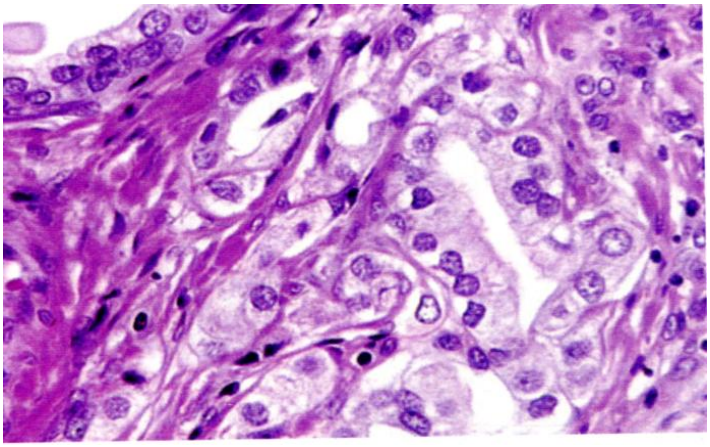
Iperplasia cribriforme

L'iperplasia delle ghiandole mucose del verumontanum è una lesione microacinare localizzata in prossimità dello sbocco al livello dell'uretra dei dotti eiaculatori e dell'utricolo. Istologicamente si osservano noduli costituiti da piccoli acini con scarso stroma interposto. Gli acini sono rivestiti da epitelio bi stratificato che comprende uno strato basale positivo per p63 ed uno luminale positivo per PSA. Il citoplasma delle cellule può essere eosinofilo, basofilo o chiaro, i nuclei sono prevalentemente regolari , rotondi e con piccoli nucleoli. Frequentemente nel lume si riscontrano corpi amilacei.

L'iperplasia dei residui mesonefrici è una lesione di rara osservazione, presente nel tessuto prostatico e nei tessuti circostanti. E' costituita da gruppi di piccoli tubuli rivestiti da epitelio cubico monostratificato, PSA negativo. Il citoplasma è scarso , mentre i nuclei sono rotondi e regolari. I nucleoli non presentano alterazioni rilevanti. Il lume degli acini è talora misticamente dilatato e può mostrare proiezioni papillari e materiale simil-colloideo.

Iperplasia adenomatosa atipica

L'iperplasia adenomatosa atipica è caratterizzata da una proliferazione circoscritta di piccoli acini con scarso citoplasma interposto. Gli acini sono rivestiti da cellule secretorie che morfologicamente sono simili a quelle dei dotti ed acini normali. I nucleoli sono generalmente prominenti, mentre lo strato basale è evidenziabile con tecniche immunoistochimiche mediante anticorpi per le citocheratine ad alto peso molecolare, mostrando unao strato basale discontinuo.



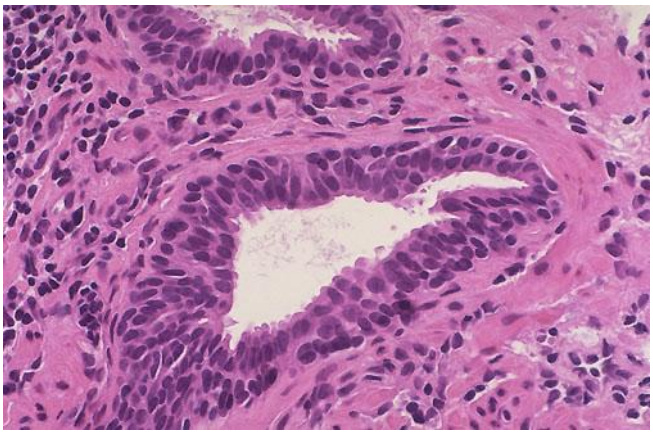
Iperplasia adenomatosa atipica

Patologie prostatiche neoplastiche

Neoplasia intraepiteliale

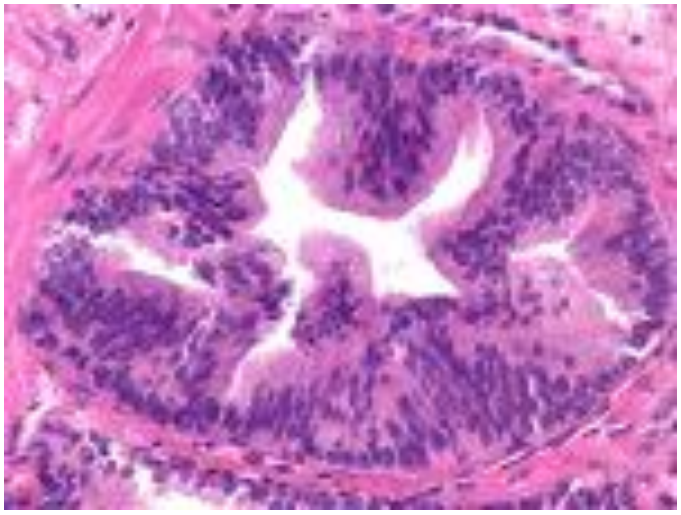
La neoplasia intraepiteliale della prostata o (PIN) è caratterizzata dalla proliferazione delle cellule secretorie di dotti ed acini pre-esistenti; le cellule che formano la PIN mostrano atipia, che può essere minima o simile a quella delle cellule neoplastiche. Lo strato basale è riconoscibile. Esiste una classificazione dei PIN in basso grado e alto grado che si basa principalmente sulle caratteristiche citologiche della lesione.

I nuclei delle cellule che compongono la *PIN di basso grado* (LGPIN) sono ampi, hanno dimensioni variabili, contenuto cromatinico normale o lievemente aumentato e possiedono piccoli nucleoli.



PIN basso grado

La *PIN di alto grado* (HGPIN)è caratterizzata da cellule con nuclei grandi e relativamente uniformi per dimensione, con contenuto cromatinico aumentato, che può essere distribuito irregolarmente, nucleoli prominenti simili a quelli delle cellule neoplastiche.



PIN di alto grado

Esistono tre varianti di PIN di alto grado con cellule di tipo non comune:

La PIN con cellule ad anello con castone, mostra un modello architetturale di crescita a ciuffo e micro papillare, le cellule che lo compongono hanno morfologia identica a quella delle cellule del carcinoma a cellule "signet-ring" infiltrante, lo strato delle cellule basali è evidente.

La PIN a piccole cellule neuroendocrine, mostra nella maggior parte dei casi un aspetto cribriforme ed è caratterizzata dalla presenza di cellule secretorie e di cellule piccole; le prime sono localizzate perifericamente, mentre le seconde sono poste centralmente e realizzano strutture tipo rosette. La componente a piccole cellule viene ritenuta espressione di differenziazione neuroendocrina.

La PIN con secrezione mucinosa, è caratterizzata da dotti ed acini con lume disteso e ripieno di mucina. Il pattern architetturale più frequente è quello piatto.

Carcinoma della prostata

Adenocarcinoma

I fattori che determinano o che contribuiscono a determinare l'insorgenza di questo tipo di carcinoma non sono ancora del tutto chiare. Sicuramente è coinvolto nel suo sviluppo il fattore ormonale, come dimostrato da Huggins, premio Nobel per aver scoperto che

gli ormoni maschili (androgeni) possono accelerare la crescita del tumore prostatico, mentre la loro eliminazione porta a
un rallentamento nella progressione dello stesso. E' stato inoltre osservato come gli eunuchi non sviluppino il carcinoma prostatico mentre, un eccesso di androgeni alla pubertà rappresenti un fattore favorente. Diversi studi, poi, sottolineano la componente familiare di questo tumore, tant'è che la neoplasia è stata riscontrata più frequentemente nei familiari di pazienti affetti da tumore
prostatico. Uomini con parenti di primo grado affetti da tumore prostatico hanno un rischio doppio di sviluppare la malattia. Sull'alimentazione troppo ricca di grassi ci sono solo sospetti, si è visto come con diete ricche di vegetali l'incidenza della malattia sia ridotta, mentre aumenterebbe con una dieta ricca di grassi e proteine animali. Anche i fattori sessuali sono stati oggetti di studio
per un loro eventuale ruolo nello sviluppo della malattia. Si è potuto constatare che il tumore è spesso associato a trasmissioni di malattie virali (virus della famiglia dei papovavirus, citomegalovirus, virus herpetico), come anche ad un comportamento sessuale non regolare, o troppo scarso o troppo abbondante. Una correlazione fra tumore prostatico e fumo non è stata provata mentre, si è visto come l'esposizione a sostanze chimiche tipo ossido di cadmio, tipico dei
lavoratori della gomma, possa favorire lo sviluppo di tale neoplasia.
Questa neoplasia è caratterizzata dall'essere, nei primi stadi, asintomatica. La comparsa di sintomi delle basse vie urinarie (disuria, pollachiuria) solitamente è ritenuta secondaria a malattia localmente avanzata. Qualche volta , parestesie, dolori o deficit motori agli arti inferiori, incontinenza fecale ed urinaria possono essere secondari a compressioni midollari da metastasi vertebrali. L a diagnosi di carcinoma prostatico si basa essenzialmente sul dosaggio del PSA sierico, esplorazione digito- rettale, ecografia trans rettale e ago biopsia prostatica.

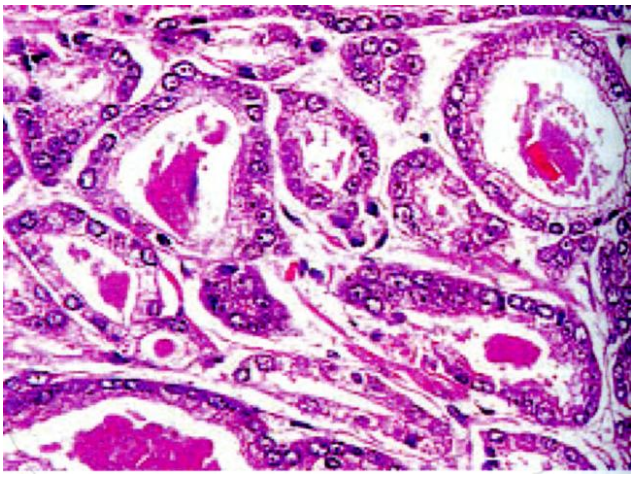
Il PSA viene generalmente valutato con riferimento a un valore soglia positivo/negativo calcolato sulla base della distribuzione del marcatore nei soggetti normali. Il valore soglia più utilizzato è 4 ng/ml. Tuttavia , un dilemma diagnostico critico è rappresentato dalla sovrapposizione fra soggetti con neoplasia confinata all'organo e soggetti con ipertrofia prostatica benigna che presentano valori di PSA compresi tra 4 e 10 ng/ml. Inoltre , è sempre più consistente il problema di porre diagnosi di carcinoma anche in

pazienti con PSA inferiore a a 4 ng/ml in quanto il 25-30% dei pazienti con neoplasia confinata all'organo presentano valori di PSA compresi tra 2,5e 4 ng/ml. Con l'esplorazione digito-rettale si riescono ad apprezzare solo i tumori che originano nella zona periferica. Sfuggono invece , quei tumori che hanno origine nella zona di transizioneo che non sono molto estesi. L'egografia trans-rettale può essere di ausilio all'esplorazione digito-rettale grazie al fatto che il 40% delle neoplasie appaiono ipoecogene. La biopsia prostatica viene eseguita in tutti i casi in cui vi sia il sospetto di neoplasia.

L a sede di origine più comune dell'adenocarcinoma è la zona periferica (circa il 70% dei tumori) ed è quindi apprezzabile all'esplorazione rettale. Meno comune è l'insorgenza dalla zona di transizione (20%) , mentre la zona centrale che costituisce la parte prevalente della base della prostata, raramente è sede d'origine del tumore (5%).

L'identificazione macroscopica del tumore non appare sempre agevole . E' possibile identificare il tumore della zona periferica e centrale, quando questo causa una deformazione del profilo o del contorno ghiandolare, mostra un colore biancastro e presenta una consistenza più compatta rispetto alla prostata normale.

Il quadro di gran lunga più frequente del carcinoma della prostata è l'adenocarcinoma acinare.



Adenocarcinoma acinare

La diagnosi si basa sulla valutazione di alcuni parametri citologici (nucleari e citoplasmatici), intra-luminari, architetturali e specifici di malignità. Dal punto di vista citologico, le cellule tumorali presentano nuclei ampi, provvisti di nucleoli prominenti, multipli e marginati e citoplasma anfofilo. Mucina acida e cristalloidi possono essere presenti nel lume. Dal punto di vista architetturale, l'adenocarcinoma è costituito da strutture acinari di dimensioni piccole o intermedie che hanno contorno esterno e distribuzione stromale irregolare. Le cellule basali sono assenti. Le indagini immunoistochimiche che possono essere d'ausilio nella definizione dell'immuno profilo del carcinoma prostatico e quindi della sua diagnosi , includono la determinazione della PSA, della fosfatasi acida prostatica specifica (PSAP) le citocheratine ad alto peso molecolare, la p63 e l'alfa-metil-CoA-racemasi (P504S) .

L' adenocarcinoma prostatico oltre all'adeno carcinoma acinare, presenta diverse varianti istologiche,:

Carcinoma squamoso, così chiamato per la degenerazione epitelioide spontante di una neoplasia a differenziazione ghiandolare o in seguito a trattamento ormonale.

Adenocarcinoma duttale, origina dalle cellule dei dotti prostatici e ha prognosi peggiore rispetto all'adenocarcinoma classico.

Carcinoma a piccole cellule, istotipo più aggressivo, origina da cellule neuroendocrine intercalate nel contesto del parenchima prostatico·

Carcinomi mucinosi della prostata, neoplasie che si caratterizzano per la tendenza a produrre elevate quantità di muco.

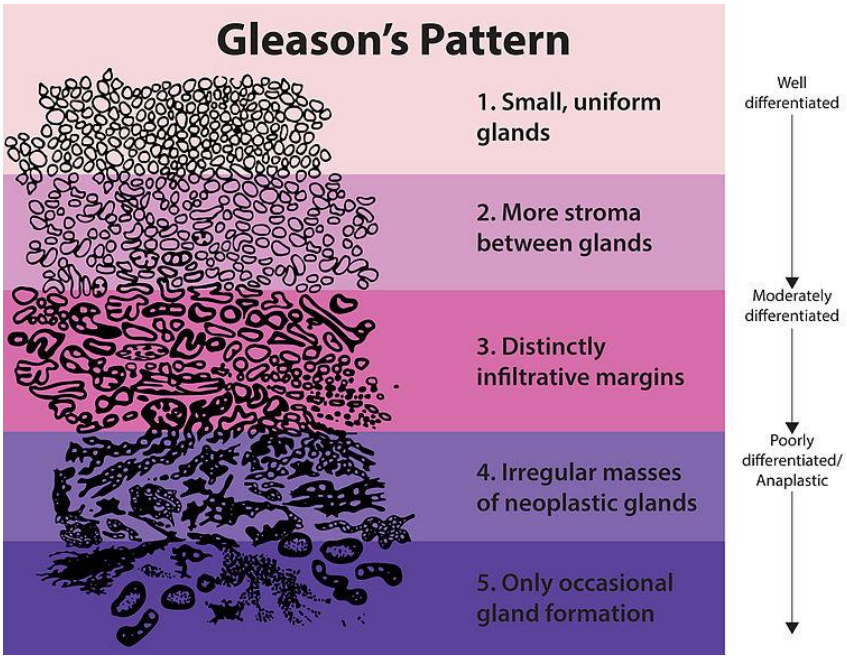
Neoplasie mesenchimali, come sarcomi o linfomi, molto rari.

Il comportamento clinico di molte di queste varianti è diverso da quello del carcinoma acinare. La loro esatta identificazione permette al clinico di impostare la terapia più appropriata.

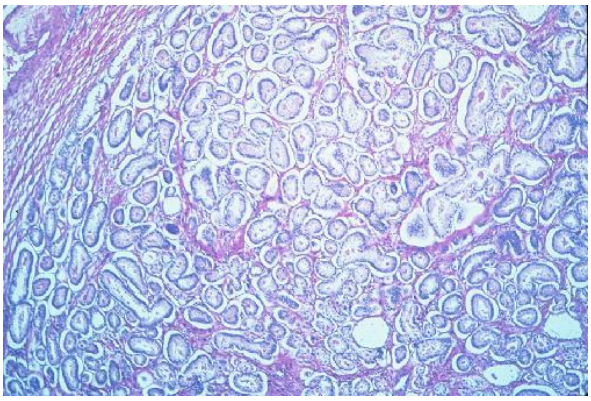
Classificazione del PCa

La prognosi nei pazienti con tumore alla prostata dipende da fattori clinici, di laboratorio, morfologici e molecolari. Per grado istologico, il sistema Gleason è

considerato il sistema di riferimento internazionale. Si basa sulla valutazione delle caratteristiche architetturali della neoplasia. Vengono riconosciuti 5 diversi pattern:

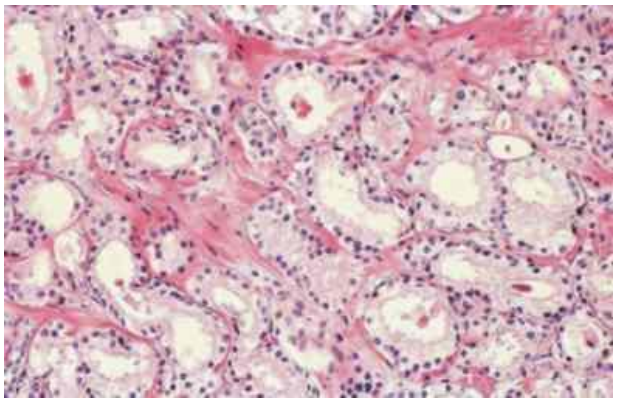


- *Gleason 1:* Tumore composto da noduli ben delimitati di ghiandole, che ricordano il tessuto prostatico normale, strettamente ravvicinate, uniformi, singole e separate l'una dall'altra. Generalmente questo quadro istologico viene riscontrato nei tumori ben differenziati

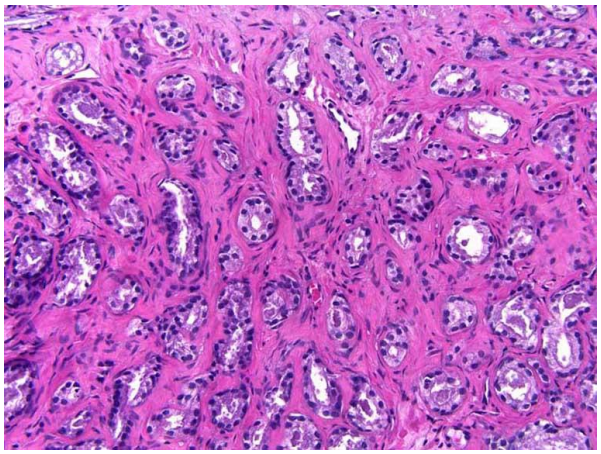


Gleason 2: Tumore ancora abbastanza circoscritto, ma con eventuale minima estensione delle ghiandole neoplastiche alla periferia del nodulo tumorale, nel tessuto prostatico non neoplastico. Le ghiandole appaiono più larghe e separate da una quantità di tessuto

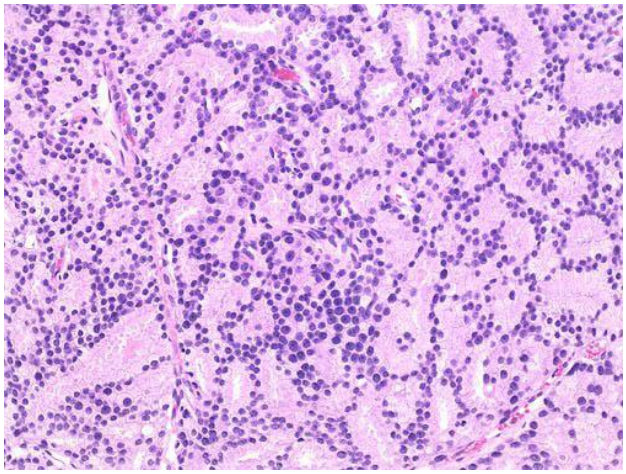
stromale maggiore rispetto alle ghiandole normali. Questo quadro è caratteristico dei tumori moderatamente differenziati.



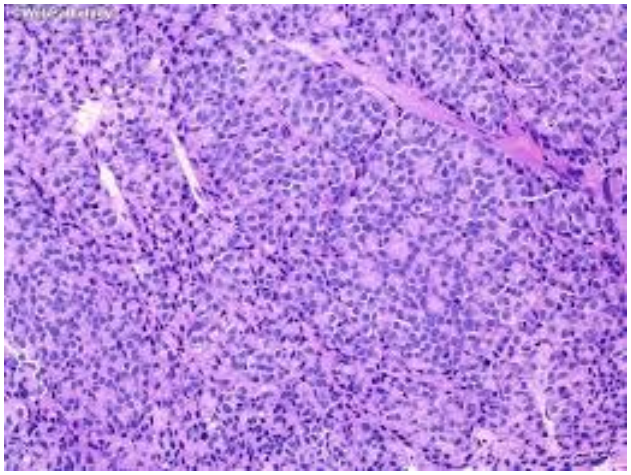
Gleason 3: In questa tipologia tumorale ancora si riesce a distinguere un aspetto ghiandolare, che infiltra il tessuto prostatico non tumorale; le ghiandole presentano notevole variabilità di forma e dimensione , ile cellule appaiono più scure. Anche questo quadro istologico costituisce un carcinoma prostatico moderatamente differenziato



Gleason 4: In questo tessuto tumorale difficilmente si distinguono le ghiandole, infatti le ghiandole tumorali presentano contorni mal definiti e generalmente appaiono fuse tra di loro; possono essere presenti ghiandole cribriformi con bordi irregolari. Questo quadro istologico è tipico di un carcinoma scarsamente differenziato.



Gleason 5: Tumore che non presenta differenziazione ghiandolare, ma è composto da cordoni solidi o da singole cellule. Questo quadro istologico è tipico di un carcinoma anaplastico



Il pattern primario o predominante e quello secondario vengono valutati separatamente e poi combinati nello score di Gleason il suo range è da 2 (1+1) a 10 (5+5) .

Il sistema di Gleason non va utilizzato nei tumori che sono stati sottoposti a trattamento ormonale neo-adiuvante; in questi casi deve essere fatto riferimento al Gleason bioptico precedente la ormonoterapia. Un sistema di grading alternativo, ma meno utilizzato , è quello proposto da Mostofi, che prevede una suddivisione in tre gradi in relazione all'entità dell'anaplasia nucleare.

Lo studio anatomo-patologico del materiale prelevato con la prostatectomia radicale deve fornire indicazioni , utilizzabili per definire lo stadio della malattia.

Questa stadi azione viene formulata secondo il sistema TNM, che comprende :

L'estensione locale del tumore (pT) che include la valutazione del rapporto della neoplasia con i limiti anatomici della prostata (cosiddetta capsula); il superamento di questi limiti anatomici è definito come estensione extra-prostatica. E' sconsigliato l'uso del termine estensione capsulare in quanto la prostata non possiede una vera e propria capsula, bensì una condensazione periferica dello stroma fibro-muscolare.E' importante che il patologo dia un'indicazione dell'estensione quantitativa della estensione extra-prostatica, cioè focale o non focale , poiché questa caratteristica ha un valore prognostico. L'estensione extra-prostatica viene definita focale , quando è uguale o inferiore a due campi ad alto ingrandimento (40X) . Invece quando lì estensione è presente in più di due campi ad alto ingrandimento , questa viene definita di tipo non focale.

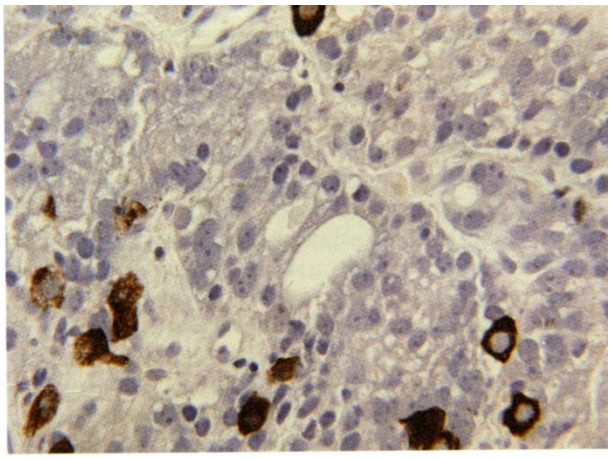
Lo stato dei margini chirurgici , definiti come positivi o negativi. Il margine è positivo quando la neoplasia raggiunge il margine utilizzato per l'identificazione dei margini chirurgici. E' importante che il patologo dia un'indicazione della sede, del numero e della estensione dei margini positivi (positività focale o non focale) in quanto hanno rilevanza prognostica.In generale la distinsione tra focale e non focale è basata sullo stesso metodo utilizzato per la definizione di estensione extra-prostatica. Il margine positivo viene ulteriormente definito come extra-prostatico o intra-prostatico. E' definito intra-prostatico quando la capsula non è presente e il piano di resezione è all'interno della prostata. La presenza di margini positivi è indicata con R (resection marging) il quale può essere 0 (cioè negativo) o R1 (positivo microscopicamente). Margini positivi sono presenti nel 59% dei casi. Negli ultimi anni questa frequenza è diventata più bassa (10%) per il miglioramento delle tecniche chirurgiche e per la precocità della diagnosi. La sede più frequente di margini positivi è l'apice.

Lo stato linfonodale (pN) ha rilievo prognostico.L'identificazione di micro metastasi (metastasi con diametro uguale o inferiore a 0,2 cm) nelle sezioni colorate con

Emastossilina e Eosina o mediante analisi immunoistochimica, nei linfonodi adiacenti è fondamentale nella determinazione della prognosi.

Il riconoscimento di metastasi parenchimali (pM) viene eseguito con tecnologie per immagini (Ecografia , Tomografia Computerizzata, Risonanza Magnetica Nucleare e PET). Tale reperto aggrava notevolmente la prognosi ed impedisce ogni trattamento locale ad intento curativo.

Il carcinoma della prostata comprende due varianti con *differenziazione neuroendocrina* , cioè il carcinoide e il carcinoma neuroendocrino. Il tipo comune contiene una proporzione variabile di cellule neuroendocrine. Si ritiene che la percentuale di cellule neuroendocrine abbia valore prognostico sfavorevole soprattutto nelle neoplasie ormono-dipendenti .



Adenocarcinoma con differenziazione neuroendocrina

Colorazione positiva alla Cromogranina A

Studi recenti hanno dimostrato che l'amplificazione del gene per il recettore androgenico abbia un ruolo prognostico importante nella progressione del carcinoma prostatico. L'amplificazione viene attualmente determinata mediante la tecnica FISH (Fluorescence in situ hybridization). Il ruolo prognostico di altri fattori con tecniche molecolari non è ancora stato definito.

Capitolo II

ANDROGENI E PROSTATA

Androgeni e recettore androgenico

Gli androgeni sono ormoni sessuali maschili responsabili sia della differenziazione e della maturazione degli organi riproduttivi maschili che dello sviluppo dei caratteri sessuali secondari.

Il testosterone (T), l'androgeno sessuale maschile più rilevante, viene prodotto a livello del testicolo dalle cellule del Leydig e rappresenta circa il 90% degli androgeni totali circolanti mentre il rimanente 10% è sintetizzato dalla corteccia del surrene come diidroepiandrosterone (DHEA), androstenediolo e androstenedione, che vengono successivamente convertiti in testosterone nei tessuti periferici.

La produzione di testosterone è regolata dall'asse ipotalamo–ipofisi–gonadi del sistema endocrino attraverso la produzione dell'ormone luteinizzante (LH) rilasciato dall'ipofisi anteriore a seguito della secrezione pulsatile dell'ormone ipotalamico Gonadotropin-releasing hormone (GnRH). Lo stesso testosterone controlla l'attività dell'asse attraverso un meccanismo di feedback negativo operato sia a livello dell'ipotalamo che dell'ipofisi.

Il testosterone è presente in circolo nella forma libera solo per il 2% circa, mentre il rimanente è veicolato nel circolo sistemico legato alle proteine plasmatiche come l'albumina ed a proteine carrier specifiche come Sex Hormone Binding Globulin (SHBG) (Hammond et al., 2003). Essendo una molecola lipofila, il testosterone è in grado di diffondere attraverso il doppio strato fosfolipidico della membrana plasmatica della cellula target, dove viene rapidamente convertito attraverso l'azione catalitica della 5α-reduttasi (Tipo I e II) a diidrotestosterone (DHT).

Sia il DHT che il testosterone possono legare il recettore degli androgeni (AR). L'AR è una fosfoproteina di 110 kDa localizzata a livello citoplasmatico appartenente alla super famiglia dei recettori per gli ormoni steroidei, che operano come fattori di trascrizione (Lamb et al., 2001).

L'AR è costituito da un'unica catena polipeptidica dotata di una struttura modulare con domini funzionali autonomi. Nella porzione N-terminale, una regione variabile e meno

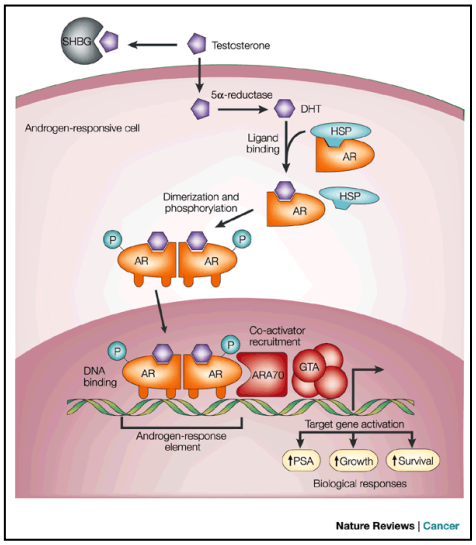
conservata, è presente il dominio di transattivazione "trascriptional activation function 1 (AF-1)", dominio bersaglio di eventi di fosforilazione che partecipa all'attivazione ligando-indipendente del recettore e permette l'interazione con i fattori dell'apparato trascrizionale influenzandone quindi l'attività.

La porzione centrale è costituita dal DNA binding domain (DBD), deputato al riconoscimento di sequenze palindroniche specifiche sul DNA responsive agli androgeni (androgen responsive element, ARE). Il DBD presenta una sequenza amminoacidica ricca di residui di cisteina che coordinano due atomi di zinco e che conferiscono al dominio la struttura spaziale (zinc-finger) necessaria per l'inserimento del recettore nel solco maggiore del DNA. All'interno di tale sequenza, inoltre, sono presenti due regioni importanti per la funzione del dominio stesso: il P-Box e il D-Box che permettono rispettivamente il legame al DNA e la dimerizzazione del recettore.

Una porzione definita "hinge region" funge da cerniera tra il DBD e la porzione C-terminale o ligand binding domain (LBD), contenente al suo interno il dominio di trans attivazione, trascriptional activation function 2 (AF-2), costituita da 12 regioni ad α-elica (H1-H12) ed un β-turn (tra H5-H6) che formano una tasca idrofobica per il legame al ligando specifico ed alle heat shock proteins (Hsp). L'AR, infatti, nello stato basale non legato, è localizzato nel citoplasma in un complesso con le Hsp e le immunofilline chaperonine come Hsp90, 70, 56 e 23 (Pratt & Toft, 1997), la cui funzione è quella di impedire la corretta conformazione recettoriale e impedirne l'attivazione. A seguito del legame del testosterone o del DHT al LBD del recettore, il complesso AR-Hsp subisce una variazione conformazionale che determina il rilascio delle Hsp, la dimerizzazione del recettore, la liberazione della porzione del DBD e la conseguente traslocazione dell'AR attivato nel nucleo e il legame al DNA a livello del promoter dei geni target (AREs : androgen responsive elements)

Una volta legato al DNA, l'AR è in grado di reclutare numerose proteine co-regolatorie che svolgono un ruolo nell'attività trascrizionale (Shang et al., 2002), come i co-attivatori appartenenti alla famiglia delle p160 (SRC-1, GRIP1/TIF2 e RAC3/pCIP/ACTR/AIB1/TRAM1), P/CAF, CBP, Tip60 e p300 ad azione istone acetil-trasferasica (HAT). La loro funzione è quella di indurre acetilazione degli istoni rendendo la cromatina accessibile alla trascrizione da parte della polimerasi e i co-attivatori AR-associated (ARA) ARA70, 55 e 54 che interagiscono con il complesso di

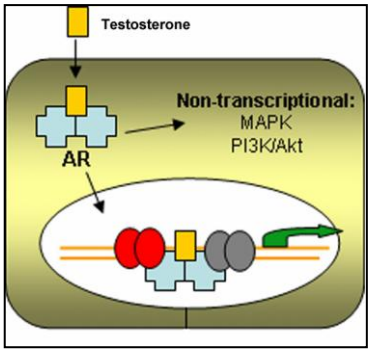
inizio trascrizione regolando in modo specifico i prodotti della trascrizione genica regolata dagli androgeni (Naar et al., 2001).



Meccanismo di azione degli androgeni

In aggiunta alle proprietà trascrizionali, l'AR esercita funzioni non-genomiche per le quali il legame testosterone/DHT-AR porta all'attivazione della via di trasduzione del segnale Ras/extracellular signal-related kinase (ERK) attraverso l'attivazione di chinasi come c-Src (Migliaccio et al., 2000; Kousteni et al., 2001; Kousteni et al., 2003; Unni et al., 2004).normalmente mantenuto inattivo mediante il legame di heat-shock proteins alla regione HBD (Hormone Binding Domain)

Il testosterone, induce la proliferazione attivando il pathway PI3K/Akt e MAPK



AR e prostata

Nella prostata il recettore per gli androgeni è localizzato sia nella porzione epiteliale che in quella stromale e svolge diverse azioni: a livello del tessuto epiteliale l'AR controlla la proliferazione cellulare, induce differenziamento e regola le funzioni metaboliche e

secretorie della prostata, mentre a livello stromale, AR regola la produzione di alcuni fattori di crescita come Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Insuline-like Growth Factor (IGF), Neuropepeptide Growth Factor (NGF), Keratinocite Growth Factor (KGF), che vanno poi ad agire in modo paracrino sullo strato epiteliale regolandone positivamente la proliferazione delle cellule basali e proteggendo dall'apoptosi le cellule secretorie luminali (Shung & Chung, 2002).

La prostata non può svilupparsi, differenziarsi, mantenere le dimensioni fisiologiche e funzionare in assenza di T e DHT. La conversione del T a DHT nel tessuto prostatico è catalizzata dall'enzima 5α-reduttasi di Tipo II soprattutto a livello della componente stromale (Silver et al., 1994). Successivamente il DHT viene trasportato a livello delle cellule epiteliali. Tra i due compartimenti esiste infatti una profonda relazione che porta ad avere uno sviluppo controllato della ghiandola prostatica. Questo "crosstalk" tra stroma ed epitelio è molto importante per la regolazione della crescita e dello sviluppo della prostata in risposta all'azione androgenica.

Diversi studi suggeriscono l'esistenza di centinaia di geni il cui livello di espressione subisce una variazione significativa in seguito all'esposizione delle cellule prostatiche agli androgeni (De Primo et al., 2002; Nelson et al., 2002; Velasco et al., 2004; Segawa et al., 2002; Waghray et al., 2001; Xu et al., 2001). Tuttavia solo 13 di questi geni svolgerebbero un ruolo rilevante nelle normali funzioni della prostata in condizioni fisiologiche (Velasco et al., 2004). Uno di questi geni androgeno responsivi è quello che codifica per il prostate specific antigen (PSA), una proteasi il cui dosaggio viene utilizzato come un importante marker clinico per lo studio dello sviluppo e dell'ipertrofia prostatica benigna (BPH) e della progressione del tumore prostatico (CaP). Oltre ad esso, attraverso questi studi si sono identificati altri geni androgeno-regolati che comprendono i geni per l'enzima regolatore del ciclo cellulare cyclin-dependent kinase (Cdk-8), la subunità catalitica p85 della phosphatidilinolitol-3-kinase (PI3K) e RAB4, membro della famiglia dell'oncogene Ras.

Androgeni e carcinoma prostatico

La crescita del carcinoma prostatico è, in fase iniziale, primariamente dipendente dagli androgeni; tali ormoni circolano legati, in modo reversibile, alla proteina SHBG, ma la frazione considerata biologicamente attiva è rappresentata da una piccola percentuale di

ormone libero. Non è stato ancora totalmente chiarito il ruolo di questa proteina nello sviluppo del tumore prostatico ma, rappresentando una riserva di testosterone, elevati livelli di SHBG sono stati associati ad un maggior rischio di tumore (Meikle et al., 1987; Geller & Vik Franson, 1989). Gli studi epidemiologici finora effettuati non hanno riportato alcuna associazione tra i livelli sierici degli androgeni e il rischio di tumore prostatico ma hanno piuttosto indicato una maggiore concentrazione di androgeni tissutali nella prostata neoplastica rispetto a quella normale (Heracek et al., 2007).

Sebbene lo specifico tipo cellulare all'interno del compartimento epiteliale prostatico che porta all'insorgenza del tumore prostatico sia ancora oggetto di discussione (Litvinov et al., 2003) ed il meccanismo molecolare genetico implicato nell'iniziazione e nello sviluppo del cancro prostatico non sia descritto con precisione, l'attività dell'AR appare di notevole importanza in tutte le fasi della progressione della patologia tumorale (Litvinov et al., 2003). Proprio per questo motivo, le attuali terapie per il tumore prostatico allo stadio avanzato prevedono o la somministrazione di agonisti di GnRH o l'orchidectomia (per rimuovere gli androgeni prodotti dal testicolo) o la somministrazione di antagonisti dell'AR come la bicalutamide, flutamide o ciproterone acetato (CPA).

L'importanza rivestita dall'azione dell'AR nelle diverse fasi dello sviluppo e della progressione nonché nel trattamento terapeutico del tumore prostatico, ha portato alla necessità di avere modelli cellulari che permettessero di studiare i meccanismi molecolari di attivazione e di regolazione dell'AR. Ad es alcuni autori (Scaccianoce et al., 2003) hanno isolato una linea cellulare di CaP androgeno-indipendente DU145 iperesprimente l'AR. Le cellule DU145 sono state trasfettate stabilmente col plasmide contenente il gene dell'AR umano e sono state selezionate le colonie cellulari esprimenti l'AR (DU145-AR). Nelle cellule DU145-AR la localizzazione dell'AR è a livello citoplasmatico in condizioni basali in assenza di androgeni, mentre in seguito al trattamento con T (0,1 μM per 48h) l'AR attivato trasloca nel nucleo. Le cellule DU145-AR trattate con T vedono aumentata la proliferazione cellulare, aumentando la crescita di circa il 30% rispetto alle cellule trasfettate con il plasmide controllo, mentre l'androgeno non esercita alcun effetto sulla proliferazione cellulare delle cellule DU145-pcDNA3. L'androgeno-dipendenza ripristinata nelle cellule DU145-AR viene

confermata dal fatto che la trasfezione delle cellule DU145 con l'AR ripristina l'espressione del PSA, assente nelle cellule parentali Le cellule DU145-AR con la loro ripristinata sensibilità agli androgeni hanno rappresentano quindi un valido modello per lo studio dei meccanismi di attivazione e regolazione dell'AR coinvolti nella progressione del CaP da androgeno-dipendente ad androgeno-indipendente.

Studi più recenti hanno indicato che l'inizio de processo di tumorogenesi prostatica può in molti casi essere attribuito all'attivazione androgeno dipendente di distinti pathways di promozione della crescita cellulare. Ad es è stata riportata la up-regolazione androgeno dipendente dei membri della famiglia di fattori di trascrizione E-twenty-six (ETS) tramite la fusione genica tra il gene promoter TMPRSS2 AR -regolato e la regione codificante di alcuni membri della famiglia ETS ,ERG e la variante 1 di ETS: la overespressione di ERG e/o di ETV1 si verifica infatti nel 50% dei tumori prostatici .Questa fusione conferisce responsività agli androgeni ai fattori di trascrizione ETS , che porta a progressione del ciclo cellulare (Rubi n et al., 2011). Altri patways di segnalazione coinvolti nell'iniziazione del tumore prostatico includono la disregolazione dei pathways PI3K and RAS/(Taylor et al 2010) In questo studio, l'analisi del patways di segnalazione di AR ha rivelato maggiori alterazioni rispetto ad altri patways, indicando AR come il. "master regulator" del carcinoma prostatico

Carcinoma prostatico androgeno resistente(CRPC)

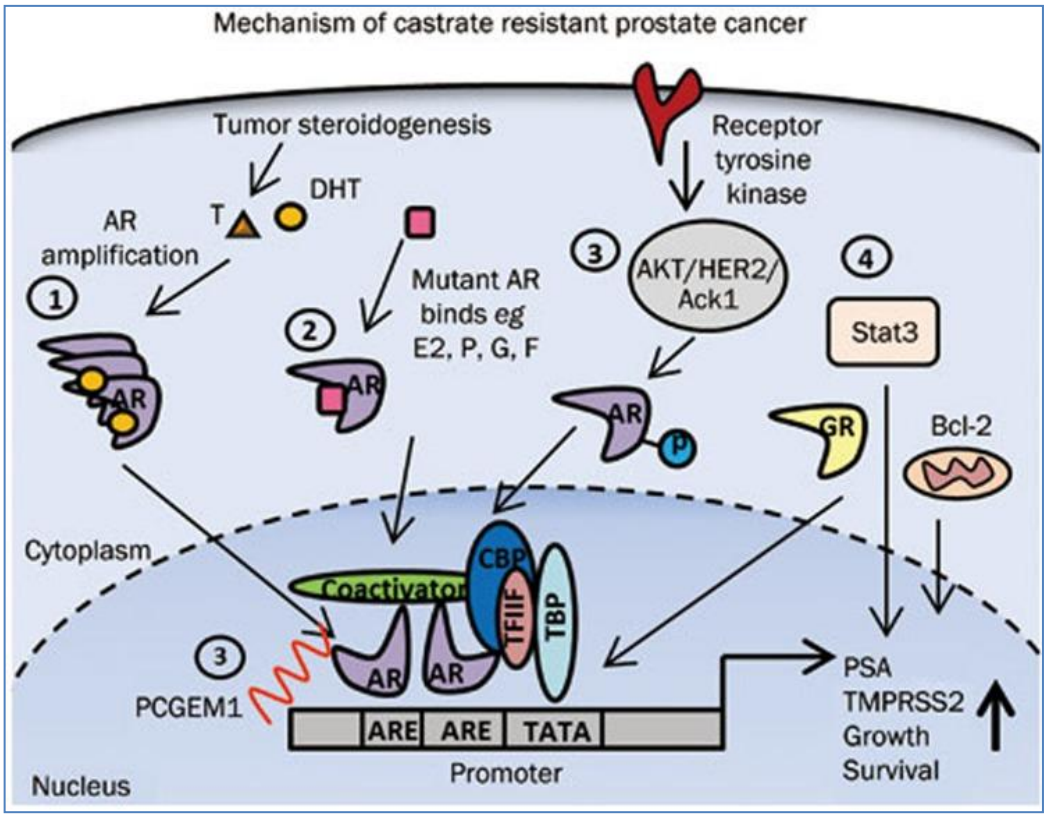
Il tumore prostatico, dopo una prima fase di responsività agli androgeni e di remissione della malattia, sviluppa androgeno resistenza, rendendo così le terapie antiandrogeniche inutili ed inefficaci. Questo è probabilmente dovuto non tanto ad una perdita di sensibilità agli androgeni, ma ad una de-regolazione della via di segnale mediata da questi ormoni (Jenster, 1999).Inizialmente, si ipotizzava che la resistenza alla terapia androgenica fosse da mettere in relazione con la perdita dell'espressione genica dell'AR (Quarmby et al., 1990; Tilley et al., 1990). Tale ipotesi era avvalorata dalla scoperta che diverse linee cellulari di tumore prostatico androgeno-indipendente comunemente studiate non esprimevano l'AR e i tumori derivanti dal modello animale di cancro prostatico (Dunning rat) mostrava la perdita dell'espressione dell'AR nella transizione da androgeno dipendenza ad androgeno-indipendenza (Buchanan et al., 2001a).

In seguito, studi più dettagliati sull'espressione dell'AR in varie fasi cliniche del tumore prostatico hanno dimostrato che la maggior parte dei carcinomi prostatici resistenti agli androgeni mantiene degli elevati livelli di espressione proteica del recettore (Buchanan et al., 2001b)

Inoltre, era stato precedentemente osservato un aumento dell'espressione del gene AR nel 22% delle metastasi di cancro prostatico (Bubendorf et al., 1999) e nel 23-28% dei casi di tumore prostatico primario (Koivisto et al., 1997) in seguito a terapia anti-androgenica

Prove dell'attività svolta dall'AR nei tumori androgeno-resistenti sono state ottenute in diversi studi. Il gene regolato dall'AR codificante il PSA, ad esempio, viene espresso nella grande maggioranza dei tumori prostatici androgeno-resistenti. Si è infatti visto che, in seguito all'impianto sottocute di cellule di CaP androgeno-dipendenti in topi immunodepressi castrati (e quindi privi di androgeni), l'attività trascrizionale del gene del PSA è inizialmente repressa, ma tale inibizione è transiente poiché viene ripristinata in seguito alla progressione di questi tumori alla fase di androgeno-indipendenza (Zhang et al., 2003). Ulteriori prove sono state ottenute attraverso l'utilizzo della linea cellulare di adenocarcinoma prostatico sia androgeno-dipendenti che androgeno-indipendenti. Il blocco selettivo dell'attività dell'AR in cellule androgeno-dipendenti LNCaP inibisce sia la proliferazione cellulare che l'espressione del PSA (Zegarra-Moro et al., 2002). Ma lo stesso risultato è raggiunto quando le cellule androgeno-indipendenti LNCaP-C4, selezionate dalla linea androgeno-dipendente LNCaP, vengono trattate con antiandrogeni (Zegarra-Moro et al., 2002). Queste evidenze sperimentali suggeriscono quindi che anche il cancro prostatico androgeno-resistente sia in qualche maniera sensibile all'azione mediata dall'AR.

I meccanismi che promuovono la resistenza alla deprivazione androgenica rimangono ancora non definiti, ma sono probabilmente diversi. In breve , sono stati ipotizzati 4 possibili meccanismi 1) mutazioni di AR che rendono il recettore responsivo a ligandi alternativi non androgenici 2) aumentata sensibilità di AR ai suoi agonisti 3) attivazione di AR ligando -indipendente e 4) meccanismi AR-independenti (Tan et al., 2015).



Androgen and AR action in castration-resistant prostate cancer. Mechanism of castration-resistant prostate cancer. Several mechanisms promote the progression of castration-resistant prostate cancer: (1) AR overexpression coupled with continued tumor steroidogenesis. (2) Promiscuous binding and activation of mutant AR by alternative ligands, such as estrogen (E2), progesterone (P), glucocorticoids (C) and flutamide (F). (3) Ligand-independent mechanisms of AR activation via crosstalk with Akt, HER2, and Ack1 kinases that phosphorylate the AR and via long non-coding RNAs (*eg*, PCGEM1) that bind to the AR to stimulate transcription of AR target genes. (4) AR-independent pathways, in which cancer cell survival and growth are directed by Stat3 signaling or by upregulation of anti-apoptotic Bcl-2.

1) Più della metà delle forme metastatiche androgeno-indipendenti presentano mutazioni nel gene codificante AR (Taplin et al., 1995). Sono state trovate mutazioni somatiche puntiformi, responsabili di sostituzioni aminoacidiche nell'"Hormone Binding Domain"; queste forme mutate producono recettori androgenici in grado di legarsi indistintamente ad androgeni, estrogeni, progesterone (Taplin et al., 1995; Tilley et al., 1996), a volte anche anti-androgeni, ribaltando gli effetti di una terapia anti-androgeni. Infatti, studi in colture cellulari in cui sono state riscontrate mutazioni a carico del gene dell'AR e dei suoi co-attivatori, hanno mostrato che l'AR verrebbe attivato anche da altri steroidi oltre che da antiandrogeni (Grossmann et al., 2001). Una mutazione di questo tipo, T877A, è stata osservata nella linea cellulare androgeno-dipendente LNCaP come anche in molti tumori resistenti a seguito del trattamento con

l'anti-androgeno idrossiflutamide: questa forma mutata dell'AR viene fortemente attivata dagli androgeni prodotti dal surrene, dall'estradiolo e dal progesterone, come anche dagli anti-androgeni idrossiflutamide e CPA (Veldscholte et al., 1990). La presenza di un AR mutato oltre a ridurre l'efficacia di una terapia di rimozione degli androgeni può selezionare cloni cellulari tumorali androgeno-indipendenti.

2). Si ritiene che i tumori androgeno resistenti richiedano ancora la presenza di androgeni per la loro crescita e sopravvivenza, ma a delle concentrazioni molto più basse. Secondo tale ipotesi l'AR diverrebbe sensibile ai livelli di testosterone o DHT tipici dell'animale castrato o risponderebbe in maniera più consistente agli androgeni prodotti dalla corteccia della ghiandola del surrene per un incremento nell'espressione di AR nelle cellule. E' stato dimostrato che il 28% dei tumori androgeno-independenti, sviluppatisi dopo terapia di deprivazione androgenic,a avevano aumentato l'espressione di AR dovuta ad amplificazione del gene AR (Koivisto et al., 1997) Quindi anche più bassi livelli di androgeni sono sufficienti per attivare il recettore androgenico overespresso. Analisi dei livelli di androgeni presenti nei tessuti di tumore prostatico androgeno resistente hanno dimostrato che le concentrazioni di DHT, DHEA e androstenedione subiscono una riduzione durante la privazione di androgeni, mentre i livelli di testosterone si mantengono simili a quelli rilevati nel tessuto prostatico di un soggetto non trattato affetto da iperplasia prostatica benigna (Mohler et al., 2004). Un'altra ipotesi proposta è che l'attivazione dell'AR possa avvenire anche in caso di assenza del proprio ligando specifico (promiscuità del ligando). Mutazioni di AR localizzate a livello del dominio di transattivazione AF-2 (aa 874-910)(es T877A, L701H, W741L, and F876L) riducono la specificità e la selettività per i ligandi e conferiscono al recettore simili proprietà di attivazione ligando-aspecifiche (Bentel & Tilley, 1996) A volte , una mutazione di AR determina un cambiamento conformazionale di un antagonista verso la forma agonista , risultando quindi in una attivazione di AR invece che in una inibizione

3)Il terzo meccanismo di sviluppo di CRPC è l'attivazione di AR ligando-indipendente. Studi hanno mostrato che i ligandi che attivano il recettore tirosina chinasi quail insulin-like growth-factor-1 (IGF-1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF), possono attivare AR in conseguenza di attivazione del patways a valle di PI3K/AKT/mTOR così creando un 'outlaw receptor'. L'attivazione di AR può

anche avvenire attraverso un crosstalk con altri pathways di segnalazione, come quelli mediati dalle tirosine chinasi (non recettore) Src and Ack1(Green et al., 2012)

4) L'ultimo pathway che porterebbe al CRPC bypassa completamente la segnalazione AR mediata. E' stato dimostrato che la terapia di castrazione nei topi induce il rilascio da parte delle cellule prostatiche cancerose morenti di fattori pro infiammatori che causano l' infiltrazione di cellule leucocitarie B e T. Le cellule B infiltranti producono linfotossina e fattori che incrementano la segnalazione STAT3 che è vitale per promuovere la sopravvivenza ormono indipendente delle cellule cancerose prostatiche.(Ammirante et a., 2010) Similarmente, la upregolazione di della proteina antiapoptotica Bcl-2 protegge le cellule tumorali dall'apoptosi indotta dalla castrazione (Setlur et al., 2005).

ESTROGENI E PROSTATA

Gli estrogeni regolano crescita e funzione della prostata in diversi stadi di sviluppo attraverso meccanismi sia diretti che indiretti. *L'azione indiretta* più importante svolta dagli estrogeni consiste nell'interferenza con la produzione degli androgeni, attraverso la repressione dell'asse ipotalamo-ipofisi-gonadi, con effetti diretti sui testicoli.

Tra *gli effetti diretti* esercitati dagli estrogeni sulla prostata vi sono, ad esempio, quelli prodotti dall'estradiolo grazie all'aromatizzazione locale del testosterone. Infatti , nello stroma della prostata normale è espresso l'enzima aromatasi, richiesto per il metabolismo degli androgeni in estrogeni, indicando una segnalazione estrogenica locale che influenza sia l'epitelio che lo stroma (Ellem et al., 2004).

Alcuni studi, eseguiti su tessuto prostatico normale di ratto e di uomo in cultura, hanno inoltre dimostrato come gli estrogeni possano stimolare la sintesi di DNA e regolare l'espressione di specifici geni prostatici quando gli estrogeni sono in combinazione con gli androgeni, gli effetti sulla sintesi del DNA e sull'espressione di specifici geni è fortemente sinergica. (Nevalainen et al., 1991; Martikainen et al., 1987)

L' identificazione dei recettori estrogenici classici ERα ed ERβ nella prostata di alcune specie di mammiferi (Latil et al., 2001; Ye et al., 2000; Lau et al., 2000) ha supportato l'azione diretta degli estrogeni nel tessuto ghiandolare. Nella prostata umana e di roditore ERβ è il sottotipo espresso nella maggioranza delle cellule epiteliali, così come in alcune cellule stromali, mentre ERα è espresso in forma più limitata ed è tipicamente localizzato solo nelle cellule stromali (Royuela et al., 2001)

Gli effetti degli estrogeni sulla prostata sembrano inoltre strettamente associati a quelli della prolattina (Härkönen, 2003). Sia la prolattina ipofisaria infatti che quella prodotta, in maniera endogena, dall'epitelio prostatico (Nevalainen et al., 1997) regolano la prostata stimolando la sua attività secretoria, oltre che la sua proliferazione e sopravvivenza (Nevalainen et al., 1991, 1993; Reiter et al., 1999; Ruffion et al., 2003).

Di recente, nuovi modelli sperimentali hanno offerto la possibilità di studiare gli effetti degli estrogeni sul tessuto prostatico umano indifferenziato. Ad esempio, studi effettuati su "prostasfere" hanno evidenziato che le cellule progenitrici prostatiche

esprimono i recettori estrogenici ERα, ERβ e GPR30 e possono essere quindi diretti targets degli estrogeni (Hu et al., 2011); in aggiunta tali cellule mostrano una risposta proliferativa a 1 nM estradiol-17β suggerendo che gli estrogeni possono regolare il normale rinnovamento delle cellule staminali /progenitrici prostatiche (Nelles 2011).

Estrogeni e patologie prostatiche

Lo sviluppo della prostata sembra essere particolarmente sensibile all'azione degli estrogeni. L'estrogenizzazione neonatale con estradiolo e DES provoca un'inibizione tempo- e dose-dipendente della crescita e della funzionalità della prostata in topi e ratti (Naslund&Coffey, 1986; Prins, 1992; Prins et al., 1993; Pylkkanen et al.,1991). Oltre all'inibizione della crescita, l'estrogenizzazione neonatale promuove le infiammazioni, l'iperplasia epiteliale e lo sviluppo di lesioni displastiche, che istologicamente assomigliano alle lesioni preneoplastiche PIN (Pylkkanen et al., 1991; Prins & Birch, 1997). Inoltre l'espressione di numerosi ed importante geni regolatori della crescita è alterato. Questo include l'up-regolazione del recettore per gli estrogeni ERα e dei proto-oncogeni responsivi agli estrogeni c-myc e c-fos (Prins & Birch, 1997; Pylkkanen et al., 1993; Salo et al., 1997) e la down-regolazione di AR ed ERβ (Prins&Birch, 1995; Prins et al., 1998). E' anche riconosciuto un ruolo importante degli estrogeni esogeni nella infiammazione cronica della prostata ed in una aberrante proliferazione dello strato basale dell'epitelio prostatico risultante in una metaplasia squamosa. Un trattamento prolungato dei roditori con dosi elevate di androgeni ed estrogeni insieme ha indotto infatti , metaplasie epiteliali, ipertrofia stromale ed una forte reazione infiammatoria nello stroma prostatatico (Andersson&Tisell, 1982; Belis et al., 1983; Risbridger et al., 2001). Questi effetti potrebbero essere anche causati dall'associazione con l'iperprolattinemia (Lane et al., 1997). Comunque, elevati livelli serici di estrogeni e prolattina associati a livelli molto bassi di testosterone in topi transgenici che iperesprimono l'aromatasi (AROM+) (Li et al., 2001) o in topi ipogonadici trattati con gli estrogeni (hpg) (Bianco et al., 2002) non inducono iperplasia prostatica, enfatizzando la necessità di un'azione combinata di androgeni ed estrogeni in alcune fasi dello sviluppo prostatico.

Gli estrogeni possono essere considerati uno dei fattori di rischio ormonale dello sviluppo dell'iperplasia prostatica benigna e del cancro prostatico (Bosland, 2000; Henderson &Feigelson, 2000; Griffiths, 2000).

Estrogeni e carcinoma prostatico

Oltre agli androgeni, crescenti evidenze indicano un ruolo chiave degli estrogeni nella alterata segnalazione intra-prostatica coinvolta nello sviluppo del carcinoma prostatico(Bonkoff and Berges 2009.) In contrasto a quanto affermato in passato, infatti, oggi diversi studi suggeriscono che gli estrogeni possono avere un ruolo critico nel predisporre e perfino causare il carcinoma prostatico (Nelles 2011).

Sembra che la presenza intraprostatica di estrogeni addizionata a quella degli androgeni sia la chiave per la carcinogenesi della prostata . Modelli animali di tumore prostatico hanno dimostrato che, almeno nei ratti, gli estrogeni sono una condizione necessaria, se non sufficiente, per la carcinogenesi prostatica. Topi Arko (aromatase knockout), che non possono produrre localmente estrogeni, sviluppano iperplasia prostatica ma non PCa (McPherson et al 2001). Il tumore prostatico è stato indotto in ratti Noble da un trattamento combinato con estradiolo-testosterone, quest'ultimo con un ruolo di supporto dal momento che la supplementazione androgenica da sola è meno efficace nel provocare la carcinogenesi (Leav et al., 1988; Ho and Roy, 1994; Bosland et al., 1995). Infatti l'induzione del carcinoma prostatico nei ratti Noble è del 100% con trattamento combinato testosterone ed estrogeni ma del 40% con il solo testosterone. Il didrotestosterone, che non è aromatizzato ad estrogeni come il testosterone, induce il tumore solo neli 4% dei ratti (Bosland, 2005). E' importante notare che l'enzima aromatasi (CYP19) è alterato nei tessuti tumorali prostatici, (Ellem et al., 2004) e l'espressione di CYP19A1 è aumentata di 30 volte nelle metastasi di tumore prostatico comparata al tumore primario (Montgomery et al., 2008). I topi ARKO presentano una ridotta incidenza di lesioni preneoplastche PIN rispetto ai topi wild-type, mostrando l'importanza della produzione in situ di E2 per la tumorogenesi. In seguito ad esposizione agli estrogeni, alcuni metaboliti degli estrogeni sono stati associati alla generazione di radicali liberi e al danno diretto genomico su modelli di prostata di ratto (Ho and Roy, 1994)

E’ stata studiata anche la correlazione tra il rischio di cancro alla prostata e il polimorfismo di geni correlati agli estrogeni. Nicolaiew e collaboratori hanno esaminato i polimorfismi nei geni di ERα e ERβ in soggetti affetti da carcinoma prostatico di etnia caucasica evidenziando la mancanza di associazione tra i polimorfismi di ERβ e il cancro alla prostata. Al contrario la ripetizione GGGA (genotipo 5/4, 6/5 e6/6) che si trova nel primo introne del gene di ERαsembra essere correlato con (un aumentato) il rischio di cancro alla prostata (Nicolaiew et al., 2009) Una più recente analisi su circa 2000 uomini francesi ha identificato un’associazione tra polimorfismi in alcuni geni correlati al metabolismo degli estrogeni (*CYP1B1* and *CYP19*) e il rischio di carcinoma prostatico. (Cussenot et al., 2007).

Alcuni autori hanno a proposto il ruolo critico svolto dagli estrogeni in combinazione con gli androgeni e possibilmente con la prolattina, nello sviluppo del cancro prostatico. Nelle cellule epiteliali prostatiche infatti gli estrogeni aumentano i livelli dei recettori per la prolattina (Nevalainen et al., 1996). E’ quindi ipotizzabile che la prolattina medi gli effetti degli estrogeni nella prostata non solo a livello sistemico ma anche a livello cellulare con un importante meccanismo di sviluppo, facilitato dagli estrogeni, che provoca dei cambiamenti displastici nella ghiandola (Härkönen, 2003)

Poichè una caratteristica istologica del tessuto prostatico trattato con estrogeni è l’infiammazione cronica, è stato anche proposto un meccanismo di carcinogenesi indotto dall’infiammazione per stress ossidativo, formazione di radicali liberi e conseguente danno al DNA (Frenkel et al., 1995; Elmets et al., 1998; Casale et al., 2000).

Infine, gli studi sperimentali hanno suggerito che un’esposizione inappropriata agli estrogeni durante le fasi critiche della differenziazione e dell’organogenesi potrebbero predisporre la prostata a cambiamenti anormali; gli estrogeni potrebbero anche avere un ruolo nella progressione di lesioni pre-maligne che possono progredire fino all’insorgenza del cancro prostatico. Altri risultati suggeriscono che l’inibizione delle vie di segnale degli estrogeni potrebbero prevenire questo processo, ipotesi che apre nuove possibilità nello studio del meccanismo di regolazione degli estrogeni nella prostata, per un’azione terapeutica e preventiva dello sviluppo e carcinogenesi prostatica

Recettori estrogenici e carcinoma prostatico

Il coinvolgimento dei recettori estrogenici nella patogenesi del cancro alla prostata e la progressione tumorale è stato suggerito in seguito a studi che hanno rilevato la presenza dei recettori classici di ERα ed ERβ nei tessuti prostatici neoplastici.

Diversi autori hanno sottolineato l'importanza ed il ruolo di ERα nella carcinogenesi prostatica.
(Singh et al., 2008; Ricke et al., 2008). Ad es, è stato condotto uno studio su linee cellulari tumorali prostatiche (NCI-H660), portatrici della fusione TMPRSS2-ERG, una traslocazione cromosomica presente nella maggior parte dei pazienti affetti da carcinoma prostatico, che esprimono sia ERα che ERβ ma mancano di AR (Setlur et al., 2008). La proliferazione di questa linea cellulare veniva soppressa da un agonista di ERβ e amplificato da un agonista di ERα indicando come l'attivazione di ERα potrebbe costituire un fattore importante nella promozione del cancro alla prostata. In aggiunta, ERα sembra essere correlato con la promozione dell'invasione del carcinoma prostatico (Takahashi et al., 2003). Yu e collaboratori hanno dimostrato che E2, così come un agonista specifico di ERα, 1,3,5-Tris (4-idrossifenil) -4-propil-1H-pirazolo stimola, attraverso ERα l'espressione di TGFβ, con conseguente aumento di invasione delle cellule tumorali prostatiche in maniera paracrina (Yu et al., 2011).

Altre investigazioni hanno mostrato la perdita di espressione di ERβ nell'HGPIN e nella displasia di alto grado rispetto alla prostata normale, suggerendo un ruolo "tumor suppressing" per questo recettore .(Leav et al., 2001; Horvath et al., 2001; Fixemer et al., 2003). In accordo , Horvath (Horvath et al. 2001) e Leav (Leav et al. 2001) hanno evidenziato una riduzione dell'espressione di ERβ durante la carcinogenesi, suggerendo che ERβ potrebbe essere importante per il mantenimento del tessuto epiteliale normale..
È interessante notare che, Hurtado e collaboratori hanno dimostrato che l'iperespressione di ERβ1 (ERβ) causava arresto del ciclo cellulare in fase iniziale in G1nella cellule neoplastiche LNCaP (Hurtado et al., 2008). Inoltre, l'over-espressione di ERβ ,utilizzando un agente demetilante il DNA, incrementava l'apoptosi nelle cellule tumorali (Walton et al., 2008). Studi condotti da Rossi (Rossi et al., 2011) hanno dimostrato che il raloxifene, aveva un effetto antiproliferativo e pro-apoptotico in vitro sul cellule di cancro alla prostata che esprimono ERβ e ERα. Nello stesso studio, è stato

dimostrato che il raloxifene attivava ERβ piuttosto che ERα, per cui la soppressione della crescita cellulare era a sostegno del ruolo antitumorale di ERβ. Questi studi unitamente ad altri condotti sul ruolo di ERα e ERβ nella carcinogenesi prostatica hanno i suggerito il paradigma che ERβ abbia un ruolo prevalentemente protettivo "tumor suppressor", mentre ERα un ruolo di induzione della neoplasia "tumor promoting" (Risbridger et al. 2007; Bonkhoff & Berges 2009; Celhay et al. 2010; McPherson et al. 2010; Nakajima et al. 2011; Attia & Ederveen 2012).Alcuni studi comunque non erano in accordo con questa teoria, ad es Horvath e collaboratori (Horvath et al. 2001) avevano ipotizzato che i tumori che mantengono l'espressione di ERβ sono associati ad un elevata possibilità di ripresa della malattia, e Leav et al. (2001) aveva riportato che ERβ è espresso nelle metastasi del CaP, cosa che potrebbe indicare che ERβ è un marker di un fenotipo altamente maligno.

Recentemente, nuove investigazioni hanno messo in discussione questo paradigma , dimostrando una variabile espressione di ERβ in diversi stadi tumorali (Zellweger et al. 2013) e un possibile ruolo oncogenico delle splice variants di ERβ, ERβ2 and ERβ 5, (Dey et al. 2012; Nelson et al. 2014).E' importante notare che la presenza di queste splice variants ad alti livelli è stata dimostrata in soggetti affetti da carcinoma prostatico con prognosi infausta (Fujimura et al., 2001). Leunge e collaboratori hanno prodotto anticorpi specifici diretti contro ERβ, ERβ2 e ERβ5, e hanno condotto studi di immunoistochimica su 144 campioni provenienti da pazienti sottoposti a prostatectomia radicale; i risultati ottenuti , correlati con follow-up clinico hanno indicato un'alta espressione di ERβ2 in correlazione con la riduzione dei valori di PSA sierici e la presenza di metastasi post-operatorie , inoltre hanno dimostrato che la presenza di una localizzazione nucleare di ERβ2 combinata ad una localizzazione citoplasmatica di ERβ5 era correlata con una più breve sopravvivenza (Leung et al., 2010). Cellule PC3 transfettate con ERβ2 e ERβ5 mostravano una maggiore invasività,. Se ERβ2 o ERβ5 formano etero dimeri con ERβ endogeno in cellule PC3 si ha di conseguenza una maggiore attività trascrizionale di ERβ, queste osservazioni sono in contraddizione con la di proprietà tumor suppressive di ERβ.(Risbridger et al., 2007; Celhay et al., 2010)

Non sono stati finora riportati dati sull'espressione di GPER nella prostata neoplastica, l'unico dato riportato è uno studio in vitro, condotto su linee cellulari di carcinoma prostatico, dove i trattamenti con un agonista di GPER, il G-1, provocava un'arresto della crescita cellulare in fase G2 (Chan *et al.* 2010).

Fitoestrogeni e carcinoma prostatico

Le differenze geografiche dei rischi di insorgenza del cancro prostatico, molto più comune nei paesi occidentali rispetto a quelli asiatici, suggeriscono che fattori ambientali possono giocare un ruolo importante nella carcinogenesi prostatica. E' stato ipotizzato che i fitoestrogeni presenti nella dieta potrebbero in parte spiegare il perchè di queste differenze. E' stato riportato che una dieta ricca di fitoestrogeni isoflavonoidi inibisce o ritarda lo sviluppo di lesioni displastiche indotte dal DES (Mäkelä et al., 1995). Studi epidemiologici mostrano come il consumo di fitoestrogeni e/o i livelli sierici di fitoestrogeni siano inversamente correlati con il rischio di insorgenza del cancro prostatico (Lee et al., 2003; Ozasa et al., 2004). Studi sperimentali supportano l'idea dei fitoestrogeni come agenti protettivi del CaP: la genisteina (un fitoestrogeno isoflavonoide) sopprime il cancro prostatico indotto chimicamente (Wang et al., 2002) e riduce l'incidenza di adenocarcinomi scarsamente differenziati nei modelli di topi transgenici (Mentor-Marcel, 2001). Comunque, gli effetti benefici dei fitoestrogeni nella prevenzione e nel trattamento dal CaP umano rimangono da dimostrare. Solo un numero limitato di trials clinici sono stati eseguiti, suggerendo un moderato effetto favorevole ma solo in un numero limitato di individui (Jarred et al., 2002; De Vere et al., 2004).

Capitolo III

GPER

Storicamente, le risposte cellulari agli estrogeni o ai composti estrogenici , sono state descritte in termini di " recettori nucleari classici ", il recettore nucleare α (ERα) (King e Greene, 1984;. Green et al.,1986) e il recettore nucleare β (ERβ) (Kuiper et al., 1996). Questi recettori mediano gli effetti cellulari sull'espressione genica nota come segnalazione "genomica", attraverso la formazione di omo o etero-dimeri, e legandosi con le sequenze ERE nelle regioni promotrici dei geni target (Edwards, 2005). I recettori nucleari ERs (anche in assenza di ligando o legando direttamente il DNA) interagiscono anche con altri fattori di trascrizione e proteine modificanti il DNA attraverso le interazioni complesso proteina-proteina , regolando così l'espressione genica e la funzione cellulare (Schultz-Norton et al., 2011). Oltre agli effetti genomici, in risposta a concentrazioni fisiologiche di estrogeni hanno luogo una varietà di risposte cellulari" rapide", che si verificano in un lasso di tempo che va da secondi a minuti, e che sono in contrasto con la trascrizione ex-novo e la sintesi proteica (Falkenstein et al., 2000). Questi effetti rapidi estrogeno-mediati avvengono attraverso vie enzimatiche e canali ionici attraverso l'attivazione di quelli che vengono genericamente indicati come ERs associati alla membrana (mER), e sono indicati come pathways" non-genomici " o " extra-nucleari " (Fu e Simoncini, 2008; Levin, 2009). Si deve tuttavia notare che ogni distinzione assoluta tra effetti genomici e non genomici è piuttosto arbitraria, dal momento che molti pathways di segnalazione intracellulare risultano nella modulazione dell'espressione genica (Ho et al.,2009). Di conseguenza, la combinazione di queste azioni cellulari multiple permette la messa a punto di una regolamentazione dell'espressione genica estrogeno-mediata (Björnström e Sjoberg, 2005). In aggiunta ERs, subiscono anche una serie di modificazioni post-traslazionali tra cui fosforilazione, l'acetilazione, la sumoilazione, e la palmitoilazione (Anbalagan et al., 2012) che modulano la loro funzione. Si può quindi concludere che la risposta cellulare mediata dalla stimolazione estrogenica è il risultato di un ampio gioco di eventi trascrizionali e non trascrizionali. Oltre ai classici recettori degli estrogeni nucleari, nel corso degli ultimi 10 anni, diversi autori hanno individuato e caratterizzato le funzioni di un recettore di membrana , GPER (G protein-coupled receptor) che sembra essere

implicato prevalentemente nelle segnalazioni rapide estrogeniche (Filardo et al., 2000; Prossnitz et al, 2008a, b.; Prossnitz e Barton, 2011; Filardo and Thomas, 2012), sebbene siano stati anche descritti suoi effetti sull'espressione genica (Prossnitz e Maggiolini, 2009;. Vivacqua et al, 2012). GPER fu identificato in diversi laboratori tra il 1996 e il 1998 come recettore orfano e fu inizialmente denominato GPR30 appartenendo alla famiglia dei recettori 7-transmembrana accoppiati alla proteina G .

Il cDNA di questo recettore è stato identificato da più fonti, tra cui i linfociti B (Owman et al., 1996; Kvingedal e Smeland, 1997) , cellule di carcinoma mammario ER-positive (Carmeci et al., 1997), cellule endoteliali umane (Takada et al., 1997). uttavia, nel 2000, studi pionieristici di Filardo e colleghi hanno dimostrato che l'espressione di GPER era necessaria per la rapida attivazione di ERK1 / 2 estrogeno-mediata (Filardo et al., 2000) e successivamente per la generazione di cAMP (Filardo et al., 2002). Nel 2005, il legame di GPER agli estrogeni è stato dimostrato da più gruppi (Revankar et al., 2005; Thomas et al., 2005) e, nel 2006, è stato descritto il primo agonista selettivo di GPER (G-1) (Bologa et al., 2006). Questo e la successiva identificazione di antagonisti selettivi di GPER (Dennis et al., 2009, 2011) ha stimolato un numero sempre maggiore di studi ad identificare le potenziali funzioni cellulari e fisiologiche di questo recettore. Ad oggi, le funzioni di GPER sono stati descritte quasi in ogni sistema fisiologico, comeil sistema endocrino, urinario, nervoso, immunitario, l'apparato riproduttivo, muscolo-scheletrico e cardiovascolare (Prossnitz e Barton, 2011). In questo modo, l'azione di GPER , combinata con quella degli estrogeni attraverso gli ER classici, serve per aggiungere complessità ai meccanismi coinvolti nelle risposte fisiologiche agli estrogeni.

Ligandi di GPER

Diversi studi hanno dimostrato il legame specifico degli estrogenici a cellule esprimenti il GPER. Nel 2005, Thomas et al. hanno dimostrato il legame selettivo dell'estradiolo triziato su preparati di membrane cellulari che esprimono in maniera endogena o mediante trasfezione GPER, con un'affinità di ~ 3 nM (Thomas et al., 2005). Nello stesso anno, Revankar e collaboratori utilizzarono un derivato fluorescente estrogenico per dimostrare il legame specifico degli estrogeni a varie linee cellulari tumorali trasfettate con GPER con un'affinità di ~ 6 nM (Revankar et al.,

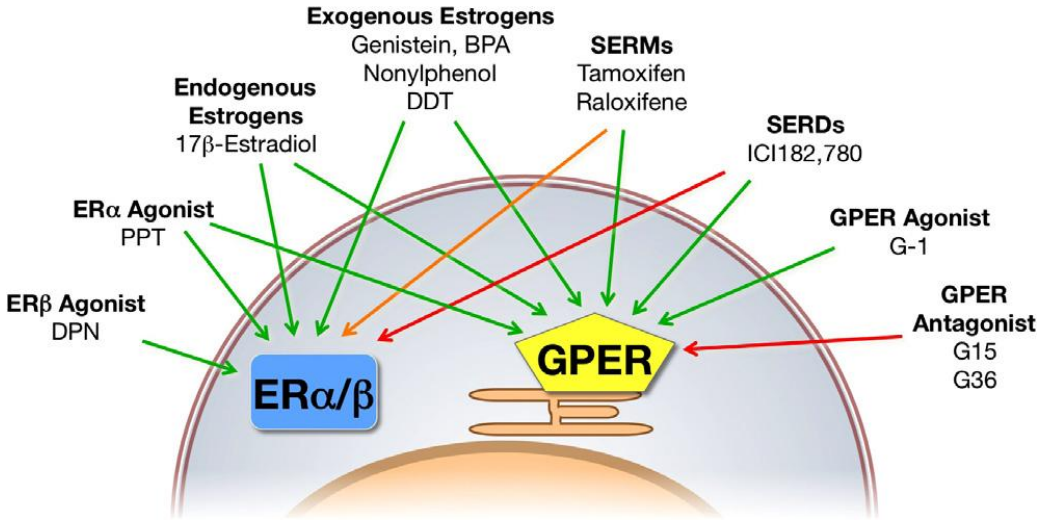
2005). Studi di imaging condotti con derivati degli estrogeni fluorescenti, hanno inoltre dimostrato una forte co-localizzazione del recettore e dell'estrogeno fluorescente, suggerendo , che GPER rappresenta il sito di legame degli estrogeni. Successivamente a questi originali studi, molti altri gruppi hanno dimostrato la capacità degli estrogeni di legarsi a cellule o preparazioni di membrane che esprimono GPER (Wang et al, 2008b;. Liu et al., 2009; Lappano et al., 2010). È importante sottolineare che all'interno della famiglia degli steroidi, gli estrogeni presentano un'elevata selettività di legame con GPER (> 1000x) superiore al testosterone, al cortisolo e al progesterone (Thomas et al., 2005). Inoltre, è stato dimostrato che l' estrone e il 17α-estradiolo mostrano una scarsa attività di legame verso GPER (Thomas et al., 2005), l' estriolo a concentrazioni μM sembra agire come un antagonista (Lappano et al., 2010); mentre, il 2-metossi-estradiolo è stato recentemente dimostrato legare il GPER (Koganti et al., 2013). Con l'abbondanza di sostanze estrogeniche sintetiche e naturali, non è sorprendente che un gran numero, di queste, ha dimostrato di interagire con GPER. Tra gli anti-estrogeni terapeutici, ICI182,780 (un down regolatore selettivo dei recettori estrogenici, SERD), che presenta un'azione antagonista verso ERα e ERβ, sembra agire come agonista di GPER (Filardo et al.,2000; Thomas e Dong, 2006). Analogamente, il 4-hydroxytamoxifene,(un metabolita attivo del tamoxifene, un modulatore selettivo del recettore estrogenico , SERM) agisce come un agonista del GPER (Revankar et al., 2005; Vivacqua et al., 2006b), e di recente è stato dimostrato che il raloxifene è in grado di attivare GPER in cellule ERα deficienti (Petrie et al., 2013).

E' stato anche dimostrato che molti composti sintetici, dai pesticidi ai derivati industriali della plastica, conosciuti per i loro effetti simil-estrogenici possono legare ed attivare il GPER, tra questi possiamo l'atrazina (Albanito et al., 2008), il bisphenolo A (Dong et al., 2011; Chevalier et al., 2012; Pupo et al., 2012; Sheng et al., 2013), la daidzeina (Kajta et al., 2013), il zearalonone, nonphenolo, kepone, p,p0-DDT, o,p0-DDE and 2,20 ,50,-PCB-4-OH (Thomas and Dong, 2006).

I fitoestrogeni inoltre mostrano attività agoniste al GPER , tra questi la genesteina (Maggiolini et al., 2004; Thomas and Dong, 2006; Vivacqua et al., 2006a), la quercitina (Maggiolini et al., 2004), l' equol (Rowlands et al., 2011), il resveratrolo (Dong et al., 2013), l' oleuropeina, e hydroxytyrosolo (Chimento et al., 2013).

Sono stati individuati numerosi composti che mostrano selettività di legame per i diversi recettori estrogenici (ERα, ERβ, e GPER). Il primo ligando selettivo per GPER ad essere identificato è stato il G-1, che mostra un'alta affinità per GPER di ~ 10 nM e con una bassissima affinità per i recettori classici ERα, o ERβ anche a concentrazioni elevate (Bologa et al, 2006;. Dennis et al, 2011). Studi successivi hanno identificato un altro composto, il G15, che sembra essere un antagonista altamente selettivo di GPER (Dennis et al., 2009). Inoltre è stato sintetizzato un successivo antagonista di GPER, il G36 (Dennis et al., 2011). Insieme, questi tre composti sono stati utilizzati in più di 150 pubblicazioni per studiare gli effetti di GPER in cellule e tessuti di animali vivi e sono serviti come base per l'analisi radioimaging con agenti selettivi per il GPER (Nayak et al, 2010;. Ramesh et al, 2010;.. Burai et al, 2012).

E'stato di recente descritto il MIBE (etil 3- [5- (2-ethoxycarbonyl-1-methylvinyloxy) -1-metil-1H-indol-3-il] but-2-enoato), un composto antagonista sia di ERα che di GPER, sebbene l' affinità per entrambi i recettori sia superiore a 10 μM (Lappano et al., 2012). Due ulteriori composti, GPER-L1 e GPER-L2 hanno mostrato avere attività di agonista selettivo del GPER, con un'affinità di legame di ~100 nM (Lappano et al., 2012). È interessante notare che il PPT, un'agonista selettivo di ERα, ha mostrato recentemente di agire come un agonista di GPER (a concentrazioni di 10-100 nM), mentre l'agonista selettivo di ERβ, il DPN, non ha mostrato attività simile con il GPER (fino a concentrazioni 10 μM) (Petrie et al., 2013). Sebbene la sequenza aminoacidica di GPER, presenta un'omologia con il recettore per chemochine, appartenente alla sottofamiglia di GPCR (Owman et al., 1996; Feng e Gregor, 1997), ci sono prove che suggeriscono che una chemochine (CCL-18) modula l'attività GPER, possibilmente attraverso un legame diretto con GPER (Feng e Gregor, 1997). Questi risultati , portano a pensare che CCL-18, potrebbe agire come un inibitore endogeno di GPER.



Ligandi di GPER e ERs: Agonisti (frecce verdi), agonisti parziali (SERMS, frecce arancio) antagonisti (frecce rosse) SERMS e SERDs funzionano come antagonisti di ERs e come agonisti di GPER in diversi tessuti. I ligandi selettivi per GPER (G-1, G15 e G36) sono altamente selettivi per GPER e poco per ERs (Meyer et al. (2011b).

Meccanismi di segnalazione mediati da GPER

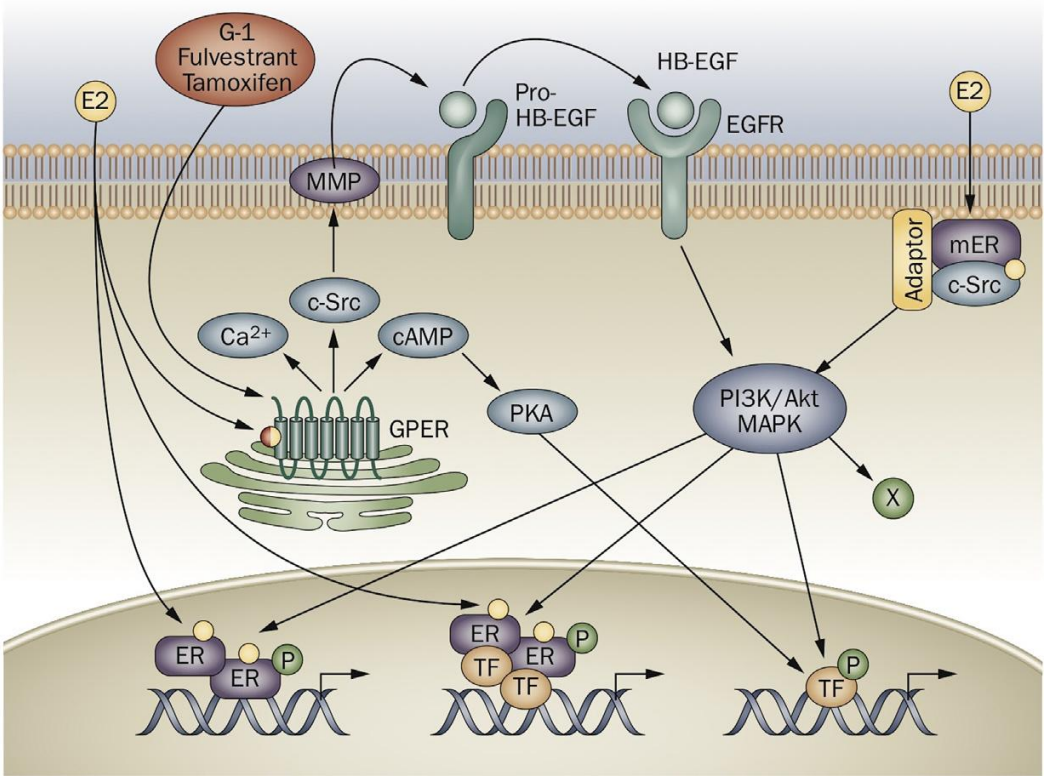
La prima rapida segnalazione estrogeno-mediata da GPER, è stata riportata da Filardo (Filardo et al. 2000) in questo studio è stato descritto un meccanismo di attivazione di ERK1 / 2 , mediato da GPER , impiegando la transattivazione di EGFR (Daub et al., 1996). In accordo con i primi eventi di segnalazione mediata da un G protein coupled-receptor, GPER agiva attraverso un pathway sensibile alla tossina della pertosse , dipendente da Gβγ, questa via di segnalazione implicava un coinvolgimento delle proteine $G\alpha i_{/o}$.

Tuttavia, inibizione dell'attività chinasica di EGFR o la down regolazione di HB-EGF mediante CRM-197 o lo scavenging libero di HB-EGF mediante l'uso di anticorpi, impedisce la segnalazione estrogeno-mediata, suggerendo il coinvolgimento di un transattivazione di EGFR mediante metallo proteinasi con il successivo rilascio di HB-EGF, un meccanismo successivamente confermato da diversi studi (Revankar et al., 2005; Peyton e Thomas, 2011;Li et al., 2013). Inoltre, è stato evidenziato un ruolo dell'integrina α5β 1 la quale sarebbe implicata nella transattivazione di EGFR (Quinn et al., 2009) stesso ruolo è stato identificato per la sfingosina chinasi (Sukocheva et al., 2006). In aggiunta a queste vie, è stato anche dimostrato che GPER media l'attivazione della proteina Gαs (Thomas et al., 2005) che porta all'attivazione adenylyl ciclasi e

l'accumulo di cAMP(Filardo et al., 2002), questo in seguito porta alla attivazione di PKA (Zucchetti et al., 2013) e all'attivazione trascrizionale di CREB (Kanda e Watanabe, 2004). Ulteriori pathways a valle attivati da GPER , comprendono la PI3K (Revankar et al., 2005; Petrie et al., 2013), PKC (Goswami et al., 2011), la mobilizzazione del calcio (Revankar et al, 2005; Tica et al, 2011) ed altri canali ionici (Fraser et al, 2010; Goswami et al, 2011) È interessante notare che molti studi hanno osservato che GPER è prevalentemente espresso sulle membrane intracellulari (Revankar et al., 2005; Otto et al., 2008; Filardo e Thomas, 2012), mentre risulta poco espresso sulla superficie cellulare della maggior parte delle cellule e linee cellulari ciò è probabilmente dovuto sia inefficienti meccanismi di esportazione, che coinvolgono la proteina 3 modificante l'attività del recettore (RAMP3) (Bouschet et al, 2012;. Lenhart et al 2013) sia ad una internalizzazione costitutiva di GPER (Cheng et al., 2011a, b). Inoltre, derivati differentemente permeabili agli estrogeni rivelano che la membrana necessita di permeabilità al ligando per la rapida segnalazione da parte di GPER, suggerendo che il pool intracellulare è funzionale (Revankar et al., 2007).Oltre agli effetti di segnalazione rapida, GPER regola anche l'espressione genica , come dimostrato da alcuni dei primi studi condotti sul recettore (Kanda e Watanabe, 2003a, b; Kanda e Watanabe, 2004; Maggiolini et al, 2004; Ylikomi et al, 2004).

Questi, insieme a successivi studi hanno evidenziato l'up-regolazione di diversi geni tra cui *c-fos* dagli estrogeni (O'Brien et al, 2006; Vivacqua et al, 2006b; Albanito et al., 2007; Prossnitz e Maggiolini, 2009). Inoltre, è stato dimostrato che gli estrogeni e l' agonista selettivo per GPER up-regolano l'espressione di proteine regolatrici del ciclo cellulare come cicline A, D1 e E (Albanito et al.,2007), del fattore di crescita del tessuto connettivo (Pandey et al., 2009), dell' Earlygrowth response-1 (Vivacqua et al., 2012), e del VEGF (De Francesco et al., 2013), in molti casi attraverso meccanismi EGFR e ERK-dipendenti. Quest'ultimo meccanismo è coinvolto nella scissione della ciclina E, generalmente associata alla progressione del tumore e alla resistenza a anti-estrogeni, indotta dal tamoxifene e del G-1 nelle cellule esprimenti il GPER(Li et al., 2013). È interessante notare che recenti studi di Maggiolini e colleghi hanno suggerito che la localizzazione nucleare di GPER, mediante un meccanismo importina-dipendente , è coinvolta nella sua attività trascrizionale (Pupo et al., 2013). Inoltre, GPER in un complesso con EGFR è stato localizzato nel promotore della ciclina D1

(Madeo e Maggiolini, 2010). Come due proteine integrali di membrana siano localizzate sul DNA all'interno del nucleo, rappresenta una teoria intrigante, che necessita di ulteriori chiarimenti.



Pathways di segnalazione di GPER e ERs (Recettori Estrogenici). Gli estrogeni endogeni, incluso il 17β-estradiol (E2), rappresentano attivatori non selettivi dei tre recettori estrogenici conosciuti, ERα, ERβ and GPER. Gli estrogeni attivano i ERs nucleari, inducendo la dimerizzazione dei recettori , legando i dimeri ai promotori dei geni target. Alternativamente, Gli ERs attivati, modulano le funzioni di alter classi di fattori trascrizionali (TFs) attraverso l'interazione protein-proteina.Sottopopolazioni di ERs, dopo l'attivazione da parte degli estrogeni, interagiscono con proteine adattatrici e molecole di segnalazione come *c-src*, il quale media segnalazioni rapide attraverso i pathways di PI3K/Akt eMAPK. GPER che è predominantemente localizzato all'interno delle cellule , può essere attivato dagli estrogeni, da agonisti selettivi come G-1, da down-regolatori dei recettori estrogenici , come il fulve strano, o da modulatori selettivi degli ERs come il tamoxifene e il raloxifene. L'attivazione di GPER stimola la produzione di cAMP, la mobilitazione del calcio e *c-src*, che vanno ad attivare le MMPs (Metallo - proteinasi della matrice). Queste MMPs causano il clivaggio di pro-HB-EGF (pro-heparin-binding epidermal growth factor), il quale viene rilasciato nella forma libera, HB-EGF free , la quale transattiva EGFR (epidermal growth factor receptor), attivando a turno i pathways di MAPK e PI3K/Akt. Questi pathways possono attivare effetti rapidi (nongenomici) effetti (X), o effetti genomici regolando la trascrizione genica.La regolazione trascrizionale mediata dagli estrogeni, può coinvolgere fenomeni di fosforilazione (P) del recettoreo di altri TFs, i quali possono reagire direttamente con ER, o legarsi alle regioni promotrici dei geni target, indipendentemente da ER. Prossnitz and Barton (2011).

Interazioni di GPER con altri recettori

Recettori Estrogenici (ERs)

La coespressione di ERα, ERβ e di GPER, suggerisce la possibilità di interazioni tra questi recettori e i loro pathways di segnalazione (Prossnitz e Barton, 2011). Infatti cross-talk funzionali sono stati riportati , dove l'espressione di GPER è necessaria

insieme a ERα per l'attività estrogeno mediata in cellule tumorali (Albanito etal., 2007), o per inibire le funzioni mediate da ERα in cellule epiteliali uterine (Gao et al., 2011). Cross-talk funzionali tra ERα e GPER sono stati evidenziati da studi funzionali condotti sulle coronarie suine, dove si osserva un'acuta vasodilatazione, NO-dipendente, solo con agonisti selettivi di ERα come PPT, ma è completamente abrogata quando ERα, ERβ, e GPER sono contemporaneamente attivati dagli estrogeni (Traupe et al., 2007). Una ridondanza sugli effetti mediati dai recettori è suggerita dal lavoro di Mauvais-Jarvis e colleghi (Tiano et al., 2011), i quali hanno riferito che gli effetti protettivi degli estrogeni sulle isole pancreatiche sono comparabili e indipendenti dall'attivazione selettiva di ERα, ERβ, o GPER (Tiano et al., 2011). Per questo motivo, il cross-talk tra GPER e recettori estrogenici (ed eventualmente altri recettori steroidei) appare essere probabile e dovrebbe essere considerato nella valutazioni di possibili questioni patogenetiche o terapeutiche relative alla attivazione o inibizione di recettori estrogenici (Barton, 2012).Il cross-talk tra i recettori e la ridondanza funzionale sono necessari per meglio comprendere le azioni di farmaci indirizzati verso i uno o più recettori estrogenici. ERα-36, una variante di splicing di membrana di ERα umano , identificato nelle cellule di cancro mammario come un effettore dominante negativo di ERα wilde-type (Wang et al., 2005, 2006), è stato indicato come effettore di segnalazione GPER-dipendente (Kang et al., 2010). La sua funzione resta controversa soprattutto perché sono scarsi i dati in vivo. Infine, anche il recettore nucleare orfano correlato con ERα (ERRα) è stato implicato nelle segnalazioni mediate da GPER , dal momento che l'attivazione di GPER causa l'attivazione trascrizionale della sintesi proteica di ERRα , e regola gli effetti a valle mediati da ERRα e la proliferazione cellulare (Li et al., 2010).

Recettori dei glucocorticoidi (GR)

I glucocorticoidi sono importanti regolatori energetici e del metabolismo osseo (Tisdale, 2002; Meyer et al, 2011a.), e un ruolo di GPER nel mantenimento del metabolismo è stato suggerito dal momento che l'attivazione di GPER riduce l'assunzione di cibo (Washburn et al., 2013) e stimola la secrezione di insulina (Sharma e Prossnitz, 2011), mentre si è visto i topi GPER-deficienti risultano obesi e insulino-

resistenti (Haas et al, 2009;. Ford et al, 2011.; Meyer et al, 2011a.; Sharma et al., 2013). GPER sembra, anche, regolare negativamente l'attività trascrizionale dei recettore dei glucocorticoidi (Ylikomi et al., 2004) che possono quindi influenzare indirettamente il metabolismo e la spesa energetica, l'omeostasi energetica globale e il peso corporeo.

Recettori dei mineralcorticoidi (MR)

L'aldosterone, è un potente vasocostrittore ed un mitogeno sintetizzato dalla ghiandola surrenale e dalle cellule muscolari lisce vascolari (Matsuzawa et al., 2013), è stato proposto che esso agisce mediante GPER per determinate funzioni (Gros et al., 2011, 2013). L'' aldosterone non si lega al GPER (Cheng et al., 2013), appare quindi da escludere la possibilità che GPER sia un recettore dell'aldosterone, è possibile che, analogamente a quanto riportato per i recettori estrogenici, esista un cross-talk funzionale tra MR e GPER.

Recettori della vitamina D (VDR)

Come un secosteroide, la vitamina D (colecalciferolo) controlla la funzione ossea e la crescita attraverso l'attivazione del recettore della vitamina D (VDR) (Norman e Powell, 2014).E'stata rilevata l'espressione di GPER nelle ossa umane (Heino et al., 2008) e è stata riportata una regolazione della crescita ossea GPER-dipendente (Heino et al., 2008; Ford et al, 2011.; Ren e Wu, 2012). Una interazionei nfiammazione-dipendente tra la segnalazione del recettore della vitamina D e GPER ha suggerito effetti inibitori estrogeno-dipendenti della vitamina D sulla encefalite autoimmune, un modello animale di Sclerosi multipla dove l'effetto della vitamina D è completamente abrogato nei topi femmina privi GPER (Subramanian et al., 2012). Interazioni GPER / VDR sembrano essere coinvolti negli effetti benefici della vitamina D sulla struttura ossea nelle donne in postmenopausa, che è stata dimostrata essere estrogeno-dipendente (Durusu Tanriover et al., 2010).

Interazioni di altri recettori con il GPER

Nell'ovaio, la funzione di GPER dipende dal GPCR transmembrana, che media gli effetti dell'ormone luteinizzante, una glicoproteina eterodimerica, che condivide la

stessa subunità α di FSH, TSH, TSH, e hCG (Dufau, 1998). E' stato recentemente dimostrato che l'ormone luteinizzante regola l'espressione di GPER , sia a livello di mRNA che di proteina, nelle cellule della granulosa umana (Pavlik et al., 2011). Interazioni e cross-talk tra GPER e recettori per i fattori di crescita, in particolare EGFR e IGFR, sono stati recentemente riassunti da Maggiolini e colleghi (Bartella et al, 2012;.. Lappano et al, 2013). Inoltre, le funzioni di GPER possono essere influenzate , sia in salute che in malattia, dalla riprogrammazione, indotta dai fattori trascrizione, del suo pattern di espressione, come è stato recentemente descritto per ERα nel cancro mammario (Ross-Innes et al.,2012).

Funzioni e localizzazione del GPER nei distretti corporei.

Sistema nervoso

Gli effetti 17β-estradiolo Nel sistema nervoso centrale e periferico riguardano principalmente il mantenimento dell'omeostasi , la regolazione della plasticità sinaptica e la modulazione della recezione dolorifica. Sebbene molti di questi effetti potrebbero coinvolgere ERα ε ERβ , crescenti evidenze indicano che GPER ha ruoli multipli nel mediare gli effetti neurologici di 17β-estradiolo. La proteina GPER e il suo corrispettivo mRNA sono stati localizzati in differenti aree del sistema nervoso di roditori di sesso maschile e femminile: ipotalamo, ippocampo, mesencefalo e midollo spinale (Brailoiu et al., 2007; Dun SL et al., 2009; Hazell et al.,2009). Inoltre il GPER ha un possibile ruolo protettivo nei confronti dei danni indotti dal glutammato (Gingerich, 2010) mentre nei cervelli di ratti di sesso femminile il trattamento con G-1 e con 17β-estradiolo ha promosso la sopravvivenza neuronale in seguito ad eventi ischemici (Lebesgue et al., 2009-2010).

Sistema immunitario

Il ruolo del GPER nella regolazione delle risposte immunitarie è ancora poco noto. L' espressione di GPER in diverse cellule immunitarie, come cellule B e T cells, monociti/macrofagi, e neutrofili, hanno suggerito che alcuni degli effetti estrogenici nel

sistema immunitario potrebbero essere mediati da GPER (Wang et al., 2008; Blasko et al., 2009; Rettew et al.,2010; Cabas et al., 2013).

Studi di Wang e collaboratori, su topi knockout per GPER, hanno evidenziato un possibile ruolo del GPER nell'atrofia del timo indotta dal 17β-estradiolo (Wang et al., 2008).

Altri studi hanno portato alla luce il ruolo antiinfiammatorio degli ormoni estrogeni nelle malattie autoimmunitarie, in particolare nella sclerosi multipla. Blasko e collaboratori che hanno messo in evidenza che GPER migliorava la funzione protettiva del 17β-estradiolo in un modello sperimentale di sclerosi multipla murina (Blasko et al., 2009).

Recenti studi hanno dimostrato come il trattamento con G-1 stimola la produzione ex-novo delle citochine antiinfiammatorie IL-10 in cellule T-helper polarizzate di tipo 17, in vivo e in vitro, tramite l'attivazione della via trasduzionale ERK1/2- dipendente (Brunsing et al., 2011).

La scoperta di questo ruolo immunomodulatore del G-1 ha così portato ad affermare un possibile ruolo clinico nel trattamento di malattie infiammatorie croniche di tutti gli agonisti del GPER (Prossnitz et al., 2011).

Apparato cardiovascolare

17β-estradiol endogeno è implicato in differenze *sesso-specifiche* osservate nella ipertensione arteriosa and nelle malattie cardiovascolari. ERα and ERβ sembrano essere implicati negli effetti protettivi cardiovascolari del17β-estradiolo ma anche GPER è stato proposto come mediatore in tali attività estrogeniche. Il GPER è espresso nei miocardiociti sia nel topo (Martensson et al., 2009) e sia nell'uomo (Patel et al., 2010) e anche nei miocardiociti in cultura.

Trattamenti con G-1 hanno prodotto effetti cardioprotettivi in animali affetti da infarto del miocardio, quali un'espressione ridotta di citochine pro-infiammatorie (Weil et al., 2010), un aumento dell'attivazione di Akt (Filice et al., 2009) e di ERK1/2 (Filice el al., 2009; Deschamps et al., 2009), un incremento della fosforilazione di eNos (Filice et al., 2009) e una diminuzione della permeabilità dei mitocondri (Bopassa et al., 2010).

E’ inoltre molto probabile il ruolo del GPER nell’aterosclerosi e nell’infiammazione associata a causa della sua espressione nell’endotelio umano, nelle cellule muscolari lisce e nei macrofagi implicati nei processi di sviluppo dell’aterogenesi stessa.
Per concludere, ulteriori studi portati avanti da Filice e collaboratori hanno rilevato l’effetto vasodilatatore degli agonisti del GPER (Filice et al., 2009), ecco perché quest’ultimi potrebbero essere utilizzati nel trattamento di infarto e di malattie cardiovascolari.

Apparato urinario

Il GPER è espresso abbondantemente nei tubuli renali (Hazell et al., 2009) e nelle cellule epiteliali renali (Sanden et al., 2011) di roditori.
In studi effettuati su ratti ipertesi, l’attivazione del recettore GPER mostra un miglioramento della clearance della creatinina e un abbassamento della proteinuria anche se l’ipertensione non viene corretta (Gilliam Davis et al., 2010).
A fronte di ciò è possibile un ruolo rene-protettivo da parte degli agonisti del GPER nella nefropatia ipertensiva.

Pancreas e metabolismo del glucosio

Gli estrogeni hanno un ruolo protettivo nell’obesità e nell’insulino-resistenza che è stato largamente attribuito aIla mediazione di ERα. ma anche il GPER sembra essere coinvolto. GPER è espresso nel tessuto adiposo dell’uomo e dei roditori (Haas et al.,2009 e Hugo et al.,2008), nelle isole pancreatiche e nel fegato umano che sono organi chiave dell’insulino-resistenza.
Una deficienza del recettore estrogenico, nell’uomo e nel topo, evidenzia una diminuzione di secrezione di insulina (stimolata da G-1, glucosio ed E2) da parte delle cellule del pancreas ma ciò non causa una variazione della morfologia delle cellule β-pancreatich, il che suggerisce il ruolo del GPER nel mantenimento delle funzioni metaboliche dell’insulina (Martensson et al., 2009; Kumar et al., 2011; Sharma et al.,2011)

Ossa e metabolismo dei condrociti

L’osso e la cartilagine sono tessuti ormono-sensibili (Weise et al., 2001), e livelli alti di 17β-estradiolo nel siero sono indice di minor rischio di fratture dell’anca nella donna e

nell'uomo (Ohlsson et al., 2009). Gli effetti preservativi della terapia estrogenica sull'osso, specialmente con SERMs and SERDs, che agiscono come agonisti di GPER, indirettamente suggeriscono un ruolo di GPER nel metabolismo dell'osso.

Nell'osso il GPER è espresso negli osteociti, osteoclasti e osteoblasti (Chagin et al., 2007; Heino et al., 2008) nonché nei condrociti dove il GPER ha un ruolo nel regolare il differenziamento (Chagin et al., 2007; Jenei Lanzl et al., 2010).

Nei ratti, ill recettore inoltre è implicato nel controllo della crescita dell'osso in maniera però sesso-dipendente: una riduzione di GPER nei topi femmina causa una diminuzione nella crescita dell'osso (Martensson et al., 2009), al contrario, una carenza di GPER nei topi maschi i produce un aumento delle dimensioni del femore e della densità ossea (Ford J et al., 2011).

Apparato riproduttivo femminile

In topi wild-type, l'agonista del GPER (G-1) induce la proliferazione uterina delle cellule endometriali e miometriali mentre nell'ovaio del criceto il GPER regola la stimolazione della formazione del follicolo primario (Prossnitz & Barton, 2011).

E' probabile inoltre un ruolo del GPER nella contrattilità uterina durante il travaglio (Maiti et al., 2011) visto e considerato che l'attivazione del recettore estrogenico migliora la risposta contrattile mediata dall'ossitocina nel miometrio.

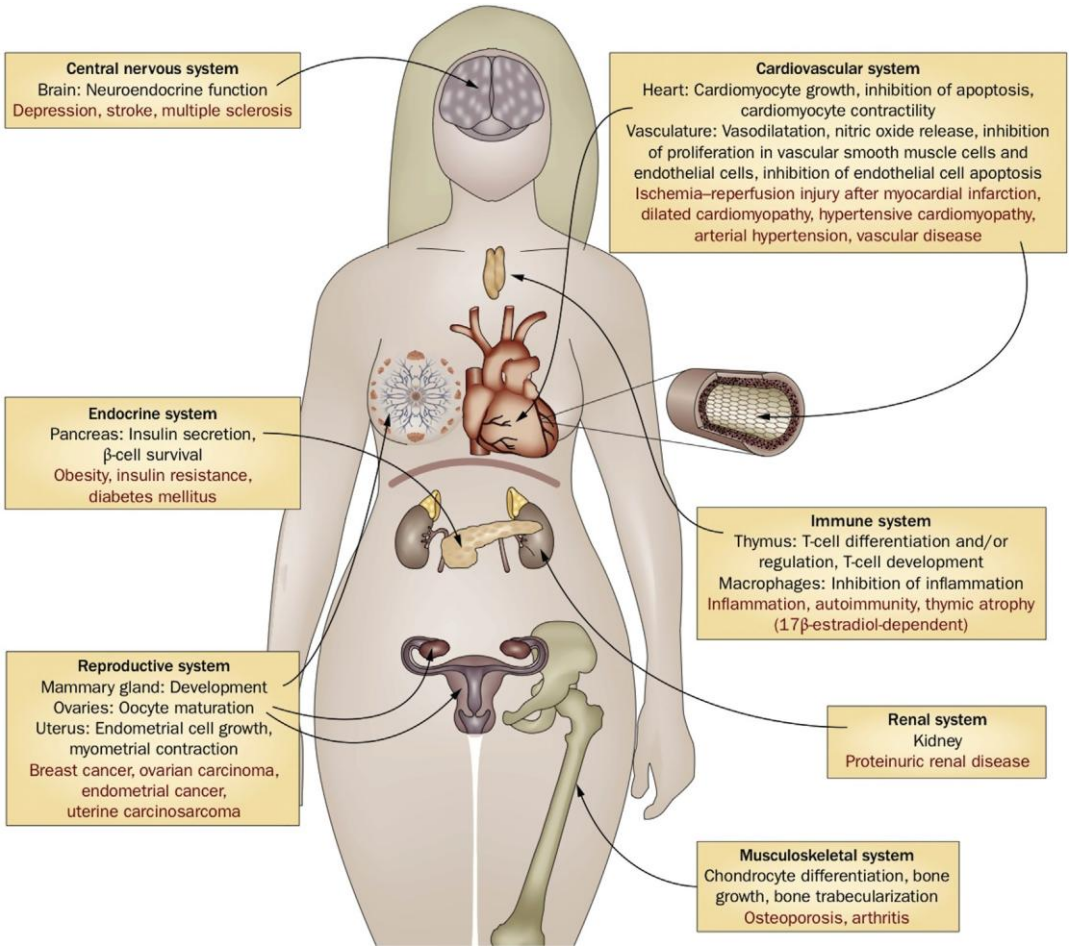
Apparato riproduttivo maschile

Testicolo E' stata evidenziata l'espressione del GPER cellule germinali testicolari in cultura quali gli spermatogoni (Sirianni et al., 2008), gli spermatociti in pachitene e gli spermatidi rotondi isolati di ratto (Chimento et al., 2010-2011).

Il recettore è stato inoltre immunolocalizzato nelle cellule somatiche e/o germinali del testicolo umano (Franco et al.,2011; Rago et al., 2011; Chevalier et al., 2012). E' stato quindi ipotizzato un possibile un ruolo del GPER nei processi di differenziamento delle cellule germinali maschili e nell'attività dello stesso testicolo.

Recentemente, tramite immmunofluorescenza,Western blot a RT-PCR, è stato identificato il GPER in spermatozoi eiaculati umani e suini evidenziando una specie-speficità della sua localizzazione (Rago et al, 2014).Ciò ha suggerito il coinvolgimento differenziale del recettore sull'influenza degli estrogeni nella reazione acrosomiale, ,

nell' attività mitocondriale e nella motilità nemaspermica. *Epididimo* I dati finora riportati riguardanti la presenza del GPER nell'epididimo sono molto limitati .Uno studio (Hess et al., 2011) ha rilevato la presenza di mRNA per GPER nei duttuli efferenti di ratto, mentre un altro studio (Joseph et al., 2011), tramite tecniche di PCR quantitativa, ha evidenziato mRNA per GPER nell'epididimo di ratto adulto quantizzandolo per regione: il *corpus* è il tratto epididimario in cui il recettore è risultato maggiormente espresso, seguito dal *segmento iniziale* ed infine da*lla cauda*.. Un altro lavoro recente (Martínez-Traverso e Pearl, 2015) ha mostrato il GPER (immunolocalizzazione e Western blot) nell'epididimo di ratti Wistar , Brown Norway e Sprague Dawley. Il recettore è risultato espresso soprattutto nelle cellule principali a livello citoplasmatico nel corpo e poi nella coda.



Questi dati fanno ipotizzare una possibile implicazione del GPER nel mediare l'azione estrogenica nelle varie regioni epididimarie, quali il riassorbimento del fluido luminale,

la maturazione funzionale degli spermatozoi e la loro protezione durante il deposito prima dell'emissione.

Prostata Limitati studi hanno finora investigato GPER nelle cellule prostatiche umane. Alcuni autori hanno valutato la presenza di GPER in linee di carcinoma prostatico LnCap, PC-3 e DU-145, dimostrando che solo le linee cellulari invasive di PC-3 e DU-145 esprimono GPR30 a differenza delle meno invasive LnCap (Maier et al.2006; Pisolato et al. 2012). Altri autori hanno dimostrato che il trattamento di cellule di adenocarcinoma prostatico con il ligando non estrogenico G1 induceva l'inibizione della crescita in vitro ed in vivo attraverso l' attivazione di GPER. (Chan et al. 2010). Recentemente è stato identificato il GPER nella prostata di coniglio (Comeglio et al., 2014) ma a tutt' oggi , non sono stati ancora riportati dati sulla espressione di GPER nei tessuti di prostatici umani.

Capitolo IV

MATERIALI E METODI

Pazienti

Il presente studio è stato condotto su 55 campioni di archivio provenienti dalla Unità di Anatomia Patologica dell'Ospedale Civile dell'Annunziata (CS), Italia. I campioni, fissati in formalina ed inclusi in paraffina, sono stati ottenuti da 5 pazienti (45-65 anni) affetti da patologie prostatiche benigne e da 50 pazienti (55-70 anni) affetti da adenocarcinoma prostatico. I campioni tumorali sono stati classificati in base alle loro caratteristiche anatomo-patologiche secondo il Gleason grading system (Gleason patterns) in: 20 tumori ben differenziati (Gleason score 2-6), 12 tumori moderatamente differenziati (Gleason score 7) e 18 tumori scarsamente differenziati (Gleason score 8-10).

Reagenti

Tutti i reagenti se non diversamente indicato sono stati acquistati dalla Sigma Aldrich (Milano, Italia). L'anticorpo policlonale di coniglio anti-GPER, LS-A4271, e il suo relative peptide bloccante (LS-P4271) sono stati acquistati dalla MBL International Corporation (Woburn, MA, USA.) Gli anticorpi policlonali di coniglio anti-GPER, K19, anti-Akt 1/2/3 (H-136), anti p-Akt1/2/3 (Ser 473) sc-33437, anti CREB-1(C-21), anti β-actin, il peptide bloccate del K19 e l'anticorpo policlonale di capra anti p-CREB-1 (Ser 133): sc-7978 sono stati acquistati dalla Santa Cruz Biotechnology (Ca, USA) L'anticorpo secondario biotilinato di capra anti- coniglio e il complesso avidina–biotina perossidasi di rafano (ABC/HRP) sono stati acquistati dalla Vector Laboratories (Ca, USA) gli anticorpi secondari IgG coniugati con la perossidasi di rafano, di capra anti-coniglio e di coniglio anti-capra sono stati acquistati dall' Amersham (USA).

Il cromogeno Diaminobenzidine (DAB) è stato acquistato dalla Zymed Laboratories (CA, USA).

Analisi Immunoistochimica

Sezioni di tessuto di 5 μm, incluse in paraffina, sono state montate su vetrini, pretrattati con poly-lysina, e successivamente sparaffinate e reidratate (7-8 sezioni

seriali).L'analisi immunoistochimica è stata condotta dopo smascheramento antigenico, mediante calore. Perossido d'idrogeno (3% in acqua distillata) è stato usato, per 30 min, per inibire l'attività delle perossidasi endogene. Mentre siero normale di capra (10%) è stato utilizzato, per 30 min, al fine di bloccare i siti non-specifici di legame. L'immuno-localizzazione è stata condotta usando separatamente due diversi anticorpi primari anti-GPER (LS-A4271 e il K19) (1:100) incubandoli a 4°C overnight. In seguito è stato utilizzato un anticorpo secondario biotilinato di capra anti-conoglio (1:600) per 1h a temperatura ambiente, seguito dall' ABC/HRP. L'immuno-reattività è stata visualizzata con la DAB. Tutte le sezioni sono state controcolorate con Ematossilina. Per i controlli per adsorbimento, sono stati utilizzati gli anticorpi primari, preadsorbiti con un eccesso del loro peptide bloccante purificato, a 4°C per 48 ore. Tessuto di carcinoma mammario è stato utilizzato come controllo positivo.

Score di Allred

I vetrini immuno-colorati di ogni campione tumorale, sono stati valutati , mediante microscopia luce, usando lo score di Allred (Allred *et al* 1998) il quale combina uno score proporzionale con uno score di intensità. Lo score proporzionale, rappresenta la proporzione stimata di cellule tumorali positivamente colorate. (0 = nessuna; 1 = 1/100; 2 = 1/100 a <1/10; 3 = 1/10 a <1/3; 4 = 1/3 a 2/3; 5 = >2/3). Lo score di intensità viene assegnato come media stimata dell'intensità di colorazione nelle cellule tumorali positive. (0 = assente ; 1 = debole; 2 = moderata; 3 = forte). Sommando lo score proporzionale e lo score di intensità si ottiene lo score totale che va da 0 a 8. In ogni vetrino sono state analizzate un minimo di 100 cellule. Per ogni campione sono state analizzate 6/7 sezioni seriali. .Infine per valutare le differenze di score tra i campioni è stata condotta un'analisi statistica mediante ANOVA ad una via.

Estrazione delle proteine.

L'estrazione di proteine da aree selezionate di sezioni fissate in formalina ed incluse in paraffina (FFPE), è stata condotta in accordo a Kawashima (Kawashima *et al* 2014). Sezioni FFPE sono state trasferite in tubi di polypropylene da microcentrifuga da 1.5 ml, sparaffinate mediante incubazione in xilolo per 10 min. a temperature ambiente, successivamente reidratate con la serie di etanolo (etanolo assoluto, etanolo al 95%,

etanolo al 70% ed etanolo al 50%), dopo ogni incubazione , i tessuti sono stati pellettati mediante centrifugazione a 16,000g per 3 min, ogni fase di incubazione/ centrifugazione è stata ripetuta 2 volte. Il pellet ottenuto è stato, brevemente seccato all'aria e pesato. Successivamente è stato omogenizzato in 100 volumi di PEB (Protein Extration Buffer) (500 mM Tris–HCl pH 8.0 e il 2% SDS). I campioni sono stati incubati a at 90°C per 90 min. Gli estratti sono stati centrifugati a 16,000g per 20 min. e il surnatante ottenuto è stato conservato a -80°C fino alle analisi biochimiche.

Western blot analysis

50 μg di lisato proteico, sono bolliti per 5 min, separati in condizioni denaturanti mediante SDS-PAGE su un gel di polyacrylamide Tris-glycina al 10%, ed elettroblottate su una membrana di nitrocellulosa. Le membrane così ottenute sono state incubate per 1 ora a temperatura ambiente, con tampone TRIS contenente il 5% di latte in polvere, senza grassi, e il 2% di Tween-20 al fine di bloccare i siti aspecifici di legame. Successivamente le membrane sono state incubate overnight con la serie di anticorpi primari policlonali (anti- GPER 1:500, anti- AKT: 1:500, anti pAKT 1:1000, anti- CREB 1:300, anti- pCREB 1: 500). Il complesso antigene–anticorpo è stato evidenziato mediante incubazione delle membrane, per 1 ora a temperatura ambiente, con gli anticorpi secondari, coniugati con la perossidasi di rafano (1-7000). Il legame con gli anticorpi secondari è stato visualizzato con il ECL Plus Western blotting detection system (GE Healthcare Europe GmbH, Milan Italy) in accordo con le istruzioni della casa produttrice. Ogni membrana è stata esposta con una lastra fotografica per 2 min. . Tutti i Blots sono stati successivamente strippati e re-incubati con la β–actina, una proteina che serve come controllo per il caricamento. I controlli negativi sono stati preparati usando tessuti lisati, nei quali gli antigeni erano stati precedentemente rimossi mediante pre-incubazione con gli anticorpi specifici (1 ora a temperatura ambiente) e successivamente immuno-precipitati con la proteina A/G-agarosio (Aquila *et al*. 2002). L'analisi Western blot è stata ripetuta 3 volte per ogni campione.

Capitolo V

RISULTATI

Analisi Morfologica

Ghiandola prostatica normale . Le aree non alterate della prostata non neoplastica mostrano la tipica struttura della ghiandola prostatica. Si osservano, infatti ghiandole tubulo-alveolari (otricoli) ramificate, immerse in uno stroma fibromuscolare. Ciascuna ghiandola otricolare è costituita da numerosi acini provvisti di papille, che riversano il loro secreto all'interno del lume. Tutte le ghiandole appaiono rivestite da uno strato basale, caratterizzato da un basso epitelio cubico e da uno strato luminale caratterizzato da un alto epitelio colonnare (Fig.1)

Adenocarcinoma. Nei campioni tumorali di adenocarcinoma, sono stati evidenziati defferenti arrangiamenti delle cellule neoplastiche (Gleason patterns). In accordo con i criteri anatomo-patologici, ad ogni pattern è stato attribuito un valore numerico da 1 a . Il valore 1 è stato attribuito all0arrangiamento cellular più simile a quello della prostata normale, mentre il valore 5 è stato attribuito a quella organizzazione cellulare totalmente diversa da un aspetto normale. Nei nostri campioni di adenocarcinoma, abbiamo prevalentemente riscontrato un Gleason pattern 2 nei tumori ben differenziati, un Gleason pattern 3 nei campioni moderatamente differenziati e un Gleason pattern 4 nei campioni di adenocarcinoma scarsamente differenziati. (Fig. 2). Inoltre nei campioni tumorali abbiamo frequentemente osservato la presenza di lesion pre-neoplastiche, high-grade prostatic intraepithelial neoplasia (HGPIN)(Fig 3A).

Immunolocalizzazione del GPER

Ghiandola prostatica normale. Nei campioni normali, noi addiamo riscontrato una forte immunolocalizzazione di GPER nel citoplasma delle cellule epiteliali basali, mentre nessuna colorazione appare nel citoplasma delle cellule epiteliali luminali. Inoltre è stata evidenziata un debole colorazione nel citoplasma delle cellule stromali. (Fig. 1).

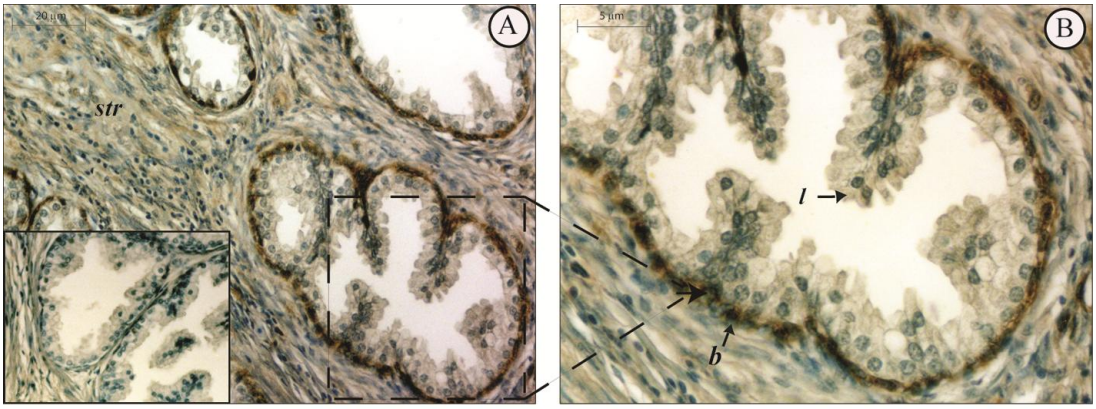


Fig 1. Immunoreattività del GPER nella prostate umana non neoplastica. A: Forte immunolocalizzazione nelle cellule basali epiteliali e debole immunoreattività nelle cellule stromali. *Scale bar 20 μm* B: Ingrandimento dell'area prostatica delineate dal tratteggio nella figura A. *Scale bar 12,5 μm* ***b***) *cellule epiteliali basali*; ***l***) *cellule epiteliali luminali* ***str***) *cellule stromali*. Inserto: controllo per adsorbimento

Adenocarcinoma. Nei campioni neoplastici, abbiamo evidenziato una immunolocalizzazione del GPER, forte nel citoplasma delle cellule organizzate secondo Gleason patterns 2 o Gleason patterns 3, mentre l'immunoreattività appariva debole o moderata nelle cellule con Gleason patterns 4. (Fig 2)

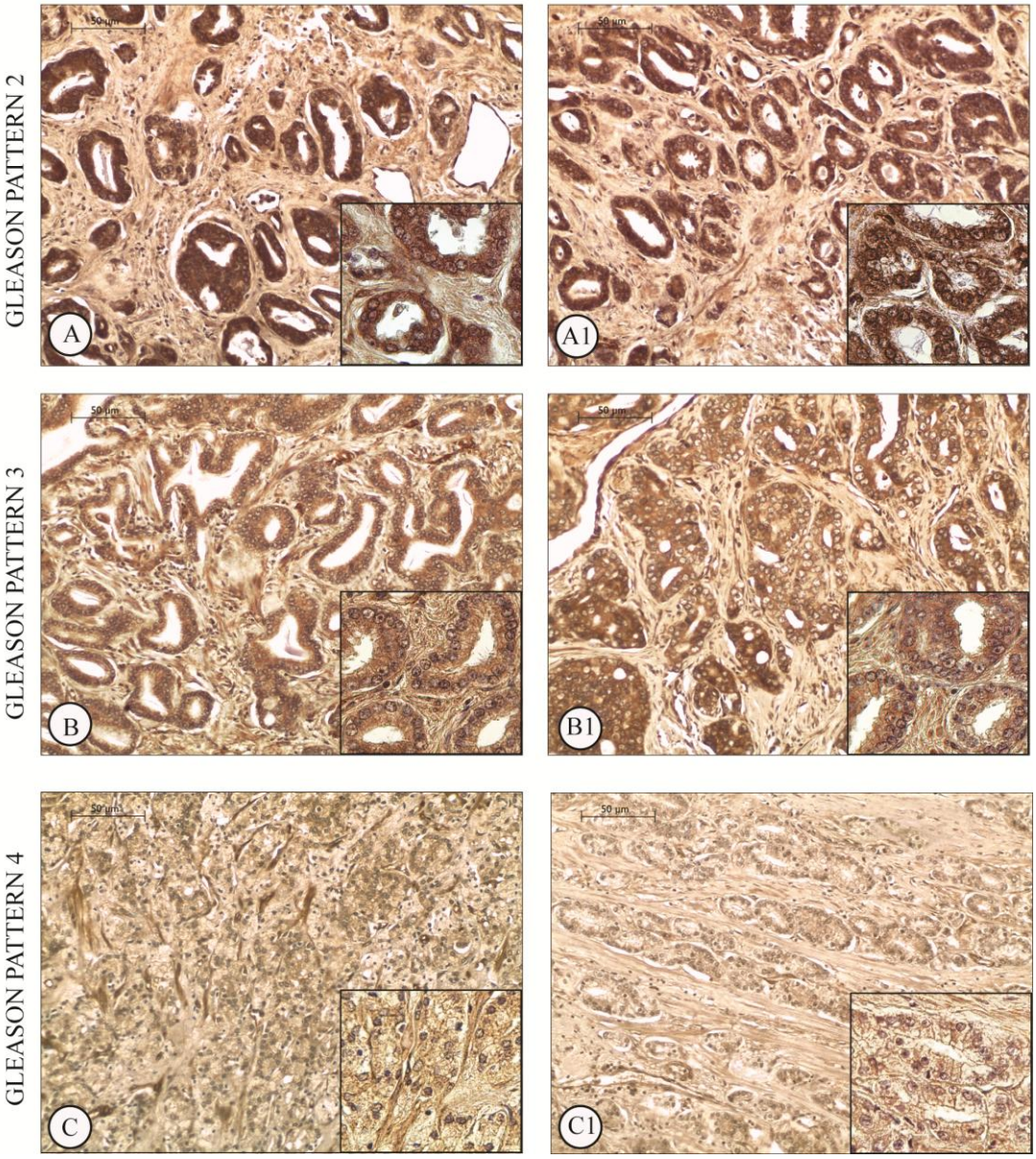


Fig 2. Immunolocalizzazione di GPER nell'adenocarcinoma prostatico. La figura mostra, aree rappresentative con organizzazione cellulare Gleason pattern 2 (A-A1), Gleason pattern 3 (B-B1) e Gleason pattern 4 (C-C1, *Scale bars 20 μm*. Gli inserti rappresentano gli ingrandimenti delle immagini, *Scale bar 12,5 μm* .

Lo score effettuato sui campioni di adenocarcinoma, secondo Allred, è stato riassunto in Tabella 1.

Table1: GPER immunoreactivity (Allred score) in Gleason patterns observed in prostatic adenocarcinoma samples.

Gleason patterns	*Allred score* (*median*)
2 (n=20/50)	7
3 (n=40/50)	6 *
4 (n=20/50)	3 **

Total adenocarcinoma samples n = 50. Total immunostaining score: Propotion score + Intensity score (range 0-8).
Significant difference: * = p<0,05 with respect to pattern 3; ** = p < 0,005 with respect pattern 2 and pattern 3

L'immunolocalizzazione di GPER nei campioni di adenocarcinoma ha evidenziato una fortissima colorazione nel citoplasma delle cellule epiteliali luminali delle lesioni pre-neoplastiche. (Allred score = 8) (Fig.3A)

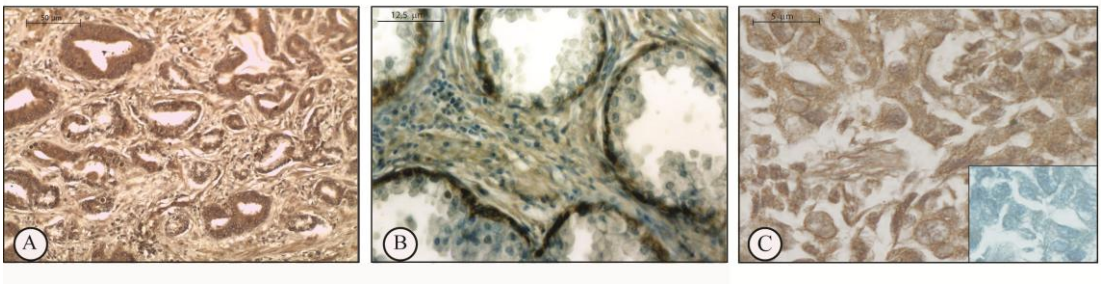


Fig 3. A: Immunolocalizzazione di GPER in HGPIN, *Scale bar 20 μm* B: Immunolocalizzazione di GPER nel tessuto prostatico di controllo, *Scale bar 12,5 μm*. C: Controllo positivo (carcinoma mammario), *Scale bar 5 μm.* Inserto : controllo per adsorbimento.

Inoltre, nonostante, abbiamo evidenziato una localizzazione citoplasmatica di GPER, nella maggior parte dei nostri campioni, in 5 dei 50 adenocarcinoma abbiamo evidenziato una localizzazione nucleare di GPER (dati non mostrati).

Analisi Western blot

GPER: L'immunoblot dei tessuti prostatici non neoplastici, ha mostrato la presenza di una singola banda con un peso molecolare di ~ 42kDa (Fig 4A: *lanes* 1,2,3). Questa banda co-migra con quella del controllo positivo (SKBR3) (Fig. 4A: *lane* C+). Bande simili, ~ 42kDa, sono state determinate nei nostri campioni di adenocarcinoma (Fig 4B). In questi campioni è stata evidenziatauna riduzione decrescente dell'intensità delle bande osservate nei campioni ben-differenziati (*lane* 1), moderatamente differenziati (*lane* 2) e scarsamente differenziati (*lane* 3) (Fig 4B).

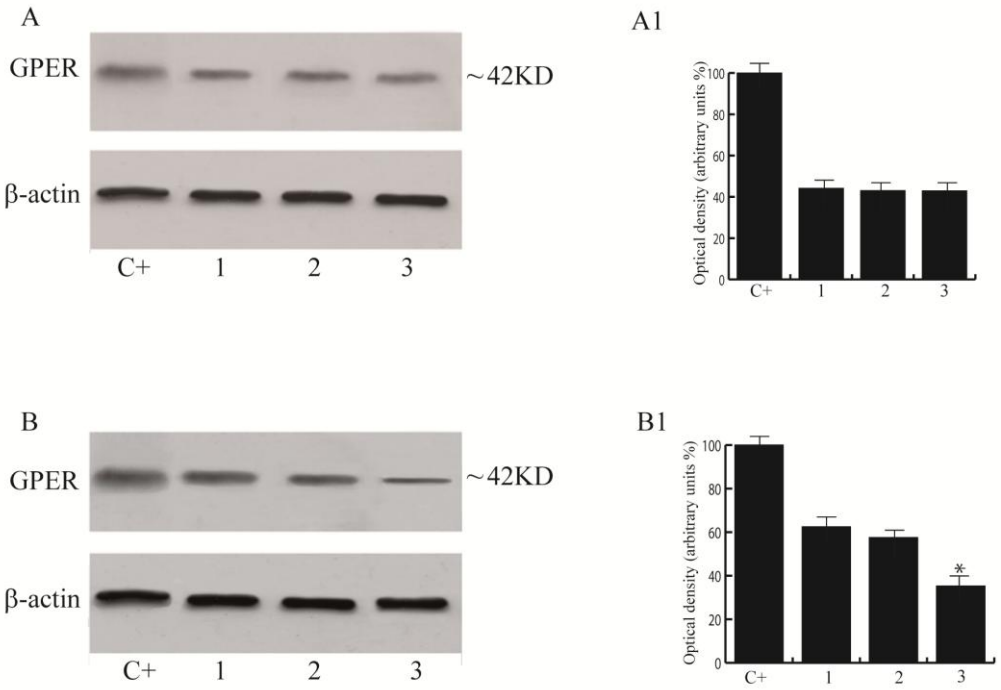


Fig 4. Immunoblot di GPER di proteine estratte dalla prostata umana. A: Campioni rappresentativi di prostate non neoplastica (*lanes* 1, 2, 3), controllo positivo (carcinoma mammario) *(lane* C+). B: Campioni rappresentativi di adenocarcinoma (*lane* 1: PCa ben differenziati, Gleason pattern 2), (*lane* 2: PCa moderatamente differenziati, Gleason pattern 3) , (*lane* 3: PCa scarsamente differenziati, Gleason pattern 4), controllo positivo *(lane* C+). A1/B1:valutazione dell'intensità delle bande in termini unità densitometrica arbitraria . I valori sono espressi come media ±S.E.M. *P<0.005 versus *lanes* 1 and 2. β-actin: loading control.

Akt/pAkt: Gli immunoblot di Akt/pAkt, dei campioni di adenocarcinoma prostatico, evidenziano la presenza di bande di ~ 55kDa (Fig 5A). Nessuna variazione nell'intensità delle bande è stata evidenziata per quanto concerne i livelli di Akt totali (Fig 5A1), mentre la forme fosforilata di Akt incrementa nei campioni di adenocarcinoma scarsamente differenziati (*lane* 3) rispetto a quelli ben differenziati (*lane* 1) e moderatamente differenziati (*lane 2*) (Fig 5A2).

CREB/pCREB: Gli immunoblot di CREB/pCREB, dei campioni di adenocarcinoma prostatico, evidenziano la presenza di bande di ~ 43kDa (Fig 5B). Nessuna variazione nell'intensità delle bande è stata evidenziata per quanto concerne i livelli di CREB totali (Fig 5B1), mentre la forme fosforilata di CREB aumenta nei campioni di adenocarcinoma scarsamente differenziati (*lane* 3) rispetto a quelli ben differenziati (*lane* 1) e moderatamente differenziati (*lane 2*) (Fig 5B2).

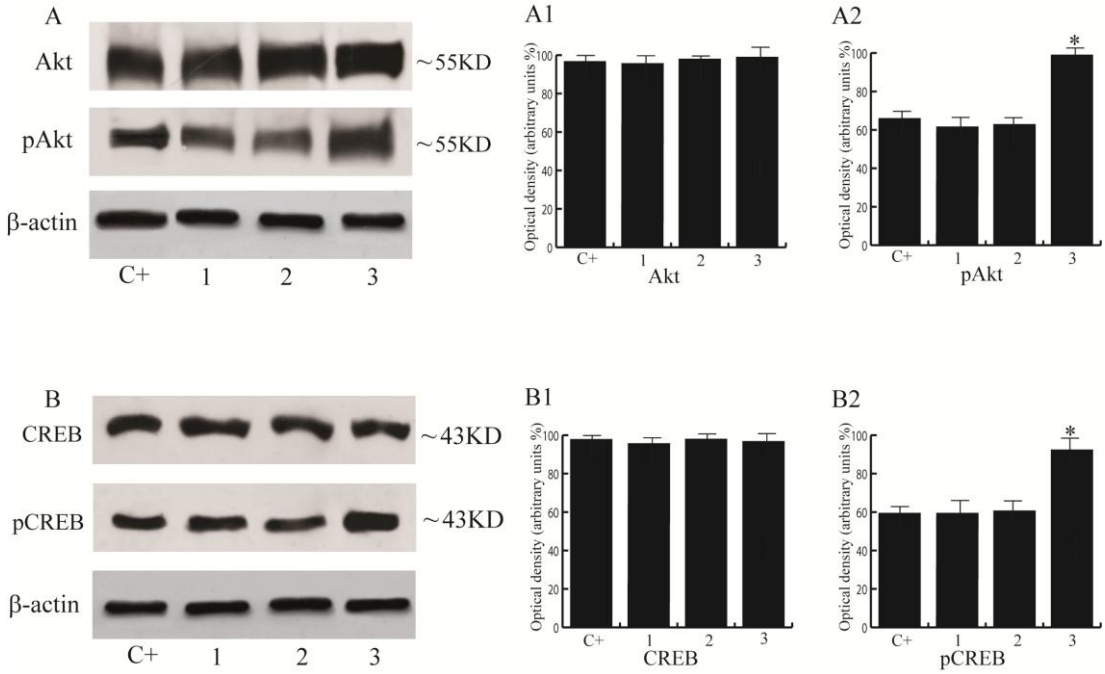


Fig 5. Immunoblots di AKT/CREB di protein estratte da campioni rappresentativi di adenocarcinoma. A: AKT/pAKT (*lane* 1: PCa ben differenziati, Gleason pattern 2), (*lane* 2: PCa moderatamente differenziati, Gleason pattern 3) , (*lane* 3: PCa scarsamente differenziati, Gleason pattern 4), controllo positive (SKBR3) *(lane* C+). B: CREB/pCREB (*lane* 1: PCa ben differenziati, Gleason pattern 2), (*lane* 2: PCa moderatamente differenziati, Gleason pattern 3) , (*lane* 3: PCa scarsamente differenziati, Gleason pattern 4), controllo positive (SKBR3) *(lane* C+). A1-A2/B1-B2: valutazione dell'intensità delle bande in termini unità densitometrica arbitraria . I valori sono espressi come media ±S.E.M. *P<0.005 versus *lanes* 1 and 2. β-actin: loading control.

DISCUSSIONE

Il recettore GPER (G-protein-coupled receptor), mostra un'elevata affinità per gli estrogeni e media le segnalazioni non genomiche degli stessi, regolando la crescita cellulare in diversi organi (Filardo et al., 2000; 2006; Prossnitz et a., 2008; Prossnitz and Maggiolini , 2009; Prossnitz and Barton , 2014) . Numerosi studi hanno dimostrato che questo recettore è implicato nella progressione di molte neoplasie , come il carcinoma mammario (Filardo et al., 2006; Tu et al., 2009) endometriale (Vivacqua et al., 2006a: Smith et al., 2007), ovarico (Smith et al., 2009) e tiroideo (Vivacqua et al., 2006b). Inoltre, GPER è stato evidenziato (Rago *et al*. 2011; Fietz *et al.* 2014) nelle cellule neoplastiche testicolari sia della linea germinale, come seminomi e carcinomi embrionali (Franco *et al.* 2011; Rago *et al.* 2011) che della linea somatica come Sertoliomi e Leydigiomi (Rago *et al.* 2011). I risultati presentati nel presente lavoro estendono le precedenti osservazioni mostrando l'espressione di GPER sia nella prostata non neoplastica che nei tessuti prostatici tumorali .

Per quanto concerne la prostata non neoplastica, il GPER è stato chiaramente identificato nel citoplasma delle cellule epiteliali basali, evidenziando un suo differente sito di espressione rispetto ai classici recettori estrogenici (ERα e ERβ). I nostri risultati suggeriscono che GPER potrebbe contribuire a mediare l'azione estrogenica in questo compartimento ghiandolare proliferativo, in accordo ad un recente lavoro sperimentale in cui è stata dimostrata l'espressione di GPER in prostasfere di cellule staminali prostatiche provenienti da una prostata adulta non neoplastica (Hu *et al.* 2011). Lo strato delle cellule epiteliali basali è costituito da una eterogenea popolazione di cellule prostatiche, contenenti cellule staminali indicate da alcuni autori come possibili cellule da cui potrebbe avere origine l'adenocarcinoma prostatico (Signoretti *et al.* 2007). Le cellule epiteliali basali sono quindi attualmente in corso di investigazione come possibili targets terapeutici.

I nostri risultati hanno inoltre mostrato una debole espressione di GPER nel compartimento stromale, il che potrebbe suggerire un possibile coinvolgimento di questo recettore nella segnalazione estrogenica tra il compartimento stromale e il compartimento epiteliale nella ghiandola prostatica.

Un altro rilevante risultato di questo studio è stata l'identificazione di GPER nelle cellule di adenocarcinoma prostatico (PCa), una forma comune di cancro negli uomini anziani, che rappresenta la seconda causa di decesso per cancro nel sesso maschile.

Il coinvolgimento degli estrogeni nella carcinogenesi prostatica è stato suggerito sia da studi sperimentali su modelli di ratto e topi Noble (Bosland 2005; Ricke et al 2008) che da studi sulla segnalazione estrogenica mediata dai recettori classici, ERα e ERβ, in tessuti e in linee cellulari tumorali (Bosland 2005; Briganti 2009; Nelles et al 2011).

Fino a poco tempo, si è creduto che ERβ avesse un ruolo prevalentemente protettivo, "tumor suppressor", contrariamente ad ERα, che invece è stato considerato un recettore coinvolto nella del carcinogenesi prostatica,"tumor promoting". Tuttavia, nuove indagini hanno messo in discussione questo paradigma, infatti è stata segnalata un'espressione di ERβ variabile in diversi stadi di differenziazione del cancro alla prostata (Zellweger et al. 2013), mentre le varianti di splicing di ERβ, ER2β e ERβ 5, hanno mostrato possibili ruoli oncogeni (Dey et al 2012;. Nelson et al 2014). E' importante notare che alti livelli di queste splice variants sono stai rilevati in soggetti affetti da carcinoma prostatico con prognosi infausta (Fujimura et al., 2001).

GPER è stato precedentemente identificato in diverse linee cellulari tumorali e i risultati ottenuti hanno chiaramente dimostrato un duplice ruolo di questo recettore, infatti è ormai chiaro che il ruolo di GPER nella proliferazione cellulare è cellula specifico. Infatti, GPER attiva la proliferazione nelle linee di carcinoma mammario ER negative, di carcinoma endometriale e carcinoma ovarico (Albanito *et al.* 2008a,b ; He *et al.* 2012) mentre inibisce la proliferazione nelle cellule di carcinoma mammario ER positive (Ariazi *et al*. 2010) e attiva l'apoptosi nelle cellule tumorali di Leydig (Chimento *et al.* 2013).

Il presente studio, ha identificato , perla prima volta, la presenza di GPER nelle cellule neoplastiche di adenocarcinoma prostatico a differenti stadi. Il carcinoma prostatico infatti è una lesione tumorale che ha origine da una lesione pre-neoplastica (neoplasia intraepiteliale prostatica di alto grado: HGPIN) e progredisce con vari gradi di differenziamento fino all' adenocarcinoma più invasivo. L'esame anatomo-patologico dei campioni ha portato ad osservare, nelle aree tumorali, regioni con differenti arrangiamenti cellulari conosciuti come "Gleason patterns" e regioni adiacenti dove erano presenti HGPIN.

L’immunoistochimica e l’analisi western blot hanno mostrato una intensa immunoreattività al GPER nelle regioni con HGPIN, mentre hanno evidenziato una progressiva diminuizione dell’espressione di GPER passando dalle aree tumorali Gleason pattern 2 a quelle Gleason pattern 4. Poiché l’invasività delle cellule tumorali aumenta dal Gleason pattern 2 al Gleason pattern 4, l’espressione di GPER appare inversamente correlata alla differenziazione delle cellule neoplastiche.

E’ stato inoltre investigato lo stato di molecole di segnalazione a valle di GPER. Abbiamo selezionato il fattore di sopravvivenza cellulare , Akt, e uno dei suoi bersagli a valle, il fattore di trascrizione CREB (Du and Montiminy 1998), poiché queste due molecole sembrano essere coinvolte nella inibizione della proliferazione di linee cellulari tumorali prostatiche dopo trattamento con Nexrutine (Garcia et al 2006). I nostri risultati hanno evidenziato un incremento delle forme fosforilate sia di Akt che di CREB nei carcinomi prostatici scarsamente differenziati rispetto a quelli poco differenziati. Pertanto, nei nostri campioni tumorali , una bassa espressione di GPER sembra essere associata ad un aumento delle forme attivate di Akt e CREB. Tuttavia, la segnalazione a valle di GPER si basa su una rete di vie di trasduzione attivata da una varietà di molecole, comprese ulteriori GPCR e fattori di crescita. Questo rende difficile la valutazione dell'azione GPER come mediatore della progressione tumorale. Ulteriori studi saranno necessari per chiarire questo punto.

CONCLUSIONI

Il presente lavoro ha dimostrato, per la prima volta, l'espressione di GPER nei tessuti prostatici umani non neoplastici e neoplastici. La presenza di GPER è stata rilevata principalmente nello strato epiteliale basale della prostatica benigna, dove questo recettore potrebbe mediare l'azione degli estrogeni sulla normale attività proliferativa e differenziativa di tali cellule. Questo risultato è degno di nota poiché le cellule epiteliali basali prostatiche sembrano contenere le cellule staminali di origine del carcinoma prostatico e, quindi, potrebbero rappresentare potenziali candidati per nuovi approcci terapeutici. Abbiamo rilevato, inoltre, l'espressione del GPER nelle cellule tumorali di adenocarcinoma , indicando una modulazione della immunoreattività a seconda della architettura delle cellule cancerose ” Gleason patterns” e quindi della progressione tumorale. I nostri risultati hanno evidenziato una espressione di GPER che sembra essere inversamente proporzionale alla differenziazione delle cellule neoplastiche.

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ORIGINAL ARTICLE

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Identification of GPER in human prostate: expression site of the estrogen receptor in the benign and neoplastic gland

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SUMMARY

Estrogens are involved in growth, differentiation and pathogenesis of human prostate through the mediation of the classical estrogen receptors ERα and ERβ. The G protein-coupled estrogen receptor (GPER) is a 'novel' mediator of estrogen signaling which has been recently recognized in some human reproductive tissues, but its expression in the prostate gland is still unknown. Here, we investigated GPER in benign (from 5 patients) and neoplastic prostatic tissues (from 50 patients) by immunohistochemical analysis and Western blotting. Normal areas of benign prostates revealed a strong GPER immunoreactivity in the basal epithelial cells while luminal epithelial cells were unreactive and stromal cells were weakly immunostained. GPER was also immunolocalized in adenocarcinoma samples but the immunoreactivity of tumoral areas decreased from Gleason pattern 2 to Gleason pattern 4. Furthermore, a strong GPER immunostaining was also revealed in cells of pre-neoplastic lesions (high-grade prostatic intra-epithelial neoplasia). Western blot analysis of benign and tumor protein extracts showed the presence of a ~42 kDa band, consistent with the GPER molecular weight. An increase in both pAkt and p cAMPresponse-binding protein (pCREB) levels was also observed in poorly differentiated PCa samples. Finally, this work, firstly, identified GPER in the epithelial basal cells of benign human prostate, with a different localization respect to the classical estrogen receptors. Furthermore, the expression of GPER in prostatic adenocarcinoma cells was also observed but with a modulation of the immunoreactivity according to tumor cell arrangements.

INTRODUCTION

A direct action of estrogen signaling in growth, function and pathogenesis of the prostate has been supported by the identification of the classical estrogen receptors (ERα and ERβ) in glandular tissue (McPherson *et al.*, 2008; Prins & Korach, 2008). The main cell types of human prostate are stromal and epithelial cells; particularly, epithelial cells consist of flat basal cells, expressing cytokeratins CK5 and CK14, and tall luminal secretory cells expressing androgen receptors and cytokeratins CK8 and CK18 (Signoretti & Loda, 2007). In the majority of reported data, the consensus is that ERα is mainly expressed in stromal cells, while ERβ is mainly expressed in epithelial luminal cells (Kawashima & Nakatani, 2012).

However, estrogen effects on target cells can be also mediated by the membrane-associated receptor G protein-coupled estrogen receptor (GPER), first named GPR30 (Prossnitz *et al.*, 2008; Maggiolini & Picard, 2010). Previous studies detected GPER in human reproductive cells, as testicular cells and spermatozoa (Rago *et al.*, 2011, 2014), but its expression in prostatic tissue has not been reported yet. Interestingly, a recent study observed GPER in rabbit prostate, suggesting it as the main mediator of estrogen action (Comeglio *et al.*, 2014).

Following previous studies, focusing on the role of androgens on the hormonal basis of prostate cancer (PCa) (Cooper & Page, 2014), increasing lines of evidence indicate that estrogens can also be involved in the development and progression of PCa (Nelson *et al.*, 2014). Estrogens could induce PCa development through different potential mechanisms (Nelles *et al.*, 2011), but a particular attention has been focused on actions mediated by estrogen receptors. Extensive studies on the roles of ERα and ERβ in prostatic neoplastic processes suggested the traditional paradigm where ERβ was considered predominantly protective, being anti-carcinogenic and pro-apoptotic, while ERα was considered tumor promoting (Risbridger *et al.*, 2007; Bonkhoff & Berges, 2009; Celhay *et al.*, 2010; McPherson *et al.*, 2010; Nakajima *et al.*, 2011; Attia & Ederveen, 2012).

On the contrary, GPER expression in human PCa tissues is, to our knowledge, still unknown. However, a possible involvement of this receptor in prostate carcinogenesis has been suggested by an in vitro study showing the inhibition of PCa cell growth by G1, a GPR30 agonist (Chan *et al.*, 2010). Therefore, with the aim to explore a possible GPER mediation in estrogen action on prostate gland, this study investigated the expression of this receptor in benign and neoplastic prostatic tissues.

MATERIALS AND METHODS

Patients

The investigation was performed on formalin-fixed and paraffin-embedded prostatic tissues obtained from five male patients (age 45–65 years) with benign pathology and from 50 patients with adenocarcinoma (age 55–70 years). Tumor samples were classified according to their prevalent neoplastic cell arrangements (Gleason patterns) (primary and secondary patterns) as follows: well differentiated (n = 20), moderately differentiated (n = 12) and poorly differentiated (n = 18). The archival cases were provided by the Pathologic Anatomy Unit (Annunziata Hospital, Cosenza, Italy). The ethical committee members of the University of Calabria approved the investigation program.

Chemicals and antibodies

Reagents were purchased from Sigma Aldrich (Milan, Italy), unless otherwise indicated. Rabbit polyclonal anti- human GPER primary antibody, LS-A4271, and its related blocking peptide (LS-P4271) were provided by MBL International Corporation (Woburn, MA, USA). Rabbit polyclonal anti human GPER primary antibody, K19, and its related blocking peptide were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-Akt 1/2/3 (H-136), rabbit polyclonal anti p-Akt1/2/3(Ser 473) sc-33437, rabbit polyclonal anti CREB-1 (C-21), goat polyclonal anti p-CREB-1 (Ser 133): sc-7978 and rabbit polyclonal anti β-actin primary antibodies were also purchased from Santa Cruz Biotechnology. Biotinylated goat-anti-rabbit IgG secondary antibody and avidin–biotin–horseradish peroxidase complex (ABC/HRP) were from Vector Laboratories (Burlingame, CA, USA), goat anti-rabbit and rabbit anti-goat horseradish peroxidase-conjugated IgG secondary antibodies were from Amersham (Pittsburgh, USA). Diaminobenzidine chromogen (DAB) was from Zymed Laboratories (South San Francisco, CA, USA).

Immunohistochemical analysis

Paraffin-embedded sections, 5 μm thick, were mounted on slides pre-coated with poly-lysine, and they were then deparaffinized and dehydrated (7–8 serial sections). Immunohistochemical experiments were performed after heat-mediated antigen retrieval. Hydrogen peroxide (3% in distilled water) was used, for 30 min, to inhibit endogenous peroxidase activity while normal goat serum (10%) was utilized, for 30 min, to block the non-specific binding sites. Immunodetection was carried out using separately the two anti-GPER (LS-A4271 and K19) (1 : 100) primary antibodies at 4 °C overnight. A biotinylated goat-anti-rabbit IgG was then applied (1 : 600) for 1 h at room temperature, followed by ABC/HRP. Immunoreactivity was visualized by using DAB. Sections were also counterstained with hematoxylin. Absorption controls utilized primary antibodies pre-adsorbed with an excess of their purified blocking peptide, at 4 °C for 48 h. Breast cancer tissue was used as the positive control.

Scoring system

The immunostained slides of tumor samples were evaluated by light microscopy using the Allred Score (Allred *et al.*, 1998) which combines a proportion score and an intensity score. A proportion score was assigned representing the estimated proportion of positively stained tumor cells (0 = none; 1 = 1/100; 2 = 1/100 to <1/10; 3 = 1/10 to <1/3; 4 = 1/3 to 2/3; 5 = >2/3). An intensity score was assigned by the average estimated intensity of staining in positive cells (0 = none; 1 = weak; 2 = moderate; 3 = strong). Proportion score and intensity score were added to obtain a total score that ranged from 0 to 8. A minimum of 100 cells were evaluated in each slide. Six to seven serial sections were scored in a blinded manner for each sample. One-way ANOVA was used to evaluate the differences in the scores between samples.

Protein extraction

Protein extraction from formalin-fixed paraffin-embedded (FFPE) sections (selected areas) was carried out according to Kawashima *et al.* (2014). FFPE sections were transferred to 1.5 mL polypropylene microcentrifuge tubes, deparaffinized, by incubation at room temperature in xylene for 10 min, and rehydrated with a graded series of ethanol (absolute ethanol, 95% ethanol, 70% ethanol, 50% ethanol); after each incubation, the tissues were pelleted at 16 000 ***g*** for 3 min, and the incubation/centrifugation steps were repeated twice. The pellets were then briefly air-dried. The tissue pellets were then weighed and homogenized in 100 volumes of PEB (500 mM Tris–HCl pH 8.0 and 2% SDS). Samples were incubated at 90 °C for 90 min. The extracts were centrifuged for 20 min at 16 000 ***g*** at 4 °C and the supernatants were stored at −80 °C until biochemical analysis.

Western blot analysis

Protein lysates (50 μg) were boiled for 5 min, separated under denaturing conditions by SDS-PAGE on 10% polyacrylamide Tris-glycine gels and electroblotted to nitrocellulose membrane. Non-specific sites were blocked with 5% non-fat dry milk in 0.2% Tween-20 in Tris-buffered saline (TBS-T) for 1 h at room temperature and incubated overnight with indicated polyclonal primary antibodies (anti-GPER 1 : 500, anti-AKT: 1 : 500, anti pAKT 1 : 1000, anti-CREB 1 : 300, anti-pCREB 1 : 500). The antigen–antibody complexes were then detected by incubation of the membranes for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibodies (1 : 7000). The bound secondary antibodies were located with the ECL Plus Western blotting detection system (GE Healthcare Europe GmbH, Milan, Italy) according to the manufacturer's instructions. Each membrane was exposed to the film for 2 min. All blots were stripped and reprobed with β-actin as loading control. Negative controls were prepared using tissue lysates, where antigens were previously removed by pre-incubation with specific antibodies (1 h at room temperature) and subsequently immunoprecipitated with protein A/G-agarose (Aquila *et al.*,

2002). Western blot analysis was repeated three times for each sample.

RESULTS

Morphological study

Benign prostate gland

Unaltered areas of benign prostate displayed features typical of normal glandular tissue, showing rounded branching glands, with inward papillary projections and stroma interposed. The glands were lined by a basal low cuboidal epithelium and a luminal layer of tall columnar epithelium (Fig. 1).

Adenocarcinoma

In adenocarcinoma samples, different cell arrangements of tumor cells (Gleason patterns) can be detected. According to the anatomo-pathological criteria, each pattern is given a grade from 1 to 5, with 1 looking the most like normal prostate tissue and 5 looking the most abnormal. In our adenocarcinoma samples, Gleason pattern 2 was detected prevalently in well-differentiated tumors, Gleason pattern 3 was detected in well and moderately differentiated tumors while Gleason pattern 4 was revealed prevalently in poorly differentiated tumors (Fig. 2). Furthermore, the presence of pre-neoplastic lesions, high-grade prostatic intraepithelial neoplasia (HGPIN) (Fig. 3A), was frequently observed in tumor samples.

GPER immunolocalization

Benign prostate gland

A strong GPER immunoreactivity was observed in the cytoplasm of basal epithelial cells while no immunostaining was observed in luminal secretory epithelial cells (Fig. 1). Furthermore, a weak staining in the cytoplasm of stromal cells was also detected (Fig. 1).

Adenocarcinoma

The immunoreactivity was very strong or intense in the cytoplasm of neoplastic cells arranged as Gleason patterns 2 or Gleason patterns 3, respectively, while immunoreactivity was weak/moderate in cells of Gleason 4 patterns (Fig. 2 & Table 1). HGPIN cells showed higher immunostaining (Allred score = 8) (Fig. 3A). Furthermore, cytoplasm GPER expression was observed in the majority of adenocarcinoma cells; however, areas with GPER nuclear staining were also detected in 5 of 50 samples (data not shown).

Western blot analysis

GPER

The immunoblot of benign prostatic tissues showed a single band at molecular weight value ~42 kDa (Fig. 4A: lanes 1–3). These bands co-migrated with the positive control (SKBR3) (Fig. 4A: lane C+). Similar ~ 42 kDa bands were also detected in adenocarcinoma samples (Fig. 4B). A decreasing band intensity was observed in well (lane 1), moderately (lane 2) and poorly (lane 3) differentiated PCa samples (Fig. 4B).

Akt/pAkt

The immunoblots of PCa samples showed Akt/pAkt bands (Fig. 5A). Furthermore, Fig. 5A1 shows no significant change in the levels of total Akt in the different PCa samples, while phosphorylated Akt increased in poorly PCa (lane 3) with respect to well (lane 1) and moderately differentiated (lane 2) samples (Fig. 5A2).

CREB/pCREB

The immunoblots of PCa extracts showed CREB/pCREB bands (Fig. 5B). The levels of total CREB did not change significantly between the different samples (Fig. 5B1), while higher band intensity of phosphorylated CREB (pCREB) was observed in poorly (lane 3) differentiated PCa samples vs. well (lane 1) and moderately (lane 2) differentiated specimens (Fig. 5B2).

DISCUSSION

Concerning benign prostate, GPER was clearly identified in epithelial basal cells, revealing a differential expression site with respect to the classical estrogen receptors. Epithelial basal layer consists of a heterogeneous population containing adult prostate stem cells, which have been also indicated as possible stem cells of PCa origin (Signoretti & Loda, 2007). Our results suggest that GPER could contribute to the mediation of estrogen action in this proliferative prostatic compartment. Furthermore, our finding agrees with an experimental study showing GPR30 expression in prostasphere from human normal adult prostate stem cells (Hu *et al.*, 2011). In addition, the weak expression of GPER in stromal cells suggests a possible involvement of this receptor in estrogen signaling between stromal and epithelial prostatic compartments.

Another relevant finding of the present study was the identification of GPER in cells of prostatic adenocarcinoma (PCa), a common form of cancer in elder men. Involvement of estrogen in prostatic carcinogenesis has been suggested by experimental studies from Noble rat and mouse models (Bosland, 2005; Ricke

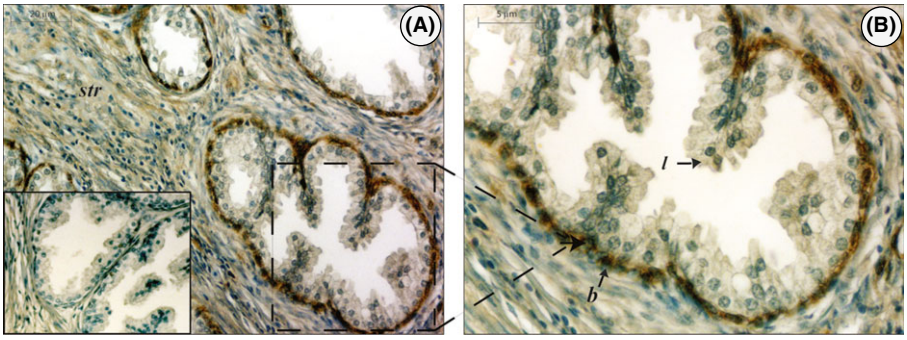


Figure 1 G protein-coupled estrogen receptor immunoreactivity in human benign prostate. (A) Strong immunostaining in basal epithelial cells and weak immunoreactivity in stromal cells. (B) Higher magnifications of prostatic areas surrounded by the dashed lines in A. *b*, basal epithelial cells; *l*, luminal epithelial cells; *str*, stromal cells. Insert: absorption control.

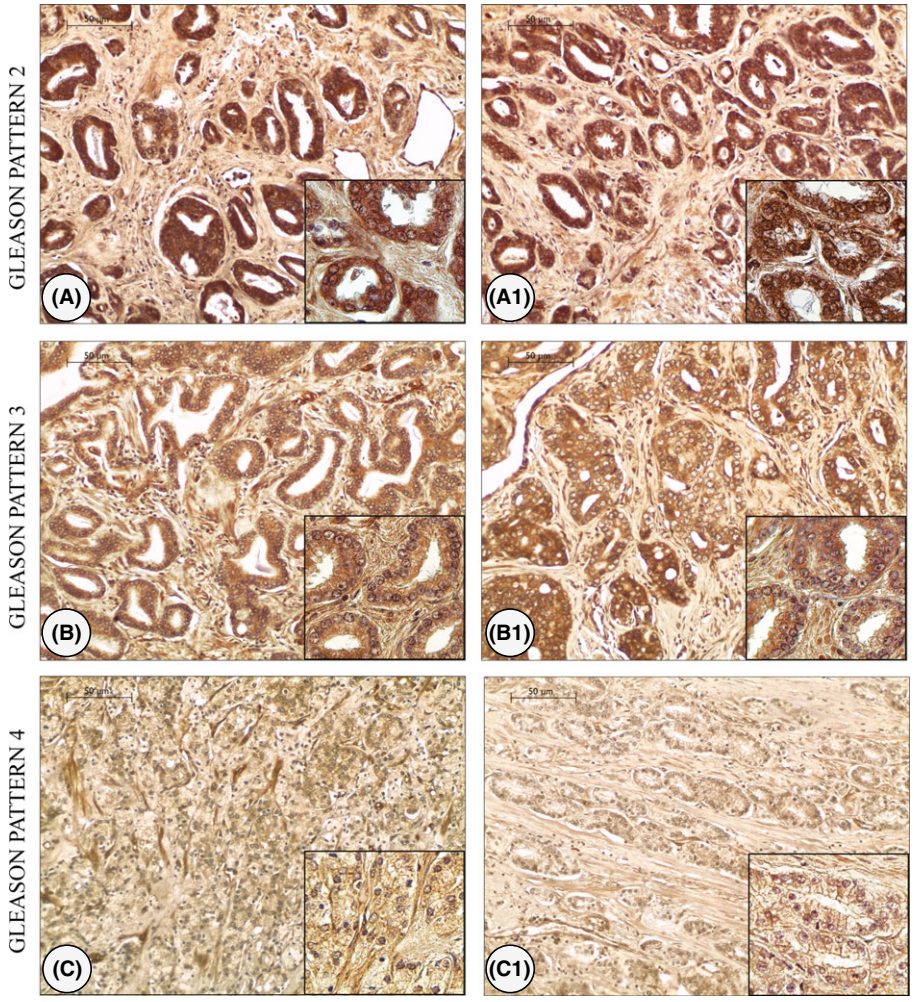
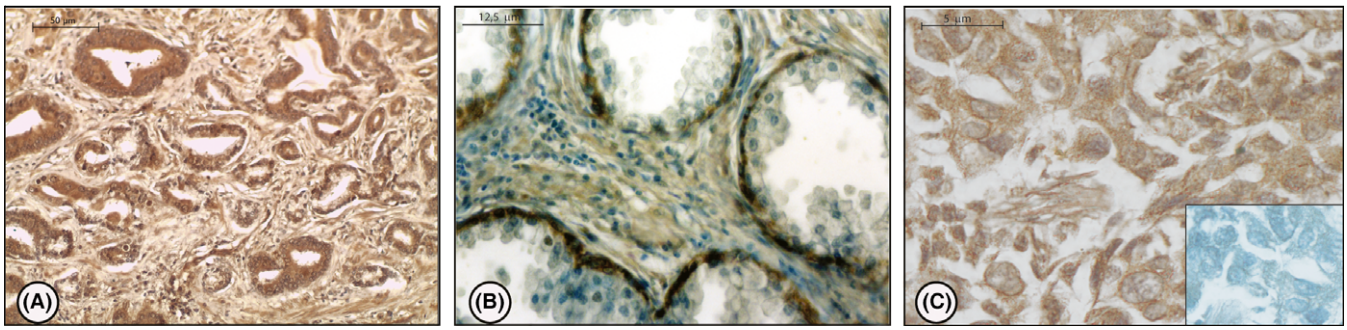


Figure 2 Immunolocalization of G protein-coupled estrogen receptor in prostate adenocarcinoma. The picture shows representative samples of tumoral areas arranged as Gleason pattern 2 (A–A1), as Gleason pattern 3 (B–B1) and as Gleason pattern 4 (C–C1). Inserts: higher magnifications of the images.

Figure 3 (A) G protein-coupled estrogen receptor (GPER) immunostaining in HGPIN. (B) GPER immunostaining in control prostate. (C) Breast cancer as positive control. Insert: absorption control.



et al., 2008); so, estrogen signaling through the classical estrogen receptors has been largely investigated in human PCa tissues and cultured PCa cells (Bosland, 2005; Briganti, 2009; Nelles *et al.*, 2011). Until recently, ERβ was believed to have a predominantly protective role, 'tumor suppressor', while ERα was considered 'tumor promoting'. However, new investigations call into question this paradigm; in fact, a variable ERβ expression was reported in different stages of PCa (Zellweger *et al.*, 2013) while ERβ splice variants, ERβ2 and ERb5, showed possible oncogenic roles (Dey *et al.*, 2012; Nelson *et al.*, 2014).

Table 1 GPER immunoreactivity (Allred score) in Gleason patterns observed in prostatic adenocarcinoma samples

Gleason patterns	Allred score (median)
2 (n = 20/50)	7
3 (n = 40/50)	6*
4 (n = 20/50)	3**

Total adenocarcinoma samples n = 50. Total immunostaining score: Proportion score + Intensity score (range 0–8). Significant difference: *$p < 0.05$ with respect to pattern 3; **$p < 0.005$ with respect to pattern 2 and pattern 3.

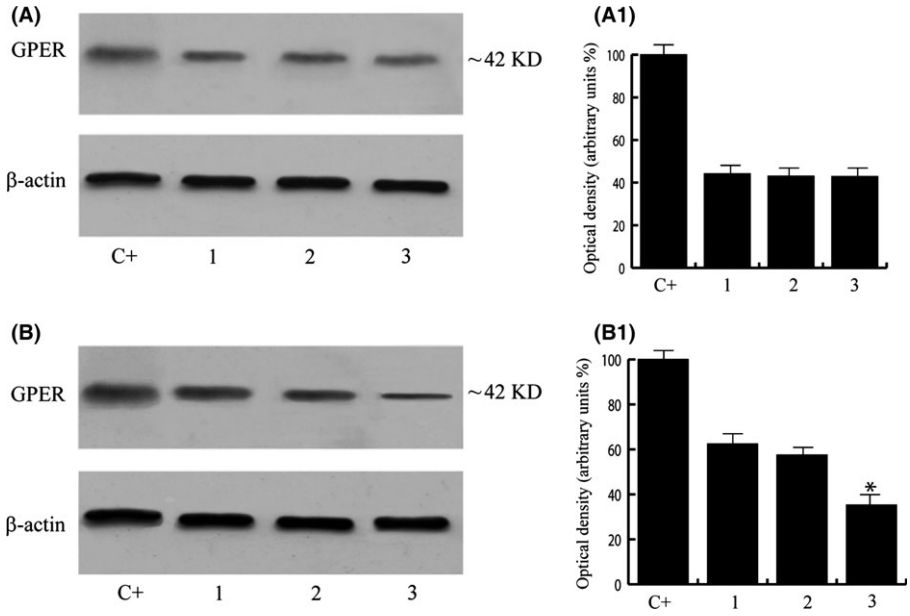


Figure 4 G protein-coupled estrogen receptor immunoblots of protein extracts from human prostate. (A) Representative samples of benign prostate (lanes 1–3), breast cancer as positive control (lane C+). (B) Representative samples of prostate adenocarcinoma (lane 1: well differentiated PCa, Gleason pattern 2), (lane 2: moderately differentiated PCa, Gleason pattern 3), (lane 3: poorly differentiated PCa, Gleason pattern 4), positive control (lane C+). A1/B1: band intensities were evaluated in term of arbitrary densitometric units. Values are mean ± SEM. *$p < 0.05$ vs. lanes 1 and 2. β-Actin serves as a loading control.

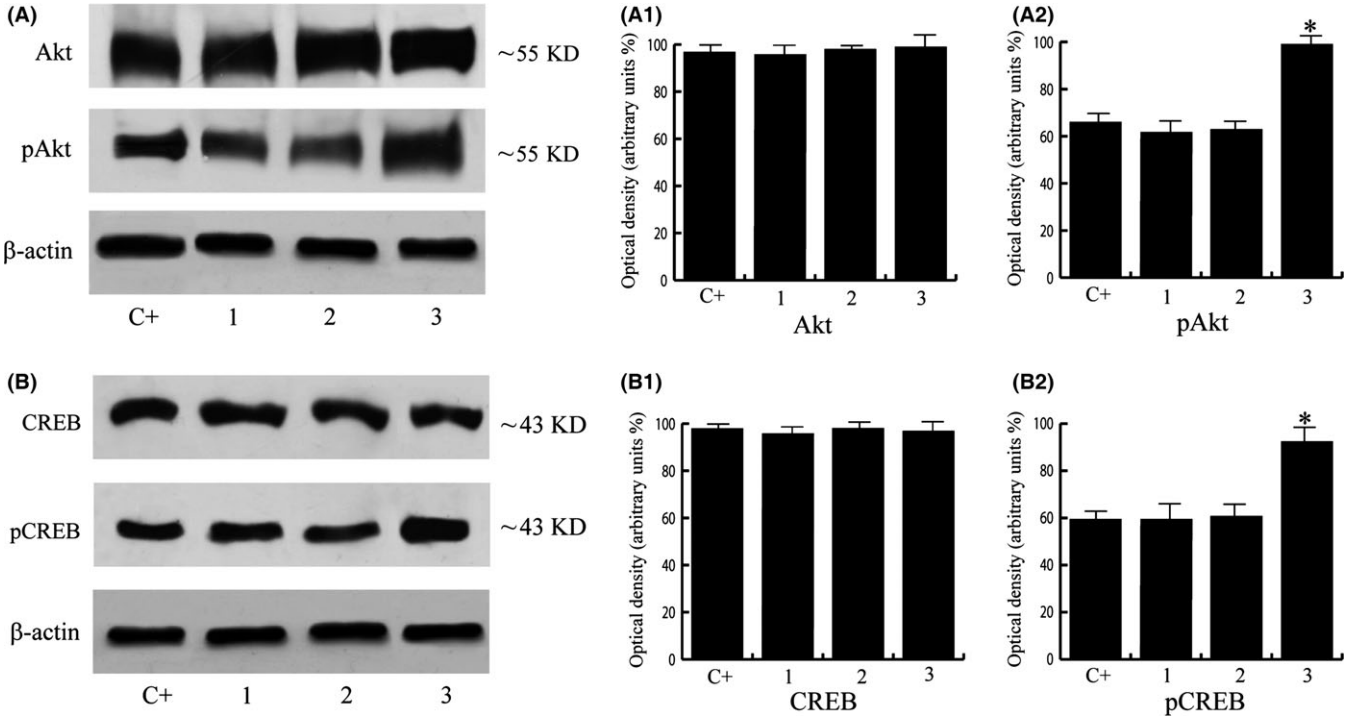


Figure 5 AKT/CREB immunoblots of protein extracts from representative samples of human adenocarcinoma. (A) AKT/pAKT (lane 1: well-differentiated PCa, Gleason pattern 2), (lane 2: moderately differentiated PCa, Gleason pattern 3), SKBR3 cells as positive control (lane C+). (B) CREB/pCREB (lane 3: poorly differentiated PCa, Gleason pattern 4), SKBR3 cells as positive control (lane C+). A1–A2/B1–B2: Band intensities were evaluated in terms of arbitrary densitometric units. Values are mean ± SEM. *$p < 0.05$ vs. lanes 1 and *2*. β-Actin serves as a loading control.

G protein-coupled estrogen receptor has been previously identified in some cultured tumor cells from reproductive tissues, showing a dual role in cell proliferation, depending on specific cells. In fact, GPER activates the proliferation in ER-negative breast cancer cells, in endometrial and in ovarian cancer cells (Albanito *et al.*, 2008a,b; He *et al.*, 2012) while it inhibits the proliferation in ER-positive breast cancer cells (Ariazi *et al.*, 2010) and activates apoptosis in tumor Leydig cells (Chimento *et al.*, 2013). Furthermore, GPER has been detected in somatic cells of normal human testes (Rago *et al.*, 2011; Fietz *et al.*, 2014) as well as in neoplastic cells of Sertoli cell tumor, Leydig cell tumor, seminoma and embryonal carcinoma (Franco *et al.*, 2011; Rago *et al.*, 2011).

The present study first identified GPER in neoplastic cells of PCa at different stages. PCa is believed to progress from a preneoplastic lesion (HGPIN) to the most invasive adenocarcinoma

in a step-like manner. So, the anatomo-pathological examination of the PCa samples led us to observe, in tumoral areas, different cell arrangements known as Gleason patterns, together with HGPIN regions. Our results showed higher GPER immunoreactivity in HGPIN regions, while immunohistochemical experiments and Western blot analysis revealed a decreasing GPER expression in tumor cells from Gleason pattern 2 to Gleason pattern 4. As the invasiveness of tumor cells increases from Gleason pattern 2 to Gleason pattern 4, GPER expression appears to be inversely related to neoplastic cell differentiation. In addition, the status of signaling molecules downstream of GPER has been investigated. We selected the cell survival factor, Akt, and one of its downstream targets, the transcription factor CREB (Du & Montiminy, 1998), because these two molecules were involved in the inhibited proliferation of Nexuritine-treated PCa cell line (Garcia *et al.*, 2006). Our results showed an increase in both pAkt and pCREB in poorly differentiated PCa. Therefore, in our tumor samples, a low GPER expression appears to be associated with an increase in Akt and CREB-activated forms. However, GPER downstream signaling relies on a network of transduction pathways that is activated by a variety of molecules, including further GPCRs and growth factors. This makes unreliable the evaluation of a few mediators of GPER action during tumorigenesis progression. Further extensive studies will clarify this point.

CONCLUSIONS

This work demonstrated the expression of GPER in benign and neoplastic human prostatic tissues. GPER was mainly confined in the basal epithelial layer of benign prostate, where this receptor could mediate estrogen action on normal cellular activity. Furthermore, our finding is noteworthy because these benign basal cells could contain stem cells of PCa origin and, therefore, they are considered potential candidates for new therapeutic approaches. In addition, GPER expression was detected in tumor cells of PCa with a modulation of the immunoreactivity according to the arrangement of neoplastic cells. This suggests that GPER expression in PCa samples could be inversely related to neoplastic cell differentiation.

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AUTHORS' CONTRIBUTIONS

V. Rago carried out immunohistochemical experiments and data analysis. F. Romeo is responsible for histopathology diagnosis. F. Giordano carried out Western blot analyses. A. Ferraro collected and analyzed archival samples. A. Carpino is responsible for conception, design, analysis and interpretation of data as well as drafting of the manuscript.

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Estrogen related receptor α (ERRα) a promising target for the therapy of adrenocortical carcinoma (ACC)

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ABSTRACT

The pathogenesis of the adrenocortical cancer (ACC) involves integration of molecular signals and the interplay of different downstream pathways (i.e. IGFII/IGF1R, β-catenin, Wnt, ESR1). This tumor is characterized by limited therapeutic options and unsuccessful treatments. A useful strategy to develop an effective therapy for ACC is to identify a common downstream target of these multiple pathways. A good candidate could be the transcription factor estrogen-related receptor alpha (ERRα) because of its ability to regulate energy metabolism, mitochondrial biogenesis and signalings related to cancer progression.

In this study we tested the effect of ERRα inverse agonist, XCT790, on the proliferation of H295R adrenocortical cancer cell line. Results from *in vitro* and *in vivo* experiments showed that XCT790 reduced H295R cell growth. The inhibitory effect was associated with impaired cell cycle progression which was not followed by any apoptotic event. Instead, incomplete autophagy and cell death by a necrotic processes, as a consequence of the cell energy failure, induced by pharmacological reduction of ERRα was evidenced.

Our results indicate that therapeutic strategies targeting key factors such as ERRα that control the activity and signaling of bioenergetics processes in high-energy demanding tumors could represent an innovative/alternative therapy for the treatment of ACC.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a very rare and aggressive disease with a high risk of relapse after radical surgery. Treatment options in advanced, metastatic stages are limited, since cytotoxic chemotherapy options are poor and radiotherapy is mostly ineffective [1]. The drug mitotane (o, p′-dichlorodiphe nyldichloroethane (o, p′-DDD)) with its adrenolytic activity is the only adrenal specific drug that is currently used for ACC treatment. However, toxicity, narrow therapeutic window and side effects are the major limitation to its use as well as therapeutic success [2]. Given the high mortality and aggressiveness of ACC, more effective and specific treatment options are needed. Recently, monoclonal antibodies targeting insulin-like growth factor II (IGFII) receptor (IGF1R) have been tested in clinical trials, however they provided a limited effectiveness in refractory patients [3]. Rationale for targeting IGF1R comes from the observation that IGFII gene is overexpressed in ACC [4]. We have recently demonstrated that IGFII/IGF1R pathway can be activated by the estrogen receptor alpha (ESR1), a gene overexpressed in ACC that mediates estrogen-dependent proliferative effects [5, 6]. ESR1 knock down was more effective than an IGF1R antibody in reducing H295R cell

proliferation *in vitro* [5] and the selective estrogen receptor modulator (SERM) tamoxifen prevented the growth of H295R both *in vitro* [7] and as xenografts *in vivo* [5]. Thus, ESR1 could be a promising target to reduce ACC growth.

Indeed, a recent study [8], investigating a large cohort of advanced ACC, confirmed the presence of a large number of potentially targetable molecules involved in ACC progression. These observations confirm that ACC is an extremely heterogeneous disease and that its pathogenesis involves integration of signals and the interplay of downstream pathways. It is currently accepted that these changes are also associated with a profound reprogramming of cellular metabolism [9]. Consequently, one potential strategy to develop an effective therapy for ACC could be the identification of a common downstream target of multiple pathways capable of controlling expression and activity of various bioenergetic factors.

Estrogen Related Receptor α (ERRα) is an orphan member of the nuclear hormone receptor superfamily of transcription factors that has been identified on the basis of its high level of sequence identity to ERα and for which an endogenous ligand has yet to be defined [10]. ERRα functions downstream of the peroxisome proliferator-activated receptor gamma coactivator-1 alpha and beta (PGC-1α and PGC-1β) and regulates the expression of genes involved in energy metabolism and mitochondrial biogenesis such as genes encoding enzymes and proteins of the tricarboxylic acid cycle, pyruvate metabolism, oxidative phosphorylation, and electron transport [11]. Research to understand how changes in cell metabolism promote tumor growth has accelerated in recent years [12]. As a consequence, research has focused on targeting metabolic dependencies of cancer cells, an approach with the potential to have a major impact on patient care. Notably, ERRα has recently been associated with dysregulated cell metabolism and cancer progression. Accordingly, increased expression of ERRα has been shown in several cancerous tissues including breast [13], ovary [14] prostate [15] and colon [16]. Several signaling pathways, also relevant to ACC development have been shown to converge upon and regulate the expression and activity of ERRα together with its coactivators such as PGC-1α and β in others tumor types [17]. Several studies have reported that ERRα inverse agonist XCT-790 [18] can induce cell growth arrest in different tumor cell lines [19, 20]. To date, few studies have investigated the role of ERRα in adrenal gland and ACC. ERRα is expressed in normal adult adrenal and regulates the expression of enzymes involved in steroidogenesis [21]. Moreover, ERRα seems to be more expressed in ACC compared to normal adrenal and adenoma [22].

The aim of this study was to establish if ERRα depletion using XCT790 can induce growth arrest in ACC cells. The data obtained support the hypothesis that ERRα could be a promising target for the treatment of adrenocortical cancer.

RESULTS

ERRα inverse agonist XCT790 decreases ERRα protein content and inhibits ACC cells proliferation *in vitro*

First, we verified that ERRα is expressed in H295R adrenocortical cancer cells. MCF-7 breast cancer cells were used as positive control [23] (Figure 1A). Moreover, we also demonstrated that in both H295R and MCF-7 cells, XCT790 treatment decreased ERRα protein levels in a dose-dependent manner (Figure 1B). The latter results confirmed the ability of XCT790 to reduce the expression of ERRα most probably by proteasome degradation [23]. Next, we evaluated the effects of different concentrations of ERRα inverse agonist XCT790 on ACC cell growth. Results from MTT assay revealed that XCT790 treatment exerted a dose- and time-dependent inhibition on H295R cell proliferation compared to vehicle-treated cells (Figure 1C). The maximum inhibitory effect on ACC cell proliferation was seen at 10 μM XCT790 that was then used for all the following experiments.

ERRα inverse agonist XCT790 inhibits ACC cells proliferation *in vivo*

We next established H295R cell xenograft tumors in immunocompromised mice to investigate the ability of XCT790 to reduce tumor growth *in vivo*. To this aim, H295R cells were injected into the intrascapular region of mice. When tumors reached an average volume of 200 mm^3, animals were randomized into two groups to be treated with either vehicle or XCT790 (2,5 mg/Kg). As shown in Figure 2A, mice treated with XCT790 displayed a significant tumor growth reduction compared to the vehicle treated control group. Accordingly, tumor reduction upon XCT790 treatment is evidenced both in terms of tumor mass (Figure 2B) and proliferation as seen in Figure 2C, showing a strong decrease in Ki67staining (value score control: 7.2 ± 0,46 (SD); value score XCT790 treated cells: 4.7 ± 0.53* (SD), *$p < 0.05$).

ERRα inverse agonist XCT790 blocks G1/S transition of ACC cells without inducing apoptosis

The observed effects of XCT790 on ACC cells proliferation led us to evaluate XCT790 action on H295R cell cycle progression.

First, by analyzing PI staining with FACSJazz flow cytometer, we investigated whether XCT790 treatment could affect the distribution of cells within the three major phases of the cycle. To this aim, H295R cells were grown for 24 h in 5% CS-FBS and then treated with either vehicle (DMSO) or 10 μM XCT790. 48 hours later, FACS analysis revealed that XCT790 treated cells accumulated

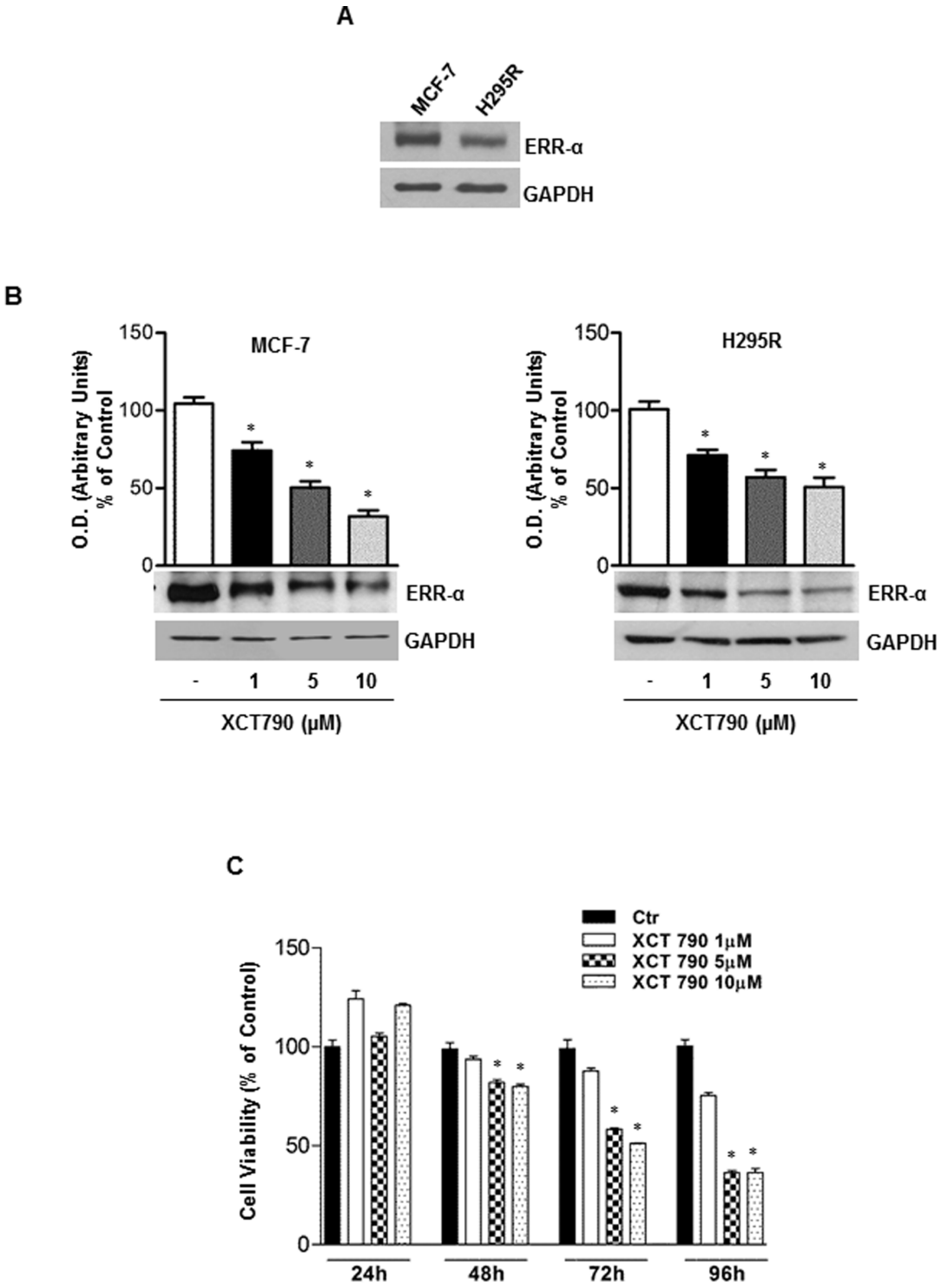

Figure 1: ERRα inverse agonist XCT790 decreases ERRα protein content and H295R cells growth *in vitro*. A. Western blot analysis of ERRα was performed on 50 μg of total proteins extracted from H295R and MCF-7 cells. Blots are representative of three independent experiments with similar results. (**B.** lower left and right panel), protein extracts from MCF-7 and H295R cells left untreated (–) or treated for 48 h with different doses of XCT790 were resolved by SDS-PAGE and subjected to immunoblot against ERRα. GAPDH served as loading control. (b, upper left and right panel), graphs represent means of ERRα optical density (O.D.) from three independent experiments with similar results normalized to GAPDH content (*$p < 0.001$ compared to untreated control sample assumed as 100). **C.** Cell viability after XCT790 treatment was measured using MTT assay. Cells were plated in triplicate in 24-well plates and were untreated (Ctr) or treated with increasing concentrations of XCT790 for the indicate times in DMEM supplemented with 2,5% Charcoal-Stripped FBS. Absorbance at 570 nm was measured on a multiwell-plate reader. Cell viability was expressed as a percentage of control, (*$p < 0.001$).

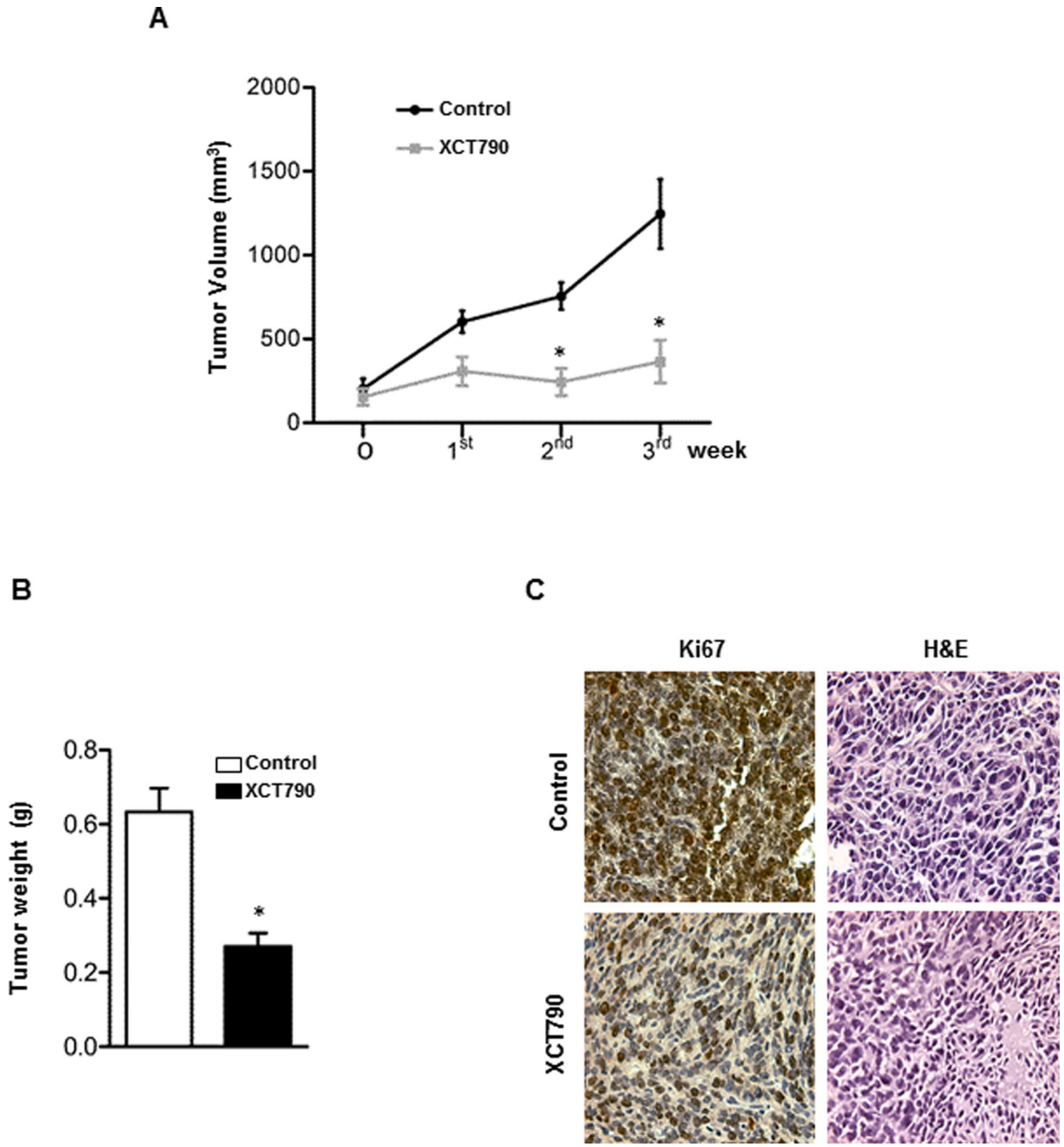

Figure 2: ERRα inverse agonist XCT790 decreases H295R cells proliferation *in vivo*. A. 6 × 10^6 H295R cells were injected subcutaneously onto the intrascapular region of immunocompromised mice and the resulting tumors were grown to an average of 200 mm^3. The animals were randomized to vehicle controls or XCT790 treatment for twenty one days. Tumor volumes were calculated, as indicated in Materials and Methods. Values represent the mean ± SE of measured tumor volume over time in the control group (filled circles, $n = 10$) and in the XCT790-treated group (filled squares, $n = 10$). **B.** After 21 days (3 weeks) tumors were harvested and weighed. Values represent the mean ± SE of measured tumour weight ($n = 10$) *$P < 0.05$ versus control at the same day of treatment. **C.** Ki67 immunohistochemical and H & E staining: histologic images of H295R explanted from xenograft tumors (magnification X 400).

in the G0/G1-phase of the cell cycle while the fraction of cells in S phase decreased compared with vehicle treated cells (Figure 3A).

In order to define the molecular mechanisms involved in XCT790-dependent cell cycle arrest, changes in levels of protein involved in cell cycle regulation were investigated by Western blotting analysis. After 48 h treatment, XCT790 reduced Cyclin D1 and Cyclin E protein content while expression levels of CDK2 and CDK4 proteins were unaffected. Consistently with the observed G1/S transition arrest of the cell cycle, Rb protein showed a hypophosphorylated status (Figure 3B–3C). As the analysis of the cell cycle revealed a minimal increase of the sub-G1 fraction (Figure 3A), a known

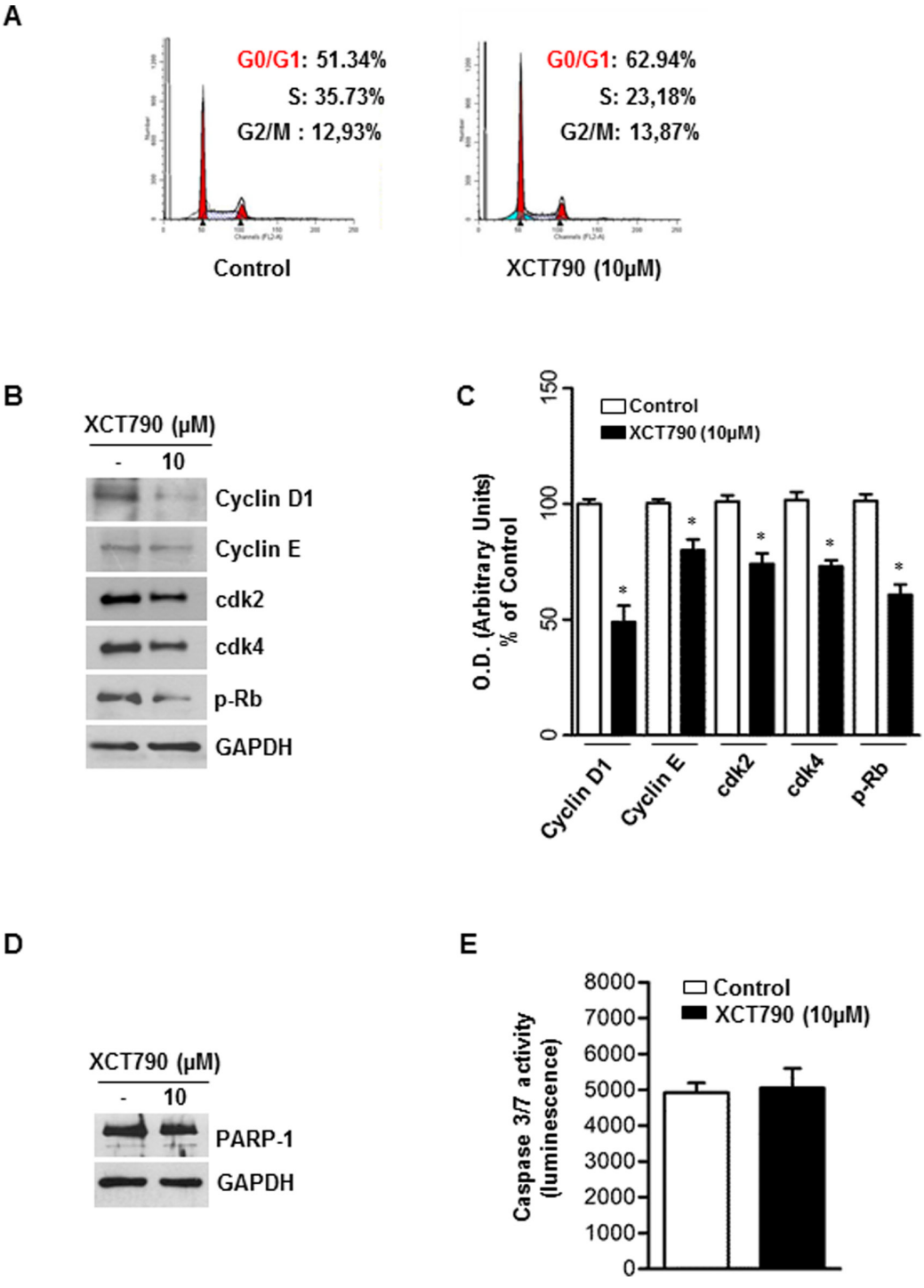

Figure 3: ERRα inverse agonist XCT790 impairs G1/S transition of ACC cells without inducing apoptosis. A. The distribution of H295R cells in the cycle was determined by Flow Cytometry using Propidium-iodide (PI) stained nuclei. The graph shows the distribution of H295R cell population (%) in the various phases of cell cycle. **B.** Total proteins from H295R cells left untreated (–) or treated with XCT790 for 48 h were resolved by SDS-PAGE and subjected to immunoblot analysis using specific antibodies against human Cyclin D1, Cyclin E, cdk2, cdk4, p-Rb. **C.** Graphs represent means of Cyclin D1, Cyclin E, cdk2, cdk4, p-Rb optical densities (O.D.) from three independent experiments with similar results normalized to GAPDH content, (*$p < 0.001$ compared to each untreated control assumed as 100); **D.** Total proteins were analyzed by Western blot for PARP-1. Blots are representative of three independent experiments with similar results. GAPDH served as loading control. **E.** Cellular caspase 3/7 activity was determined by Caspase-Glo assay system using the substrate Ac-DEVD-pNA and expressed as relative luminescence units (RLU) of treated cell to untreated control cell. Each column represents the mean ± SD of three independent experiments (*$p < 0.001$ compared to untreated control sample).

marker of apoptotic events, we next attempted to verify the presence of apoptotic features such as PARP-1cleavage and caspase 3/7 activation, all well-known biochemical markers of programmed cell death. Surprisingly, results from Western blotting analysis for PARP-1 (Figure 3D) and caspase 3/7 activity assay (Figure 3E) clearly showed that XCT790 did not activate an apoptotic pathway.

XCT-790 decreased mitochondrial mass and function in ACC cells

The activity of ERRα is highly dependent on the presence of coactivator proteins, most notably PGC-1α and PGC-1β [24], both known for their crucial role in regulating energy metabolism and mitochondrial biogenesis [24]. Moreover, it has been observed that XCT790 treatment, causing ERRα proteasome degradation, also down-regulates PGC1-α [24]. Based on these observations, we first checked if XCT790 treatment regulates PGC1-α expression in H295R cells. To this aim, ACC cells were left untreated or treated with 10 μM XCT790 for 48 h. Results from Western blotting showed (Figure 4A–4B) that XCT790 treated cells display a reduced expression of PGC1-α, with no effect on PGC-1β levels. We then asked whether reduced levels of PGC1-α would lead to reduction of mitochondrial mass. To this purpose we treated cells with MitoTracker deep red FM that stains specifically mitochondria independently of their membrane potential. Using flow cytometric analysis (Figure 4C), fluorescent imaging (Figure 4D) and fluorescent plate reader (Figure 4E), we found that XCT790 significantly decreased mitochondrial mass.

The mitochondrial citrate carrier CIC is a protein that belongs to a family of metabolites transporters embedded in the inner mitochondrial membrane [25, 26] and has been recently highlighted as important component in maintaining mitochondrial integrity and bioenergetics in normal and particularly in tumor cells [27]. We used CIC protein expression as a marker of both mitochondrial mass and function and found that XCT790 decreased mitochondrial CIC expression (Figure 4F–4G) as well as its transport activity (Figure 4H) in H295R-treated cells compared to vehicle-treated control cells.

To extend these findings, we used immunoblotting to monitor the abundance of a known reliable marker of mitochondrial mass, TOM20, in response to 10 μM XCT790 treatment. We found that XCT790 treated-H295R cells displayed a reduced expression of ERRα, as expected, concomitantly with a drastic decline of TOM20 protein expression (Figure 5A–5B). Similarly, the analysis of the expression of the mitochondrial oxidative pathway (OXPHOS) enzymes showed a substantial reduction of all the complexes (Figure 5C). In agreement with these findings, the reduction in the ATP content reveals a bioenergetics failure induced by XCT790 in treated cells (Figure 5D).

XCT790 induce cell death by necrosis in ACC cells

Very recent data revealed that low levels of CIC or its impaired expression induce mitochondrial dysfunction followed by enhanced mitochondrial turnover via autophagy/mitophagy mechanism [27]. Based on this observation and accordingly to our above reported results showing the ability of XCT790 to down-regulate CIC expression in H295R cells, we wanted to verify if autophagic features were detected in our experimental conditions. Autophagy is characterized by acidic vacuoles (AVO) formation, which can be measured by acridine orange (AO) vital staining. AO moves freely to cross biological membranes and accumulates in acidic compartment, where it is seen as bright red fluorescence [28]. As shown in Figure 6A (upper panel), AO vital staining of 48 h XCT790-treated H295R cells showed the accumulation of AVO in the cytoplasm. To quantify the accumulation of the acidific component, we performed FACS analysis of acridine orange-stained cells using FL3 mode (> 650 nm) to quantify the bright red fluorescence and FL1 mode (500–550 nm) for the green fluorescence. As shown in Figure 6A (lower panel), XCT790 treatment raised the strength of red fluorescence from 7,5% to 51%. These results corroborate the observation that XCT790, increases the formation of AVOs which suggests autophagy/mitophagy as possible mechanisms to explain the reduced mitochondrial mass. This latter event could be responsible for the inhibitory effects on cell growth elicited by XCT790 on adrenocortical cancer cells. A careful evaluation of the autophagic/mitophagic process by investigating changes in autophagic markers such as Beclin 1, LC3B, BNIP3 and Cathepsin B (Figure 6B), suggested that XCT790 treatment promotes the initial stages of the autophagic process. This is supported by the evidence of increased Beclin 1 expression and the presence of the cleaved LC3B form [29]. However, autophagy fails to terminate as indicated by decreased BNIP3, Cathepsin B and Lamp1 proteins expression [29]. Therefore, we evaluated XCT790 ability to induce H295R cells death by necrosis. To this aim, Trypan blue exclusion test was performed after 48 h of XCT790 treatment. As shown in Figure 6C, H295R displayed a significant increase in the number of positive stained cells compared to control cells indicating that membrane integrity and permeability were lost accounting for a necrotic event following a bionergetic failure triggered by ERRα depletion.

DISCUSSION

The molecular heterogeneity and complexity that characterize adrenocortical cancer biology combined with lack of an effective treatment, drive towards the discovery of new therapeutic targets. Advances in the understanding of the molecular pathogenesis of ACC have

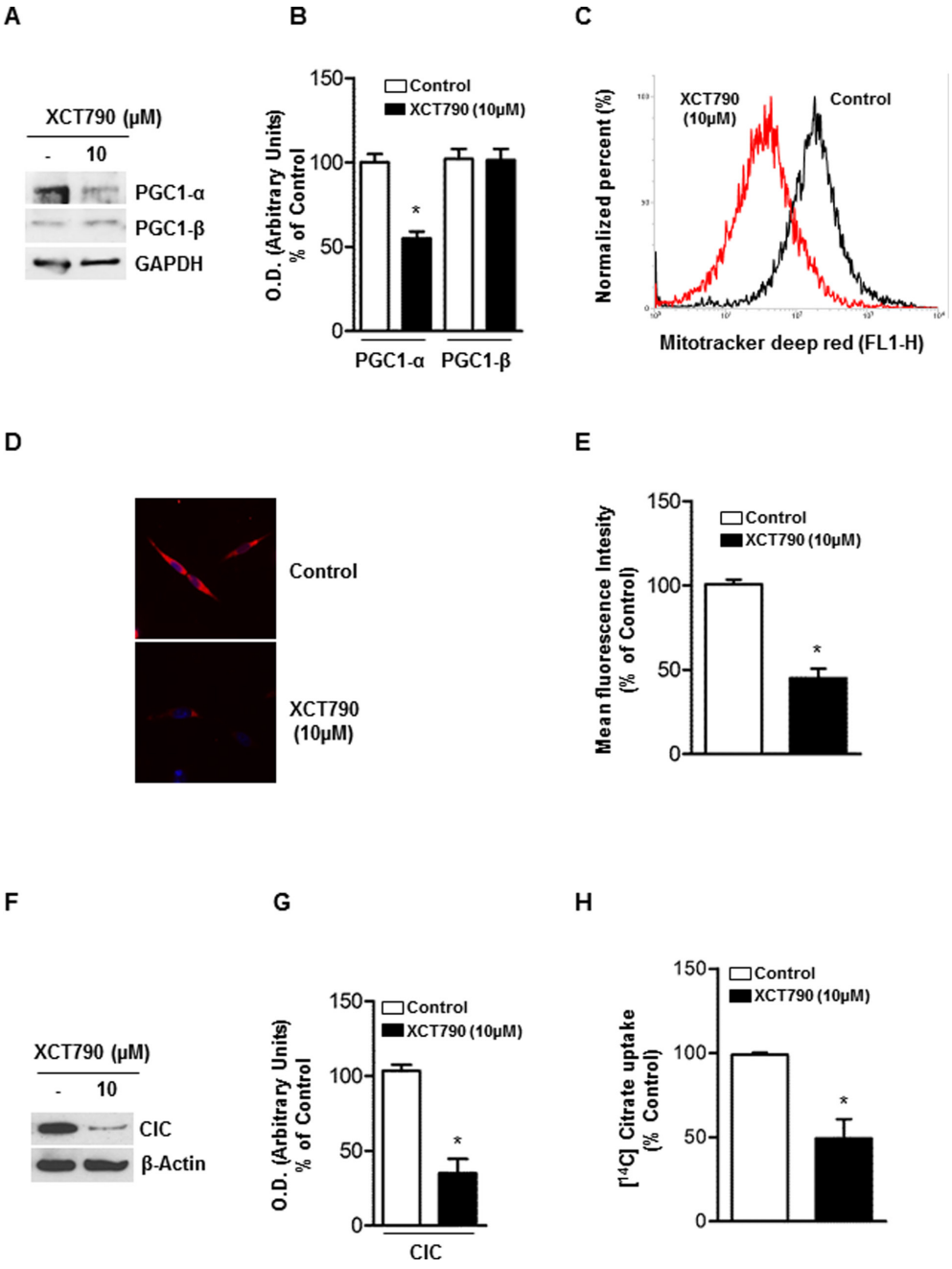

Figure 4: XCT-790 decreases mitochondrial mass and function in H295R cells. A. Total protein extracts from H295R cells, left untreated (–) or XCT790 treated in 2.5% DCC-FBS medium for 48 h were analyzed by Western blot with antibodies against PGC-1α and PGC-1β. GAPDH was used as loading control. **B.** Graphs represent means of PGC-1α and β optical densities (O.D.) from three independent experiments with similar results normalized to GAPDH content (*$p < 0.001$ compared to each untreated control sample assumed as 100). **C.** H295R cells were right untreated (control) or treated with XCT790. 48 h later, absorption of MitoTracker deep red FM was determined by FACS analysis. The uptake of MitoTracker was used as an indicator for the mitochondrial mass. **D.** Reduction in mitochondrial mass was further evaluated by fluorescence microscopy of MitoTracker-stained cells. **E.** Quantification of Mito-Tracker fluorescent signal intensity in untreated (control) or XCT790-treated H295R cells was evaluated measuring red fluorescent signal by a fluorescent plate reader (ex. 644; em. 665) *$p < 0{,}001$ compared to untreated control sample. **F.** Immunoblots for CIC expression from mitochondrial extracts in untreated (–) or XCT-790 treated H295R cells for 48 h. β-Actin served as loading control. Blots are representative of three independent experiments with similar results. **G.** Graph represent means of CIC density (O.D.) from three independent experiments with similar results normalized to β-Actin content (*$p < 0.001$ compared to untreated control sample assumed as 100). **H.** CIC activity was measured at 20 min as steady-state levels of citrate/citrate exchange. Transport was started by adding 0.5 mM [^{14}C]Citrate to proteoliposomes preloaded internally with 10 mM citrate and reconstituted with mitochondria isolated from untreated H295R cells (Control; white column) and H295R-treated cells (black column). The transport reaction was stopped at 20 minutes. Results are expressed as percentage of the control. The data represent means ± SD of at least three independent experiments.

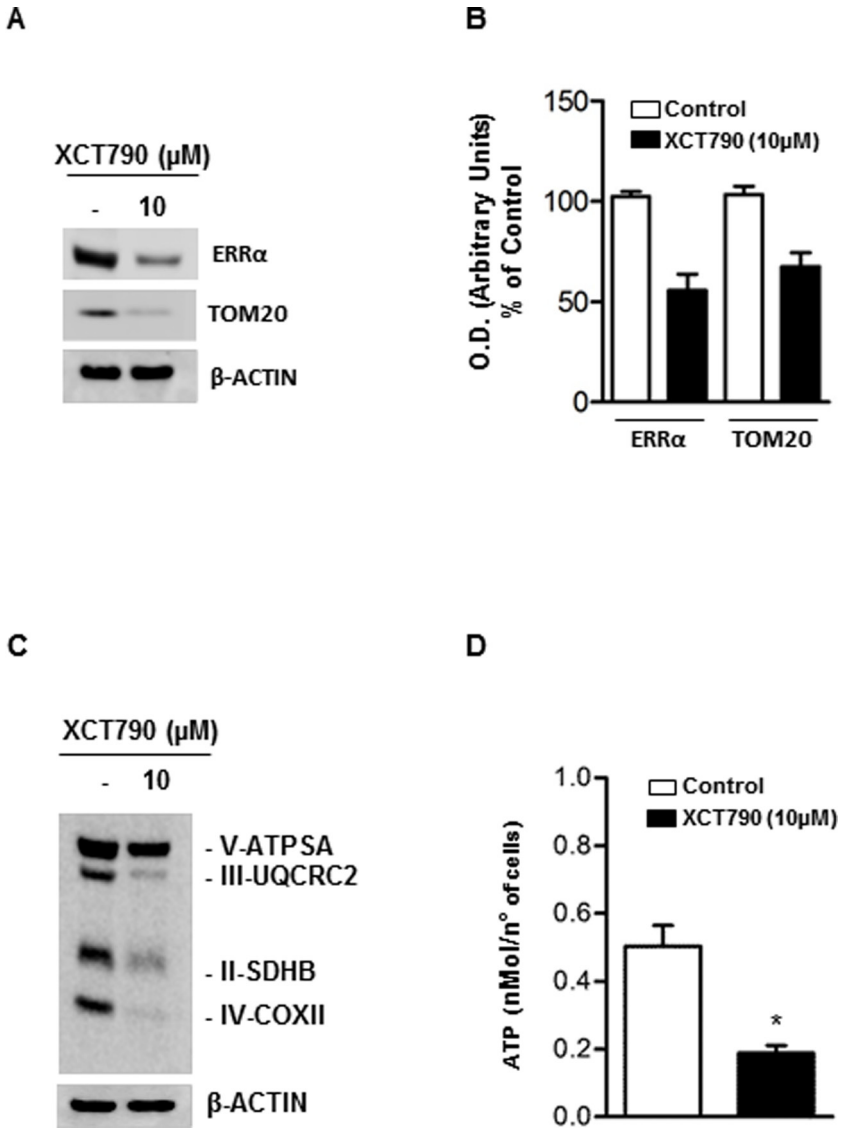

Figure 5: XCT790 decreased OXPHOS protein content and ATP concentration in H295R cells. A. Total protein extracts from H295R cells, left untreated (–) or treated for 48 h in 2.5% DCC-FBS medium with 10 μM XCT790, were analyzed by Western blot using antibodies against ERRα and TOM20. β-actin was used as loading control. **B.** Graphs represent means of ERRα and TOM20 optical densities (O.D.) from three independent experiments with similar results normalized to β-Actin content (*$p < 0.001$ compared to each untreated control sample assumed as 100). **C.** Total protein extracts from H295R cells left untreated (–) or treated for 48 h in 2.5% DCC-FBS medium with 10 μM XCT790, were analyzed by Western blot experiments using antibodies against OXPHOS subunits. β-Actin was used as loading control. Blots are representative of three independent experiments with similar results. **D.** ATP concentrations in H295R cells untreated (–) or treated with XCT790 were determined as described in Material and Methods and expressed as nmol/number of cells. Each column represents the mean ± SD of three independent experiments (*$p < 0, 001$).

been made based on studies of gene expression profiling and genetic syndromes associated with the development of ACC [30]. Results from these studies have highlighted the presence of different and important modifications such as somatic TP53 mutations, alterations at 11p15, a chromosomal locus of IGFII, H19 and cyclin-dependent kinase inhibitor 1C, β-catenin accumulation and activation of the Wnt signaling pathway and overexpression of SF-1 protein [30]. Moreover we have recently demonstrated the involvement of ESR1 in ACC cell growth regulation [5]. Genetic modifications and molecular pathways alterations have as a common purpose the survival and proliferation of the transformed phenotype. It is currently accepted that these changes are associated with a concurrent adaptation and reprogramming of cellular metabolism [31]. In this scenario adrenocortical tumors are not an exception and the metabolic receptor ERRα represents a good therapeutic target. In fact, ERRα is a common downstream target of multiple pathways and a key factor in controlling the expression and activity of various bioenergetics processes. Indeed, it has already been observed that high ERRα gene expression correlates with unfavorable clinical outcomes

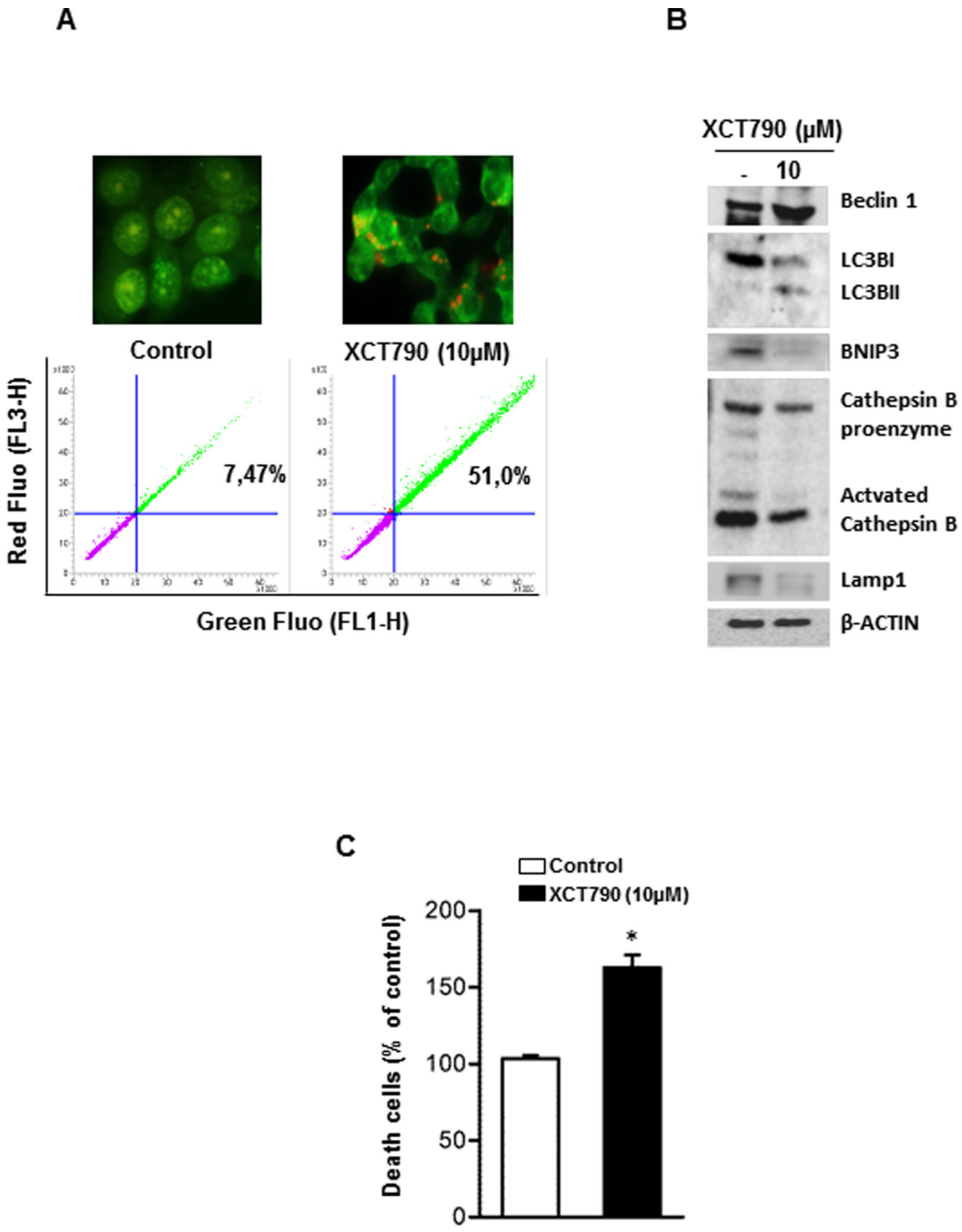

Figure 6: XCT790 induces necrosis in H295R cells. A. H295R cells were left untreated (control) or treated with XCT790 10 µM. After 48 h, cells were incubated with (1 µg/mL) acridine orange (AO) solution for 30 min at 37°C. Absorption of AO was determined by FACS analysis (lower panel). In the same experimental conditions, treated or untreated H295R cells were stained with acridine orange, mounted and immediately analyzed by fluorescent microscope (upper panel). **B.** Total protein extracts from H295R cells left untreated (–) or treated in 2.5% DCC-FBS medium with XCT790, as indicated, for 48 h were analyzed by Western blot experiments using antibodies against Beclin 1, LC3B, BNIP3, Cathepsin B, Lamp1. β-Actin was used as loading control. Blots are representative of three independent experiments with similar results. **C.** Cell death by necrosis was assessed by Trypan blue-exclusion assay in H295R cells untreated (–) or treated with XCT790. The mean ± SD of three replicates are shown. Cell death was expressed as a percentage of control, (*$p < 0.001$).

in breast [32] and ovarian cancer [14, 33] and that breast cancer cells exhibiting high ERRα activity are more sensitive to growth inhibition by an ERRα inverse agonist such as XCT790 [34]. Consistent with this findings and with very recent data reporting high ERRα expression in adrenal tumors compared to benign and normal adrenal gland [22], here we report that ERRα is expressed in H295R cells, the most valid cell model to study ACC

biology. Moreover, our data show that pharmacological down-regulation of ERRα expression impaired H295R cell proliferation *in vitro* in a dose-dependent fashion. Most importantly, the same inhibitory effect was obtained also in *in vivo* experiments using H295R cells as xenograft model. At the molecular level, the growth inhibition is associated with a G0/G1 cell cycle arrest and by the decreased levels of G1-phase markers such as Cyclin D1 and pRb while CDKs protein levels were unaffected. Noteworthy, cell cycle arrest was not followed by any apoptotic event since we were unable to detect any morphological data (data not shown) or biochemical events such caspase activation and PARP-1 cleavage.

Accumulating data provide evidence that a caspase-independent form of programed cell death such as autophagy can be at play under certain conditions [35]. Therefore we investigated whether the inhibitory effects induced by XCT790 treatment could be linked to autophagy. Our results indicated that XCT790 caused a significant increase in autophagic vesicles. Concomitantly, we observed a drastic reduction in the expression of PGC1-α protein, which plays a key role in mitochondrial biogenesis, and of mitochondrial carrier CIC. The reduction of mitochondrial mass, also confirmed by the reduction of TOM20 protein expression, is followed by a considerable and significant decrease in the ATP concentration. Despite the presence of some autophagic markers such as the up-regulation of Beclin 1 and the cleaved form of LC3 protein, the formation of autophagolysosomes seems to be incomplete as evidenced by the reduction in LAMP1 protein, known to play an important role during the final steps of autophagy process [36]. A possible explanation could be a considerable reduction in the availability of intracellular ATP, required to drive forward the active cell death mechanism including autophagy. On the other hand, we cannot exclude that the observed initial steps of autophagy are a defense cell response to keep cells alive during energy failure to counteract the reduced expression and activity of the master bioenergetic executor ERRα. Moreover, the bioenergetics crisis following treatment with ERRα inverse agonist might be responsible for the loss of plasma membrane integrity, a key signature for a necrotic cell death, allowing the significant increase in the number of Trypan blue stained cells.

However, our most significant finding is that in ACC cells ERRα depletion after XCT790 treatment clearly caused a reduction of mitochondrial function and mass leading to the activation of a number of cellular mechanisms that result in tumor cell death.

It's now well known that mitochondria with its direct involvement in bioenergetics, biosynthesis and cell signaling are mandatory for tumorigenesis. Thus, it's not surprising that many studies have begun to demonstrate that mitochondrial metabolism and signaling is potentially a successful avenue for cancer therapy. Moreover, ACC is (in most cases) characterized by steroids producing/secreting cancer cells highly dependent on functioning mitochondria to ensure steroidogenic processes. For these reasons, strategies using mitochondrial metabolism and signaling as targets should be particularly effective for ACC treatment. Moreover, our current data obtained performing *in vivo* experiments by using H295R cells as xenograft model and according to previous *in vivo* studies performed in breast [37] and leukemia [38] tumor cells also suggest that chemical depletion of ERRα may be specific for high energy demanding cells such as tumor cells without exerting any toxic effect on other tissues.

In conclusion, our study supports the hypothesis that ERRα represents a valid innovative/alternative target for the treatment of adrenocortical cancer.

MATERIALS AND METHODS

Cell culture

H295R adrenocortical cancer cells were obtained from Dr. Antonio Stigliano (University of Rome, Italy) and cultured in DMEM/F12 supplemented with 1% ITS Liquid Media Supplement, 10% fetal bovine serum (FBS), 1% glutamine, 2% penicillin/streptomycin (complete medium) MCF7 breast cancer cells were maintained in monolayer cultures DMEM/F12. supplemented with 10% FBS, 1% glutamine, 2% penicillin/streptomycin. Both cell lines were cultured at 37°C in 5% CO_2 in a humidified atmosphere. All media and supplements were from Sigma-Aldrich, Milano, Italy.

Western blot analysis

Whole cell lysate were prepared in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS and a mixture of protease inhibitors) or in ice-cold lysis buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 60 mM octylglucoside). Samples were analyzed by 11% SDS-PAGE and blotted onto a nitrocellulose membrane. Blots were incubated overnight at 4°C with anti-ERRα polyclonal antibody, anti-cyclin D1, anti-cyclin E, anti-cdk2, anti-cdk4, anti-p-Rb, anti-PARP, anti-cathepsin B, anti-LAMP1, anti-Tom20 (all from Santa Cruz Biotechnology), anti-Beclin 1 (Novus Biological), anti-LC3B antibody, anti-BNIP3 antibody, Mitoprofile Total OXPHOS Human WB Antibody Cocktail (Abcam) and then incubated with appropriate horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. The immunoreactive products were detected by the ECL Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). GAPDH antibody (Santa Cruz Biotechnology) or anti-β-Actin antibody (Sigma-Aldrich) were used as internal control.

Cell viability assay

H295R cells were seeded in 12-well plates at a density of 1×10^5 cells per well and cultured in complete medium overnight. Before treatment culture medium was switched into in DMEM F-12 supplemented with 2.5% charcoal stripped (CS) FBS and cells were untreated or treated with different concentration of XCT790 (Tocris Bioscience, Bristol, UK) for the indicated time. DMSO (Sigma-Aldrich) was used as vehicle control. Cell viability was measured using MTT assay (Sigma-Aldrich). Each experiment was performed in triplicate and the optical density was measured at 570 nm in a spectrophotometer. Experiments were repeated three times.

Trypan blue assay

Trypan blue stain was prepared freshly as a 0.4% solution in 0.9% sodium chloride before each experiment. After trypsinization, 20 µl cell suspension was added to 20 µl of Trypan blue solution and mixed thoroughly. Triplicate wells of dye positive cells from untreated or XCT790 treated were counted using a hemocytometer and the experiment was repeated three times.

Xenograft model

Athymic Nude- Foxn1^{nu} mouse 4–6 weeks old) from Charles River Laboratories [Calco (LC), Italy] were maintained in groups of five or less and quarantined for one week. Mice were kept on a 12 h light/dark cycle with ad libitum access to food and water.

6×10^6 H295R cells suspended in 100 µl of sterile PBS (*Dulbecco's* Phosphate Buffered Saline) and mixed with 100 µl of matrigel, were injected subcutaneously into the intrascapular region of each animal. When tumor size reached a volume of about 200 mm^3 mice were randomly divided in 2 groups. Animals were injected every other day with vehicle (soy oil) or XCT790 (2,5 mg/Kg) over a 21 day period. Tumors were measured with a caliper every two days, volumes were calculated using the formula $V = a\ b^2/2$ (*V:volume; a* is the length of the long axis, and *b* is the length of the short axis. At the end of the treatment period tumors were harvested and tumor weight and volumes were evaluated. All animal procedures were approved by the local Ethics Committee for Animal Research.

Immunohistochemical analysis

5 µm thick paraffin-embedded sections were mounted on slides precoated with poly-lysine, and then they were deparaffinized and dehydrated (seven to eight serial sections). Immuno-histochemical experiments were performed using rabbit polyclonal Ki67 primary antibody (Dako, Denmark) at 4°C over-night. Then, a biotinylated goat-anti-rabbit IgG was applied for 1 h at room temperature, followed by avidin biotin-horseradish peroxidase reaction (Vector Laboratories, CA). Immunoreactivity was visualized by using the diaminobenzidine chromogen (Sigma-Aldrich). Counterstaining was carried out with methylene-blue (Sigma-Aldrich). Hematoxylin and eosin Y staining was performed as suggested by the manufacturer (Bio-Optica, Milan, Italy).

Scoring system

The immunostained slides of tumor samples were evaluated by light microscopy using the Allred Score [39] which combines a proportion score and an intensity score. A proportion score was assigned representing the estimated proportion of positively stained tumor cells (0 = none; 1 = 1/100; 2 = 1/100 to <1/10; 3 = 1/10 to <1/3; 4 = 1/3 to 2/3; 5 = >2/3). An intensity score was assigned by the average estimated intensity of staining in positive cells (0 = none; 1 = weak; 2 = moderate; 3 = strong). Proportion score and intensity score were added to obtain a total score that ranged from 0 to 8. A minimum of 100 cells were evaluated in each slide. Six to seven serial sections were scored in a blinded manner for each sample.

Cell cycle analysis

H295R cells treated with different doses of XCT790 were fixed, treated with RNase A (20 µg/ml), stained with Propidium iodide (100 µg/ml) (Sigma-Aldrich) and analyzed by Flow Cytometry using BD FACSJazz™ Cell Sorter (Becton, Dickinson and Co) for DNA content and cell cycle status.

Caspases 3/7 activity assay

Caspases activity was measured with Caspase-Glo Assay Kit (Promega Italia SRL, Milano, Italy) following the manufacturer instruction. The luminescence of each sample was measured in a plate-reading luminometer (Gen5 2.01) with Synergy H1 Hybrid Reader. Each experiment was performed on triplicate wells per condition.

Mitochondrial mass determination

XCT790 treated or untreated H295R cells were incubated in serum free medium with 200 nM Mitotracker deep red (Invitrogen, USA) for 30 min at 37°C in the dark. After staining, cells were washed twice with cold PBS, trypsinized, centrifuged at 1200 rpm for 5 min and then resuspended in PBS. Absorption of MitoTracker deep red FM was determined by FACS analysis and by fluorescence microscopy. In the same experimental conditions, fluorescent signal intensity was also assessed using a fluorescent plate reader (ex. 644 nM; em. 665 nM).

Detection of acidic vesicular organelles (AVOs) with acridine orange

H295R cells were cultured on 6 well plates and treated in 2.5% CS-FBS with or without 10 μM XCT790. After 48 h, cells were washed with PBS and stained for 30 min at 37°C with 1 μg/mL acridine orange solution (Sigma-Aldrich). Cells were then washed three times with cold PBS and one drop of mounting solution was added. Cell were observed and imaged by an inverted fluorescence microscope (100X magnification). Accumulation of the acidic vacuoles was also determined by FACS analysis.

ATP Determination

1×10^5 cells were seeded in 96 white clear bottom multi-well plates in complete medium. Two days later, cells were treated in DMEM F-12 supplemented with 2.5% CS FBS containing 10 μM XCT790. After 48 h, ATP concentrations were determined using the CellTiter-Glo luminescent cell viability assay (Promega) following the manufacturer instruction. Results were normalized to the cell number evaluated by HOECHST staining (Sigma-Aldrich) and expressed as nMol/number of cells.

Mitochondria reconstitution and transport measurements

The transport activity was carried out as described previously [40]. Briefly, isolated mitochondria from untreated (control) or XCT790 treated H295R cells were solubilized in a buffer containing 3% Triton X, 114, 4 mg/ml cardiolipin, 10 mM Na2SO4, 0.5 mM EDTA, 5 mM PIPES pH 7. The mixture was incubated for 20 min and centrifuged at 138,000 × g for 10 min. The supernatant was incorporated into phospholipid vesicles by cyclic removal of the detergent [41]. The reconstitution mixture consisted of 0.04 mg protein solution, 10% Triton X-114, 10% phospholipids (egg lecithin from Fluka, Milan, Italy) as sonicated liposomes, 10 mM citrate, 0.85 mg/ml cardiolipin (Sigma) and 20 mM PIPES, pH 7.0. The citrate transport was measured after external substrate removal from proteoliposomes on Sephadex G-75 columns, pre-equilibrated with buffer A (50 mM NaCl and 10 mM PIPES, pH 7.0). Transport at 25°C was started by the addition of 0.5 mM [^{14}C] citrate (Amersham) to the eluted proteoliposomes and terminated by the addition of 20 mM 1,2,3-benzene-tricarboxylate. Finally, the external radioactivity was removed from the Sephadex G-75 columns, liposomes radioactivity was measured and transport activity was calculated [41].

Statistics

All experiments were performed at least three times. Data were expressed as mean values ± standard deviation (SD), statistical significance between control and treated samples was analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA) software. Control and treated groups were compared using the analysis of variance (ANOVA). A comparison of individual treatments was also performed, using Student's t test. Significance was defined as $p < 0.05$.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Androgens Inhibit Aromatase Expression Through DAX-1: Insights Into the Molecular Link Between Hormone Balance and Leydig Cancer Development

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Leydig cell tumors (LCTs) of the testis are steroid-secreting tumors associated with various steroid biosynthetic abnormalities and endocrine dysfunctions. Despite their overall rarity, LCTs are still of substantial interest owing to the paucity of information regarding their exact nature and malignant potential. In the present study, we disclose the ability of androgens to inhibit Leydig tumor cell proliferation by opposing to self-sufficient in situ estrogen production. In rat Leydig tumor cells, R2C, androgen treatment significantly decreases the expression and the enzymatic activity of cytocrome P450 aromatase, responsible for the local conversion of androgens into estrogens. This inhibitory effect relies on androgen receptor (AR) activation and involves negative regulation of the *CYP19* gene transcriptional activity through the nuclear orphan receptor DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1). Ligand-activated AR up-regulates the expression of DAX-1 and promotes its increased recruitment within the steroidogenic factor-1 site-containing region of the aromatase proximal promoter II in association with the nuclear receptor corepressor. The biological relevance in LCTs of the newly highlighted functional interplay between AR, DAX-1, and aromatase is underlined by our in vivo observations, revealing a marked down-regulation of AR and DAX-1 expression and a strong increase in aromatase levels in testes tissues from old Fischer rats with spontaneously developed Leydig cell neoplasia, compared with normal testes tissues from younger animals. In elucidating a mechanism by which androgens modulate the growth of Leydig tumor cells, our finding support the hypothesis that maintaining the adequate balance between androgen and estrogens may represent the key for blocking estrogen-secreting Leydigioma development, opening new prospects for therapeutic intervention. **(*Endocrinology* 156: 1251–1262, 2015)**

Testicular Leydig cell tumors (LCTs) are the most common neoplasms of male gonadal interstitium (1) and account for about 3% of all testicular cancers. Most frequently diagnosed in 5- to 10-year-old prepubertal boys and 30–60 aged adult men (2, 3), LCTs are commonly benign, but about 10% of them reveal a malignant phenotype in adult patients (4). Metastatic LCTs are resistant to irradiation and to most of the chemotherapic agents (5,

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Abbreviations: AR, androgen receptor; Bic, bicalutammide; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; DAPI, 4′,6-diamidino-2-phenylindole; DAX-1, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; DHT, dihydrotestosterone; E_2, estradiol; ER, estrogen receptor; FRNT, Fischer rats normal testes; FRTT, Fischer rats tumor testes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ki-67, Ki-67 antigen; LCT, Leydig cell tumor; LRH-1, liver receptor homolog-1; Mb, mibolerone; MTT, 3-[4,5-dimethylthiaoly]-2,5-diphenyltetrazolium bromide; NC, negative control; N-CoR, nuclear receptor corepressor; OH-Fl, hydroxyflutamide; PII, promoter II; PII-Arom, PII P450 aromatase promoter; PRF, phenol red free; PRF-CT, PRF media containing 2.5% charcoal-treated horse serum; SF-1, steroidogenic factor-1; T, testosterone.

6), rendering it necessary to individuate as many new therapeutic target as possible, in the perspective of a multifaceted treatment.

Because LCTs are steroid-secreting tumors, they are often associated with endocrine disturbance (7–10). It is now apparent that the adequate balance in the androgen/estrogen ratio is necessary for normal testicular development and function (11) and may potentially represent a clinical central factor in LCTs growth and progression. Androgens, which are mainly produced in testicular Leydig cells, drive the expression of the male phenotype, including male sexual differentiation, development of secondary sex characteristics, and manteinance of spermatogenesis (12). Androgen action is mediated by the androgen receptor (AR) that acts as a ligand-inducible transcription factor (13). In testicular cell-specific AR knockout mice, the lack of the receptor in Leydig cell compartment mainly affects steroidogenic functions (12). Mutations in the AR gene are responsible for the onset of varying levels of androgen insensitivity syndrome (14, 15) that, as result of the hormonal imbalance, is associated to a higher risk of developing testicular tumors (16). Immuno-histochemical findings in the testes of androgen insensitivity syndrome patients suggest the high expression of the cytochrome P450 aromatase as one of the molecular changes responsible for the increased risk of LTCs (17). The P450 aromatase enzyme is crucially involved in the maintenance of the androgen/estrogen essential balance, because it governs estrogen biosynthesis within the testis by catalyzing the irreversible conversion of C_{19} androgenic substrates, testosterone (T) and androstenedione, into the C_{18} estrogens, estradiol (E_2) and estrone (18). Several observations suggest that estrogens can elicit proliferative effects in human and rodents tumor Leydig cells through an autocrine mechanism (19). Findings in transgenic mice show that increased E_2 to T ratio, including excessive estrogen exposure, disturbs Leydig cell function and might cause hyperplasia, hypertrophy, and development of Leydig cell adenomas (20–22). In humans, elevated P450 aromatase expression, with consequent high plasma E_2 levels, has been described in patients with testicular LCTs, further substantiating the role played by P450 aromatase on the pathogenesis of leydigiomas (23–26). All these experimental and clinical observations strongly support that abnormalities of the male gonadal functions may be associated with the decreased ratio of androgen/estrogen levels. So far, it has been demonstrated that androgens are able to suppress the expression of a number of steroidogenic enzyme genes eventually resulting in decreased testicular steroidogenesis (27, 28). However, information regarding the precise function of AR in Leydig tumor cells is still lacking.

Here, we investigated the effect of androgens on the expression of the P450 aromatase in rat Leydig tumor cells R2C, a well-documented experimental model for leydigioma (19, 29–31). We demonstrated the existence of a novel mechanism, through which androgens are able to down-regulate the in situ estrogen production and Leydig tumor cell proliferation by inhibiting the expression of the P450 aromatase gene. This occurs through a functional cross talk between the AR, the orphan nuclear receptor DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) and the P450 aromatase.

Materials and Methods

Reagents and antibodies

Mibolerone (Mb) was from PerkinElmer; bicalutammide (Bic) (Casodex) was from Astra-Zeneca; hydroxyflutamide (OH-Fl) and 4′,6-diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich; BSA and AR (C-19), DAX-1 (K-17), nuclear receptor corepressor (N-CoR) (H-303), β-actin (AC-15), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FL-335), cyclin D1(M-20), p21 (H-164), and Ki-67 antigen (Ki-67) (M-19) antibodies were from Santa Cruz Biotechnology, Inc; cytochrome P450 aromatase antibody was from Serotec.

Cell cultures

Authenticated rat Leydig tumor cells R2C were acquired from ATCC (LGC Standards), stored according to supplier's instructions and used within 4 months after frozen aliquots resuscitations. Cells were cultured in Ham's F-10 (Sigma) medium supplemented with 15% horse serum, 2.5% fetal bovine serum, and antibiotics (Invitrogen, S.R.L.).

Before each experiment, cells were synchronized in phenol red-free (PRF) serum-free media for 24 hours. All the experiments were performed in PRF media containing 2.5% charcoal-treated horse serum (PRF-CT).

Animal studies

Male Fischer 344 rats were a gift of Sigma-Tau. 6-month-old Fischer rats (n = 3) and 24-month-old Fischer rats (n = 4) were used for in vivo studies. Twenty-four-month-old animals presented spontaneously developed LCTs (Fischer rats tumor testes [FRTT]), which were absent in younger animals (Fischer rats normal testes [FRNT]). Testes of all animals were surgically removed by qualified, specialized animal care staff in accordance with the recommendation of the Guidelines for the Care and Use of Laboratory Animals (NIH) and with the Animal Care Committee of University of Calabria.

Cell viability and proliferation assays

Cell viability was evaluated by 3-[4,5-dimethylthiaoly]-2,5-diphenyltetrazolium bromide (MTT) and cell counting assays, as previously described (32). Briefly, for MTT (Sigma) assay, a total of 5×10^4 cells were seeded onto 24-well plates and treated for 6 days with increasing concentrations of Mb. The MTT assay was performed as the following: 100-mL MTT stock solution in PBS (2 mg/mL) was added into each well and incubated at 37°C

for 2 hours followed by media removal and solubilization in 500-mL dimethyl sulfoxide. After shaking the plates for 15 minutes, the absorbance in each well, including the blanks, was measured at 570 nm in Beckman Coulter.

For cell counting, R2C cells were seeded on 6-well plates (2×10^5cells/well) in 2.5% PRF-CT. After 24 hours, cells were exposed for 6 days to 10^{-8}M Mb or vehicle treated. The effects of Mb on cell proliferation were measured 0, 3, and 6 days after initial exposure to treatments by counting R2C cells using a Burker's chamber, with cell viability determined by trypan blue dye exclusion.

Anchorage-independent soft agar growth assays

R2C cells (10^4/well) were plated in 4 mL of Ham's F-10 with 0.5% agarose and 5% charcoal-stripped fetal bovine serum, in 0.7% agarose base in 6-well plates. After 2 days, media containing vehicle or treatments were added to the top layer and replaced every 2 days. Anchorage-independent growth was assessed as previously described (31).

Immunocytochemical staining

Immunocytochemical staining of R2C was performed as previously described (33). Cells were fixed for 30 minutes in freshly prepared para-formaldehyde (2%) and incubated for further 30 minutes with 10% normal rabbit serum to block the nonspecific binding sites. Immunocytochemical staining was performed using an affinity purified goat anti-Ki-67 antibody (Santa Cruz Biotechnology, Inc). The cells were then incubated with the secondary antibody biotinylated rabbit-antigoat IgG (Vector Laboratories) for 1 hour at room temperature followed by incubation with avidin/biotin/horseradish peroxidase complex (Vector Laboratories). The peroxidase reaction was developed using Stable 3,3′-diaminobenzidine (Sigma Chemical) for 3 minutes.

RNA extraction, RT-PCR, and real-time PCR

Cells were maintained overnight in serum-free medium and then treated for 24 or 48 hours in 2.5% PRF-CT medium. RNA extraction and reverse transcription were performed as previously described (33) with minor modifications. For RT-PCR, primers were *DAX-1* (forward), 5′-CAGGCCATGGCGTTCCTGTA-3′ and (reverse), 5′-TCCTGCCGCCTGGTGGTGAG-3′; and *CYP19* (forward), 5′-CAGCTATACTGAAGGAAATCC-3′ and (reverse), 5′-AATCGTTTCAAAAGTGTAACCA-3′.

Quantitative PCR was performed using SYBR Green universal PCR master mix (Bio-Rad Laboratories). Primers used were *CYP19* (forward), 5′-GAGAAACTGGAAGACTGTATGGATTTT-3′ and (reverse), 5′-ACTGATTCACGTTCTCCTTTGTCA-3′; and DAX-1 (forward), 5′-CTGGGTGGGGAGGGACTGC-3′ (reverse), 5′-CCTGGCGCGGGTGGTTCTC-3′. PCRs were performed in the iCycler iQ Detection System (Bio-Rad), using 0.1μM each primer in a total volume of 30 μL of reaction mixture following the manufacturer's recommendations. Each sample was normalized on the basis of its 18S ribosomal RNA content. The 18S quantification was performed using a TaqMan Ribosomal RNA Reagent kit (Applied Biosystems) following the method provided in the TaqMan Ribosomal RNA Control Reagent kit. The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold differences in gene expression relative to 18S ribosomal RNA and calibrator, calculated following the ΔΔThreshold cycle (Ct) method, as published previously (19).

Immunoblotting analysis

R2C cells or total tissue of FRNT and FRTT were lysed for protein extraction (31). Total and cytosolic/nuclear extracts were prepared and subjected to SDS-PAGE as described (34). The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program.

Immunofluorescence

Immunofluorescence analysis was performed as previously described, with minor modifications (35). Briefly, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, followed by blocking with BSA (3%, 30 min), and incubated with anti-P450 aromatase or anti-DAX-1 antibodies (4°C, overnight) and with fluorescein-conjugated secondary antibody (30 min, room temperature). IgG primary antibody was used as negative control (NC). DAPI (Sigma) staining was used for nuclei detection. Protein cellular localization was observed under a fluorescence microscope (Olympus BX51 fluorescence microscope; Olympus Italia S.R.L.). Cells were photographed at ×100 magnification using ViewFinder Software, through an Olympus camera system dp50 and the optical densities of stained proteins were analyzed by ImageJ software (NIH).

P450 aromatase activity assay

The P450 aromatase activity in subconfluent R2C cells culture medium was measured by the tritiated water release assay using 0.5μM [$^{1\beta 3}$H]androst-4-ene-3,17-dione as substrate (36). The incubations were performed at 37°C for 2 hours under an air/CO_2 (5%) atmosphere. The results, obtained as picomoles per hours and normalized to milligrams of protein (pmol/h·mg of protein), were expressed as fold change over control.

Radioimmunoassay

R2C cells were seeded on 6-well plates (2×10^5cells/well) in 2.5% PRF-CT. After 24 hours, cells were exposed for 72 hours to vehicle or 10^{-8}M Mb and/or 10^{-6}M OH-Fl. The E_2 content of medium recovered from each well was determined against standards prepared in low-serum medium using a RIA kit (DSL 43100; Diagnostic System Laboratories).

Plasmids, transfections, and luciferase reporter assays

The plasmids containing different segments of the rat P450 aromatase promoter II (PII) sequence ligated to a luciferase reporter gene were kindly provided by Dr M.J. McPhaul and were −1037/+94 (p-1037), −688/+94 (p-688), and −688/+94 mut (5′cAMP response element (CRE)m, 3′CREm, XCREm, and steroidogenic factor-1 (SF-1)m) containing mutations of 5′CRE, 3′CRE, XCRE and SF-1, respectively (29). Firefly luciferase reporter plasmid XETL is a construct containing an estrogen-responsive element from the *Xenopus* vitellogenin promoter (37). Cells were transfected using FuGENE HD Transfection Reagent (Promega) according to manufacturer's instructions. *Renilla reniformis* luciferase expression vector pRL-Tk (Promega) was used for transfection efficiency. Empty pGL2-basic vector was used as a control to measure basal activity. Luciferase activity was measured using Dual Luciferase Assay System (Promega).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as described (38) using anti-DAX-1 and anti-N-CoR antibodies. Normal rabbit or mouse IgGs were used as NCs. A 3-μL volume of each sample and DNA input were used for PCR using the primers flanking SF-1 sequence in the rat P450 aromatase promoter region (forward), 5′-ATGCACGTCACTCTACCCACTCAA-3′ and (reverse), 5′-TAGCACGCAAAGCAGTAGTTTGGC-3′. The amplification products were analyzed in a 2% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

All data were expressed as the mean ± SD of 3 independent experiments. Statistical significances were analyzed using Student's *t* test or one-way ANOVA, where appropriate. *, $P < .05$ was considered as statistically significant.

Results

Androgens reduce cell proliferation rate in estrogen-dependent R2C Leydig tumor cells

To investigate whether androgens might play a role in the modulation of estrogen-dependent Leydig tumor cell growth, we used the rat Leydig tumor cell line R2C, a well-documented experimental model for Leydigioma (19, 29–31). In the present study, experiments were carried out using the synthetic AR agonist Mb to minimize the metabolic conversion of androgen to estrogenic compounds by cultured cells. The effect of increasing concentrations of Mb on R2C Leydig tumor cell viability was evaluated by MTT assay. As shown in Figure 1A, Mb treatment significantly decreased R2C cell viability. The inhibitory effect of 10^{-8}M Mb was further confirmed by trypan blue exclusion test, showing that it began 2 days after androgen administration and persisted thereafter. The inhibitory effect exerted by 10^{-8}M Mb was abrogated by the addition of the AR antagonist OH-Fl (Figure 1B). In addition, Mb treatment was also able to decrease the number of colonies present in soft agar (Figure 1C). Reduction of cell proliferation after Mb administration was further proved by evaluating the expression levels of the proliferation marker Ki-67 (Figure 1D), the cyclin-dependent kinase inhibitor p21, which is also a well-known androgen-dependent gene (39), and cyclin D1 (Figure 1E), which represents a key factor in the E_2-induced proliferative effects in estrogen-responsive tissues (40). According to the reduced cell number, Mb-treated R2C cells exhibited a decreased expression of Ki-67 and cyclin D1 but increased levels of p21.

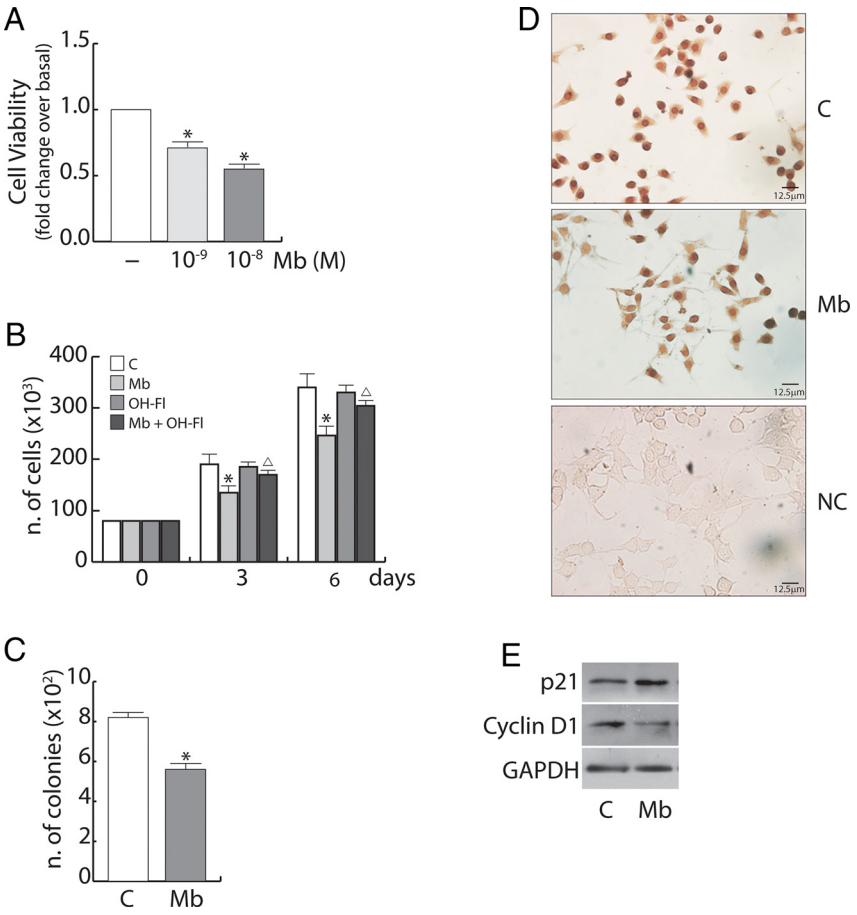

Figure 1. Mb treatment reduces proliferation in R2C cells. A, MTT assays in R2C cells treated with vehicle (−) or different Mb concentrations (M) as indicated for 6 days. B, Cells were treated with vehicle or 10^{-8}M Mb and/or 10^{-6}M OH-Fl. Drug effects on cell proliferation were measured at day 0, 3, and 6 after initial exposure to treatments by cell counting using a Burker's Chamber, with cell viability determined by trypan blue dye exclusion. C, R2C cells were seeded (1×10^4/well) in 0.5% agarose and treated as described above. Cells were allowed to grow for 14 days and then the number of colonies more than 50 μm were quantified and the results graphed. The results represent the mean ± SD of 3 different experiments each performed with triplicate samples. *, $P < .05$ vs vehicle; Δ, $P < .05$ vs Mb treated. D, Immunocytochemical staining of proliferation marker Ki-67 in R2C cells treated with vehicle (C) or 10^{-8}M Mb for 72 hours. No immunoreactivity was detected when R2C cells were incubated without the primary antibody (NC). Scale bar, 12.5 μm. E, Immunoblotting of p21 and cyclin D1 in R2C cells treated with vehicle (C) or 10^{-8}M Mb for 72 hours. GAPDH, loading control. Data from D and E are representative of 3 separate experiments.

Similar results on cell proliferation (eg, R2C cell number and Ki-67 expression) were obtained by treating R2C cell with the AR natural ligand dihydrotestosterone (DHT) (Supplemental Figure 1, A and B).

Androgen administration decreases P450 aromatase expression and activity in R2C cells

Local estrogen production by highly expressed P450 aromatase

represents a major feature involved in the positive control of R2C cell proliferation (19, 23). Thus, we explored the possibility that androgen treatment might impact on P450 aromatase gene expression in R2C cells. Mb administration was able to reduce the cellular content of the enzyme at both mRNA and protein levels, as detected by real-time PCR (Figure 2A) and immunoblotting (Figure 2B) assays, respectively. Similar findings were obtained after DHT administration (Supplemental Figure 1, C and D). These results were further confirmed by immunofluorescence analysis detecting a reduced P450 aromatase immunoreactivity in the cytoplasm of R2C cells as well as in the perinuclear region, when cells were treated with Mb. No reaction was noticed in the nuclei and in the cells processed without primary antibody (NC) (Figure 2C). Notably, the reduction of P450 aromatase protein levels upon Mb treatment was also reflected by a change in its enzymatic activity, as measured by the tritiated water release assay (Figure 2D) and a reduction in E_2 production (E_2 production in vehicle-treated samples was 3138 ± 140 pg/mL) (Figure 2E). The inhibitory effect exerted by androgen treatment on P450 aromatase expression and activity was abrogated by the contemporary addition of AR antagonists, OH-Fl or Bic, indicating that it was mediated by AR activation (Figure 2). Having demonstrated the ability of Mb to down-regulate aromatase expression and activity in R2C cells, we investigated whether it was able to affect E_2/estrogen receptor (ER) signaling in R2C cells. To this aim, we performed a transient transfection experiment using the XETL plasmid, which carries firefly luciferase sequences under control of an estrogen-response element upstream of the thymidine kinase promoter. As shown in Figure 2F, we observed that R2C cell exposure to Mb significantly reduced XETL activation. As expected, addition of OH-Fl completely reversed this effect.

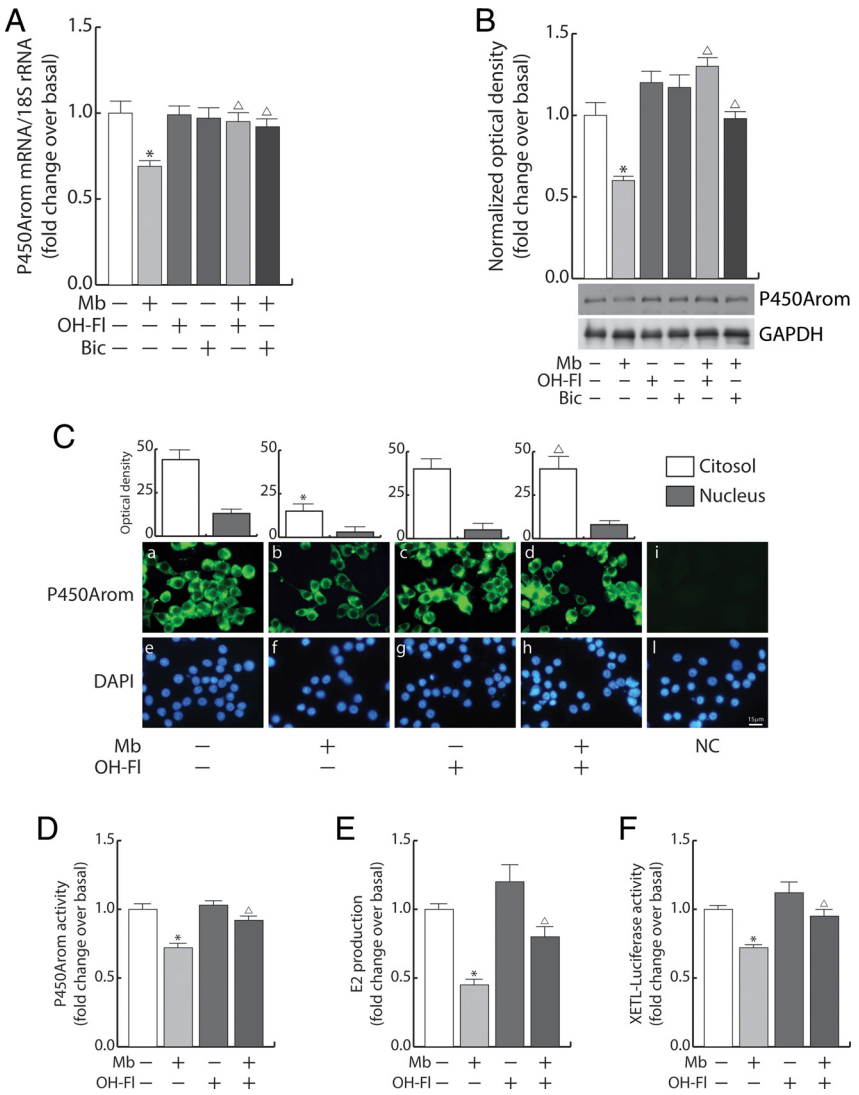

Figure 2. Effects of Mb on P450 aromatase expression and activity in R2C cells. A, P450 aromatase mRNA content evaluated by real-time RT-PCR in cells treated for 24 hours with vehicle (−), or 10^{-8}M Mb and/or 10^{-6}M OH-Fl or 10^{-6}M Bic as indicated. Each sample was normalized to its 18S rRNA content. The values represent the mean ± SD of 3 different experiments each performed in triplicate. B, Total protein extracts from R2C cells treated with vehicle (−), 10^{-8}M Mb and/or 10^{-6}M OH-Fl or 10^{-6}M Bic as indicated for 48 hours were used for immunoblotting analysis of P450 aromatase expression. GAPDH, loading control. Histograms represent the mean ± SD of 3 separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold change over basal, which was assumed to be 1. C, Immunofluorescence of P450 aromatase (a–d) in cells treated for 48 hours with vehicle (−), 10^{-8}M Mb and/or 10^{-6}M OH-Fl. DAPI staining was used to visualize the cell nucleus (e–h). Scale bar, 15 μm. NC (i and l). Histograms represent the mean ± SD of 4 separate experiments, in which stained P450 aromatase protein was evaluated in terms of optical density arbitrary units by ImageJ software (NIH). P450 aromatase activity (D) and E_2 production (E) in cells treated for 72 hours with vehicle (−), 10^{-8}M Mb and/or 10^{-6}M OH-Fl. F, R2C cells were transiently cotransfected with XETL (0.5 μg/well) and treated for 48 hours with vehicle (−), 10^{-8}M Mb, and/or 10^{-6}M OH-Fl. Activation of reporter gene expression XETL in vehicle-treated cells is arbitrarily set at 100%. Data represent the mean ± SD of 3 different experiments each performed in triplicate. *, $P < .05$ vs vehicle; Δ, $P < .05$ vs Mb treated.

Ligand-activated AR down-regulates the transcriptional activity of P450 aromatase PII-proximal promoter

Next, we investigated whether the down-regulatory effect of Mb on P450 aromatase expression could be

due to a negative influence on P450 aromatase gene transcriptional activity. In human fetal and adult testes, R2C and H540 rat Leydig tumor cells and in purified preparation of rat Leydig, Sertoli, and germ cells, P450 aromatase expression is regulated by a promoter proximal to the translational starting site named PII (41–43).

Thus, R2C cells were transiently transfected with a luciferase reporter plasmid containing the PII P450 aromatase promoter (PII-Arom) sequence (−1037/+94). As shown in Figure 3A, a significant reduction in PII-Arom activity could be observed upon Mb administration. This effect was no longer detectable after the addition of the AR antagonist OH-Fl, further confirming the involvement of AR activation. In addition, no inhibitory effects were observed in Mb-treated cells transfected with the empty-luciferase reporter plasmid (data not shown). PII-regulated P450 aromatase expression is driven by specific response elements: a nuclear receptor half-site binding SF-1/liver receptor homolog-1 (LRH-1) (44) and CRE-like sequences binding CRE binding protein/activating transcription factors protein family members (29). Therefore, to characterize PII-Arom regions that are functionally important for transcriptional regulation by androgens, transient transfection experiments were performed by using different sized or mutated PII-Arom fragments fused to the luciferase reporter gene (29), as schematically exemplified in Figure 3B, left panel.

Similarly to what observed for the PII-Arom (−1037/+94), the transcriptional activity of the PII-Arom construct (−688/+94), which still includes the 5′CRE, 3′CRE, XCRE, and SF-1 binding sites, was decreased in response to Mb stimulation, thus confirming the importance of these sites for PII-driven regulation of P450 aromatase expression (Figure 3B, right panel). Mutations of the 5′CRE (5′CRE m), the 3′CRE (3′CRE m), or the XCRE (XCREm) did not influence the response to androgens, because PII-Arom transcriptional activity was still reduced after Mb administration, mirroring wild-type PII-Arom activity. On the contrary, we evidenced the loss of the inhibitory effect exerted by Mb in the presence of the construct bearing a SF-1/LRH-1 mutated binding site (SF-1m), demonstrating that P450 aromatase regulation by androgens requires the integrity of the SF-1 motif.

Mb induces DAX-1 expression and its recruitment within the P450 aromatase gene promoter in R2C cells

One of the major repressors of SF-1-mediated transactivation of target genes in steroidogenic tissues is the nuclear orphan receptor DAX-1 (28, 45–47). Moreover, previous findings demonstrated that P450 aromatase is a physiologic target gene of the nuclear orphan receptor DAX-1 that, acting as an adaptor molecule, represses P450 aromatase transactivation through a variety of mechanisms (48–50). Thus, we investigated, in our experimental system, whether DAX-1 may represent a molecular link between androgens and P450 aromatase.

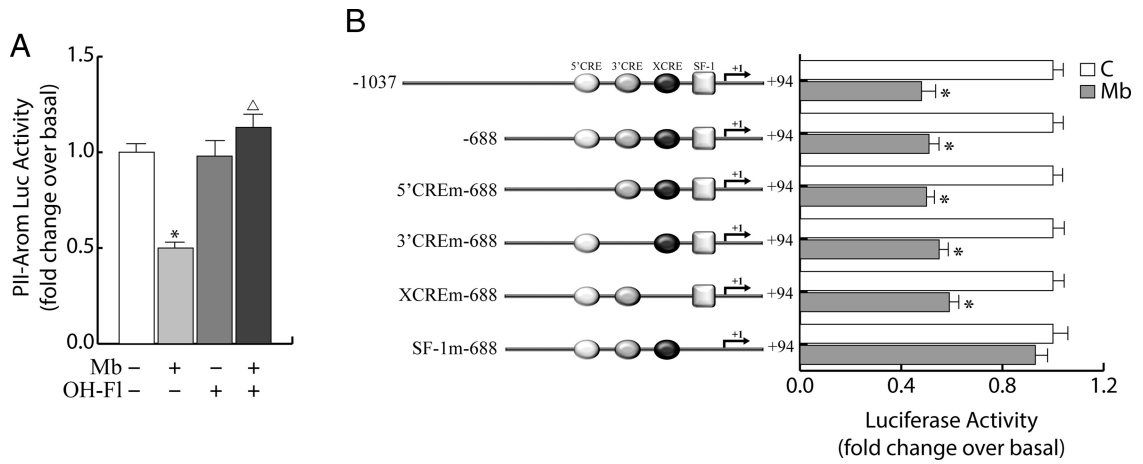

Figure 3. Effects of Mb on P450 aromatase promoter activity in R2C cells. A, R2C cells were transiently transfected with a luciferase reporter plasmid containing the PII-Arom (−1037/+94) and treated for 24 hours with vehicle (−), 10^{-8}M Mb, and/or 10^{-6}M OH-Fl. Activation of reporter plasmids in vehicle treated samples is arbitrarily set at 1. B, Schematic representation of the PII P450 aromatase proximal promoter constructs used in this study (left panel). All of the promoter constructs contain the same 3′ boundary (+94). The 5′ boundaries of the promoter fragments varied from −1037 to −688. Three putative CRE motifs (5′-CRE at −335; 3′-CRE at −231; XCRE at −169) are indicated as *circles*. The SF-1 motif at −90 is indicated as a square. A mutated 5′CRE, 3′CRE, XCRE, and SF-1-binding site is present, respectively, in 5′CREm, 3′CREm, XCREm, and SF-1m constructs. PII P450 aromatase transcriptional activity in R2C cells, transfected with the above described promoter constructs and treated with vehicle (C) or 10^{-8}M Mb for 24 hours, is shown (right panel). Activation of reporter plasmids in vehicle treated samples is arbitrarily set at 1. Results represent the mean ± SD of 3 different experiments each performed in triplicate. *, $P < .05$ vs vehicle; Δ, $P < .05$ vs Mb treated.

First, we evaluated, in R2C cells, the effects of Mb administration on DAX-1 levels.

By using quantitative real-time PCR, we performed a time-course study to evaluate DAX-1 mRNA levels in R2C cells. As shown in Figure 4A, a Mb-dependent induction of DAX-1 mRNA levels could be already observed after a 12-hour treatment and persisted thereafter. The enhanced DAX-1 mRNA levels were concomitant with a decreased P450 aromatase mRNA content (Figure 4B). Induction of DAX-1 expression by Mb (Figure 4C) or DHT (Supplemental Figure 1, E and F) was confirmed by Western blot analysis, also revealing that DAX-1 protein localizes prevalently into the nucleus wherein it was further enhanced upon Mb administration. Immunofluorescence analysis confirmed these observations (Figure 4D). DAX-1 regulation involves AR activation, because addition of the AR antagonists OH-Fl reduced the Mb-dependent up-regulation of DAX-1 (Figure 4C).

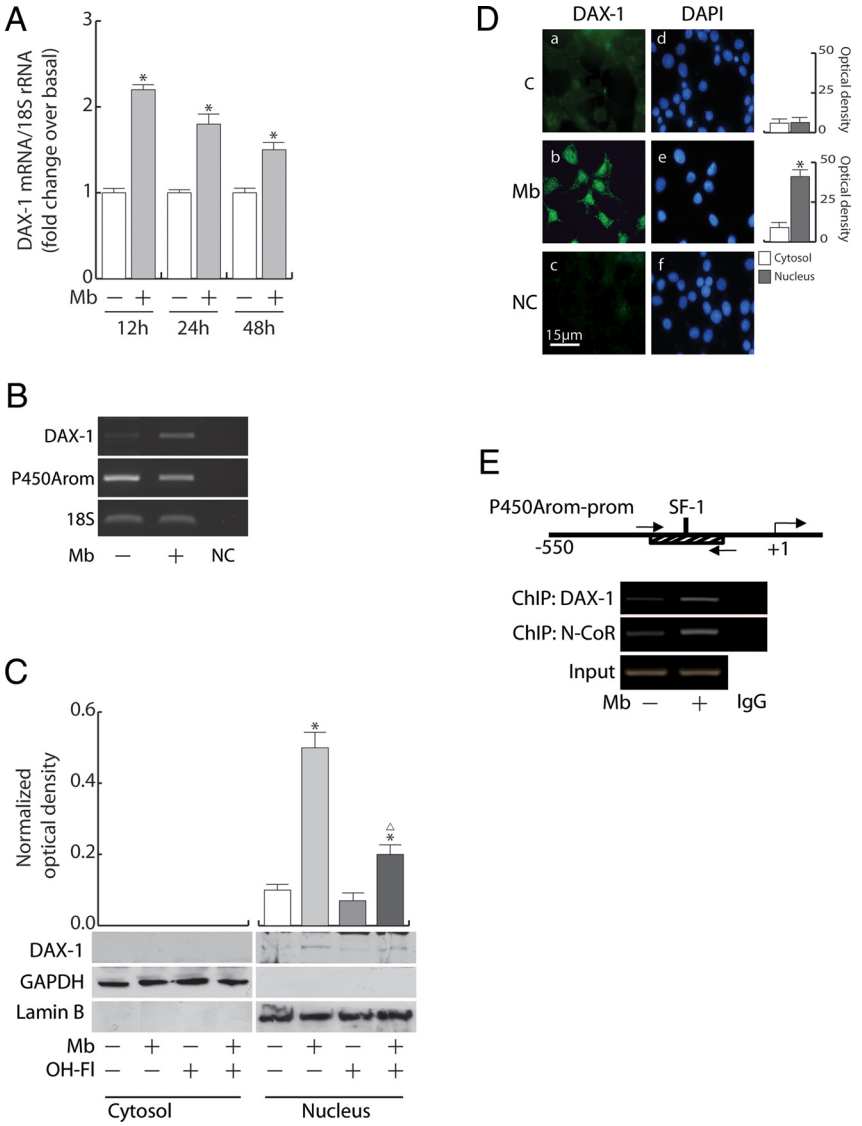

Figure 4. Mb up-regulates DAX-1 expression in R2C cells. A, Time-course analysis of DAX-1 mRNA content evaluated by real-time RT-PCR in cells treated with vehicle (−), or 10^{-8}M Mb, as indicated. Each sample was normalized to its 18S rRNA content. Values represent the mean ± SD of 3 different experiments each performed in triplicate. B, RT-PCR for DAX-1 and P450 aromatase mRNA expression in R2C cells treated with vehicle (−) or 10^{-8}M Mb for 48 hours. 18S, internal standard. C, Immunoblotting for DAX-1 expression in cytosolic or nuclear extracts from cells treated for 48 hours with vehicle (−), 10^{-8}M Mb, and/or 10^{-6}M OH-Fl. GAPDH and Lamin B were assessed as control of protein loading and purity of lysate fractions. Histograms represent the mean ± SD of 3 separate experiments, in which band intensities were evaluated in terms of optical density arbitrary units. D, Immunofluorescence of DAX-1 (a and b) in cells treated for 48 hours with vehicle (C) or 10^{-8}M Mb. DAPI staining was used to visualize the cell nucleus (d and e). Scale bar, 15 μm. NC (c and f). Histograms represent the mean ± SD of 4 separate experiments, in which stained proteins were evaluated in terms of optical density arbitrary units by ImageJ software (NIH). E, Sheared chromatin from R2C cells treated with vehicle (−) or 10^{-8}M Mb for 2 hours was precipitated using anti-DAX-1 or anti-N-CoR antibodies. The 5′-flanking sequence of the PII P450 aromatase proximal promoter containing the SF-1 site was detected by PCR with specific primers listed in Materials and Methods. Inputs DNA were amplified as loading controls. IgG, control samples. *, $P < .05$ vs vehicle; Δ, $P < .05$ vs Mb treated.

To provide evidence of the participation of DAX-1 in the androgen-dependent modulation of P450 aromatase expression, we assessed, by ChIP assay, the ability of DAX-1 to associate to the SF-1/LRH-1-containing region of the PII P450 aromatase proximal promoter upon androgen treatment. According with previous findings, both DAX-1 and N-CoR were recruited at the SF-1/LRH-1 motif-containing region of the PII P450 aromatase gene promoter (Figure 4E). Interestingly, DAX-1 occupancy of the SF-1/LRH-1 site-containing sequence of the PII promoter was induced in a ligand-dependent manner, because DAX-1 recruitment was enhanced by Mb administration. Concomitantly, recruitment of the steroid receptor corepressor N-CoR within the same promoter region was also significantly increased by Mb treatment (Figure 4E).

These results demonstrate, for the first time, how DAX-1 enhancement by androgens may negatively affect the modulation of the P450 aromatase gene in Leydig tumor cells.

AR/DAX-1/P450 aromatase expression pattern in male Fischer rats

As a final step of the current study, we evaluated the expression pattern of DAX-1, P450 aromatase, and AR in testis tissues from younger (FRNT) and older (FRTT) Fischer rats. Aged animals presented spontaneous LCTs, a phenomenon not observed in younger animals (51, 52), allowing us to use them as a good in vivo model useful to corroborate our in vitro observations. As shown in Figure 5, DAX-1 protein was expressed in FRNT but scarcely detectable in FRTT. A similar expression pattern was displayed by AR. On the contrary, a strongly increased P450 aromatase immunoreactivity could be observed in FRTT compared with FRNT. The AR/DAX-1/P450 aromatase protein expression pattern in normal (FRTT) and tumoral (FRNT) testis tissues was mirrored by the corresponding mRNA content, as detected by RT-PCR analysis (data not shown). Thus, our in vivo results indicate that the occurrence of Leydigioma is associated to a significant decrease in AR and DAX-1 levels and to a remarkable enhancement of P450 aromatase expression, confirming results obtained in R2C cell line.

Discussion

In the present study, we highlighted, in Leydig tumor cells, the existence of a novel functional interplay between the AR, the orphan nuclear receptor DAX-1, and the P450 aromatase enzyme that could play a role in leydigiomas' development and progression.

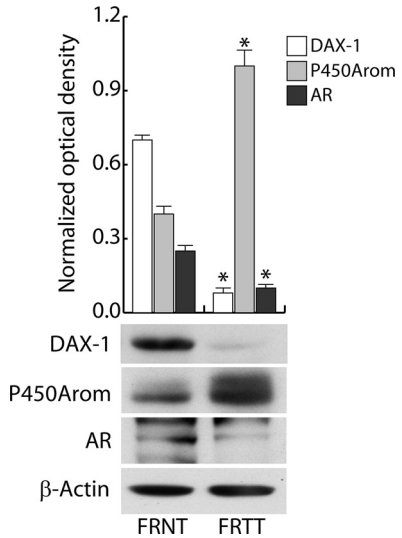

Figure 5. DAX-1, P450 aromatase, and AR expression in Fischer rat testes. Immunoblotting of DAX-1, P450 aromatase, and AR in tissues from normal (FRNT) and tumor (FRTT) Fischer rat testes. β-Actin, loading control. Immunoblots show a single representative result. Histograms represent the mean ± SD of 3 separate experiments, in which band intensities were evaluated in terms of optical density arbitrary units. *, $P < .05$ vs FRNT.

Testicular sex steroid synthesis is an elaborated dynamic process that, in addition to the negative feedback throughout the hypothalamic-pituitary-gonadal axis, is also finely regulated by various autocrine/paracrine factors (53, 54). In this scenario, the maintenance of a delicate balance between androgens and estrogens appears to have a fundamental role for male gonadal integrity and function (55). This balance is governed by cytochrome P450 aromatase, encoded by the *CYP19* gene, which catalyzes the local conversion of androgens to estrogens and whose expression has been detected in Leydig cells and some population of germ cells (56). Interestingly, the expression of AR at the same site of P450 aromatase and ER in the testis (23, 57–59) suggests a possible cross-regulation between the 2 steroid-induced signaling pathways.

It is now generally accepted that locally secreted estrogens may act as autocrine factors exerting proliferative effects on human and rodents Leydig cells (10, 19). Increased E_2 to T ratio disrupts Leydig cell function and might cause hyperplasia, hypertrophy, and Leydig cell adenomas (20, 21). Indeed, in mice, chronic estrogen treatment induces LCTs (60), eventually regressing after estrogen withdrawal with cellular alterations suggestive of apoptosis (61). On the other hand, in male occult LCTs patients, gynecomastia, the more frequent clinical sign observed, is accompanied by increased E_2 and decreased T levels, strongly suggesting the presence of an estrogen-secreting tumor (7, 8, 10, 62). However, less is known regarding the role of AR in the modulation of the complex testis paracrine/autocrine signaling especially concerning the molecular mechanism for androgen/AR regulation of Sertoli cell, Leydig cell, and peritubular myoid cell proliferation and/or differentiation (12). Here, we demonstrate that prolonged administration of the synthetic AR agonist Mb is able to reduce proliferation rate in a well-documented in vitro model for Leydig cell neoplasms, such as rat Leydig tumor cells R2C (63). Our study indicates that androgen-dependent reduction of cell growth is consequent to the decrease of the self-sufficient in situ estrogen production, which represents a major feature of R2C cells (19, 23). Indeed, in this cell type, androgen treatment negatively impacts on P450 aromatase by down-regulating its expression at both mRNA and protein levels and determining a decrease of its enzymatic activity. It has been shown that androgens are able to affect the expression of steroidogenic enzymes via an AR-mediated (64) or a local feedback mechanism (65, 66). Our results indicate that the inhibitory effects induced by androgen administration on P450 aromatase expression involve AR activation, because they disappear in the presence of the AR antagonist

OH-Fl or bicalutamide. These data confirm previous in vitro and in vivo findings that AR signaling in Leydig cells displays autocrine regulation and that lacking functional AR in Leydig cells has a major influence on Leydig cell steroidogenic function (12, 27). The observed AR-mediated inhibition of P450 aromatase cellular levels involves regulation of *CYP19* gene transcriptional activity. Distinctive tissue-specific *CYP19* promoters are employed to direct the expression of P450 aromatase mRNA (67). The main regulator of normal testicular cell aromatization is the *CYP19* proximal PII, located immediately upstream of the transcriptional initiation site. PII is also been reported as the main active promoter in LCTs and in R2C cells (24, 25, 31, 42, 43). Different response elements have been identified in PII: 3 motifs resembling CRE and an SF-1 binding site (29, 68), but no androgen-response element has been defined. By functional studies, using constructs containing wild-type or different 5′-deleted regions of rat PII-Arom, we demonstrated the ability of androgens to decrease PII transcriptional activity. This inhibitory effect was abrogated when a promoter fusion containing a mutated SF-1 element was employed, suggesting that the integrity of the SF-1 sequence is a prerequisite for the down-regulatory effects of the AR ligands on P450 aromatase promoter activity. The above described observations are consistent with recent studies showing that AR inhibits the transactivation of SF-1 in Leydig cells (27) as in gonadotrope-derived cells (69). The importance of the androgen-induced inhibition of SF-1-dependent P450 aromatase gene transactivation in Leydig tumor cells is highlighted by a recent case report of a 29-year-old male diagnosed with an estrogen-producing LCT, severe gynecomastia, and elevated plasma E_2 levels. In fact, in this report, authors demonstrate elevated estrogen synthesis in LCT to be consequent to enhanced PII-driven *CYP19* transcription, most likely caused by elevated levels of the transcription factor SF-1 (26). Thus, our findings are suggestive of the possibility that androgen action on testicular estrogen production might be accomplished by cross talk between androgen signaling and major transcription factors responsible for P450 aromatase gene expression.

In the aim of getting insights into this issue, we investigated the involvement of the nuclear orphan receptor DAX-1 that has key roles in the development and the maintenance of reproductive function, because it is a crucial regulator of steroidogenesis in mammals. Indeed, DAX-1 has a restricted expression pattern to tissues directly involved in steroid hormone production (70). Within these tissues, DAX-1 colocalizes with SF-1 (45) and acts as a global antisteroidogenic factor, likely by suppressing SF-1-mediated transcription through a variety of mechanisms (28, 46, 47, 49, 50, 71). For instance, DAX-1 is a repressor of several steroidogenic enzymes (28, 70, 72), blocking steroid production at multiple levels and decreasing the formation of steroids that are precursors of estrogens. In Y-1 adrenocortical cells, DAX-1 blocks the rate-limiting step in steroid biosynthesis by repressing steroidogenic acute regulatory protein expression (46) as well as by inhibiting both P450 cholesterol side-chain cleavage (*CYP11A*) and 3-β-hydroxysteroid dehydrogenase cellular levels (50). Moreover, it has also been shown that in insulin-treated MA-10 Leydig cells, triggering of the insulin receptor signaling pathway decreases cAMP-induced steroidogenic acute regulatory protein, P450 cholesterol side-chain cleavage, and 3-β-hydroxysteroid dehydrogenase via induction of DAX-1, without any change in serum LH or FSH levels (73). Worthily the aromatase coding gene, *CYP19* represents a physiological target for DAX-1 in Leydig cells. A very elegant study examining the consequences of DAX-1 deficiency on Leydig steroidogenesis in vivo demonstrated that there was no alteration in the expression of the 5 steroidogenic genes required for T biosynthesis, whereas P450 aromatase mRNA, protein, and enzymatic activity was increased significantly in DAX-1-deficient Leydig cells. Enhanced P450 aromatase expression was accompanied by a 40-fold increase in intratesticular E_2 (48). Consistently, in a patient with adrenal insufficiency and hypogonadotropic hypogonadisms, deletion of the exon 2 of *DAX-1* gene, dramatically affecting its function (74), has been associated with disturbed biological function of testis, especially hyperplasia of Leydig cells (75). Our findings indicate that, in rat Leydig tumor cells R2C, DAX-1 expression is regulated by androgens. In our experimental system, androgen-dependent up-regulation of nuclear DAX-1 is associated to a significant increase of its recruitment within the SF-1 site-containing region of the PII P450 aromatase proximal promoter. According to previous observations (49), DAX-1 occupancy of the PII promoter is concomitant with the enhanced recruitment of the steroid receptor corepressor N-CoR. Therefore, the mechanism we described might contribute to explain the negative influence of androgens on rat Leydig tumor cell proliferation and well correlates with observations in endometrial carcinoma, where loss or decreased DAX-1 expression results in increased intratumoral steroid production and enhanced estrogen-dependent proliferation of cancer cells (76).

Thus, in Leydig tumor cells, the induction of DAX-1 expression and the consequent modulation of P450 aromatase expression and activity exerted by androgens may represent an in situ defensive mechanism for modulating unopposed estrogenic effects. Thus, the maintenance of a proper ratio between androgen and estrogen testicular levels appears to be fundamental. As shown in the present

study, long-term administration (3 and 6 d) of low doses of androgens (in the nanomolar range) decreases R2C cell number by binding the AR and reducing the effect of locally produced E_2. On the contrary, high doses (in the micromolar range) of aromatizable and nonaromatizable anabolic androgenic steroids induce, after a 24-hour treatment, proliferation of R2C cells as a consequence of the binding to the ER (32). Although reinforcing the concept that the finely tuned balance between androgens and estrogens is critical in the development of proliferative diseases, these observations make it appealing to speculate whether such a mechanism can also exist in other endocrine-related cancers. In fact, the aberrant expression of P450 aromatase is believed to contribute to the development and progression of breast cancer (18, 36, 77–79). To this regard, we recently demonstrated that ligand-activated AR negatively regulates in situ estrogen production by activating *DAX-1* gene transcription in estrogen-related MCF-7 breast cancer cells, providing new clues for the inhibitory role exerted by androgens on estrogen-dependent cancer cell proliferation (79). More surprisingly, emerging evidences indicate the prostate as a target for locally produced estrogens (80, 81). Specifically, P450 aromatase is expressed in nonmalignant prostatic stroma but not in normal epithelial cells. In contrast, the onset and/or progression of malignancy is accompanied by the appearance and increase of P450 aromatase expression and activity within the prostate epithelium together with potential alteration of P450 aromatase promoter usage, similarly to that occurred in breast cancer (82, 83). Besides, prostate epithelium phenotypic changes are also associated to dysregulation of DAX-1, whose expression is considerably reduced in benign prostate hyperplasia compared with normal prostate tissue (84, 85), thus leaving the issue opened for future challenges. Direct confirmation of the biological relevance of our findings comes from results obtained in normal (FRNT) and tumoral (FRTT) testes tissues from younger and older, respectively, Fischer rats, which are characterized by exceptionally high incidence of spontaneous Leydig cell neoplasm associated with aging (51, 52). It appears extremely interesting to observe that tumor development is concomitant with a marked downregulation of AR and DAX-1 expression and a strong increase in P450 aromatase levels, as it emerges comparing young normal rat testes with the older ones showing the presence of the neoplasia. Specifically, FRNTs are characterized by high expression levels of DAX-1 and very low levels of P450 aromatase enzyme. On the contrary, Leydig tumor development in FRTT is associated with an opposite expression pattern showing a barely detectable DAX-1 content and a strongly increased P450 aromatase expression. Importantly, DAX-1 expression pattern is mirrored by AR expression.

Collectively, our study, for the first time, identifies the existence of a functional AR/DAX-1/P450 aromatase interplay involved in the inhibition of the estrogen-dependent testicular cancer cell proliferation. In elucidating a mechanism by which androgens modulate the growth of Leydig tumor cells, these findings reinforce the hypothesis that restoring the correct balance between androgen and estrogen levels may open new opportunities for therapeutic intervention in this type of endocrine-related cancer.

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Human sperm liver receptor homolog-1 (LRH-1) acts as a downstream target of the estrogen signaling pathway

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Abstract

In the last decade, the study of human sperm anatomy, at molecular level, has revealed the presence of several nuclear protein receptors. In this work, we examined the expression profile and the ultrastructural localization of liver receptor homolog-1 (LRH-1) in human spermatozoa. We evidenced the presence of the receptor by Western blotting and real time-RT-PCR. Furthermore, we used immunogold electron microscopy to investigate the sperm anatomical regions containing LRH-1. The receptor was mainly located in the sperm head, whereas its expression was reduced in the neck and across the tail. Interestingly, we observed the presence of LRH-1 in different stages of testicular germ cell development by immunohistochemistry. In somatic cells, it has been suggested that the LRH-1 pathway is tightly linked with estrogen signaling and the important role of estradiol has been widely studied in sperm cells. To assess the significance of LRH-1 in male gametes and to deepen understanding of the role of estrogens in these cells, we investigated important sperm features such as motility, survival and capacitation. Spermatozoa were treated with 10 nM estradiol and the inhibition of LRH-1 reversed the estradiol stimulatory action. From our data, we discovered that human spermatozoa can be considered a new site of expression for LRH-1, evidencing its role in sperm motility, survival and cholesterol efflux. Furthermore, we may presume that in spermatozoa the LRH-1 effects are closely integrated with the estrogen signaling, supporting LRH-1 as a downstream effector of the estradiol pathway on some sperm functions.

Key words: estrogen receptors; human sperm anatomy; human testis; liver receptor homolog-1; male genital tract; nuclear receptors.

Introduction

Numerous studies have demonstrated the involvement of sex hormones in the regulation of reproductive system functions, in particular the role of estrogens in spermatogenesis and male fertility (O'Donnell et al. 2001; Carreau et al. 2002). The physiological responses to estrogens in target cells are known to be mediated by at least two distinct receptor subtypes, estrogen receptor (ER) α and β. Both receptors seem to be expressed in germ cells in various stages of development from spermatogonia to elongated spermatids and spermatozoa (Durkee et al. 1998; Aquila et al. 2004; Guido et al. 2011). When estradiol occupies its ligand-binding domain, the ERs can act through distinct 'genomic' and 'non-genomic' pathways. The occurrence of non-genomic effects is now established in different tissues and cell lines, including spermatozoa (Morley et al. 1992; Cato et al. 2002; Aquila et al. 2006, 2007). The ER non-genomic actions involve interactions with intracellular pathways, where the ligand–ER complex modulates the activity of several kinases and structural proteins, such as PI3K–Akt–GSK3 and MAPK. Previous data have demonstrated that several nuclear receptors, linked to rapid non-genomic actions, are expressed in human spermatozoa. Progesterone receptor is involved in the acrosomal reaction (Tesarik et al.

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1993; De Amicis et al. 2011, 2012), whereas androgen receptor, ERα and ERβ evidenced their interaction with the phosphatidylinositol-3OH kinase (PI3K)/Akt pathway (Aquila et al. 2004, 2007). In addition, estradiol [estra-1,3,5,(10)-triene-3,17β-diol] (E2) significantly stimulates *in vitro* capacitation, acrosome reaction and fertilizing ability of mammalian spermatozoa (Adeoya-Osiguwa et al. 2003; Aquila et al. 2003, 2004).

The liver receptor homolog (LRH-1, also referred to as NR5A2, FTZ-F1, FTF or CPF), such as ER, is a member of the nuclear receptor superfamily; it is expressed in liver, intestine and exocrine pancreas where it plays vital roles in early development and cholesterol homeostasis (Annicotte et al. 2003). Recently, LRH-1 has been identified in many steroidogenic tissues such as ovary and testis in several species, suggesting that this receptor may play a critical role in development and differentiation of the endocrine and reproductive systems (Boerboom et al. 2000; Sirianni et al. 2002; Hinshelwood et al. 2003; Liu et al. 2003; Pezzi et al. 2004). LRH-1 has been reported to be expressed in some steps of spermatogenesis but in this context its role remains unknown. Several studies have showed LRH-1 expression in rat round spermatids and pachytene spermatocytes as well as an LRH-1 relationship with aromatase (Pezzi et al. 2004; Sierens et al. 2010). Aromatase is a critical enzyme, required for the conversion of androgens to estrogens, which was identified also in spermatozoa (Aquila et al. 2002, 2003). In breast cancer cells the expression of LRH is regulated by estrogens. In somatic cells, it was suggested that the LRH-1 signaling is tightly linked with the estrogen pathway (Annicotte et al. 2005). The structure of LRH-1 has been determined recently and much remains to be learned about this nuclear receptor.

In this work, we show, for the first time, the presence of LRH-1 in human ejaculated spermatozoa, providing evidence of its ultrastructural compartmentalization. Intriguingly, LRH-1 appears to mediate, as a downstream target of estrogenic signaling, the E2-induction of crucial sperm biological functions such as motility, survival and cholesterol efflux.

Material and methods

Chemicals

PMN Cell Isolation Medium was from BIOSPA (Milan, Italy). Total RNA Isolation System kit, enzymes, buffers, nucleotides 100-bp ladder used for RT-PCR were purchased from Promega (Milan, Italy). Moloney Murine Leukemia Virus (M-MLV) was from Gibco BRL-Life Technologies Italia (Milan Italy). Oligonucleotide primers were made by Invitrogen (Milan, Italy). Bovine serum albumin (BSA) protein standard, Laemmli sample buffer, prestained molecular weight markers, Percoll (colloidal PVP-coated silica for cell separation), hematoxylin, eosin, dimethylsulfoxide (DMSO), Earle's balanced salt solution (EBSS), estradiol [estra-1,3,5,(10)-triene-3,17β-diol] (E2), 17alpha-estradiol (EA) and all other chemicals were purchased from Sigma-Aldrich (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100 and Eosin Y were from Farmitalia Carlo Erba (Milan, Italy). Gel band purification kit, Enhanced chemiluminescence (ECL) Plus Western blotting detection system, Hybond™ ECL™, Hepes Sodium Salt were purchased from Amersham Pharmacia Biotech (Amersham, Buckinghamshire, UK). The cholesterol-oxidase (CHOD)-peroxidase (POD) enzymatic colorimetric kit was from Inter-Medical (Biogemina Sas, Catania, Italy). Monoclonal anti-human LRH-1/NR5A2 antibody (mouse anti-human LRH-1) was from R&D Systems (Minneapolis, MN, USA). Santa Cruz Biotechnology (Santa Cruz, CA, USA) provided LRH1/NR5A2 (N-16), an affinity purified goat polyclonal antibody raised against a peptide mapping at the N-terminus of NR5A2 of human origin (goat anti-human LRH-1), biotinylated anti-goat IgG, avidin-biotin-horseradish peroxidase (ABC) complex, normal goat serum, normal mouse serum, diaminobenzidine (DAB), goat polyclonal anti-actin Ab, anti-goat secondary Ab, anti-mouse secondary Ab, LRH-1 blocking peptide. A cell-permeable pyrazolylbiphenylethanone compound (specific antagonist of LRH-1) (LA) was from Calbiochem (Milan, Italy).

Semen samples and spermatozoa preparations

Human semen was collected, according to the World Health Organization (WHO, 2010) recommended procedure by masturbation from men undergoing semen analysis in our laboratory. This study included sperm samples with normal parameters of semen volume, sperm count, motility, vitality and morphology, according to the WHO Laboratory Manual (WHO, 2010). In each experiment three normal samples were pooled. The study was approved by the local medical-ethics committee and all participants gave their informed consent.

Processing of ejaculated sperm

After liquefaction, three normal semen samples were pooled and subjected to centrifugation (800 *g*) on a discontinuous Percoll density gradient (80 : 40% v/v) (WHO, 2010). The 80% Percoll fraction was examined using an optical microscope, equipped with a ×100 oil objective, to ensure that a pure sample of sperm was obtained. An independent observer, who observed several fields for each slide, inspected the cells. Percoll-purified sperm were washed with unsupplemented EBSS medium and incubated in the same medium (uncapacitating medium) for 30 min at 37 °C and 5% CO_2, without (control) or with treatments (experimental). The treatments were as follows: 10 nM E2 or 5 μM LA or 10 nM EA or 10 nM E2 plus 5 μM LA. The LA exhibited no inhibitory effect against AR (androgen hormone receptor), ERα, or TRβ (thyroid hormone receptor β) at concentrations of up to 10 μM (Benod et al. 2013). When the cells were treated with the LA, a pretreatment of 15 min was performed and subsequently the spermatozoa were incubated with E2 for 30 min. E2 and LA were dissolved in dimethylsulfoxide (DMSO, 0.1% final concentration in culture); 17EA was dissolved in ethanol (EtOH, 0.02% final concentration in culture). Neither DMSO nor EtOH, when used as solvent controls, induce any positive results in any of the *in vitro* assays.

RNA extraction, cDNA synthesis and real time RT-PCR

Total RNA was isolated from purified human ejaculated spermatozoa as previously described (Aquila et al. 2002). RNA was treated with DNase, and the purity and integrity of the RNA was confirmed

spectroscopically and by gel electrophoresis before use. DNase-treated RNA 2-μg samples were transcribed by 200 IU M-MLV reverse transcriptase in a reaction volume of 20 μL (0.4 μg oligo-dT, 0.5 mM deoxy-NTP and 24 IU RNasin) for 30 min at 37 °C, followed by heat denaturation for 5 min at 95 °C. PCRs were performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a total volume of 30 μL reaction mixture, following the manufacturer's recommendations, using the SYBR Green Universal PCR Master Mix 2× (Applied Biosystems) and 0.1 μM of each primer. Negative controls contained water instead of first-strand cDNA. Each sample was normalized on the basis of its 18S ribosomal RNA content. The relative LRH-1 gene expression levels were normalized to a calibrator that was chosen to be the sample with the highest threshold cycle (Ct). Final results, expressed as *n*-fold differences in orphan nuclear receptor gene expression relative to 18S rRNA and calibrator, were calculated following the ΔΔCt method as follows: $n\text{-fold} = 2^{-(\Delta Ctsample - \Delta Ctcalibrator)}$, where ΔCt values of the sample and calibrator are determined by subtracting the average Ct value of the nuclear receptor gene from the average Ct value of the 18S rRNA reference gene. Before using the ΔΔCt method for relative quantification, we performed validation experiments to demonstrate that efficiencies of target and reference were approximately equal, following Applied Biosystems instructions [http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf (page 14)]. Primer pairs were as follows: LRH-1 (GenBank accession number NM_003822), Forward: 5′-TACCGACAAGTGGTACATGGAA-3′ (exon 6), Reverse: 5′-CGGCTTGTGATGCTATTATGGA-3′ (exon 7), giving an 89-bp fragment. TM3 mouse Leydig cells were used as positive control (Pezzi et al. 2004).

Western blot analysis of sperm proteins

Sperm samples, washed twice with EBSS (uncapacitating medium), were incubated without or with the indicated treatments, and then centrifuged for 5 min at 5000 *g*. The pellet was resuspended in lysis buffer as previously described (Aquila et al. 2007, 2008). Equal amounts of protein (70 μg) were boiled for 5 min, separated by 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and probed with an appropriate dilution of the LRH-1 Ab. The bound of the secondary Ab was revealed with the ECL Plus Western blotting detection system according to the manufacturer's instructions. As internal control, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) of the first antibody and reprobed with anti-β-actin Ab.

Immunogold labeling of LRH-1

Immunogold labeling for LRH-1 was performed as previously reported (Aquila et al. 2010a). Briefly, spermatozoa fixed overnight in 4% paraformaldehyde were washed in phosphate-buffered saline (PBS) to remove excess fixative. Then they were dehydrated in a graded alcohol, infiltrated in LR white resin, polymerized in a vacuum oven at 45 °C for 48 h, and 60-nm ultrathin sections were cut and placed on coated nickel grids for post-embedding immunogold labeling for anti-human LRH-1 Ab. Potential non-specific labeling was blocked by incubating the sections in PBS containing 5% normal goat serum, 5% BSA, and 0.1% cold water fish gelatine at room temperature for 1 h. Sections were incubated overnight at 4 °C with anti-LRH-1 primary Ab (1 : 500) in PBS buffer and then incubated in 10 nm colloidal gold-conjugated anti-goat secondary Ab (1 : 50) for 2 h at room temperature. The sections were subsequently washed in PBS, fixed in glutaraldehyde, counterstained with uranyl acetate and lead acetate, and examined using a Zeiss EM 900 transmission electron microscope (TEM). To assess the specificity of the immunolabeling, a negative control was performed in sperm sections incubated with the colloidal gold-conjugated secondary Ab and with normal goat serum instead the primary Ab.

Immunohistochemistry

Paraffin-embedded human testis sections, 5 μm thick, were mounted on slides precoated with poly-lysine, and then de-paraffinized and dehydrated (seven to eight serial sections). Immunohistochemical experiments were performed after heat-mediated antigen retrieval in 0.01 M citrate buffer, pH 6. Hydrogen peroxide (3% in distillate water) was used for 30 min to inhibit endogenous peroxidase activity, whereas normal goat serum (10%) was used for 30 min to block the non-specific binding sites. Immunodetection was carried out using anti-LRH-1 (1 : 50) primary Ab at 4 °C overnight. A biotinylated anti-goat IgG was then applied (1 : 600) for 1 h at RT, followed by the avidin- biotin-horseradish peroxidase complex (ABC/HRP). Immunoreactivity was visualized using the diaminobenzidine chromogen (DAB). Testis sections were also counterstained with hematoxylin. The primary Ab was replaced by normal goat serum in negative control sections. Absorption controls utilized primary Ab preabsorbed with an excess (5 nmol mL^{-1}) of the purified respective blocking peptides, at 4 °C for 48 h. Unaltered human testicular tissues were provided by the archives of the Anatomy-Pathological Unit of Annunziata Hospital, Cosenza, Italy. Three different testicular tissues were used in the experiments and each analysis was repeated three times. Six to seven serial sections were processed for each sample.

Evaluation of sperm motility and viability

Sperm motility and viability were assessed by means of light microscopy examining an aliquot of each sperm sample which had been incubated in the absence (NC) or in presence of 10 nM E2 or 5 μM LA or 10 nM EA or 10 nM E2 plus 5 μM LA. We decided to treat spermatozoa with E2 at 10 nM concentration since it was the most effective in our previous study (Guido et al. 2011). Sperm motility was expressed as percentage of total motile spermatozoa, including the rapid progressive (PR) plus the slow progressive (NP) cells (normal values: PR + NP > 40% as reported by WHO 2010). Viability was assessed by red-eosin exclusion test using Eosin Y to evaluate potential toxic effects of the treatments. An independent observer scored 200 cells for stain uptake (dead cells) or exclusion (live cells). Sperm viability was expressed as the percentage of total live sperm. Viability was evaluated before and after pooling the samples and there were no adverse effects among the different treatments on human sperm survival (Aquila et al. 2003; Cappello et al. 2012).

Measurement of cholesterol in the sperm culture medium

Cholesterol was measured in duplicate by a CHOD – POD enzymatic colorimetric method according to manufacturer's instructions in the incubation medium from human spermatozoa as previously reported (Aquila et al. 2010b; Cappello et al. 2012). Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control, NC) for 30 min at 37 °C and 5% CO_2. Other samples were incubated in the presence of 10 nM E2 or 5 μM LA or

10 nM EA or 10 nM E2 plus 5 μM LA. At the end of the sperm incubation, the culture media were recovered by centrifugation, then lyophilized and dissolved in 1 mL of buffer reaction. The samples were incubated for 10 min at room temperature, and the cholesterol content was measured at 505 nm. Cholesterol standard used was 200 mg dL^{-1}. Cholesterol results are presented as mg per 10×10^6 number of spermatozoa.

Statistical analysis

The experiments for real time RT-PCR, immunogold labeling and immunohistochemistry were repeated on at least three independent occasions, and Western blot analysis was performed in at least four independent experiments. The data obtained from motility, viability (six replicate experiments using duplicate determinations) and the CHOD – POD enzymatic colorimetric method (six experiments using duplicate determinations) were presented as the mean ± SEM. The differences in mean values were calculated by the one-way analysis of variance (ANOVA). The Wilcoxon test was used after ANOVA as a *post-hoc* test.

Results

LRH-1 protein is expressed in human spermatozoa

To address a possible role of LRH-1 in human spermatozoa, we investigated the presence of LRH-1 in normal human ejaculated spermatozoa by Western blot analysis. Two different antibodies detected a band of the expected size at about 64 kDa for LRH-1 protein (Fig. 1), according to the results obtained in TM3 Leydig cells, used as positive control (Pezzi et al. 2004).

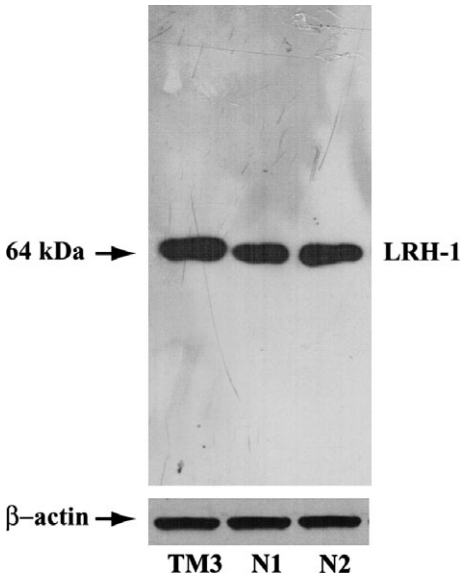

Fig. 1 LRH-1 is present in human ejaculated spermatozoa. Western blot of LRH-1 protein in human sperm, expression in two samples of ejaculated spermatozoa from normal men (N1, N2). TM3 extract was used as positive control and β-actin as internal control. The experiments were repeated at least four times and the autoradiograph of the figure shows the results of one representative experiment.

LRH-1 mRNA is expressed by human spermatozoa

To quantify mRNA levels of LRH-1 in human spermatozoa, RNA was isolated from Percoll-separated spermatozoa of different normozoospermic samples (N1, N2) followed by quantitative real-time PCR. As shown in Fig. 2, LRH-1 mRNA was expressed at appreciable levels in TM3 cells, as previously reported (Pezzi et al. 2004), and in ejaculated spermatozoa.

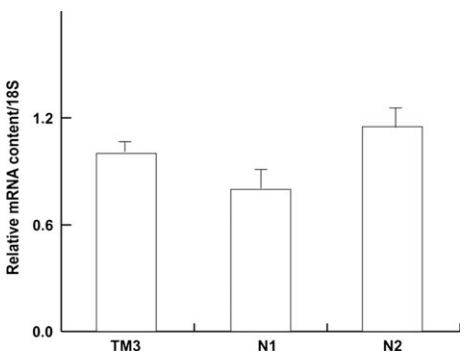

Fig. 2 Real time RT-PCR analysis of human LRH-1 gene in human spermatozoa. Real time RT-PCR was used to quantify the level of LRH-1 mRNA obtained from ejaculates of two normozoospermic patients (N1, N2) and TM3 Leydig cells, used as positive control for LRH-1. Data represent the mean ± SEM of RNA samples obtained from TM3 cells, while the columns of the different ejaculates from normozoospermic patients were repeated at least three times, both expressed as relative difference from the calibrator.

Immunogold localization of LRH-1 in human sperm

Different findings on human sperm anatomy suggest the sperm molecules are compartmentalized in the regions where they are needed. Thus, sperm morphological features, at molecular levels, may be indicative of the function carried out. In the present study, post-embedding immunogold labeling was used to characterize the distribution of LRH-1 at the ultrastructural level. In the ultrathin sections of sperm from normozoospermic donors, LRH-1 immunolabeling was predominantly situated in the sperm head (Fig. 3A-C), whereas only low labeling was observed in both the midpiece and the tail (Fig. 4A-C). The high resolution and magnification of the TEM allowed us to identify the presence of gold particles not only in sperm nuclei but also around the sperm head. Negative controls have been also used to confirm the specificity of the method (primarily the specificity of the LRH-1 antibody) and to exclude the presence of nonspecific staining (Figs 3D and 4D). To further examine the region-specific expression of LRH-1 across the sperm cell, we performed the quantification of gold particles. The results were expressed in terms of the number of gold particles in different regions of the sperm cell: the

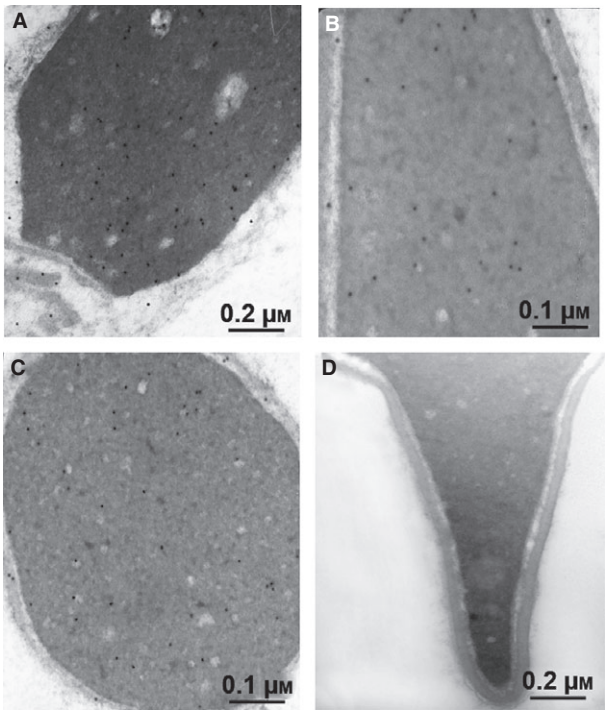

Fig. 3 Immunoelectron microscopy demonstrated LRH-1 in the head of human ejaculated spermatozoa. Sperm from normozoospermic subjects was processed as reported in Materials and methods. Electron micrographs of sperm allowed to react with Ab directed against LRH-1. (A,B) Longitudinal section of the head (×50 000 and ×85 000, respectively)/ (C) Transversal section of the head (×50 000). (D) Negative control performed as described in Material and methods (×50 000).

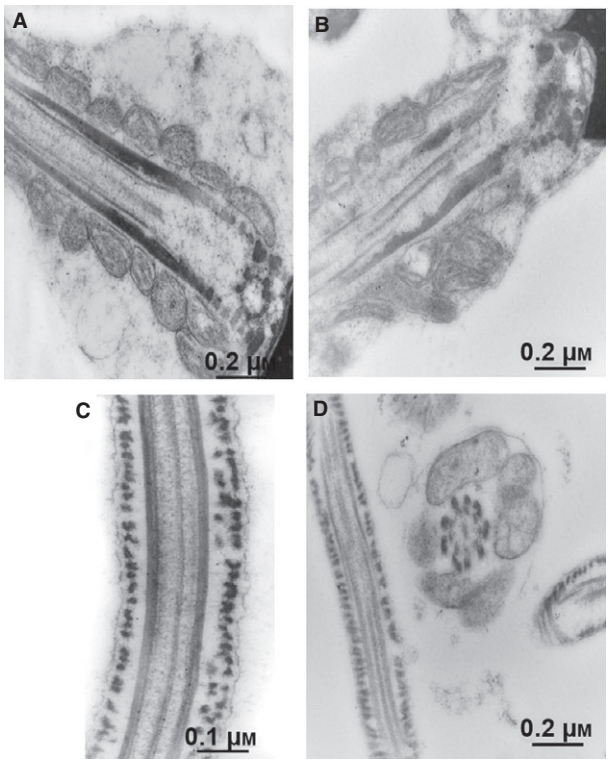

Fig. 4 Immunoelectron microscopy did not show LRH-1 in the sperm tail. Sperm from normozoospermic subjects was processed as reported in Material and Methods. Electron micrographs of sperm allowed to react with Ab directed against LRH-1. (A,B) Electron micrographs of sagittal section of the midpiece with its mitochondria (×40 000). (C) Longitudinal section of the tail (×50 000). (D) Negative control performed as described in Material and methods; longitudinal section of the end piece and a cross-section of the midpiece with mitochondria (×40 000).

nucleus, the nuclear membrane, the midpiece and the tail (Supporting Information Fig. S1).

Immunohistochemistry for LRH-1 in human testis

We performed immunohistochemistry in human testis sections to determine the expression site of LRH-1 in the different stages of germ cell development. Our results showed a nuclear sub-cellular localization of LRH-1 in developing germ cells of human testes, whereas a cytoplasmic immunoreactivity was revealed predominately in somatic testicular cells (Sertoli and Leydig cells) together with nuclear staining (Fig. 5). No immunoreactivity was observed in the negative (data not shown) and absorption controls (inserts).

LRH-1 inhibition abrogates the stimulatory effect of E_2 on human sperm motility and survival

Assessment of the motile sperm fraction is perhaps the most widely used measure of semen quality, since motility describes the ability of sperm to move properly towards an egg. Therefore, we next investigated the effect of E2, as well as its combination with 5 μM LA, on this important sperm feature. As shown in Fig. 6A, sperm motility was significantly enhanced after 10 nM E2 treatment, whereas the co-treatment with 5 μM LA reduced the effect. By treating sperm with 10 nM EA, an inactive isomer of E2, no effect was observed, as previously reported (Cheng & Boettcher, 1979). Another important parameter of human sperm quality consists in the capacity of sperm to survive as long as possible to have the chance to find and fertilize the oocyte. LRH-1 inhibition reduced the E2-effect, suggesting its role in the estrogen signaling (Fig. 6B). Neither DMSO nor EtOH, when used as solvent controls, induced any positive results.

LRH-1 partly mediated E_2-induced cholesterol efflux in human sperm

In vivo, the fertilization process is completed in the female reproductive tract, where spermatozoa undergo biochemical modifications, such as membrane cholesterol efflux. The latter is an important step in initiating transmembrane signaling events to complete the sperm maturation process

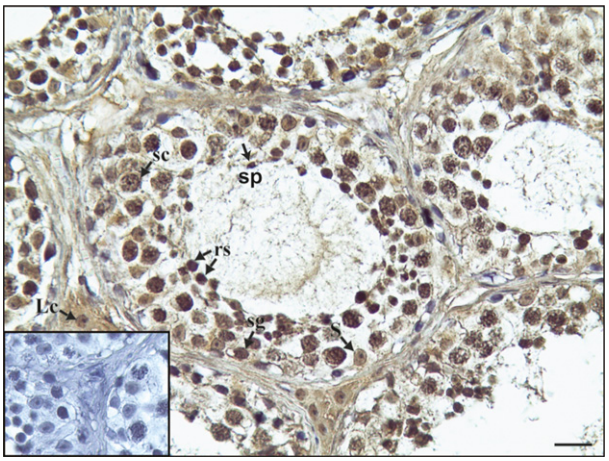

Fig. 5 LRH-1 immunoreactivity in human testis. Sg, Spermatogonium; sc, spermatocyte; rs, round spermatid; sp, spermatozoon, Lc, Leydig cell; S, Sertoli cell. Scale bar: 12.5 μm. Insert: immunonegative absorption control.

and became able to fertilize (Travis & Kopf, 2002). LRH-1 has an important role in cholesterol homeostasis in somatic cells and therefore might be important in sperm. Pooled sperm from normal samples were treated as indicated in Material and methods, and centrifuged. The upper phase of the sample was used to determine the cholesterol levels. As shown in Fig. 7, the cholesterol efflux increased with 10 nM E2, in agreement with our previous studies, whereas LA partly reversed this effect, suggesting an involvement of LRH-1 in the estrogen-induction of sperm capacitation. EA 10 nM had no effect. Neither DMSO nor EtOH, when used as solvent controls, induced any positive results.

Discussion

The role of estrogen in spermatogenesis and male reproduction is now shown taking into account the simultaneous presence of a biologically active aromatase and the widespread distribution of estrogen receptors in germ cells (Carreau et al. 2012). The presence of several nuclear receptors, including ERs, has been widely demonstrated in human spermatozoa (Tesarik et al. 1993; Aquila et al. 2004, 2007). In this study we have evaluated the presence of LRH-1 in human ejaculated spermatozoa, evidencing its new role in the regulation of sperm function. Interestingly, LRH-1 appears to be able to mediate the responsiveness to E2 of human male gamete on motility, survival and cholesterol efflux.

We showed the presence of LRH-1 in human male gamete by Western blotting and real time-RT-PCR. Besides, by immunogold analysis, we demonstrated that LRH-1 is abundantly localized in the sperm head, both on the membrane and in the nucleus. The gold particles were present but reduced in the midpiece and the tail. Immunohistochemical analysis of the human testis has shown that the LRH-1 is

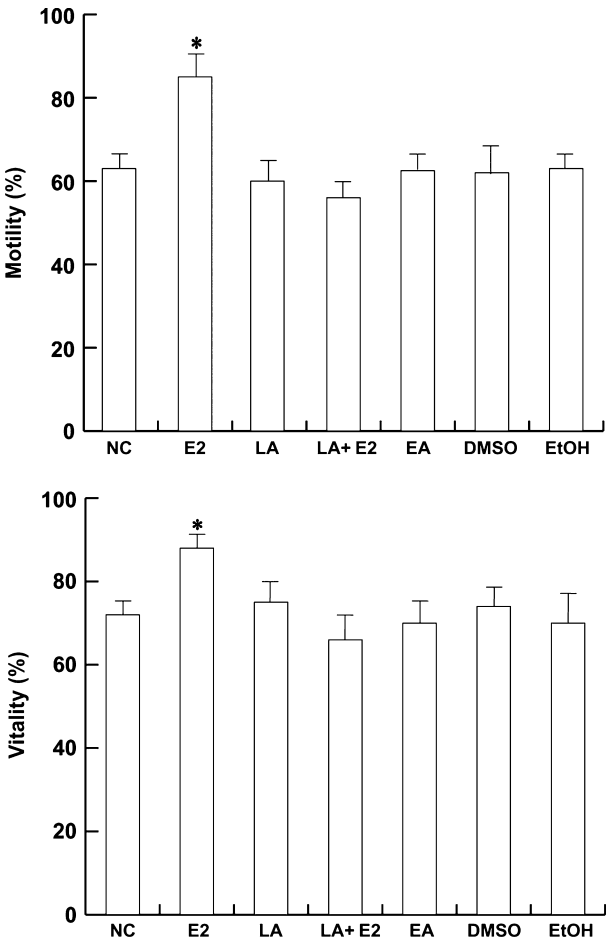

Fig. 6 Estradiol effect on sperm motility and viability is mediated by LRH-1. Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or in the presence of 10 nM E2 or 5 μM LA or 10 nM EA or 10 nM E2 plus 5 μM LA. Top panel: Sperm motility was expressed as the percentage of total motile sperm including the rapid progressive (PR) plus slow progressive (NP) sperm. Bottom panel: Sperm viability was assessed using Eosin Y as described in Material and Methods. DMSO and EtOH were used as solvent controls. Columns are mean ± SEM of six independent experiments performed in duplicate. *$P < 0.05$ vs. control.

expressed in seminiferous tubules and in the interstitium. The presence of LRH-1 led us to investigate whether it may be involved in sperm biology.

In somatic cells, it was suggested that the LRH-1 pathway is tightly associated with estrogen signaling (Annicotte et al. 2003; Lee et al. 2006). So, to investigate a functional role for LRH-1 in spermatozoa, we hypothesized a possible link between the E2/ER pathway and LRH-1 in male gametes. We therefore studied some sperm representative events such as motility and survival, by treating the cells with estradiol and inhibiting LRH-1 signaling. Our data confirmed the stimulatory estrogen action on motility and viability (Aquila et al. 2003, 2004) and demonstrated that LRH-1 is part of estrogen signaling, since its inhibition

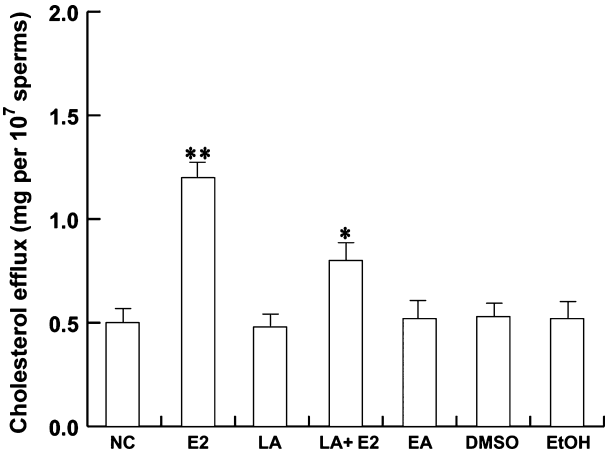

Fig. 7 LRH-1 is involved in the estradiol-induced cholesterol efflux. Washed spermatozoa were incubated in unsupplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or presence of 10 nM E2 or 5 μM LA or 10 nM EA or 10 nM E2 plus 5 μM LA. Cholesterol in culture medium from human ejaculated spermatozoa was measured by enzymatic colorimetric assay. DMSO and EtOH were used as solvent controls. Columns are mean ± SEM of six independent experiments performed in duplicate. Data are expressed in mg/10^7 sperm. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

reduced the E2-induced effects. Even if the receptor is mainly located in the sperm head, on the basis of our results, we can speculate that LRH-1 influences the sperm motility through a link with the ERβ shown on the sperm tail (Aquila et al. 2004; Guido et al. 2011), although the molecular mechanism needs to be defined.

Because of the critical roles of this receptor in human physiology and pathophysiology, identification of specific regulatory ligands, modulators of LRH-1 activity, is extremely important. Recently, a synthetic antagonist of LRH-1 has been described (Benod et al. 2013). This cell-permeable pyrazolylbiphenylethanone compound targets the LRH-1/NR5A2 ligand binding domain via direct affinity interaction, preventing LRH-1 from assuming an active conformation. Estrogenic signaling in spermatozoa has been shown to have a number of rapid effects, including the activation of phosphatidylinositol 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (Aquila et al. 2003, 2004). It was shown that LRH-1 activity can be also modulated by posttranslational events and it was reported that ERK enhances human LRH-1 activation (Lee et al. 2006). Nonetheless, the stimulation of MAPK and PI3K signaling pathways by estrogen coincides with mechanisms proposed to activate NR5A nuclear receptors, including LRH-1, in different cell types (Lin et al. 2009). LRH-1 has also been shown to be a downstream effector of estrogen-mediated cell proliferation (Annicotte et al. 2005). From the above observations we assume that the effects of LRH-1 are closely integrated with the estrogen signaling pathway in spermatozoa, too.

Morphologically mature spermatozoa leaving the testis undergo several functional maturation steps. They acquire the potential for motility in the epididymis, and complete fertilization capacity *in vivo* during their migration through the female reproductive tract (Austin, 1951; Chang, 1951). The biological processes that allow the ejaculated spermatozoa to be able to penetrate and fertilize an egg have been termed capacitation (Yanagimachi, 1994; Visconti & Kopf, 1998).

Undoubtedly, the membrane cholesterol efflux is important in starting transmembrane signaling events and successive intracellular signal transduction, leading to the acquisition of fertilizing capacity (De Lamirande et al. 1997; Travis & Kopf, 2002). LRH-1 plays important roles in metabolism, being involved in the regulation of cholesterol homeostasis and steroidogenesis (Fayard et al. 2004), so the evidence of new regulators of cholesterol efflux in spermatozoa is of great interest. On the basis of our data, we discovered in human spermatozoa a new expression site for LRH-1 which appears partly to mediate E2 action on cholesterol efflux. Therefore, in spermatozoa, estradiol may modulate the activity of LRH-1, which can be considered a downstream target of the estrogenic stimuli.

Previous studies have evidenced LRH-1 expression in human and rat testis (Sirianni et al. 2002; Pezzi et al. 2004) as well as in rat round spermatids and pachytene spermatocytes (Pezzi et al. 2004; Sierens et al. 2010). In spermatozoa, estrogens activate the ERs and consequently initiate several intracellular signaling enzymes such as receptor tyrosine kinase, phosphatidylinositol 3-kinase, Akt, Src, MAPK, protein kinase C, phospholipase D and phospholipase C. From our data, it appears that estrogens also are able to activate LRH-1 signaling in male gamete.

The identification of signaling pathways that modulate the LRH-1 activity as well as further functional studies are of great importance to investigate LRH-1 biology. Our study may represent a good starting point for further exploration that will help identify the role of this nuclear receptor in health and disease, validating LRH-1 as a novel therapeutic target for the treatment of male fertility disorders.

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Author contributions

Daniela Montanaro: acquisition and critical evaluation of Western blotting data; Marta Santoro: culturing of the cells, performing experimental conditions, survival and motility analysis, interpretation of data and critical

revision for intellectual content; Amalia Carpino: acquisition, interpretation and critical revision of immunohistochemical data; Ida Perrotta: acquisition and interpretation and critical revision of immunoelectron microscopy data; Francesca De Amicis: interpretation of data; Rosa Sirianni: acquisition and critical interpretation of RT-PCR data; Vittoria Rago: acquisition of immunohistochemical data; Serena Gervasi: interpretation of data and revision of English language; Aquila Saveria: conception, design, analysis, interpretation of data, critical revision for important intellectual content, final approval of the submitted version of the article.

Disclosure of interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The histograms show quantification of gold particles associated with the different regions of the sperm cell. Data are presented as mean ± SEM of the gold particles counted in 100 cells from six fields. $*P < 0.01$ vs. nucleus.

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Identification of G protein-coupled estrogen receptor in human and pig spermatozoa

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Abstract

Estrogens are known to influence functional properties of mammalian spermatozoa inducing rapid responses through the classical estrogen receptors (ERα and ERβ). Recently, the G protein-coupled estrogen receptor (GPER) has been identified as mediator of fast non-genomic estrogen effects in different cells. This work investigated the expression of GPER in human and pig spermatozoa using immunofluorescence, Western blot analysis and RT-PCR. GPER was found to be confined to the mid-piece of human sperm cells, whereas it was detected in the acrosomal region, the equatorial segment and the mid-piece of pig spermatozoa. Furthermore, in the male gametes of both species, the immunoblots of sperm extracts revealed a band at ~42 kDa, consistent with the GPER molecular weight, and RT-PCR detected the GPER transcripts. This is the first report demonstrating the expression of GPER in human and pig mature sperm cells and evidencing its species-specific cellular localization.
Key words: estrogens; G protein-coupled estrogen receptor; human spermatozoa; pig spermatozoa.

Introduction

Estrogens are able to control capacitation and acrosome reaction in different mammalian spermatozoa through rapid pathways (Luconi, 2006). The effects of estrogens on target cells can be mediated by the classical estrogen receptors (ERs), which act either as transcriptional factors in the nucleus or by inducing rapid non-genomic responses through intracellular secondary messengers (Acconcia & Kumar, 2006; Pedram et al. 2007). To date, the expression of ERα and ERβ in ejaculated spermatozoa has been reported only in the human and in the pig (Aquila et al. 2004; Solakidi et al. 2005; Rago et al. 2007) Therefore, the ERs are candidates to mediate the fast estrogen signalling in both these male gametes. However, the presence of alternative estrogen receptors in mature sperm cells has also been hypothesized. In fact, some biological effects of estradiol on human (Adeoya-Osiguwa et al. 2003) and mouse (Rossato et al. 2005) spermatozoa were not inhibited by tamoxifen, ruling out the involvement of ERs. Besides the ERs, estrogens can elicit rapid effects through the seven-transmembrane receptor associated to a G protein, GPER (GPR30). GPER has been recognized as mediator of rapid responses to estrogens in different cell types (Olde & Leeb-Lundberg, 2009) but its expression in sperm cells is still unknown. In addition, the signalling cascade activated by GPER includes the release of intracellular calcium (Prossnitz et al. 2008), an event which can be strictly related to sperm capacitation and acrosome reaction (Publicover et al. 2007); this consideration supports a possible GPER presence in mature sperm cells. The aim of the present paper was to investigate the expression of GPER in human and pig spermatozoa at different levels: immunolocalization, mRNA and protein level.

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Material and methods

Chemicals

Reagents were purchased from Sigma Aldrich (Milan, Italy), unless otherwise indicated. Two anti-GPER primary antibodies were used: rabbit polyclonal LS-A4271 (MBL International Corporation, USA) mapping within the 3rd extracellular domain of human GPER and rabbit polyclonal K19 (sc-48524-R) mapping within an internal region of human GPER (Santa Cruz Biotechnology, USA). Blocking peptides were LS-P4271 and sc-48524P, respectively. Goat anti-rabbit fluorescein isothiocyanate (FITC) conjugated IgG and horseradish peroxidase conjugated IgG (Santa Cruz Biotechnology) were used as secondary antibodies. Rabbit polyclonal anti β-actin (Santa Cruz Biotechnology) was also used as loading control. TRIzol RNA isolation reagent was provided from Invitrogen Life Technologies, Italy. Oligonucleotide primers for GPER were purchased from Dasit Science, Italy. Oligonucleotide primers for GPHD were provided by Invitrogen Life Technologies.

Semen samples

Human semen samples from six health voluntary donors with normal semen parameters (World Health Organization, 2010) were provided by UNICAL, Health Center (Rende, CS, Italy). Individual samples were centrifuged on a discontinuous Percoll density gradient (80 to 40%) to remove bacteria and debris (World Health Organization, 2010).

Pig semen samples were obtained from six fertile male pigs (*Sus scrofa domestica,* Large White) kept at the Swine Artificial Insemination Center (Rende, CS, Italy). Individual fresh ejaculates were collected by the gloved hand method and filtered immediately by Universal Semen bags (Minitub, Tiefenbech, Germany). Semen was transported within ½ h to the laboratory, diluted 1 : 10 with Earle's balanced salt solution and centrifuged on a discontinuous Percoll density gradient (72%/90%) to remove bacteria and debris (Kuster et al. 2004).

Percoll-purified human and pig spermatozoa were diluted in Earle's balanced salt solution and specifically treated for immunofluorescence analysis, Western blot analysis, RNA isolation, reverse transcriptase-PCR.

Immunofluorescence labelling

Sperm cells were rinsed three times with 0.5 mM Tris-HCl buffer, pH 7.5 and allowed to settle onto slides in a humid chamber. The overlying solution was carefully pipetted off and replaced by absolute methanol for 7 min at −20 °C. After methanol removal, sperm cells were washed in Tris-buffered saline (TBS) containing 0.1% Triton X-100 and were treated for immunofluorescence. Two anti-human GPER, LS-A4271 (1 : 50) and K19 (1 : 200), were utilized as primary antibodies, and the anti-rabbit FITC conjugated IgG (1 : 80) as secondary antibody. Propidium iodide (PI) (12 μm L^{-1}) was used for nuclear counterstaining. The specificity of anti-GPER antibodies was tested by pre-absorption of each primary antibody with an excess of the respective blocking peptide for 48 h at 4 °C (negative controls).

The slides were examined under a Leica TCS Saronno Palermo 2 confocal laser scanning microscope (LSM), observing a minimum of 200 spermatozoa × slide.

Western blot analysis

After Percoll removal, sperm samples were re-suspended in lysis buffer: 62.5 mM Tris-HCl (pH 6.8), 150 mM NaCl, 2% sodium dodecylsulphate (SDS), 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 0.2 mM Na_3VO_4, 1% aprotinin. Lysates were quantified using Bradford protein assay reagent. Equal amounts of protein (50 μg) were boiled for 5 min, separated under denaturing conditions by SDS-PAGE on 10% polyacrylamide Tris-glycine gels, and then electroblotted to nitrocellulose membrane. Non-specific sites were blocked with 5% non-fat dry milk in 0.2% Tween-20 in Tris-buffered saline (TBS-T) for 1 h at room temperature and incubated overnight with anti-human GPER (1 : 500) and anti-human β-actin (1 : 1000). Then, antigen-antibody complexes were detected by incubation of the membranes with the secondary antibody (anti-rabbit horseradish peroxidase-conjugated) at 22 °C for 1 h. The bound secondary antibodies were detected with the ECL Plus Western blotting detection system (Amersham, USA) according to the manufacturer's instructions. Each membrane was exposed to the film for 2 min. Protein extract from cultured SKBR3 (breast cancer cell line) was used as positive control for GPER.

RNA isolation, RT-PCR

Total RNA was extracted from human and pig spermatozoa with TRIzol RNA isolation reagent according to the manufacturer's protocol (homogenization, phase separation, RNA precipitation, RNA wash and dissolving) with minor differences between the two sperm species.

Before RT-PCR, RNA was incubated with ribonuclease-free deoxyribonuclease (DNAse) I in single-strength reaction buffer at 37 °C for 15 min. This was followed by heat inactivation of DNAse I at 65 °C for 10 min. Two micrograms of DNAse-treated RNA samples were reverse transcribed using M-MLV reverse transcriptase (Promega, USA) and 2 μL of RT products were then amplified. The applied PCR primers and the expected lengths of the resulting PCR products are the following: 5′ CTGGGGAGTTTCCTGTCTGA 3′ (forward) and 5′ GCTTGGGAAGTCACATCCAT 3′ (reverse) for human GPER with a product size of 155 bp; 5′ CCTGGACGAGCAGTATTACGATATC 3′ (forward) and 5′ TGCTGTACATGTTGATCTG 3′ (reverse) for mouse GPER with a product size of 380 bp. Cycling conditions were: 94 °C per 1 min, 58 °C per 1 min, 72 °C per 1 min for human GPER and 94 ° C per 1 min, 57 °C per 1 min, 72 ° C per 1 min for mouse GPER. Furthermore, GAPDH was amplified as internal control gene with a product size of 450 bp using the following primers: 5′ GACAACTTTGGTATCGTGGA 3′ (forward) and 5′ TACCAGGAAATGAGCTTGAC 3′(reverse). The PCR-amplified products were subjected to electrophoresis in 2% agarose gels, stained with ethidium bromide and visualized under UV transillumination.

Results

Immunolocalization

A green intense fluorescence revealed GPER in the sperm mid-piece of human spermatozoa; the tail and the head were unstained (Fig. 1A). A green brilliant light localized GPER in the mid-piece, in the equatorial segment and in the acrosomal region of pig sperm cells (Fig. 2A). The nuclear region and the tail showed no fluorescence. Negative controls (inserts) were immunonegative in both species.

The same results were obtained using the two different primary antibodies (LS-A4271 and K19). The immunofluorescence assays were repeated six times with similar results.

Western blot

One band corresponding to the molecular weight value ~42 kDa has been revealed by the anti-GPER antibody in both human (Fig. 1B) and pig (Fig. 2B) sperm samples (lanes 1–6). This band co-migrated with the positive control (SKBR3) (lane C).

RT-PCR

Reverse PCR detected GPER mRNA in both human (Fig. 1C) and pig (Fig. 2C) spermatozoa. The primer sequences were based on the human gene sequence in human sperm cells and on the mouse gene sequence in pig spermatozoa.

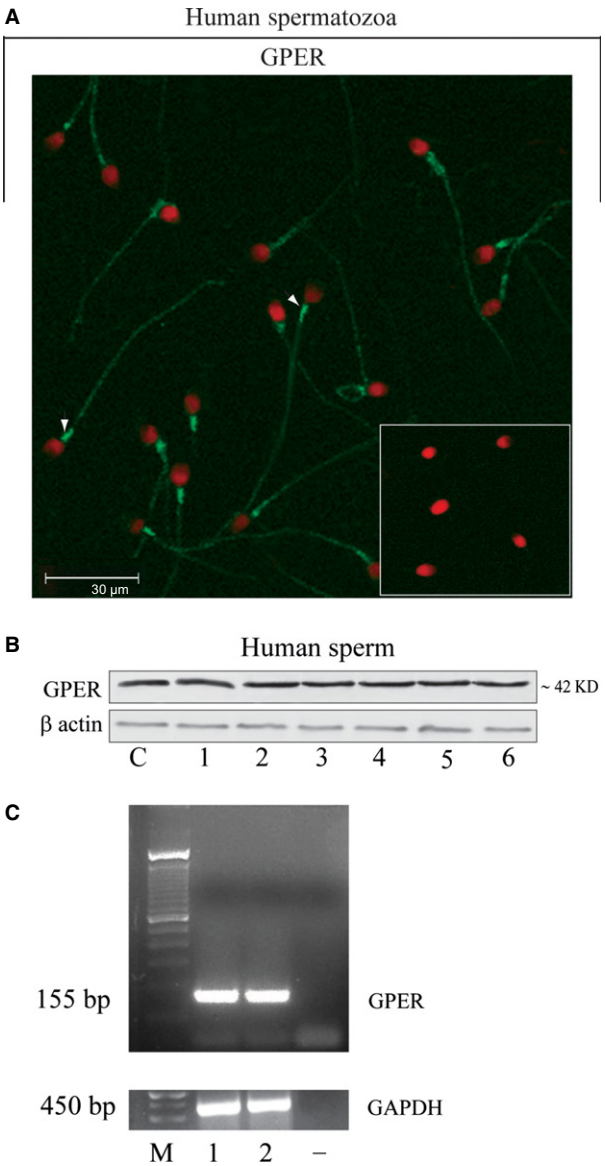

Fig. 1 Expression of GPER in human spermatozoa (A) Green immunofluorescence labeling in the sperm mid piece (arrow heads). Sperm heads show the iodure propidium red stain. The insert show the control spermatozoa. (B) Iimmunoblots of protein extracts from the six human sperm samples (lane 1–6), positive control (lane C). β actin as loading control. (C) RT-PCR results in two representative human sperm samples (lane 1–2), negative control (lane-), and markers (lane M). GAPDH (450 bp) is the internal control.

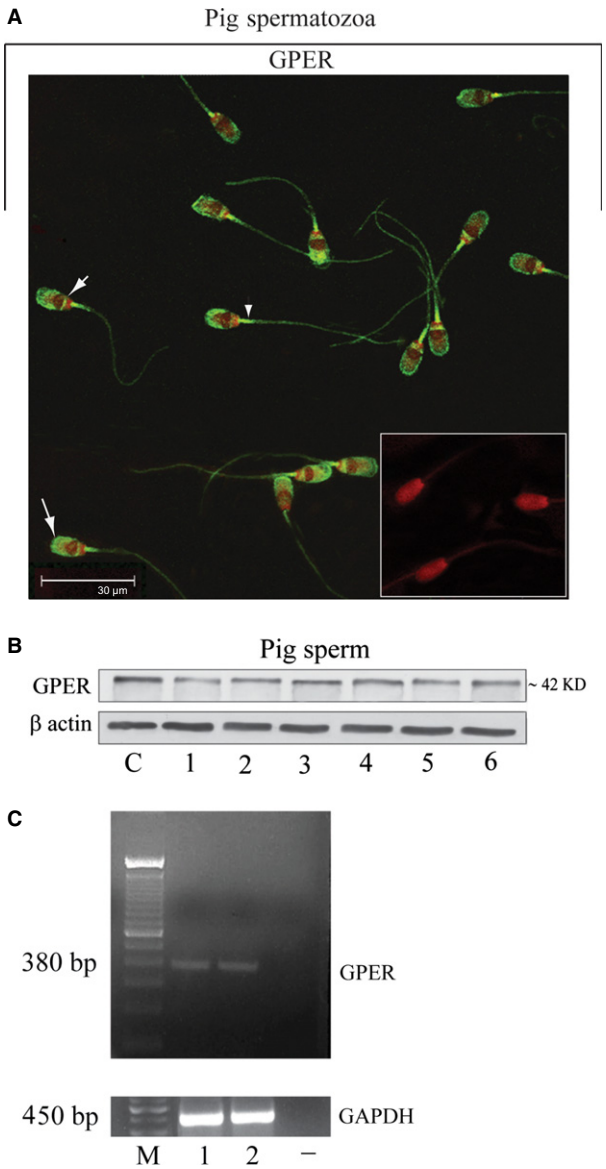

Fig. 2 Expression of GPER in pig spermatozoa (A) Green immunofluorescence in the mid-piece (arrowhead), in the equatorial segment (short arrow) and in the acrosome (long arrow) of sperm cells. Sperm heads show the propidium iodide red stain. The insert show the control spermatozoa. (B) Immunoblots of protein extracts from six pig sperm samples (lane 1–6), positive control (lane C), β-actin as loading control. (C) RT-PCR results in two representative pig sperm samples (lane 1–2), negative control (lane-), and markers (lane M). GAPDH (450 bp) is the internal control.

RT-PCR amplification revealed the expected PCR product sizes of 155 and 380 bp in human and pig spermatozoa, respectively.

Discussion

Rapid responses to estrogens are involved in the regulation of the main mammalian sperm functional properties. Estrogen promotion of capacitation and acrosome reaction have been reported in mouse (Adeoya-Osiguwa et al. 2003) and in boar ejaculated sperm cells (Ded et al. 2010), while an inhibitory role of estradiol on acrosome reaction was evidenced in human spermatozoa under physiological conditions (Vigil et al. 2008, 2011). However, whether these fast responses are mediated only by the classical ERs or also by alternative receptors remains to be elucidated.

Previous studies demonstrated that human and pig male gametes are a target for estrogens, evidencing the

expression of the classical ERs in these cells. In the human, ERβ was prevalently identified in the sperm mid-piece, whereas some discrepancies were reported for ERα which was localized in the sperm mid-piece or the equatorial region (Aquila et al. 2004; Solakidi et al. 2005). In the pig, ERα was found in the sperm mid-piece and ERβ in the acrosome region (Rago et al. 2007).

In this work we demonstrated the expression of the G-protein coupled receptor, GPER, in human and in pig spermatozoa using different approaches. Immunolocalization experiments revealed GPER in different sperm compartments of both species, whereas the immunoblots from sperm protein extracts showed one band at ~42 kDa, consistent with GPER molecular weight. These data were confirmed by RT- PCR analysis which detected GPER mRNA in both human and pig spermatozoa, evidencing the GPER transcripts at 155 and 380 bp, respectively.

To our knowledge, this is the first report on GPER expression in mammalian mature male gametes, as GPER was previously localized only in testicular germ cells of some mammalian species. In rodent testis, GPER was evidenced in mouse germ cells (Sirianni et al. 2008) and in rat isolated pachitene spermatocytes and round spermatids (Chimento et al. 2010, 2011). Conversely, conflicting results were reported in germ cells of normal human testis, where GPER was not found (Rago et al. 2011), weakly expressed (Franco et al. 2011) or clearly identified (Chevalier et al. 2012).

The present study identified GPER exclusively in the sperm mid-piece of human male gametes. The mid-piece is formed by a mitochondrial spiral around the proximal axoneme and it can be considered the powerhouse of a sperm. Therefore, the GPER presence in this cellular site could be involved in the estrogen signalling related to the sperm energy status which, in turn, modulates the main functional properties of sperm.

Concerning pig spermatozoa, GPER was identified in different cell compartments: mid-piece, acrosomal region and equatorial segment. Therefore, in pig spermatozoa GPER is expressed in cell compartments, where the estrogen signalling can be related to the acquisition of fertilizing ability. In fact, the mid-piece may be associated to the sperm energy requirement, the acrosome cap contains lysosomal enzymes dissolving the matrix around the egg, and the equatorial segment is a post-acrosomal region involved in sperm–egg plasma membrane fusion.

As reported above, the classical ERs were identified in the mid-piece/acrosome region of human and pig spermatozoa (Aquila et al. 2004; Solakidi et al. 2005; Rago et al. 2007) and GPER could be specifically co-localized in some sperm compartments of both species. This consideration leads to the hypothesis that there are interactions between GPER and classical ERs, as a cross-talk between the two receptor systems appears to be possible (Prossnitz & Barton, 2009). In fact, GPER and ERs are co-expressed in rat testicular germ cells; particularly in primary rat pachytene spermatocytes, GPER and ERα are both involved in the estrogen activation of rapid signalling pathways controlling apoptosis (Chimento et al. 2010). In addition, the inter-play between GPER and ERα in epithelium and stroma of normal human endometrium has suggested a functional collaboration of the two receptors in endometrial biology (Dennis et al. 2009).

Finally, considering our results and previous reports, it is possible to hypothesize that rapid responses to estrogens in sperm cells may be mediated either by GPER and ERs, or by a combination of both receptor types. The use of selective GPER ligands (agonists/antagonists such as G1, G15 (Rosano et al. 2012) or MIBE (Lappano et al. 2012) could help clarify the involvements of specific receptors in estrogen modulation of the functional properties of sperm.

Furthermore, a new attention should be paid to the effects of estrogenic compounds (phytoestrogens, xenoestrogens, environmental estrogens) on sperm physiology. Humans and pigs are chronically exposed to these endocrine disruptors, which are known to bind classical ERs, influencing sperm capacitation and acrosome reaction (Adeoya-Osiguwa et al. 2003; Mohamed et al. 2011; Park et al. 2011). Some of them, such as soy isoflavones (included in conventional pig feed and present in human diet), have been recognized as GPER activators in cultured cells (Maggiolini et al. 2004; Thomas & Dong, 2006). Therefore, estrogenic compounds could also bind GPER in sperm cells with the potential to alter sperm functions.

Conclusion

The present work demonstrated the expression of the estrogen receptor GPER in human and pig spermatozoa, suggesting a possible involvement of this receptor in the mediation of rapid responses to estrogens. GPER cellular expression sites were species-specific but they were related to the acquisition of sperm fertilizing ability in both species.

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Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Authors' contributions

V.R. carried out immunofluorescence expriments and data analysis. F.G. carried out Western blot analysis. E.B. carried out microscopical analysis. D.Z. carried out RT-PCR analysis. S.A. performed a critical revision of the manuscript. A.C. is the author responsible for the conception, design, analysis and interpretation of data as well as drafting the manuscript.

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REPRODUCTION

RESEARCH

Human sperm anatomy and endocrinology in varicocele: role of androgen receptor

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Abstract

The study of androgens involved in male reproduction has been object of intense efforts, while their reported action on human male gametes is limited. We previously described the presence of androgen receptor (AR) in sperm with a role related to the modulation of the PI3K pathway. In the present study, we investigated the expression of AR and its ultrastructural location in normal sperm as well as in spermatozoa obtained from varicocele patients. We observed a reduced AR content in varicocele sperm with respect to healthy sperm by western blot analysis and transmission electron microscopy (TEM). The ultrastructural location of AR was detected mainly on the head membrane as well as in the nucleus, neck, and mitochondria. Influence of dihydrotestosterone (DHT) treatment on cholesterol efflux was increased in normal sperm, while it was reduced or absent in varicocele sperm. To better understand DHT/AR significance in human male gametes, we evaluated triglyceride content and lipase, acyl-CoA dehydrogenase, and glucose-6-phosphate dehydrogenase activities upon DHT treatment. The metabolic outcome glimpsed in normal sperm was an increased metabolic rate, while 'varicocele' sperm economized energy. Taken together, our results reveal DHT and AR as new players in sperm endocrinology, indicating that varicocele sperm may have difficulty in switching to the capacitated status. A decreased AR expression and a consequent reduced responsiveness to DHT in sperm may represent molecular mechanisms involved in the pathophysiology of varicocele leading to male infertility. This study revealed new detrimental effects of varicocele on sperm at the molecular level.

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Introduction

Androgens, which are mainly produced in testicular Leydig cells, are essential throughout male life, as they play a fundamental role in sexual differentiation, maintenance of spermatogenesis, and expression of secondary sexual characteristics (Wang *et al.* 2009). The action of androgens is mediated by androgen receptor (AR), a member of the nuclear receptor superfamily, which functions as a ligand-inducible transcription factor (Wang *et al.* 2009). Naturally occurring AR ligands are testosterone and its more active metabolite dihydrotestosterone (DHT). Activated AR may trigger either genomic or rapid, nongenomic signaling pathways, and a crosstalk between the two AR functional pathways has also been proposed (Bonaccorsi *et al.* 2008, Grosse *et al.* 2012). Several findings indicate androgens and AR to be fundamental actors in the regulation of normal spermatogenesis and fertility. The function of AR has been shown to be required for the completion of meiosis and the transition of spermatocytes to haploid round spermatids, and the replacement of androgens alone is able to initiate qualitatively complete spermatogenesis in gonadotropin-deficient mice (Singh *et al.* 1995, De Gendt *et al.* 2004). Conversely, *AR* gene inactivation severely disrupts spermatogenesis by interrupting meiosis completion, thereby abolishing the mature sperm production and leading to male sterility (Walters *et al.* 2010). Nevertheless, mechanisms by which androgens regulate male fertility still need to be clarified and are the subject of intense efforts. Indeed, there are few reports on the role of androgens in the ejaculated sperm physiology. Ejaculated mammalian spermatozoa express AR, playing a role in the modulation of sperm survival and capacitation by the regulation of the PI3K/AKT signaling pathway (Aquila *et al.* 2007, Zalata *et al.* 2013). However, the presence and function of AR in

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spermatozoa are not universally accepted. It has been shown that male total *Ar* knockout (*T-Ar*$^{-/y}$) mice exhibit incomplete germ cell development and lowered serum testosterone levels, which result in azoospermia and infertility (Zhang *et al*. 2006). It has also been reported that the deletion of *Ar* gene in mouse germ cells does not affect spermatogenesis and male fertility (Tsai *et al*. 2006). Besides, the consequences of *AR* loss in particular types of testicular cells remain unclear.

Ejaculated mammalian spermatozoa are fascinating, highly differentiated cells characterized by extremely polarized cellular architecture and function. During their lifespan, spermatozoa are exposed to different physiological conditions. In the male genital tract, they remain in a quiescent state accumulating and/or economizing energy substrates (uncapacitated spermatozoa), while in the female genital tract, they undergo a functional maturation process, known as capacitation, acquiring the capability to fertilize an oocyte (Suarez 2008). Capacitation is a complicated process regulated by different molecules and several signaling pathways and involves many physiological changes including increased metabolic rate and energy expenditure (Blackmore *et al*. 1990, Baldi *et al*. 2000, De Amicis *et al*. 2012*a*, 2012*b*, Perrotta *et al*. 2012, Aquila *et al*. 2013). Different hormones, such as insulin and leptin, as well as sex steroids, namely estrogens and progesterone, have been reported to affect testicular function (Guido *et al*. 2012) and human sperm metabolism (De Amicis *et al*. 2011, Guido *et al*. 2011). In normal androgen-responsive tissues *in vivo*, androgens stimulate lipogenic gene expression (Heemers *et al*. 2003). Furthermore, they are widely regarded as important anabolic agents in the muscle or with lipolytic effects in adipose tissue (Xu *et al*. 1990, Elashoff *et al*. 1991). A role for androgens/AR in the management of energy metabolism in spermatozoa has not been addressed yet.

In the present study, to gain insight into the biological significance of the DHT/AR pathway in human male gametes, we investigated the expression of AR and its ultrastructural location in normal sperm as well as in spermatozoa obtained from varicocele patients. The influence of DHT/AR on capacitation and lipid and glucose metabolism was also evaluated.

Materials and methods

Chemicals

Percoll (colloidal PVP-coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethyl sulfoxide (DMSO), Earle's balanced salt solution (EBSS), DHT, goat polyclonal actin antibody (Ab), monoclonal mouse anti-AR Ab, and all other chemicals were purchased from Sigma Chemical Industries. Casodex (Cax), a specific antagonist of AR, was from AstraZeneca. Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100 and eosin Y were from Farmitalia Carlo Erba (Milan, Italy). ECL Plus Western blotting detection system, Hybond ECL, and HEPES sodium salt were from Amersham Pharmacia Biotech. EBSS without calcium, without magnesium, without phenol red, and without $NaHCO_3$ (uncapacitating medium) was from Genaxxon Bioscience (Milan, Italy). Colloidal gold-conjugated goat anti-mouse IgG secondary Ab was from Sigma–Aldrich, and peroxidase-coupled anti-mouse, anti-rabbit, and anti-goat IgG secondary Abs were from Santa Cruz Biotechnology. Cholesterol oxidase (CHOD)–peroxidase (POD) enzymatic colorimetric kit, triglyceride assay kit, lipase activity kit, and glucose-6-phosphate dehydrogenase (G6PDH) activity assay kit were from Inter-Medical (Biogemina Italia Srl, Catania, Italy). DHT was dissolved in ethanol (EtOH), while Cax in DMSO, and the experiments carried out with the vehicles alone did not reveal differences between treated and untreated samples (data not shown).

Semen samples and spermatozoa preparations

Human semen samples were collected, according to the World Health Organization (WHO)-recommended procedure, from healthy normozoospermic volunteer donors of proven fertility. Briefly, semen samples with normal parameters of volume, sperm count, motility, morphology, and vitality, according to the WHO Laboratory Manual (WHO 2010), were used in this study. Samples from patients affected by varicocele who consulted us for fertility investigation were also used in the study. Spermatozoa preparations were obtained as described previously (De Amicis *et al*. 2011). Reflux of blood in the pampiniform plexus was determined by palpation employing the Valsalva maneuver. Physical examination is the reference standard to diagnose varicoceles in subfertile men. Additional radiological imaging is not necessary to diagnose subclinical varicocele, because only a varicocele detected by physical examination should be considered potentially significant (Pryor & Howards 1987). Varicocele samples used in this study were from patients with diagnosed varicocele of grade III (visible without palpation) on the left testis, and their ejaculates were found to have total sperm count of 16×10^6 sperm cells per ejaculate, percentage of motility of 32%, percentage of normally formed features of 27%, and percentage of viability of 60%. The study was approved by the local medical–ethical committee, and all participants gave their informed consent.

Processing and treatments of ejaculated sperm

Each sperm sample was obtained by pooling three (normozoospermic) or four (varicocele) ejaculates of different subjects. In our experience, this was necessary to obtain enough cells to carry out all the tests (Aquila *et al*. 2007, De Amicis *et al*. 2012*a*, 2012*b*). The final sperm concentration of the resuspended samples during treatments was 20×10^6/ml. Each assay was carried out at least three times using at least three different sperm samples. Therefore, we evaluated at least a total of nine different normozoospermic specimens and 12 different varicocele specimens.

The samples were then subjected to centrifugation (800 ***g***) on a discontinuous Percoll density gradient (80:40% v:v) (Guido *et al*. 2011). The 80% Percoll fraction was examined using an optical

microscope equipped with a 100× oil objective to ensure that a pure sample containing only spermatozoa was obtained. These sperm had a motility of about 65% and a viability of 80% in both normal and pathological samples. An independent observer inspected several fields for each slide. Particularly, the same number of Percoll-purified sperm from both normal and pathological samples were washed with unsupplemented Earle's medium and incubated for 30 min at 37 °C and 5% CO_2, without (control, NC) or with the following treatments: increasing DHT concentrations (0.1, 1, and 10 nM) according to previous studies (Aquila *et al.* 2007) and Cax (10 μM) alone or combined with 1 nM DHT. When the cells were treated with the inhibitor Cax, a pretreatment of 15 min was performed.

Immunogold labeling for AR

Immunogold labeling assay was carried out as reported previously (De Amicis *et al.* 2013). Briefly, sperm fixed overnight in 4% paraformaldehyde were washed in PBS to remove excess fixative, dehydrated in graded alcohol, infiltrated in LR White resin, and polymerized in a vacuum oven at 45 °C for 48 h, while 60 nm ultrathin sections were cut and placed on coated nickel grids for post-embedding immunogold labeling with the rabbit polyclonal Ab against human AR. Potential nonspecific labeling was blocked by incubating the sections in PBS containing 5% normal goat serum, 5% BSA, and 0.1% cold-water fish gelatine at room temperature for 1 h. The sections were then incubated overnight at 4 °C with mouse polyclonal AR Ab at a dilution of 1:500 in PBS buffer. The sections were incubated in 10 nm colloidal gold-conjugated anti-mouse IgG secondary Ab at a dilution of 1:50 for 2 h at room temperature. The sections were then washed in PBS, fixed in glutaraldehyde, counterstained with uranyl acetate, and examined under a Zeiss EM 900 transmission electron microscope. To assess the specificity of immunolabeling, negative control assays were carried out for the corresponding sections of sperm that were labeled with colloidal gold-conjugated secondary Ab with normal mouse serum instead of the primary Ab.

Western blot analysis of sperm proteins

Percoll-purified sperm samples, washed twice with uncapacitating medium, were incubated as described above and then centrifuged for 5 min at 5000 ***g***. The pellet was resuspended in lysis buffer as described previously (Aquila *et al.* 2013). An equal amount of protein (80 μg) was boiled for 5 min, separated on an 11% PAGE, transferred onto nitrocellulose membranes, and probed with an appropriate dilution of the indicated primary Ab. The binding of the secondary Ab was revealed with the ECL Plus Western blotting detection system, according to the manufacturer's instructions. Western blot analysis was carried out in at least four independent experiments, and more representative results are presented.

Measurement of cholesterol content in the sperm culture medium

Cholesterol content in the culture medium in which human spermatozoa were incubated was measured in duplicate using a CHOD–POD enzymatic colorimetric method according to the manufacturer's instructions, as described previously (De Gendt *et al.* 2004). Percoll-purified sperm samples, washed twice with uncapacitating medium, were incubated for 30 min at 37 °C and 5% CO_2 in the same medium (control) or in the presence of increasing DHT concentrations. Other samples were incubated in the presence of 10 μM Cax alone or combined with 1 nM DHT. At the end of the sperm incubation period, culture medium was recovered by centrifugation, lyophilized, and subsequently dissolved in 1 ml of the reaction buffer. The samples were incubated for 10 min at room temperature, and then the cholesterol content was measured at 505 nm. Cholesterol content results are presented as mg per 10×10^6 spermatozoa as reported in our previous studies (De Gendt *et al.* 2004, Wang *et al.* 2009).

Measurement of triglyceride content in the sperm

Triglyceride content in sperm lysates was measured in duplicate with a GPO–POD enzymatic colorimetric method according to the manufacturer's instructions and as described previously (De Amicis *et al.* 2012*a*, 2012*b*). Percoll-purified sperm samples, washed twice by centrifugation with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37 °C and 5% CO_2. Other samples were incubated in the presence of the treatment agents at the indicated concentrations. At the end of the sperm incubation period, 10 μl of lysate were added to 1 ml of the reaction buffer and incubated for 10 min at room temperature. Then, triglyceride content was measured at 505 nm. Data are presented as $\mu g/10^6$ sperm.

Lipase activity assay

Lipase activity was evaluated as reported previously using the method of Panteghini *et al.* (2001) based on the use of 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester (DGGR) as substrate. Sperm extracts weighing 50 μg were loaded into individual cuvettes containing a buffer for spectrophotometric determination (Panteghini *et al.* 2001). DGGR is cleaved by lipase, resulting in an unstable dicarbonic acid ester, which is spontaneously hydrolyzed to yield glutaric acid and methylresorufin, a bluish-purple chromophore with peak absorption at 580 nm. The absorbance of samples was read every 20 s for 1.5 min. The rate of methylresorufin formation is directly proportional to lipase activity in the sample. The estimated reference interval was 6–38 U/l (μmol/min per mg protein). Enzymatic activity was determined with three control media: one without the substrate, another without the coenzyme (colipase), and one without either the substrate or the coenzyme (data not shown).

Acyl-CoA dehydrogenase activity assay

Acyl-CoA dehydrogenases are a class of enzymes that catalyze the initial step in each cycle of fatty acid β-oxidation in the mitochondria of cells. Acyl-CoA dehydrogenase activity assay was carried out on sperm samples using a modification of the method described by Lehman *et al.* (1990) and

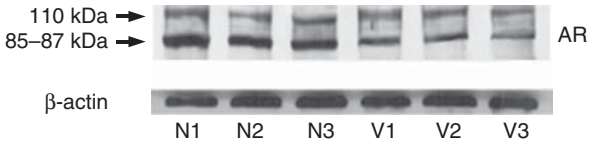

Figure 1 Reduction of AR expression in varicocele sperm. N1, N2, and N3 expression of AR in ejaculated sperm from three different samples of normal men and V1, V2, and V3 expression of AR in ejaculated sperm from three different samples of varicocele-affected men. The number on the left corresponds to molecular masses of the marker proteins. β-actin was used as loading control. The experiments were repeated at least four times, and the autoradiographs are the results of one representative experiment.

De Amicis *et al.* (2011). In brief, after cell lysis, 70 µg of sperm proteins were added to the buffer containing 20 mM MOPS (3-(N-morpholino)propanesulfonic acid), 0.5 mM EDTA, and 100 µM FAD^+ at pH 7.2. The reduction of FAD^+ to FADH was read at 340 nm upon the addition of octanoyl-CoA (100 µM) every 20 s for 1.5 min. Data are expressed as nmol/min per mg protein. Enzymatic activity was determined with three control media: one without octanoyl-CoA as the substrate, another without the coenzyme (FAD^+), and one without either the substrate or the coenzyme (data not shown).

G6PDH activity assay

The conversion of $NADP^+$ to NADPH, catalyzed by G6PDH, was measured by the increase in absorbance at 340 nm (De Amicis *et al.* 2011). Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37 °C and 5% CO_2. Other samples were incubated in the presence of the treatment agents at the indicated concentrations. After incubation, 50 µl of sperm extracts were loaded into individual cuvettes containing a buffer (100 mM triethanolamine, 100 mM $MgCl_2$, 10 mg/ml glucose-6-phosphate, and 10 mg/ml $NADP^+$, pH 7.6) for spectrophotometric determination. The absorbance of samples was read at 340 nm every 20 s for 1.5 min. Data are expressed as nmol/min per 10^6 sperm. Enzymatic activity was determined with three control media: one without glucose-6-phosphate as the substrate, another without the coenzyme ($NADP^+$), and the last one without either the substrate or the coenzyme (data not shown).

Statistical analysis

Transmission electron microscopy (TEM) was performed in at least three independent experiments, and statistical analysis was carried out by counting the numbers of gold particles over cross-sectional profiles of 80 sperm from both normozoospermic (healthy control) and varicocele samples, and the difference between the means was calculated using Student's *t*-test. Western blot analysis was carried out in at least four independent experiments. Data obtained from cholesterol assay, triglyceride assay, and lipase activity, acyl-CoA dehydrogenase activity, G6PDH activity assays (ten replicate experiments using duplicate determinations) are presented as means ± S.E.M. Differences in mean values were calculated using ANOVA with a significance level of $P \leq 0.05$. The Wilcoxon test was used after ANOVA as a *post hoc* test.

Results

AR is differentially expressed in the spermatozoa of healthy or varicocele-affected subjects

The expression of AR protein in the ejaculated sperm of healthy or varicocele-affected subjects was investigated by western blot analysis using a MAB raised against the AR epitope mapping at the 299–316 aa in the N-terminus region of AR of human origin. According to our previous study (Aquila *et al.* 2007), two protein bands with molecular weights of 110 and 87 kDa respectively were detected. The mainly expressed isoform was the 87 kDa form, corresponding to AR-A (Fig. 1). Interestingly, AR content was strongly reduced in 'varicocele' samples (Fig. 1), suggesting a role for AR in varicocele pathophysiology.

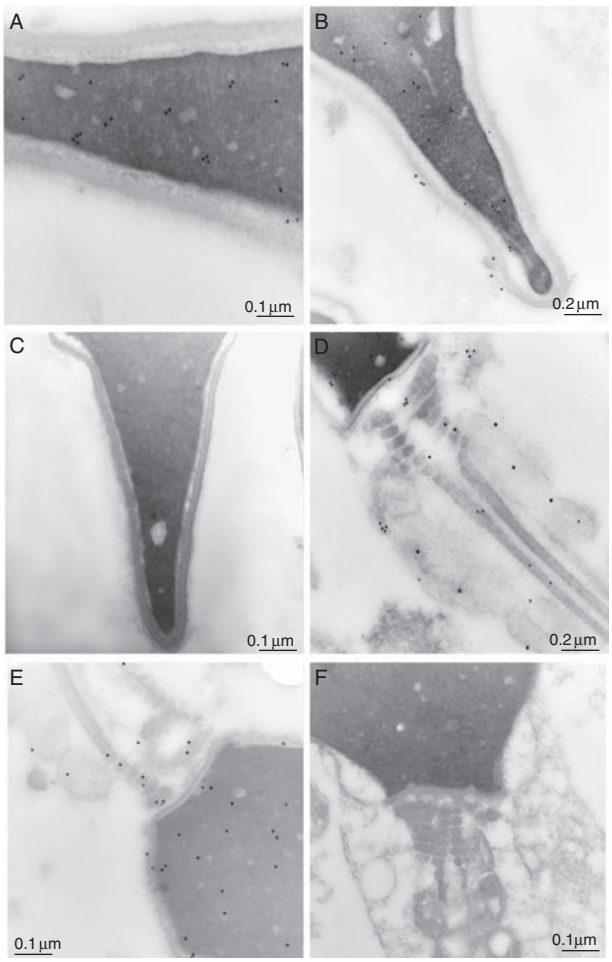

Figure 2 AR ultrastructural location in the head and neck of sperm of normozoospermic subjects. Sperm samples were collected and prepared as described in the Materials and methods section. Micrographs of the sections of ejaculated sperm of normozoospermic subjects probed with mouse monoclonal Ab against human AR: (A and B) sperm head; (D and E) neck and midpiece; and (C and F) results of the negative control (N) experiments carried out on the corresponding sections of sperm where normal mouse serum instead of the primary Ab was used. Results are representative of three similar experiments.

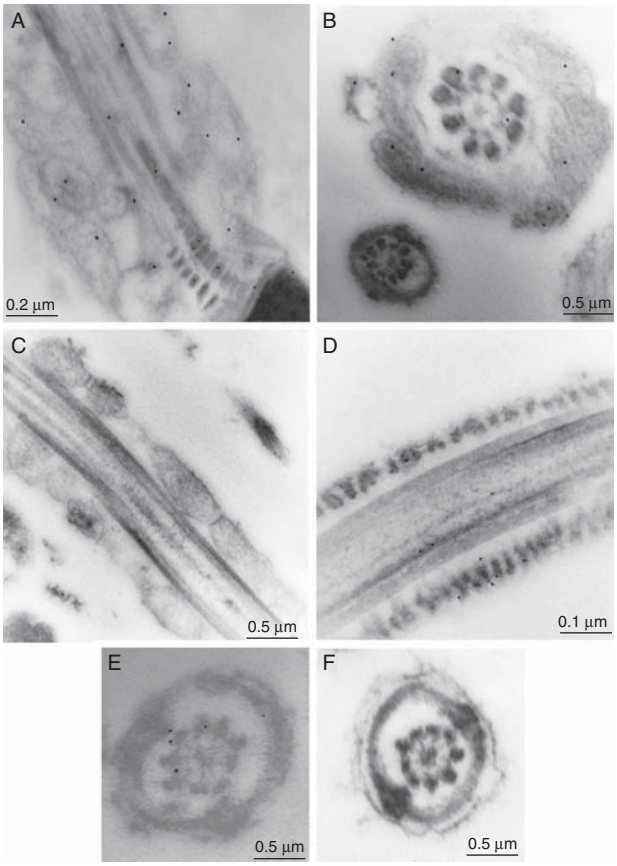

Figure 3 AR ultrastructural location in the midpiece and tail region of sperm of normozoospermic subjects. Sperm samples were collected and prepared as described in the Materials and methods section. Micrographs of the sections of ejaculated sperm of normozoospermic subjects probed with mouse MAB against human AR: (A and B) longitudinal and cross sections of the midpiece; (D and E) longitudinal and cross sections of the tail region; and (C and F) results of the negative control (N) experiments carried out on the corresponding sections of sperm where normal mouse serum instead of the primary Ab was used. Results are representative of three similar experiments.

Specifically, the expression of the short AR variant was reduced in varicocele samples, whereas the expression of the long variant remained unaffected.

Immunogold localization of AR in human sperm

Ultrastructural analysis of spermatozoa by TEM was carried out to determine the localization of AR in human sperm. As shown in Fig. 2, in healthy samples, the label was present mostly within the head, decorating the plasma membrane as well as the nucleus (Fig. 2A and B). Appreciable evidence of gold particles was also observed in the neck and the mitochondria-containing midpiece sperm compartments (Fig. 2D and E). On the contrary, AR was only faintly present as a component of the sperm flagellum, between the ribs of the fibrous sheath, outer dense fibers, and axoneme (Fig. 3A, B, D, and E). Negative control experiments with normal rabbit serum did not reveal any label in the corresponding sperm regions (Figs 2C and F and 3C and F). According to the above-described western blot data, in 'varicocele' samples, a reduction in the number of gold particles was evident in the head (Fig. 4A and B), along the neck and lower down the midpiece (Figs 4D and E and 5A and B). Moreover, labeling was absent in the tail of sperm in varicocele samples (Fig. 5D and E). Negative control experiments with normal mouse serum did not reveal any signal in the corresponding sperm regions (Figs 4C and F and 5C and F). Statistical elaboration of labeling density as the numbers of gold particles over the cross-sectional profiles of sperm from both normozoospermic and varicocele samples carried out with Student's *t*-test showed a significance of $P<0.0001$ (data not shown).

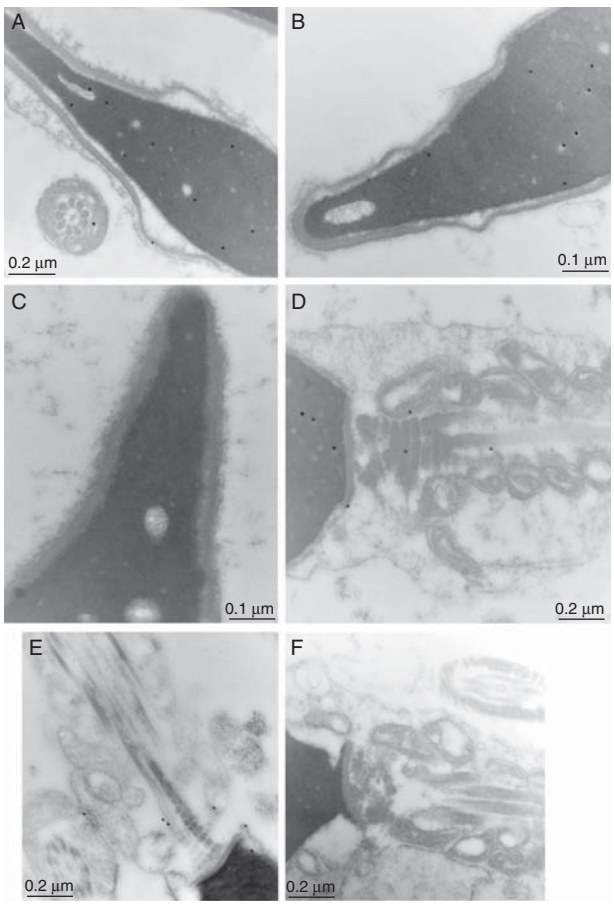

Figure 4 AR ultrastructural location in the head and neck of sperm of varicocele patients. Sperm samples were collected and prepared as described in the Materials and methods section. Micrographs of the sections of ejaculated sperm of varicocele patients probed with mouse MAB against human AR: (A and B) sperm head; (D and E) neck and midpiece; and (C and F) results of the negative control (N) experiments carried out on the corresponding sections of sperm where normal mouse serum instead of the primary Ab was used. Results are representative of three similar experiments.

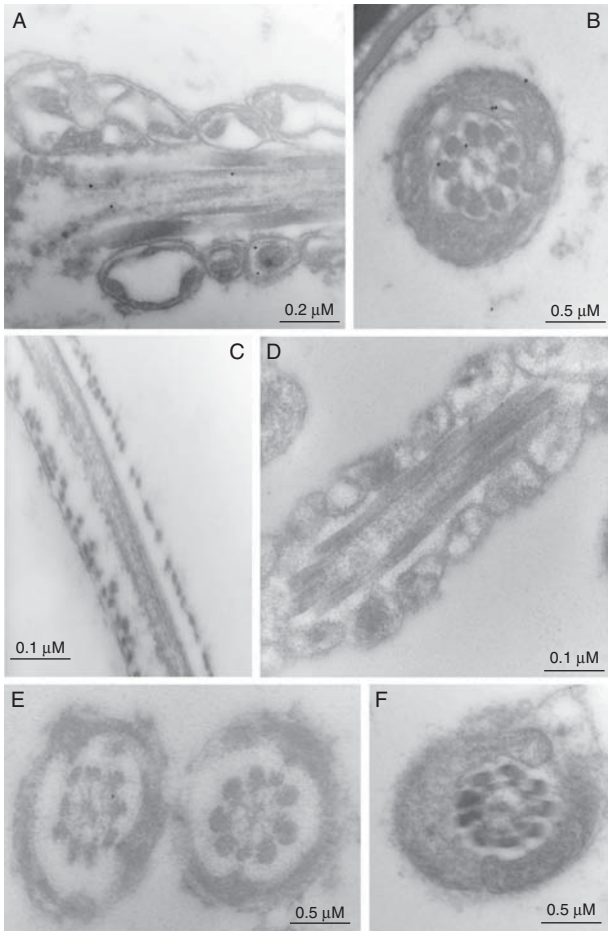

Figure 5 AR ultrastructural location in the midpiece and tail region of sperm of varicocele patients. Sperm samples were collected and prepared as described in the Materials and methods section. Micrographs of the sections of ejaculated sperm of varicocele patients probed with mouse MAB against human AR: (A and B) longitudinal and cross sections of the midpiece; (D and E) longitudinal and cross sections of the tail region; and (C and F) results of the negative control (N) experiments carried out on the corresponding sections of sperm where normal mouse serum instead of the primary Ab was used. Results are representative of three similar experiments.

DHT induces cholesterol efflux in human sperm

Our previous study has demonstrated the involvement of AR in capacitation (Aquila *et al*. 2007); therefore, to better understand its role in fertilization potential, we examined the effects of DHT/AR on cholesterol efflux, using three different normozoospermic samples as well as three different varicocele samples. Treatment with DHT (at concentrations ranging from 0.1 to 10 nM) increased cholesterol efflux with a maximum effect at 1 nM compared with the untreated samples (NC, Fig. 6). Co-treatment with 10 μM Cax, a specific AR antagonist, abrogated this DHT action, indicating that it is mediated by AR. In varicocele samples, under control conditions, cholesterol efflux was remarkably lower than that in the normal samples. DHT at a concentration of 1 nM was able to induce cholesterol efflux, although to a lesser extent than that in the normozoospermic samples.

DHT increases G6PDH activity in human sperm

Glucose is metabolized through glycolysis and the pentose phosphate pathway (PPP) in spermatozoa, the latter producing NADPH to achieve fertilization (Urner & Sakkas 1999). The possible role of DHT in this context was investigated by evaluating the activity of G6PDH, the key rate-limiting enzyme in the PPP. In healthy samples, DHT was able to greatly induce G6PDH enzymatic activity, and 10 μM Cax reverted the effect of 1 nM DHT. On the contrary, treatment with DHT, at all the tested concentrations, was unable to affect G6PDH activity in varicocele samples (Fig. 7).

DHT interferes with lipid metabolism in human sperm

Testosterone is a fat-reducing hormone that promotes lipolysis and reduces fatty acid synthesis (De Pergola 2000). As a role for this steroid in lipid metabolism in sperm is yet to be defined, we evaluated intracellular triglyceride content upon treatment with increasing DHT concentrations. As shown in Fig. 8A, DHT was able to decrease triglyceride content in a dose-dependent manner, and the combination of Cax with 1 nM DHT reverted the effect. On the contrary, in varicocele samples, we observed an increase in triglyceride content. To determine the mechanism through which DHT alters sperm lipid metabolism, we evaluated its action on lipase and acyl-CoA

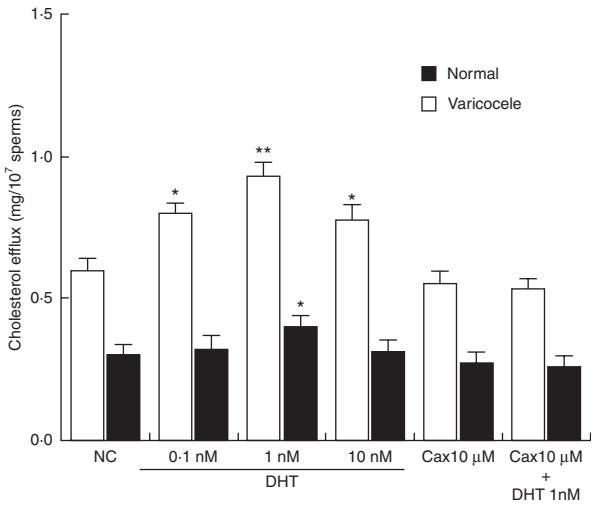

Figure 6 Influence of DHT on cholesterol efflux in human sperm. Purified spermatozoa (normal and varicocele affected) were incubated in unsupplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or in the presence of treatment agents at the indicated concentrations. Cholesterol content in the culture medium in which human ejaculated spermatozoa were incubated was measured using an enzymatic colorimetric assay. Columns represent means ± S.E.M. of ten independent experiments carried out in duplicate. Data are expressed as mg/10^7 sperm. *P=0.05 vs control and **P<0.02 vs control.

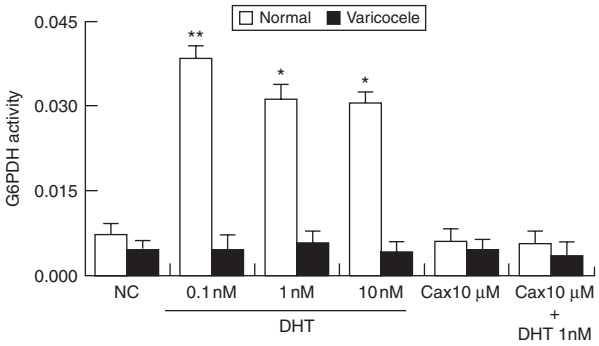

Figure 7 Effects of DHT on glucose metabolism in human sperm. Washed spermatozoa (normal and varicocele affected) were incubated in unsupplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or in the presence of treatment agents at the indicated concentrations. G6PDH activity assay was carried out as described in the Materials and methods section. Columns represent means ± S.E.M. *$P<0.005$ vs control and **$P<0.001$ vs control.

dehydrogenase activities. Interestingly, lipase activity was induced upon DHT treatment, with the maximum effect being observed at the concentration of 1 nM, while on using 10 μM Cax, the 1 nM DHT-induced action was revoked (Fig. 8B). Concomitantly in normal samples, DHT at all the tested concentrations induced acyl-CoA dehydrogenase activity, and the combination of Cax with 1 nM DHT abolished the effect (Fig. 8C). In varicocele samples, DHT was effective at the concentration of 1 nM producing a significant induction reverted by 10 μM Cax co-treatment.

Discussion

To date, varicocele represents one of the most common causes of male infertility, and although this pathology has been studied extensively, the mechanisms through which it can influence male fertility are not fully defined. Although it is well known that androgens are involved in the development of male sexual characteristics as well as in spermatogenesis, their action in the context of male infertility is not fully clarified. In the present study, we investigated the anatomical location of AR and its possible action on energy management in sperm at the ultrastructural level. Our data strengthen the functional role of AR in human sperm outcomes and underline the molecular basis of male infertility in human varicocele.

The presence of AR has been shown previously by immunofluorescence in the head and in the midpiece, corresponding to the site of the mitochondria, in human sperm (Solakidi *et al.* 2005, Aquila *et al.* 2007). In this study, by TEM with immunogold labeling analysis, we analyzed the anatomical regions containing AR in 'healthy' human sperm. Interestingly, numerous gold particles decorated the head at both the membrane and the nucleus. Labeled regions corresponding to the connecting piece (neck), especially in the segmented columns, were observed. Elevated AR expression was detected in the mitochondria, whereas its expression

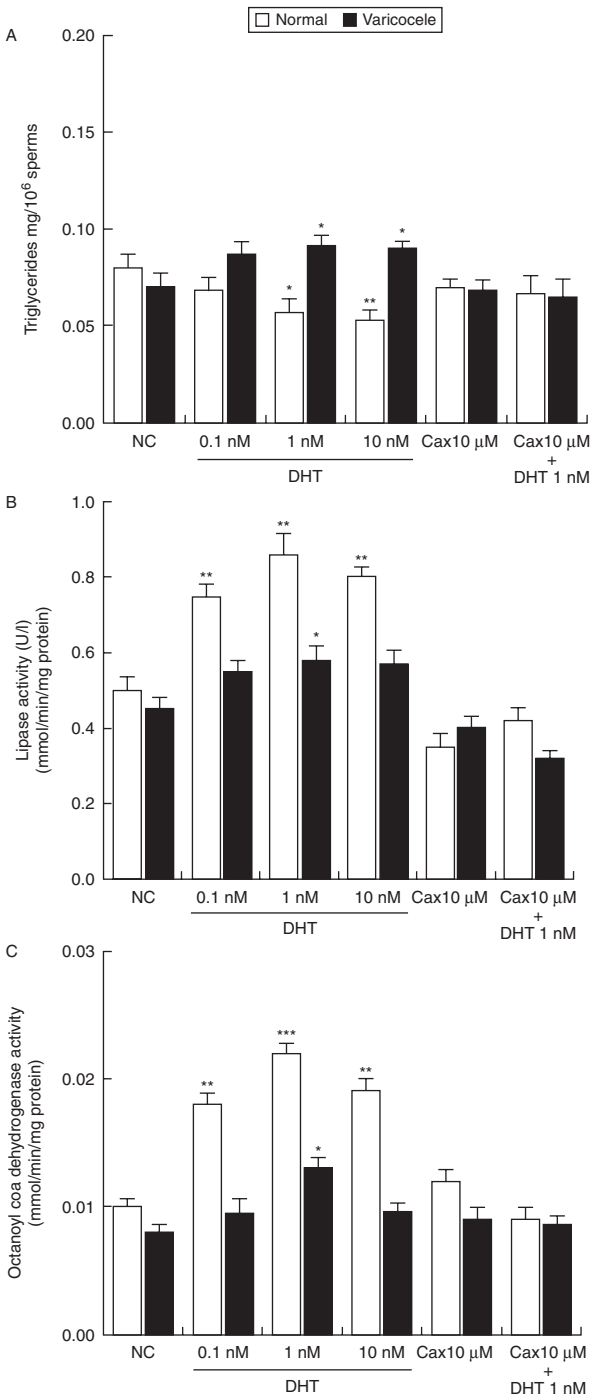

Figure 8 Effects of DHT on lipid metabolism in human sperm. Washed spermatozoa were incubated in unsupplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or in the presence of treatment agents at the indicated concentrations. (A) Triglyceride assay was carried out as described in the Materials and methods section. Columns represent means ± S.E.M. *$P<0.05$ vs control and **$P<0.01$ vs control. (B) Lipase activity assay was carried out as described in the Materials and methods section. Columns represent means ± S.E.M. *$P<0.05$ vs control and **$P<0.01$ vs control. (C) Octanoyl-CoA dehydrogenase activity assay was carried out as described in the Materials and methods section. Columns represent means ± S.E.M. *$P=0.05$ vs control; **$P<0.01$ vs control; and ***$P<0.005$ vs control.

was slowly reduced along the flagellum to the end piece. It is worth noting that ultrastructural analysis revealed that the expression of AR in varicocele samples was decreased. Particularly, rare gold particles were detected on the entire sperm from the head to the tail region, demonstrating that low levels of AR are present in this pathological condition. These data are consistent with the results of the western blot analysis, where a reduced expression of both AR isoforms was observed, and with recent data reported by Zalata *et al.* (2013).

A functional role for steroid receptors in capacitation, acrosome reaction, motility, and survival in sperm has been reported (Adeoya-Osiguwa *et al.* 2003). It is well known that human ejaculated sperm are not capable of fertilizing oocyte as they need two important sequential steps: capacitation and acrosome reaction. During capacitation, sperm undergo final maturation by increasing cholesterol efflux, which in turn alters sperm membrane fluidity, tyrosine phosphorylation of sperm proteins, and metabolic rate.

In this study, we demonstrated that DHT through AR is able to induce a capacitation marker such as cholesterol efflux, accordingly with an increase in protein phosphorylation reported previously (Aquila *et al.* 2007). The importance of androgens in the completion of male gamete maturation process during epididymal transit has been proved by the presence of AR in epididymal tissues (Zhou *et al.* 2002). Therefore, our data sustain the concept that AR influences sperm capacitation together with estrogen receptor (ER) and progesterone receptor (PR) (Guido *et al.* 2011, De Amicis *et al.* 2012*a*, 2012*b*). Interestingly, basal cholesterol efflux was reduced in varicocele sperm with respect to healthy sperm, and the effect of DHT was abated in the former. This result may probably be due to the reduced expression of AR determining a decreased responsiveness to DHT. Besides, our data are in agreement with the idea that grade II/III infertile varicocele presents an alteration in sperm plasma membrane dynamics due a decreased cholesterol mobility (Buffone *et al.* 2006).

The presence of AR in sperm mitochondria allowed us to hypothesize that this receptor could be involved in energy metabolism. It has been demonstrated that testosterone deficiency leads to increased fat deposition, whereas high testosterone levels inhibit adipocyte development, promoting lipolysis and reducing fatty acid synthesis (Salam *et al.* 2012). In normal sperm, DHT was able to reduce triglyceride content, while it induced lipase and acyl-CoA dehydrogenase activities, suggesting a lipolytic effect. Capacitated sperm imply

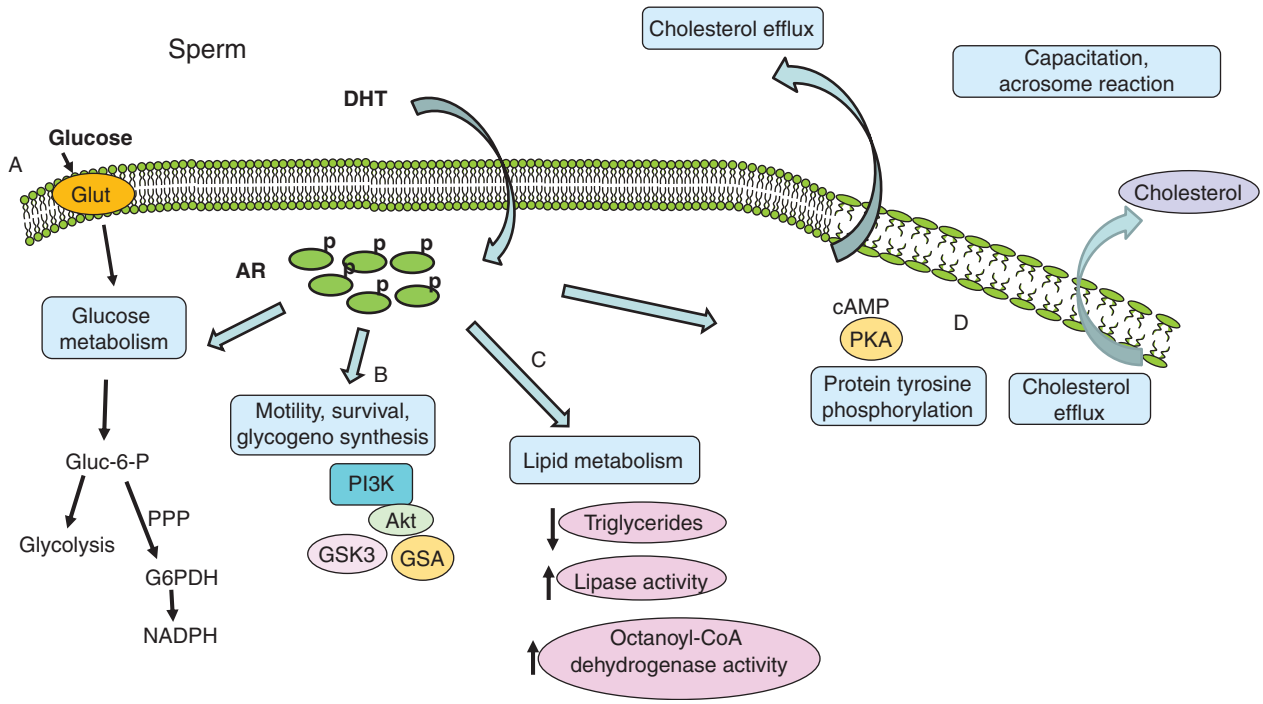

Figure 9 Roles of AR in sperm. DHT goes across the sperm membrane and binds to AR inducing its phosphorylation and different effects. (A) Glucose enters into sperm via glucose transporter (Glut), and it is phosphorylated to glucose-6-phosphate (Gluc-6-P) to be used in the PPP and in glycolysis, producing NADPH and ATP respectively. Upon DHT treatment, AR induces the activity of G6PDH, the first enzyme of the PPP, contributing to the regulation of glucose metabolism in the sperm; (B) AR upon DHT treatment is strictly involved in the PI3K/AKT pathway in the regulation of sperm survival; (C) DHT/AR signaling is able to regulate lipid metabolism inducing lipase and octanoyl-CoA dehydrogenase activities, thus reducing triglyceride levels; and (D) AR upon DHT treatment induces cholesterol efflux and tyrosine phosphorylation of sperm proteins, both closely related to and representing the main events of the capacitation process. During capacitation, an increase in membrane fluidity occurs, and cholesterol escapes from the cell after the activation of PKA that induces tyrosine phosphorylation of sperm proteins.

increased metabolism and overall energy expenditure, and previous studies have demonstrated that hormones induce capacitation together with energy expenditure (Aquila *et al.* 2009, Goodson *et al.* 2012).

Therefore, we retain our previous finding that DHT interacting with AR may activate the receptor through phosphorylation (Aquila *et al.* 2007), increasing metabolic activities and fueling capacitation in sperm. Interestingly, we observed a reduced response to DHT in varicocele sperm; therefore, it could be speculated that varicocele sperm are affected by a dismetabolic syndrome due to inefficacious enzymatic activities leading to the accumulation of triglycerides. Accordingly, a strong association of testosterone with lipogenesis has been observed at the systemic level during dismetabolic syndrome (Salam *et al.* 2012). Although the regulation of sperm energy metabolism is not well known, it may be generalized that uncapacitated sperm are associable with an anabolic metabolism, while capacitation with a catabolic metabolism. In this context, our data on varicocele sperm might indicate that they have difficulty in switching to the capacitated status.

It has been found that testosterone can modulate metabolic enzymes through a rapid nongenomic action in rat hepatocytes and also acts on G6PDH activity in fish (Baque *et al.* 1996, Sunny *et al.* 2002). Consistently, in normal sperm, we observed that DHT induced G6PDH activity, while the effect did not occur in varicocele sperm. Previous data from our laboratory have widely confirmed that nuclear receptors in sperm mediate functional maturation by regulating their metabolic status (De Amicis *et al.* 2012*a*, 2012*b*, Santoro *et al.* 2013). In this study, we defined the role of AR in healthy and varicocele samples, supporting the importance of the balance of steroids/receptors in human sperm physiology. The reduction of AR expression and responsiveness to DHT in varicocele sperm allowed us to define AR as a key player in the functional maturation of human male gametes. In Fig. 9, we present the proposed roles for the AR in sperm.

In conclusion, varicocele affects testicular function in a variety of ways including spermatogenesis, semen quality, sperm functions, and morphology. From our data, it emerges that this pathology induces damage in the male gamete at the molecular level, opening a new chapter in the already multifactorial pathophysiology of the varicocele.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SHORT COMMUNICATION

Insulin affects sperm capacity in pig through nitric oxide

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Insulin (Ins) has recently been demonstrated to have the ability to induce the capacitation process in pig spermatozoa. In various mammalian species, capacitation has been linked to the nitric oxide (NO) signalling; therefore, this study investigated NO production in Ins-treated pig spermatozoa by fluorescence-activated cell sorting. For the same samples, sperm capacitation was evaluated by chlortetracycline staining, protein tyrosine phosphorylation pattern and acrosomal status. A significant increase of the intrasperm NO level and the activation of three capacitation indices were detected in response to Ins treatment. Conversely, sperm preincubation with an NO synthase inhibitor (*N*-nitro-*L*-arginine methyl ester) or with the anti-Ins receptor β (IRβ) antibody reversed all of the Ins-related effects. These results suggest that Ins has the capacity to enhance intracellular NO concentrations in pig spermatozoa and indicate a possible NO implication upon Ins promotion of capacitation.
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Keywords: acrosome reaction; insulin; nitric oxide; pig sperm capacitation

INTRODUCTION

A potential new role of insulin (Ins) in sperm physiology has emerging in recent years. Ins expression and secretion have been demonstrated in human male gametes,[1] which have been found to be damaged in men affected by Ins-dependent diabetes.[2] In addition, Ins treatment can enhance motility and the extent of the acrosome reaction[3] of human spermatozoa.

In our previous study, the expression of Ins and Ins receptor β (IRβ) in pig spermatozoa and the ability of Ins to induce capacitation process were determined.[4] Furthermore, in pig spermatozoa, we recently reported the expression levels of the three isoforms of nicotinamide adenine dinucleotide phosphate-dependent nitric oxide (NO) synthases (inducibile NOS, endothelial NOS and neuronal NOS)[5] which are responsible for the synthesis of intracellular NO.[6] Because a link between capacitation and NO signalling has been suggested in some mammalian spermatozoa, the aim of the present study was to investigate the capacity of pig male gametes to produce NO in response to Ins treatment. Furthermore, a possible role of the NO free radical in the Ins-promotion of capacitation was also evaluated.

MATERIALS AND METHODS

Percoll-purified spermatozoa from five fertile male pigs (*Sus scrofa domestica*, Large White) were incubated with uncapacitating Earle's medium (Sigma Chemical, Milan, Italy) for 30 min at 39 °C and 5% CO_2 with or without 0.1 nmol l^{-1} porcine Ins (Sigma Chemical) and 0.7 mmol l^{-1} *N*-nitro-*L*-arginine methyl ester (*L*-NAME) (Vinci Biochem, Firenze, Italy). Because Ins action is mediated by its receptor, some spermatozoa were also pretreated (15 min) with the monoclonal mouse anti-IRβ antibody (C18C4 from Stressgene, Bologna, Italy) prior to the addition of 0.1 nmol l^{-1} Ins in order to perform an autocrine blockage. Untreated spermatozoa were capacitated (C) by incubation in Earle's capacitating medium for 120 min at 39 °C and 5% CO_2 as a positive control.

Intracellular NO concentrations were measured according to the protocol of Lampiao.[7] Briefly, Ins-treated sperm were loaded with 10 μmol l^{-1} of the fluorescent probe 4,5-diaminofluorescein-2/diacetate (DAF-2/DA; Vinci Biochem) and incubated (120 min and 37 °C) in the dark. Some of the samples were loaded with the NOS inhibitor, *N*-nitro-*L*-arginine methyl ester (*L*-NAME) (0.7 mmol l^{-1}) 30 min prior to DAF-2/DA loading. After incubation with DAF-2/DA, the cells were analysed by fluorescence-activated cell sorting (FACS analyser) at a single-cell level, and data were analysed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

The chlortetracycline (CTC) staining assay was performed according to Wang *et al.*[8] Briefly, 45 μl of the sperm suspension (5×10^6 ml^{-1}) was treated with an equal volume of a CTC solution for 30 s, followed by the addition of 8 μl of 12.5 paraformaldehyde in 0.5 mol l^{-1} Tris-HCl (pH 7.4). Ten microlitres of the fixed sperm suspension were placed on a microscope slide and examined with an epifluorescence microscope (Olimpus BX41) with multiple fluorescent filters. One hundred spermatozoa were examined and classified according to the following CTC staining patterns: uncapacitated spermatozoa (fluorescence uniformly distributed over the head), capacitated spermatozoa (a fluorescence-free band in the post-acrosome region) and acrosome-reacted spermatozoa (almost no fluorescence over the sperm head except for a thin band of fluorescence in the equatorial segment).

For western blot analysis, spermatozoa were washed and centrifuged for 5 min at 5000*g*. The pellet was resuspended in lysis buffer, and equal amounts of proteins (80 μg) were boiled for 5 min, separated by 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and probed with rabbit antiphosphotyrosine antibody

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(PY99; Santa Cruz, CA, USA) (1 : 500). The bound fraction of the secondary antibody (peroxidase-coupled anti-rabbit antibody; Santa Cruz) was revealed using an enhanced chemiluminescence plus western blot detection system.

The acrosome reaction assay was carried out as previously reported by Carpino *et al.*[4] Acrosomal status was monitored by the acrosome-specific fluorochrome fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin in conjunction with DNA-specific fluorochrome propidium iodide to test viability. Staining patterns: Live spermatozoa (without nuclear red propidium iodide staining) were classified into two main categories as follows: (i) acrosome-reacted cells with uniform green fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin fluorescence of the acrosomal cap; and (ii) acrosome-intact cells without any fluorescence. Values were expressed as percentage. Four replicate experiments were performed for each semen sample.

The data, presented as mean±s.e.m., were evaluated using a one-way ANOVA. The differences in mean values were calculated at significance level of $P<0.05$.

RESULTS

Incubation of spermatozoa with 0.1 nmol l^{-1} Ins induced a significant NO increase (**Figure 1a and 1b**), while sperm pre-treatment with the NOS inhibitor *L*-NAME reversed the effect of Ins (**Figure 1c**). The DAF fluorescence data are expressed as the mean fluorescence (percentage of control, control adjusted to 100%) (**Figure 1d**).

Ins treatment increased the amount of spermatozoa showing a capacitated CTC staining pattern (**Figure 2a**), induced the phosphorylation pattern of the sperm protein tyrosine (**Figure 2b**) and enhanced the percentage of acrosome-reacted cells (**Figure 3**). Sperm preincubation with *L*-NAME or anti-IRβ autocrine blockage, reversed all of the hormone-related effects (**Figure 3**).

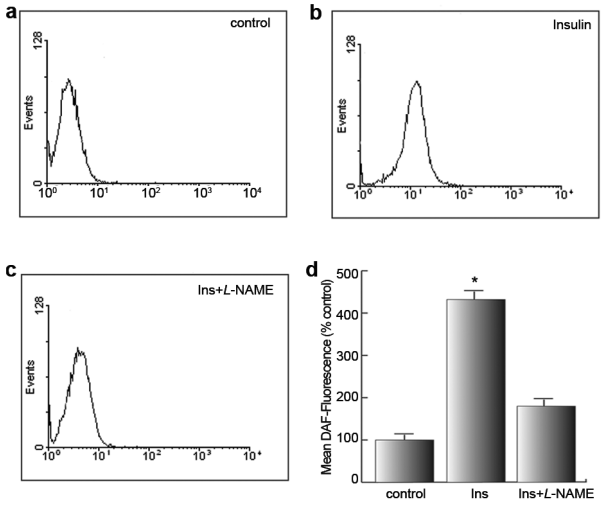

Figure 1 Representative histograms of DAF fluorescence in pig spermatozoa. (**a**) Uncapacitated spermatozoa. (**b**) Ins-treated spermatozoa. (**c**) Ins-treated sperm in presence of *L*-NAME. (**d**) Summary of the data reported above, expressed as the mean fluorescence (Mean DAF-fluorescence of the control was set to 100%. All other treatments were normalized accordingly). Values of percentage are expressed as mean ±s.d. (*$P<0.05$ *vs.* control). DAF, 4,5-diaminofluorescein-2/diacetate; Ins, insulin; *L*-NAME, *N*-nitro-*L*-arginine methyl ester.

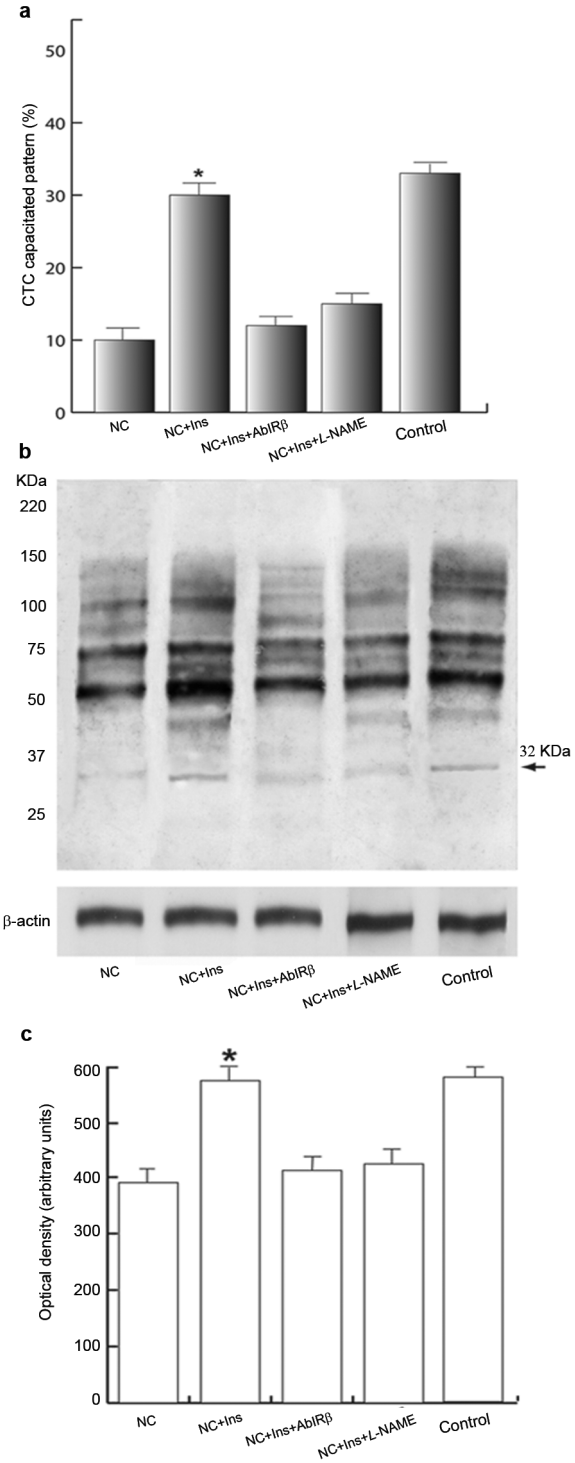

Figure 2 Effects of Ins treatment on the acquisition of CTC capacitated pattern and protein tyrosine phosphorylation in pig spermatozoa. Uncapacitated spermatozoa were incubated in the absence (NC) or presence of Ins, as well as in presence of *L*-NAME+Ins or anti-IRβ Ab+Ins. Capacitated spermatozoa (C) as positive control. (**a**) CTC pattern of percent capacitated sperm. (**b**) Western blot analysis of protein tyrosine phosphorylation from sperm lysates. (**c**) Band intensities were evaluated in term of arbitrary densitometric units. Values are as mean±s.d. (*$P<0.05$ *vs.* control). anti-IRβ Ab, insulin receptor β antibody; CTC, chlortetracycline; Ins, insulin; *L*-NAME, *N*-nitro-*L*-arginine methyl ester.

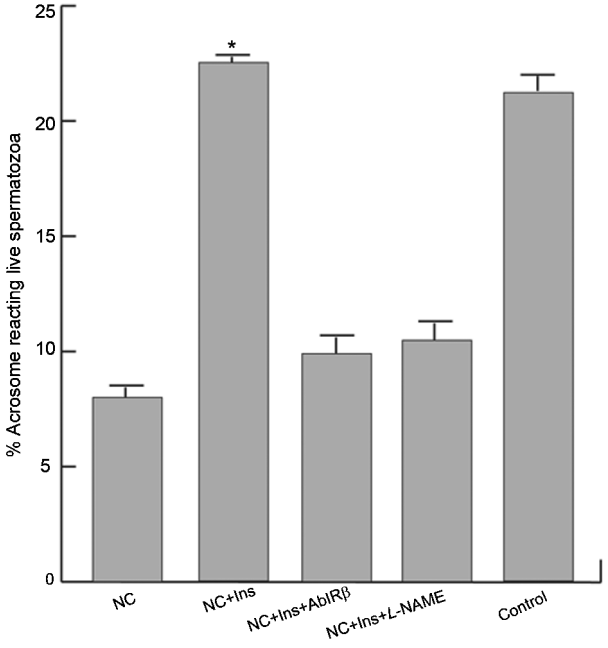

Figure 3 Acrosome reaction in Ins-treated pig sperm. Uncapacitated sperm were incubated in the absence (NC) or in the presence of Ins, as well in presence of anti-IRβ+Ins or *L*-NAME+Ins. Capacitated sperm as positive control (C). Values of percentage are expressed as mean ±s.d. (**P*<0.05 *vs.* control). anti-IRβ, insulin receptor β; Ins, insulin; *L*-NAME, *N*-nitro-*L*-arginine methyl ester.

DISCUSSION

NO is a highly reactive free radical that regulates a vast number of functional properties in mouse, hamster, bovine and human spermatozoa. Furthermore, in porcine male gametes, NO was implicated in the promotion of capacitation and/or acrosome reaction through induction by *L*-arginine, geldanamycin and leptin.[5,9,10] We previously demonstrated the ability of Ins to promote pig sperm capacitation.[4] Thus, in the present study, we have investigated NO production in Ins-treated pig spermatozoa. We used the Ins dose that induced the best stimulatory response in male gametes, based on our previous data. Our results revealed a significant increase in the level of intrasperm NO in response to Ins treatment, and this effect was reversed by sperm pre-treatment with an NOS inhibitor.

Furthermore, we have investigated the possibility of NO involvement in the Ins-mediated promotion of capacitation. Capacitation is a signal transduction-mediated event that modifies the sperm plasma membrane and leads to the acquisition of fertilizing ability. Firstly, we have observed the acquisition of a CTC capacitated pattern by the sperm membrane. We have also evaluated two cellular events associated with capacitation: protein tyrosine phosphorylation and the acrosome reaction. In fact, sequential protein tyrosine phosphorylation of the sperm compartments (midpiece, flagellum and head) occurs during capacitation and it appears to be related to the acquisition of hyperactivated motility, the capacity to bind the zona pellucida and acrosome reaction competence. In addition, capacitation prepares the sperm cells for the acrosome reaction, a process of specialized exocytosis that enables sperm cells to penetrate the female gamete. As expected, Ins treatment increased the amount of capacitated spermatozoa and induced the protein tyrosine phosphorylation pattern, including the sp-32 protein which is considered a marker of pig sperm capacitation.[11] At the same time, the acrosome reaction extent was enhanced in the male gametes stimulated by Ins. However, the inhibition of each of these indices by *L*-NAME (NOS inhibitor) and anti-IRβ autocrine blockage indicated the involvement of NO in the hormonal action.

Finally, these findings suggest that Ins can affect pig sperm capacitation through the NO pathway and provide new evidences concerning the relationships between sperm physiology and hormonal molecules involved in metabolic processes.

COMPETING FINANCIAL INTERESTS

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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