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TITOLO TESI

PELP1, EGR-1 and ERRα as potential targets for new therapies against adrenocortical carcinoma

Settore Scientifico Disciplinare BIO13

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A te che sei stato la luce nel buio,

a te che mi hai accompagnato sempre,

a te che hai mi hai riservato il tuo caldo sorriso quando ero in difficoltà,

a te che hai gioito per ogni mio attimo di felicità,

a te che hai tenuto stretta la mia mano per sempre,

a te che mi hai lasciato troppo presto,

a te papà

e

Al senso della mia vita…..

ai miei figli Lorenzo e Rodolfo

INDEX

ABSTRACT

I Carcinomi surrenalici (ACC) sono tumori rari e altamente aggressivi, associati a una prognosi molto sfavorevole, principalmente a causa di un alto rischio di recidiva e di opzioni terapeutiche limitate (Stojadinovic et al. 2002). L'escissione chirurgica completa offre le migliori possibilità di sopravvivenza a lungo termine, ma abbastanza spesso, nonostante la resezione completa, l'incidenza della recidiva è molto frequente (Glover et al. 2013). L'eziologia del cancro surrenale rimane ancora ignota, ma studi negli ultimi 10 anni suggeriscono che alcune mutazioni genetiche nella ghiandola surrenale sono alla base dell'iniziazione di un tumore maligno (Libe et al. 2007; Soon et al. 2008; Giordano et al. 2009). Tuttavia, l'ACC è una malattia estremamente eterogenea e sta diventando chiaro che la patogenesi dell'ACC determina l'alterazione di diversi segnali cellulari e l'interazione di alcuni pathways. Tra questi, il sistema dell'IGF e le vie estrogeno-dipendenti sembrano essere implicati. L'individuazione di fattori coinvolti negli eventi regolatori di tali pathways cellulari può contribuire a chiarire i meccanismi molecolari alla base delle alterazioni ed a individuare potenziali target terapeutici. Il laboratorio di Biologia Applicata e Cellulare dell'Unical, sta lavorando da alcuni anni per chiarire il coinvolgimento dei pathway IGF dipendenti ed estrogeno dipendenti nell'insorgenza e nella progressione del carcinoma surrenalico.

In questo lavoro di tesi si è voluto approfondire il ruolo di tre fattori coinvolti in questi pathways:

- PELP1 (Scaffold Protein Proline-, Glutamic Acid-, and Leucine-Rich Protein 1);
- EGR-1 (Early growth response gene-1);
- ERRα (Estrogen Related Receptor α).

PELP1 agisce come un coattivatore del recettore dell'estrogeno (ER) e quindi esercita un ruolo essenziale nella modulazione delle funzioni di ER. I coregolatori di ER hanno un ruolo fondamentale nella progressione e nella risposta al trattamento ormonale dei tumori estrogeno-dipendenti. In precedenza il laboratorio di Biologia Applicata e Cellulare dell'Unical ha dimostrato che, nel carcinoma adrenocorticale (ACC), ER è *up-regolato* e l'estradiolo attiva le vie di segnalazione IGF-II/IGF1R definendo il ruolo di questo cross-talk funzionale nella proliferazione delle cellule di ACC, H295R. Lo scopo della prima parte di questo studio è stato determinare se PELP1 è espresso nell'ACC e se possa giocare un ruolo nel promuovere l'interazione tra ER e IGF1R che consente

l'attivazione di vie importanti per la crescita delle cellule di ACC. L'espressione di PELP1 è stata rilevata tramite analisi Western blot nei tessuti di ACC e nelle cellule H295R. La riduzione della proliferazione cellulare delle H295R è stata valutata mediante il saggio A3-(4,5-Dimetilthiaoly)-2,5-difeniltetrazolio bromuro (MTT) e mediante l'incorporazione della timidina [3H]. PELP1 è espresso nei tessuti di ACC e nelle cellule H295R. Inoltre, il trattamento delle H295R con E2 o IGF-II ha indotto la una formazione di un complesso multiproteico costituito da PELP1, IGF1R, ER e Src, coinvolto nell'attivazione rapida di ERK1/2. Il silenziamento di PELP1 spegne tale via di segnalazione e riduce la crescita delle cellule H295R indotta da E2 e da IGF-II. L'identificazione del complesso PELP1/ER/IGF1R/c-Src come parte della via di segnalazione E2- e IGF-II-dipendente nell'ACC suggerisce che PELP1 è un nuovo ed efficiente potenziale bersaglio, per ridurre la crescita dell'ACC.

Nella seconda parte di questo lavoro di tesi abbiamo focalizzato la nostra attenzione sul recettore EGR-1, perché in uno studio precedente si è dimostrato che il trattamento della linea cellulare di carcinoma adrenocorticale, H295R con G-1, l'agonista non steroideo, ad alta affinità per GPER **(**G protein-coupled estrogen receptor 1) ha ridotto la crescita tumorale *in vitro* ed *in vivo* attraverso un meccanismo GPER-indipendente. Inoltre, abbiamo osservato che il trattamento con G-1 induce arresto del ciclo cellulare ed apoptosi a seguito di un'attivazione sostenuta di ERK1/2. A partire dai nostri risultati pubblicati, abbiamo eseguito uno studio di microarray che ha chiaramente evidenziato una forte e significativa *up-regolazione* del gene EGR-1 nelle cellule H295R trattate per 24 ore con la concentrazione 1 micromolare di G-1. I risultati di microarray sono stati confermati dalla RT-PCR e dall'analisi Western-blot, nonché dall' immunofluorescenza che ha rivelato una significativa colorazione nucleare per EGR-1 dopo il trattamento con G-1. EGR-1 è un punto di convergenza di molte cascate di segnali intracellulari che controllano la crescita e la proliferazione delle cellule tumorali così come altri segnali che riguardano il meccanismo di morte cellulare. Abbiamo rilevato che l'aumento dell'espressione di Egr-1 era una conseguenza dell'attivazione di ERK, ROS-dipendente mediata da G-1, prontamente invertita dalla presenza dell'antiossidante n-acetil-cisteina. Infine, abbiamo osservato che il silenziamento dell'espressione del gene EGR-1 ha invertito gli effetti principali indotti da G-1 nelle cellule di ACC, inclusa la *up-regolazione* del regolatore negativo del ciclo cellulare, $p21^{Waf1/Cip1}$ e del regolatore positivo della via apoptotica mitocondriale, BAX, così come l'inibizione della crescita

cellulare. L'identificazione della via di segnalazione ROS/MAPK/Egr-1/BAX come potenziale effetto off-target del G-1 potrebbe essere utile nell'implementazione dell'approccio farmacologico per la terapia dell'ACC.

Nell'ultima parte di questo lavoro di tesi abbiamo concentrato la nostra attenzione sul ruolo dell'ERRα, un target a valle di diversi pathways molecolari coinvolti nella patogenesi dell'ACC, la cui attività può essere modulata da diversi co-regolatori, tra cui il colesterolo. In un recente studio del laboratorio del Prof. Pezzi stati testati gli effetti dell'agonista inverso ERRα, XCT790, sulle H295R. Il trattamento con XCT790 (1-5-10 μM) ha diminuito i livelli della proteina ERRα in modo dose-dipendente causando anche un'inibizione dose-dipendente della crescita cellulare dopo 48-72 ore di trattamento. E' stato inoltre studiato l'effetto *in vivo* dell'XCT790 su xenotrapianti di H295R. Topi trattati con XCT790 (2,5 mg / Kg) presentavano una significativa riduzione della crescita del tumore rispetto al gruppo di controllo trattato col veicolo. Inoltre, un aumento dei livelli di colesterolo nel siero è stato riscontrato in uno studio condotto su 152 pazienti con ACC. La maggior parte di questi pazienti sono stati trattati con mitotano, noto per indurre epatotossicità e disregolazione del metabolismo dei lipidi, eventi che potrebbero determinare l'aumento di Chol. Inoltre, i nostri dati sul siero di 28 pazienti con ACC trattati con il mitotano mostrano livelli di Chol superiori ai normali valori fisiologici (248 ± 11 mg / dl, media ± se). L'analisi di questi campioni ha mostrato che i livelli di Chol totali in terapia erano significativamente superiori a quelli prima della terapia (da 167 ± 21 a 229 ± 25 mg / dl). l nostri dati *in vitro* mostrano che la sovraespressione di ERRα in un modello di cellule tumorali corticosurrenali ($H295R^{ERR\alpha}$) è in grado di aumentare la proliferazione cellulare solo quando il siero conteneva lipoproteine. Questi dati suggerisco che ERRα richiede Chol come un agonista funzionale nelle cellule di ACC. Basandoci su questi dati, abbiamo ipotizzato che il trattamento a lungo termine con mitotano, aumentando i livelli di Chol, favorisca l'attivazione di ERRα selezionando un fenotipo di ACC più invasivo e metastatico. Questi dati combinati con i nostri risultati suggeriscono che il complesso Chol/ERRα/PCG1α è un target idoneo a controllare la crescita dell'ACC. Noi ipotizziamo che l'ERRα potrebbe essere un "regolatore principale" di riprogrammazione del metabolismo nell'ACC. In effetti, la deplezione di ERRα nelle cellule H295R ha causato una riduzione della massa e della funzione mitocondriale. Inoltre, i nostri risultati dimostrano che la sovraespressione di ERRα aumenta drasticamente

l'assorbimento di glucosio nelle cellule H295R, supportando l'ipotesi che ERRα sia coinvolto nell'adattamento metabolico dell'ACC. Inoltre il trattamento delle cellule H295R con XCT790 per 24 ore (un tempo inferiore a quello richiesto per causare la morte cellulare), riduce significativamente la migrazione cellulare, al contrario, la sovraespressione di ERRα aumenta questo processo. Di conseguenza, l'espressione ectopica di ERRα aumenta i livelli dei marker dell'EMT (N-Cadherin, Vimentin, Slug), mentre l'XCT790 blocca la loro up-regulazione. Un'altra osservazione interessante è l'aumento delle cellule *anoikis-resisistenti* in presenza della sovraespressione di ERRα. Inoltre la sovraespressione di ERRα nelle H295R aumenta la crescita in condizioni di aderenza indipendente, consente la formazione e aumenta il numero di sferoidi 3D ($H295R^{Sph}$). Abbiamo sviluppato H295R come sferoidi per 5 giorni, dissociati e riseminati settimanalmente nel mezzo per gli sferoidi per 5 settimane ($H295R^{Sph-5}$), prima di testare le cellule per la motilità. La migrazione di queste cellule era superiore alle cellule aderenti. Le $H295R^{Sph-5}$ hanno una maggiore espressione di marcatori mesenchimali come Vimentin, N-cadherin e Slug se confrontati con le H295R coltivate in adesione. Abbiamo valutato gli effetti metabolici indotti dal trattamento con l'agonista inverso del recettore ERRα, XCT790, sulle cellule H295R, tramite l'analisi del tasso di consumo di ossigeno (OCR) e del tasso di acidificazione extracellulare (ECAR). Abbiamo dimostrato che il trattamento con XCT790 influenza profondamente il metabolismo ossidativo e glicolitico delle cellule H295R, infatti è in grado di ridurre il consumo di ossigeno, la capacità glicolitica e la riserva glicolitica, in maniera dose-dipendente. Similmente agli effetti metabolici indotti dall'XCT790, abbiamo osservato che anche le cellule H295R trattate con Simvastatina presentato una funzione mitocondriale ridotta come dimostrato dalla diminuzione dei valori OCR ed ECAR.

In conclusione, nel nostro studio abbiamo valutato gli effetti del trattamento con l'agonista inverso di ERRα e del silenziamento stabile di ERRα in un modello sperimentale di carcinoma adrenocorticale. Complessivamente, questi risultati suggeriscono che ERRα svolge un ruolo fondamentale nella transizione epiteliale-mesenchimale e nella resistenza all'anoikis nelle cellule tumorali di ACC. I risultati ottenuti hanno confermato, nel nostro modello sperimentale, il ruolo funzionale di ERRα nell'attività mitocondriale e nel metabolismo ossidativo e glicolitico cellulare, suggerendo che ERRα svolge un ruolo fondamentale nella riprogrammazione

metabolica delle cellule tumorali di ACC proponendolo come potenziale bersaglio terapeutico del carcinoma adrenocorticale.

Il lavoro è stato oggetto di due pubblicazioni scientifiche:

1. CASABURI, IVAN*****, AVENA, PAOLA*****, **DE LUCA, ARIANNA***, SIRIANNI, ROSA, RAGO, VITTORIA, CHIMENTO, ADELE, TROTTA, FRANCESCA, CAMPANA, CARMELA, RAINEY, WILLIAM E., PEZZI, VINCENZO (2017). *GPER-independent inhibition of adrenocortical cancer growth by G-1 involves ROS/Egr-1/BAX pathway*. ONCOTARGET, vol. 8, p. 115609-115619, ISSN: 1949-2553, doi: 10.18632/oncotarget.23314
*These authors contributed equally to this work.

2. **DE LUCA ARIANNA**, AVENA PAOLA, SIRIANNI ROSA, CHIMENTO ADELE, FALLO FRANCESCO, PILON CATIA, CASABURI IVAN, PEZZI VINCENZO. (2017). *Role of Scaffold Protein Proline-, Glutamic Acid-, and Leucine-Rich Protein 1 (PELP1) in the Modulation of Adrenocortical Cancer Cell Growth.* CELLS, vol. 6, ISSN: 2073-4409, doi: 10.3390/cells6040042

Inoltre, questo lavoro ha contribuito alla realizzazione di due REVIEWS:

1. CASABURI IVAN, CHIMENTO ADELE, **DE LUCA ARIANNA**, NOCITO MARTA, SCULCO SARA, AVENA PAOLA, TROTTA FRANCESCA, RAGO VITTORIA, SIRIANNI ROSA, PEZZI VINCNZO. (2018). *Cholesterol as an Endogenous ERRα Agonist: A New Perspective to Cancer Treatment.* Front Endocrinol (Lausanne). Sep 11;9:525. doi: 10.3389/fendo.2018.00525. eCollection 2018. Review.

2. CHIMENTO ADELE, CASABURI IVAN, AVENA PAOLA, TROTTA FRANCESCA, **DE LUCA ARIANNA**, RAGO VITTORIA, PEZZI VINCENZO, SIRIANNI ROSA. (2018). *Cholesterol and Its Metabolites in Tumor Growth: Therapeutic Potential of Statins in Cancer Treatment.* Front Endocrinol (Lausanne). 2019 Jan 21;9:807. doi: 10.3389/fendo.2018.00807. eCollection 2018. Review.

RATIONALE

It is known that the pathogenesis of adrenal carcinoma is due to an abnormal expression of different genes that can determine the dysregulation of many cellular pathways promoting tumor initiation and progression. The identification of molecule/s involved in the regulatory events of these cellular pathways can help to clarify the altered molecular mechanisms that could also become potential therapeutic targets. The Laboratory of Applied and Cellular Biology of Unical has been working for some years to clarify the involvement of IGF1R and estrogen-dependent pathways in the onset and progression of adrenal carcinoma.

In this thesis work we wanted to investigate the role of three main factors involved in these pathways:

- PELP1 (Scaffold Protein Proline-, Glutamic Acid-, and Leucine-Rich Protein 1);
- EGR-1 (Early growth response gene-1);
- ERRα (Estrogen Related Receptor α).

During the first part of the PhD program, it was investigated the role of Scaffold Protein Proline-, Glutamic Acid-, and Leucine-Rich Protein 1 (PELP1) in the modulation of adrenocortical cancer cell growth.

The second part of the program was focused on the involvement of ROS/Egr-1/BAX pathway in GPER-independent inhibition of adrenocortical cancer growth by GPER-agonist, G-1.

In the last part of the program it was elucidated the molecular mechanisms activated by the Estrogen Related Receptor α (ERRα), a key cellular metabolic factor, that represent a novel potial target to prevent adrenocortical cancer progression. A part of the final stage of the work was completed at the University of Bari Aldo Moro, Department of Biosciences, Biotechnologies and Biopharmaceuticals (Bari, Italy), where a set of different experiments was performed by using the Seahorse XFe96 Extracellular Flux Analyzer, able to monitor in living cells and, at the same time, cellular oxygen consumption rate (OCR), as a measure of mitochondrial respiration capability, and the extracellular acidification rate, (ECAR), as a measure of glycolytic flux within live cells.

Adrenocortical carcinomas (ACC) are rare and highly aggressive tumors, associated with a very poor prognosis, mostly due to a high risk of recurrence and limited therapeutic options (Stojadinovic et al. 2002). Complete surgical excision offers the best chance of long term survival but quite often, despite the complete resection, the tumor reinstates very recurrently (Glover et al. 2013). The cause of adrenal cancer remains elusive, but studies in the past 10 years suggest genetic mutations in the adrenal gland lead to the initiation of a malignant tumor (Libe et al. 2007; Soon et al. 2008; Giordano et al. 2009). However, ACC is an extremely heterogeneous disease and the majority of currently published studies have analyzed only single pathways of signal transduction. It is becoming clear that ACC pathogenesis involves integration of signals and the interplay of downstream pathways. Among these, the IGF system and estrogen-dependent pathways appear to be of particular interest. It has been demonstrated that the insulin-growth factor 2 (IGF-II) gene is strongly over-expressed in adrenocortical carcinomas, representing one of the most commonly identified mutations in ACC (Lafemina and Brennan 2012). IGF-II indicates a reliable prognostic marker in this disease, suggesting that it can be used to identify patients with a high risk of recurrence (Giordano et al. 2003; Samani et al. 2007; Ribeiro and Latronico 2012). In ACC cells, IGF-II induces mitogenic effects through the interaction with the IGF1 receptor (IGF1R), resulting in the activation of the PI3K/AKT/mTOR signaling cascade, as well as RAS/MAPK and the PLC/PKC pathways (Pollak 2008). It is known that estrogens are produced by the enzyme aromatase using androgens as substrate, and we have already shown that ACC is characterized by aromatase overexpression (Barzon et al. 2008). Thus, we can speculate that, in ACC patients, despite the normal circulating estrogen levels, a higher local estrogen production can occur. The classical mechanisms of estrogen action are mainly mediated by two members of the nuclear receptor superfamily, the estrogen receptor (ER) α and β. In ACCs, we previously demonstrated that ERα is upregulated (Barzon et al. 2008) and that estradiol enhances H295R cell proliferation (Montanaro et al. 2005). Recently, the team of Prof. Pezzi demonstrated, both *in vitro* and *in vivo,* the potential involvement of G-coupled-estrogen receptor (GPER) in H295R cell growth that was strongly inhibited by GPER agonist, G1 (Chimento et al. 2015). Furthermore, the growth inhibitory effect was also achieved using XCT790, the ERRα inverse agonist (Casaburi et al. 2015). These results suggest that estrogen signaling and the related nuclear receptors are involved in ACC cell growth. Prof. Pezzi'lab also demonstrated that the existence of a functional interplay

between the IGF-II/IGF1R axis and the estrogen signaling which turned out to be essential in controlling intracellular pathways crucial for ACC proliferation. In particular, it has been demonstrated that IGF-II caused ERα phosphorylation on serine 118 and serine 167 residues, activating ERα in a ligand-independent manner and increasing cell proliferation. On the other hand, activation of IGF-II/IGF1R pathways could be also triggered by ERα. Accordingly, ERα knock-down was more effective than an IGF1R antibody in controlling H295R cell proliferation (Sirianni et al. 2012). However, the molecular mechanisms involved in IGF-II-induced ERα phosphorylation and in E2/ERα activation of IGF-II/IGF1R-dependent pathways in ACC are not completely clearified.

A large number of studies highlighted that, in cancer cells, ER coregulators play a critical role in hormonal responsiveness and tumor progression (Mishra et al. 2004). PELP1/MNAR is a novel ER coactivator that exerts an essential role in ER's actions and its expression is deregulated in hormone-driven cancers. PELP1 appears to function as a scaffolding protein, coupling ER with several proteins including growth factors supporting oncogenesis in terms of cell proliferation and metastasis (Vadlamudi et al. 2004; Migliaccio et al. 2005; Vadlamudi et al. 2005b; Manavathi and Kumar 2006; Rajhans and Vadlamudi 2006). These regulatory interactions have important functional implications in the cross-talk of ER and growth factors signaling (Nagpal et al. 2008; Chakravarty et al. 2010b). Taking into account all of these observations, the main purpose of the first part of this study was to define if PELP1 is expressed in ACC and if it is able to play a role in ACC growth by promoting cross-talk between ERα and IGF1R.

Limited therapeutic options are the main features to deal with when addressing adrenocortical cancer (ACC). Mitotane is the drug that is currently used for the treatment of advanced and metastatic ACC (Glover et al. 2013). However, toxicity, narrow therapeutic window and unwanted side effects represent major limitations to its use as well as therapeutic success (Else et al. 2014; Ronchi et al. 2014). As above reported, we found that ERα expression is up-regulated in ACC and estradiol enhances proliferation of H295R cells (Montanaro et al. 2005; Barzon et al. 2008). Moreover, tamoxifen, a selective estrogen receptor modulator (SERM), inhibits estrogen-and IGF-II-stimulated H295R adrenocortical cancer cell proliferation *in vitro* and reduces H295R xenografts growth (Sirianni et al. 2012). However, in addition to ERα modulation, it has been demonstrated that tamoxifen can act as full agonist on the G protein-coupled

estrogen receptor (GPER) (Vivacqua et al. 2006). G-1 (1-[4-(6-bromobenzo [1, 3]dioxol-5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta-[c]quinolin-8-yl]-ethanone), a non-steroidal GPER agonist, has been developed to dissect GPER-mediated estrogen responses from those mediated by ERα and β (Bologa et al. 2006). Since its discovery, G-1 has been used in a large number of studies to investigate the role of GPER in several systems including the nervous, immune, reproductive and vascular systems as well as cancer (Chimento et al. 2013; Chimento et al. 2014; Prossnitz and Barton 2014). It is worth mentioning that the biological activities triggered by G-1-mediated GPER activation, such as cell proliferation (Vivacqua et al. 2006; Albanito et al. 2015) and/or cell death (Chen et al. 2005; Chimento et al. 2013), appear to be cell type specific and dependent on the ERs expression pattern (Ariazi et al. 2010). The picture becomes even more complex considering the effects elicited by G-1 in a GPER-independent manner (Wang et al. 2012). According to a Prof.'s Pezzi team previous study, G-1 is able to inhibit ACC cell growth both *in vitro* and *in vivo* (Chimento et al. 2015). In particular, cell cycle arrest and activation of the intrinsic apoptotic pathway were triggered by G-1 via long-term sustained ERK phosphorylation in a GPER-independent fashion. The aim of the second part of this study was to define in the G-1-activated pathways in adrenocortical cancer. Transcription analysis defined the gene expression alternations in H295R cells exposed to G-1, that were here investigated.

The study of De Martino et al. (De Martino et al. 2013) investigated a large cohort of advanced ACC and confirmed the presence of a large number of potentially targetable molecules such as mutation of TP53 and CTNNB1. These studies suggest that ACC is a disease extremely heterogeneous and that ACC pathogenesis involves integration of signals and the interplay of downstream pathways. Consequently, one useful strategy to develop an effective therapy for ACC will be to identify downstream target of multiple pathways. A good target could be the ERRα. ERRα is an orphan member of the superfamily of hormone nuclear receptors and it is expressed in several high energy demanding tissues, including heart, skeletal muscle and brain. In addition to its control of energy metabolism and mitochondrial biogenesis, ERRα has recently been associated with cancer progression in which it requires an elevated cell metabolism (Chang and McDonnell 2012). Notably, increased expression of ERRα has been shown in several cancerous tissues, including breast, ovary and colon (Bernatchez et al. 2013). An association between elevated expression of ERRα and a poor clinical outcome in both breast and ovarian tumors was observed in several independent studies (Ariazi et al.

2002; Fujimoto et al. 2007). Several studies suggested that peroxisome proliferator-activated receptor γ coactivator-1 α and β (PGC-1α or PGC-1β) expression level and/or activity could regulate the transcriptional activity of ERRα. The ERRα/PGC-1 complex is a downstream target of multiple signaling pathways in cancer. Several signaling pathways relevant to cancer pathogenesis have been shown to converge upon and regulate the expression and activity of PGC-1α and β, two key ERRα coactivators. It has been shown recently that activation of HER2 and insulin-like growth factor (IGF)-I receptor signaling pathways increase the expression of PGC-1β through induction of c-MYC. ERRα has also been shown to interact with the β-cat/TCF complex and with HIF-1 and reciprocally modulate each other's transcriptional activities to affect cell migration and angiogenesis (Chang and McDonnell 2012). In a recent work (Wei et al. 2016) cholesterol (Chol) has been identified as potential natural ERRα ligand able to increase the recruitment of PGC-1s to ERRα up-regulating its transcriptional activity. This new scenario, characterized by the ability of Chol ability to regulate ERRα/PGC1α complex activity, needs to be investigated particularly in the adrenal gland, a high Chol demanding tissue for steroidogenesis. Moreover, it has been demonstrated in breast cancer that ERRα expression can be regulated by estrogen through ERα and it contributes to regulate aromatase expression (Rajhans et al. 2008). ERRα shares significant sequence homology and structural similarity to ER (Giguere et al. 1988) and recognizes the same responsive elements (Johnston et al. 1997). It was initially considered, therefore, that ERRα might exhibit similar activities as ER and that it would play a role in breast cancer. However, a comprehensive evaluation of the impact of ERRα activation on ERα-dependent transcriptional regulation in MCF-7 breast cancer cells revealed surprisingly few genes that were coregulated by these receptors (Stein et al. 2008). Several studies have reported that ERRα inverse agonist, XCT790, can induce cell growth arrest in different tumors cell lines (Chisamore et al. 2009b; Wang et al. 2010). Few studies have investigated the role of ERRα in adrenal and ACC. ERRα is expressed in normal adult adrenal and regulates the expression of enzymes involved in steroidogenesis (Seely et al. 2005). Moreover, ERRα seems to be more expressed in ACC respect to normal adrenal and adenoma (Felizola et al. 2013). Interestingly, ERRα recognizes the same responsive elements bound by SF-1, a nuclear receptor overexpressed in 90% of ACC (Faria and Almeida 2012). In our recent study (Casaburi et al. 2015) we demonstrated that a dose dependent decrease of ERRα protein content caused also a dose-dependent inhibition of cell growth after 48-72h treatment. We also

investigated the *in vivo* effects of XCT790 on H295R xenografts. Mice treated with XCT790 (2.5 mg/Kg) displayed a significant tumor growth reduction compared to the vehicle treated control group. These data suggest that Chol/ERRα/PCG1α complex is an eligible target to control ACC growth. We hypothesize that ERRα could be a "master regulator"of reprogramming metabolism in ACC. In fact, ERRα depletion in H295R cells caused a reduction of mitochondrial mass and function (Casaburi et al. 2015).

Tumor metastasis involves a series of interrelated events including angiogenesis, epithelial to mesenchymal transition (EMT) and invasion. The ability of cancer cells to switch from a predominantly oxidative metabolism to glycolysis, even when oxygen is plentiful, causes lactate production and consequently an acidic environment (Gatenby and Gawlinski 2003). This leads to extracellular matrix degradation by proteolytic enzymes (Lardner 2001) and normal cell death (Williams et al. 1999) enhancing cancer cell migration and invasion. Experimental observations support the hypothesis for a role of ERRα in invasion, angiogenesis and metastasis in several tumors (Deblois et al. 2013). The absence of ERRα is able to impair tumorigenic potential in aggressive xenografted breast cancer cells (Stein et al. 2009). In addition, ERRα/PGC-1α complex binds to the promoter of VEGF regulating its expression, promoting tumor angiogenesis and invasion (Stein et al. 2009).

The aim of the last part of this study is to elucidate the role of ERRα in the reprogramming cellular metabolism associated with ACC cell motility, invasion, angiogenesis and metastases.

Background

1. Human adrenal gland

1.1 The adrenal gland: general structure

In mammals, the adrenal glands (also known as suprarenal glands) are endocrine glands that sit at the top of the kidneys (Figure 1.1).

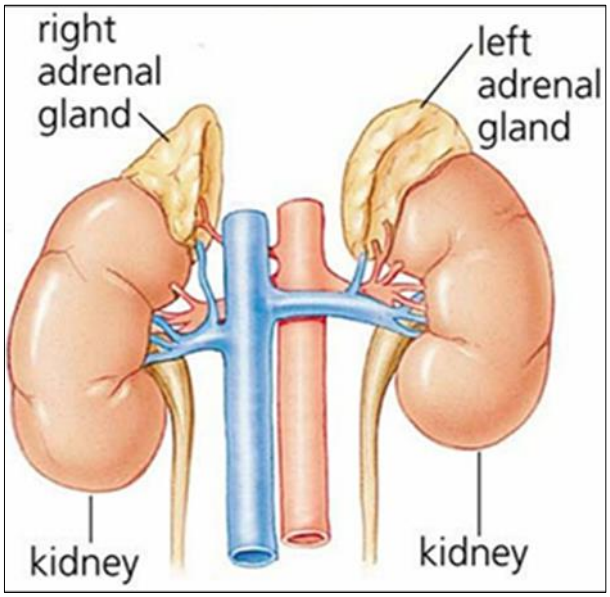


Fig. 1.1 Human adrenal gland.

The adrenal glands are located bilaterally in the retroperitoneum superior and slightly medial to the kidneys. In humans, the right adrenal gland is triangular in shape, whereas the left adrenal gland is semilunar in shape; in non-humans, they are quadrilateral in shape. The combined weight of the adrenal glands in an adult human ranges from 7 to 10 grams. They are surrounded by an adipose capsule and renal fascia.

It is now known that the adrenal gland consists of two ontogenetically, structurally and functionally distinct endocrine tissues, the cortex and the medulla. The cortex is mesodermal in origin and derived from proliferation of the coelomic epithelium. It produces various steroids with specific functions as will be described later. The medulla, on the other hand, is ectodermal in origin and neural crest-derived. It secretes

catecholamines, *i.e.*, adrenaline and noradrenaline, that facilitate the acute mammalian stress or "fight-or-flight" response.

The adrenal glands affect kidney function through the secretion of aldosterone, and recent data suggest that adrenocortical cells under pathological as well as under physiological conditions show neuroendocrine properties; within normal adrenal glands, this neuroendocrine differentiation seems to be restricted to cells of the zona glomerulosa and might be important for an autocrine regulation of adrenocortical function.

1.2 Embriology and development

The adrenal gland is two distinct endocrine organs that have separate embryological origins and physiologic functions; the mesoderm-derived cortex secretes steroid hormones while the neural crest-derived medulla secretes catecholamines (Else and Hammer 2005). Formation of the adrenal gland occurs in several distinct developmental events (Else and Hammer 2005; Kim and Hammer 2007) (Figure 1.2). During the 4th week of gestation in humans (E9.0 in mice), proliferation of mesoderm-derived cells of the coelomic epithelia and underlying mesonephros results in coalescence of the adrenogonadal primordium (AGP), defined by expression of the nuclear receptor NR5a1 (Steroidogenic factor 1, Sf1) (Hatano et al., 1996; Luo et al., 1994). At the 8th week of gestation in humans (E10.5 in mice), the bipotential AGP separates into discrete adrenal primordia (fetal adrenal zone) and gonadal primordial (Hatano et al. 1996; Kim and Hammer 2007). The segregation of a discrete adrenal primordia from the AGP involves a Wilm's tumor 1 (Wt1) and Cited2-mediated upregulation of Sf1 expression (Val et al. 2007). Once separated from the AGP, the adrenal primordial activates Sf1 expression through an entirely different mechanism – the recruitment of the homeobox protein PKNOX1 (Prep1), homeobox gene 9b (Hox) and pre B-cell leukemia transcription factor 1 (Pbx1) to a fetal adrenal-specific Sf1 enhancer (FAdE) (Zubair et al. 2008). Sf1 itself maintains FAdE-dependent expression of Sf1 in the adrenal primordia over time through autoregulation of Sf1 expression. Proliferation of fetal adrenocortical cells is believed to be under control of fetal pituitary-derived adrenocorticotropic homormone (ACTH) (Mesiano et al. 1993). However, insulin like growth factor 2 (IGF2) is expressed throughout the fetal adrenal cortex and several studies have suggested ACTH mediates some of its effects on proliferation through

IGF2 action (Ilvesmaki et al. 1993; Stratta et al. 2003; Coulter 2005). Concurrent with activation of FAdE-driven Sf1 expression at embryonic day E11.5-12.5 in mice (equivalent to 8–9th week of gestation in humans), neural-crest-derived chromaffin progenitor cells migrate into the central fetal gland. These cells form the adrenal medulla followed by the coalescence of the mesenchymal capsule around the fetal adrenal gland (Else and Hammer 2005). Before encapsulation is complete, the development of the definitive cortex (definitive zone or adult cortex) is initiated between the capsule and the fetal zone. While the fetal cortex ultimately regresses in all species, the timing of regression is species-specific; in humans the fetal zone regression occurs at birth while in mice the zone persists until puberty in males or the first pregnancy in females (Kim et al. 2009). In humans, functional zonation of the adult cortex into unique concentric steroidogenic regions initiates at birth concurrent with the coalescence of the adrenal medulla (Beuschlein et al. 2002).

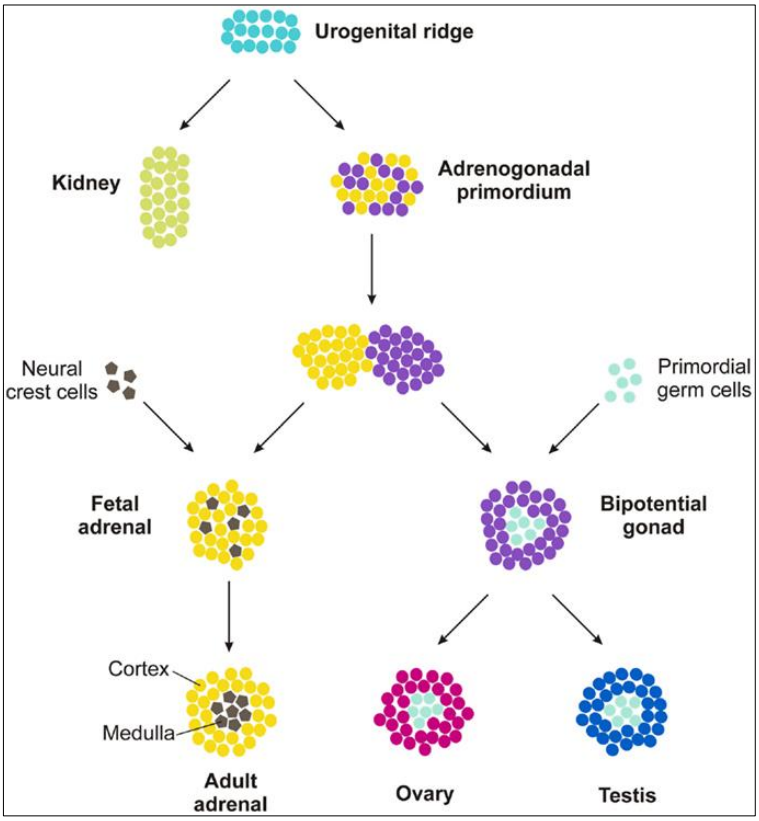


Fig. 1.2 Adrenal and ganad early development.

1.3 Histology

The adrenal cortex is composed of three functionally distinct regions, the *zona glomerulosa* (ZG) lying immediately below the capsule and corresponding to approximately 15% of cortical volume characterized by cells organised in rounded clusters around capillary coils or glomeruli, *zona fasciculata* (ZF) corresponding to up to 75% of cortical volume, characterized by cells arranged in radial rows separated by trabeculae and by blood vessels and *zona reticularis* (ZR) that lies next to the medulla, in which cells are located within a uniform reticular net of connective tissue and blood vessels (Miller WL, 2008). The ZG synthesizes mineralocorticoids; the ZF produces cortisol and the ZR secretes the so called adrenal androgens, DHEA and DHEA-sulfate. Each zone is preferentially regulated by different circulating factors that include angiotensin II (Ang II) and potassium (K+) for the ZG, adrenocorticotropic hormone (ACTH) for the ZF, and ACTH plus other yet to be determined factors for the ZR (Wang and Rainey 2012) (Figure 1.3). It has been established that the reason each zone secretes a unique set of steroids is related to the selective expression of steroid-metabolizing enzymes within each zone (Rainey 1999; Rainey et al. 2002; Nguyen and Conley 2008). However, the molecular mechanisms that cause zone-specific expression patterns of enzymes are yet to be resolved. Adrenal steroid production remains an area of active research, which supports the need to develop appropriate cell models that can mimic adrenal physiology or pathology. Primary cultures of adrenocortical cells have proven to be useful for examining the mechanisms controlling many aspects of adrenal physiology (Chen et al. 2006; Kuulasmaa et al. 2008; Cardoso 2009; Xing et al. 2010; Xing et al. 2011). However, several issues have limited the use of primary adrenal cells as in vitro models. The most common limitations are the constant requirement for fresh tissue and the difficulties associated with the isolation of adequate cortical cells. In addition, cells from different human donors are subject to considerable variability; whereas cells from rodents do not produce cortisol or adrenal androgens due to the lack of steroid 17ahydroxylase (CYP17)expression. To overcome the problems with tissue accessibility and quality, many groups have attempted to establish cell lines from adrenocortical carcinomas. This approach has been somewhat successful leading to adrenal cell lines from several species and we have previously reviewed the overall development of these models (Mountjoy et al. 1994; Rainey et al. 2004).

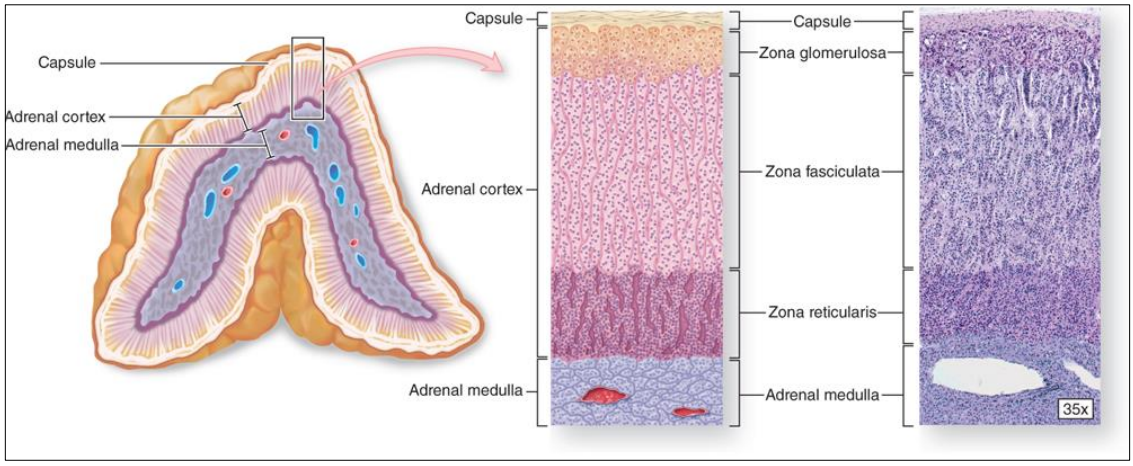


Fig. 1.3 Adrenal cortex regions.

1.4 Adrenalcortical steroidogenesis

Steroid hormones regulate a wide variety of developmental and physiological processes from fetal life to adulthood. Steroid hormones are all synthesized from cholesterol and hence have closely related structures based on the classic cyclopentanophenanthrene 4-ring structure. The human adrenal can synthesize cholesterol *de novo* from acetate (Mason and Rainey 1987), but most of its supply of cholesterol comes from plasma low-density lipoproteins (LDLs) derived from dietary cholesterol (Gwynne and Strauss 1982). By contrast, rodent adrenals derive most of their cholesterol from high-density lipoproteins via a receptor termed scavenger receptor B1, but this pathway appears to play a minor role in human steroidogenesis. The intracellular cholesterol economy is largely regulated by the sterol response element binding protein (SREBPs), a group of transcription factors that regulate genes involved in the biosynthesis of cholesterol and fatty acids (Horton et al. 2002). Adequate concentrations of LDL will suppress 3-hydroxy-3-methylglutaryl co-enzyme A reductase, the rate-limiting enzyme in cholesterol synthesis. ACTH also stimulates the activity of 3-hydroxy-3-methylglutaryl co-enzyme A reductase, LDL receptors, and uptake of LDL cholesterol. LDL cholesterol esters are taken up by receptor-mediated endocytosis, and are then stored directly or converted to free cholesterol and used for steroid hormone synthesis (Brown and Goldstein 1979). The first step in steroidogenesis takes place within mitochondria. The mechanisms by which cholesterol is transported to and loaded into the outer mitochondrial membrane (OMM) remain an active area of research (Chang et al. 2006;

Miller 2007) the principal action of StAR is to facilitate the movement of cholesterol from the OMM to the inner mitochondrial membrane (IMM). Some cholesterol may be incorporated into vesicular membranes that then fuse with other membranes, thus delivering cholesterol from one intracellular compartment to another, but this appears to be a minor pathway (Soccio and Breslow 2004). Instead, cholesterol is solubilized by binding to proteins. A steroidogenesis abnormality can often be life threatening. Congenital adrenal hyperplasia (CAH) is one of the most common disorders caused by deficiency of any enzyme involved in steroidogenesis in adrenal glands (Claahsen-van der Grinten et al. 2011; White and Bachega 2012), Impaired cortisol and aldosterone production increases adrenocorticotropic hormone (ACTH) secretion from the pituitary gland, leading to adrenal hyperplasia and accumulation of adrenal androgens. Female patients are prenatally virilized because of excess androgen and neonates of both genders may suffer from a life-threatening Addisonian crisis. Steroid hormone deficiency also occurs in aging people by hypogonadism. Most enzymes involved in steroid biosynthesis are either cytochrome P450s (CYPs) or HSDs. These steroidogenic enzymes are functionally, if not absolutely, unidirectional, so the accumulation of products does not drive flux back to the precursor. All P450-mediated hydroxylations and carbon-carbon bond cleavage reactions are mechanistically and physiologically irreversible (Hall et al. 1986) (Figure 1.4). Cytochrome P450 is a generic term for a group of oxidative enzymes, all of which have about 500 amino acids and contain a single heme group (Gonzalez 1988). The human genome includes genes for 57 cytochrome P450 enzymes (Lander et al. 2001; Venter et al. 2001). The genes are now formally termed *CYP* genes. Seven human cytochrome P450 enzymes are targeted to the mitochondria and are termed “type 1”; the other 50 human P450 enzymes are targeted to the endoplasmic reticulum and are termed “type 2.” All P450 enzymes activate molecular oxygen using their heme center and add electrons from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). Type 1 enzymes receive electrons from NADPH via a flavoprotein termed ferredoxin reductase and a small iron-sulfur protein termed ferredoxin, whereas type 2 P450 enzymes receive electrons from NADPH via a single 2-flavin protein termed P450 oxidoreductase (POR) (Miller 2005). Six P450 enzymes are involved in steroidogenesis.

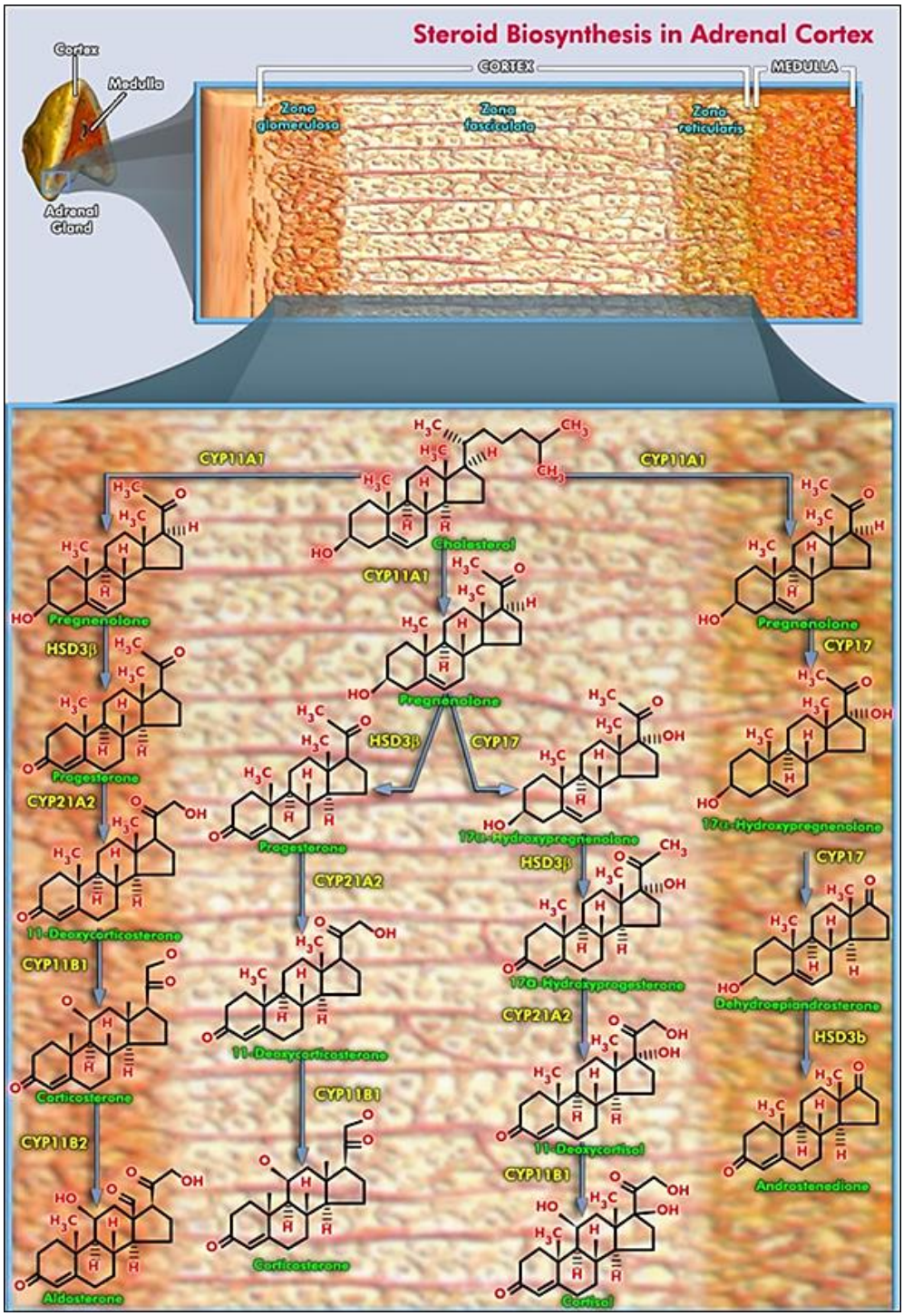


Fig. 1.4 Pathway of steroid biosynthesis in adrenal cortex.

Mitochondrial P450scc is the cholesterol side-chain cleavage enzyme catalyzing the series of reactions formerly termed "20,22 desmolase." The two isozymes of mitochondrial P450c11, P450c11β (11β-hydroxylase) and P450c11AS (aldosterone synthase), catalyze 11β-hydroxylase, 18-hydroxylase, and 18-methyl oxidase activities. In the endoplasmic reticulum, P450c17 catalyzes both 17α-hydroxylase and 17,20-lyase activities, P450c21 catalyzes 21-hydroxylation in the synthesis of both glucocorticoids

and mineralocorticoids, and P450arom catalyzes aromatization of androgens to estrogens. The HSDs have molecular masses of about 35 to 45 kDa, do not have heme groups, and require nicotinamide adenine dinucleotide (phosphates) (NADH/NAD+ or NADPH/NADP+) as cofactors; based on their activities, it is physiologically more useful to classify the HSDs as dehydrogenases or reductases. The dehydrogenases use NAD+ as their cofactor to oxidize hydroxysteroids to ketosteroids, and the reductases mainly use NADPH to reduce ketosteroids to hydroxysteroids (Agarwal and Auchus 2005; Sherbet et al. 2007).

1.5 The steroidogenic regulatory protein

Unlike cells that produce polypeptide hormones, which store large amounts of hormone in secretory vesicles ready for rapid release, steroidogenic cells store very little steroid. Thus, a rapid steroidogenic response (*e.g.*, adrenal secretion of aldosterone and cortisol in response to stress or the "pulsing" of sex steroids in response to an LH surge) requires rapid synthesis of new steroid. ACTH promotes adrenal steroidogenic cell growth. This growth occurs primarily by ACTH stimulating the production of cAMP, which in turn promotes the synthesis of IGF-II (Mesiano et al., 1993; Voutilainen and Miller, 1987), basic fibroblast growth factor (Mesiano et al. 1991), and epidermal growth factor (Coulter et al., 1996). Together, these growth factors stimulate adrenal cellular hypertrophy and hyperplasia, determining the amount of steroidogenic tissue. Second, acting over days, ACTH acts through cAMP, and angiotensin II acts through the calcium/calmodulin pathway to promote the transcription of genes encoding various steroidogenic enzymes and electron-donating cofactor proteins, thus determining the amount of steroidogenic machinery in the cell. Third, ACTH rapidly stimulates StAR gene transcription (Stocco et al., 2005) and phosphorylation of Ser195 in extant StAR (Arakane et al., 1997) to increase the flow of cholesterol from the OMM to the IMM, where it becomes substrate for the first and rate-limiting enzyme, P450scc. This acute response occurs within minutes and is inhibited by inhibitors of protein synthesis (*e.g.*, puromycin or cycloheximide), indicating that a short-lived protein species mediates this process. All microsomal (type 2) cytochrome P450 enzymes, including steroidogenic P450c17, P450c21, and P450aro, receive electrons from POR, a membrane-bound flavoprotein that is a different protein from the mitochondrial flavoprotein, ferredoxin reductase (Miller, 2005). Nuclear magnetic resonance and x-ray scattering data have

recently confirmed this view that POR undergoes these dramatic conformational changes while receiving and then transferring electrons (Ellis et al., 2009).

1.5.1 StAR protein

For steroidogenesis, free cholesterol transport across mitochondrial membranes into the mitochondria is facilitated by the steroidogenic acute regulatory protein (STAR). The role of this protein has been well demonstrated in patients with mutations in the gene encoding STAR in the disorder termed congenital lipoid adrenal hyperplasia wherein the mitochondria from the adrenals and gonads of these patients are unable to convert cholesterol to pregnenolone (Lin et al. 1995). It has been suggested that the protein StAR put directly in contact, in the mitochondria, the inner membrane with the external one, allowing the passage of cholesterol, according to the concentration gradient (Bose et al. 1999).

1.5.2 P450$_{SCC}$

The initial and rate-limiting step in the pathway leading from cholesterol to steroid hormones is the cleavage of the side chain of cholesterol to yield pregnenolone. This step is catalysed by the inner mitochondrial membrane bound cholesterol side chain cleavage enzyme (P450scc, CYP11A1) (Morohashi et al. 1987), and involves three distinct chemical reactions: 20α-hydroxylation, 22-hydroxylation, and scission of the cholesterol side-chain to yield pregnenolone and isocaproic acid (Lambeth and Pember 1983). In human adrenal gene transcription is regulated by ACTH, by gonadotropins in testis and ovary and by unknown factcors in placenta all activated through cAMP as intracellular second messenger (Kimura and Suzuki 1967). Each catalytic cycle requires a molecule of NADPH and one molecule of oxygen (Figure 1.5).

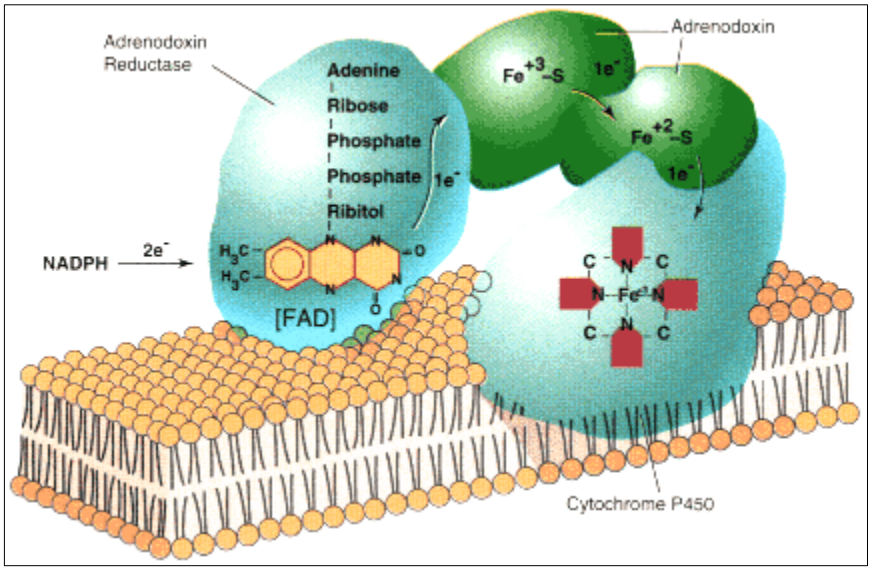


Fig. 1.5 Electron transport to mitochondrial forms of cytochrome P450.

1.5.3 P450$_{C17}$

P450$_{C17}$ is the steroidogenesis qualitative regulator. It presents both 17-α-hydroxylase and C-17, 20-lyasic activities and represents a strategic point in the synthesis of steroid hormones as it can direct pregnenolone toward mineralocorticoids, glucocorticoids or sex steroids synthesis. Pregnenolone and progesterone can form respectively, 17-α-hidroxy-pregnenolon (17-OH-Preg) and 17-α-hydroxyprogesterone (17-OH-Prog) after 17-α-hydroxylation. These 17-hydroxylated steroids then can be cleaved to give C17/20 DHEA and androstenedione, respectively. When the P450$_{C17}$ is absent, as in the zona glomerulosa, the products are C-21 17-deoxy steroids such as aldosterone. When the activity of 17-α-hydroxylase is present products are C-21 17-hydroxysteroids such as cortisol. Instead, when there are 17-α-hydroxylase and 17, 20 P450$_{C17}$ liasica activities the products are C-19 precursors of sex steroid hormones.

1.5.4 P450$_{C21}$

Progesterone and 17-OH-Prog, once synthesized, are hydroxylated in position 21 to give rise respectively to DOC (deoxycorticosterone) and 11-deoxycortisol. The P450 reductase P450$_{C21}$ uses the same used by P450$_{C17}$ for the transport of electrons from NADPH and is encoded by the gene called CYP21.

1.5.5 $P450_{C11}$ - $P450_{C18}$

P450C11 and P450C18 are located in the inner mitochondrial membrane. The human genome has two P450 genes located on chromosome 8 between bands q13 and q22 (Kawainoto et al. 1990). These two genes encode P450 proteins that have 93% amino acid sequence identity. P450C11 is encoded by the gene CYP11B1; it is significantly expressed in the fasciculata zone and is the only with 11-β-hydroxylase activity. The related gene, the P450C18, is encoded by CYP11B2 and expressed, at very low level, only in the zona glomerulosa. The P450C18 has 11-β-hydroxylase, 18-hydroxylase and 18-oxidase activities (Malee and Mellon 1991). The gene CYP11B1, which encodes for P450C11, required for the synthesis of cortisol, is regulated by ACTH, whereas CYP11B2 gene, which encodes the P450C18 required for the synthesis of aldosterone, is regulated by angiotensin II (Ang II), sodium and potassium.

1.5.6 P450arom: Aromatase

Estrogens are produced by the aromatization of androgens by a complex series of reactions catalyzed by a single microsomal aromatase, P450aro (Grumbach and Auchus, 1999; Simpson et al., 2002; Simpson et al., 1994). This typical cytochrome P450 is encoded by a single gene on chromosome 15q21.1. This gene uses several different promoter sequences, transcriptional start sites, and alternatively chosen first exons to encode aromatase mRNA in different tissues under different hormonal regulation. The *CYP19A1*gene for P450aro spans over 75 kb (Mahendroo et al., 1991) and contains five different transcriptional start sites (Mahendroo et al., 1993) with individual promoters that permit the tissue-specific regulation of its expression in diverse tissues. P450aro is a glycoprotein, but glycosylation *per se* does not appear to affect activity (Shimozawa et al., 1993). The p450aro oxidative demethylation action of C19 steroids, mainly androstenedione and testosterone, consumes three equivalents of molecular oxygen and NADPH, yielding formic acid and C18-steroids with an aromatic A-ring (Simpson et al., 1994).

1.5.7 Isozymes of 5α-Reductase

The 5α-reductases are important beyond the context of male genital differentiation and androgen action because both isozymes reduce a variety of steroids in degradative pathways. Progesterone, 17OHP, and related C21steroids are excellent substrates for both 5α-reductases, particularly the type 1; cortisol, cortisone, corticosterone, and

related compounds are also good substrates (Frederiksen and Wilson, 1971). Such 5α- (and 5β-) reduced steroids may be metabolized further and conjugated for excretion in the urine. Inhibitors of the type 2 enzyme have been developed for the treatment of prostatic hyperplasia and the prevention of its recurrence after surgery(McConnell et al., 1998): finasteride selectively inhibits human 5α-reductase type 2, whereas dutasteride inhibits both isoenzymes. These drugs are approved for treatment of prostatic hyperplasia in the United States.

1.5.8 3βHSD

Once formed, pregnenolone can be converted into 17- idrossipregnenolone by P450C17 or in progesterone by 3-β-hydroxysteroid dehydrogenase Δ4-5Δ isomerase, encoded by the HSD3B gene.

This enzyme presents two activities: 3-β-hydroxysteroid dehydrogenase and isomerase activities. In humans there are at least two forms of HSD3B, encoded by different genes:
- the gene for HSD3B type I (HSD3B1) is expressed in placenta, skin, mammary gland;
- the gene for HSD3B type II (HSD3B2) is expressed in adrenal glands and gonads. Both genes are on band p13 of chromosome 1 (Berube et al. 1989).

1.5.9 β-steroid-sulfotransferase-sulfatase

The steroid sulfates can be synthesized directly from cholesterol sulfate or may be formed by sulfation of steroids such as DHEA, by means of a cytosolic sulphate transferase leading to DHEA-S, encoded by the gene SULT2A1.

The steroid sulfates can also be converted by hydrolysis in native form using a steroid-sulfatase.

1.5.10 17-chetosteroid-reductase

In adrenal, DHEA can be converted in Δ5-androstenediol and Δ4-androsterone in testosterone through 17-chetosteroid-reductase (17-CHSR) activity. Δ5-androstenediol, testosterone and estradiol can also be converted respectively in DHEA, Δ4-androstenedione and estrone by the same enzyme, tank to a reversible activity known as 17-β- hydroxysteroid dehydrogenase (17-β-HSD). So this enzyme presents both androgenic and estrogenic 17-CHSR characteristics.

2. Adrenocortical cancers

2.1 Introduction

Tumors that originate from the adrenal cortex can be divided into benign adenomas and malignant adenocarcinomas. They differ from other cancers because the cancer may be associated to an endocrine component (Allolio and Fassnacht 2006).

Secreting forms are responsible for the onset of endocrine syndromes which vary depending on the type of hormone produced in excess: • Cushing's syndrome, caused by hypersecretion of cortisol; • Conn's syndrome, caused by aldosterone hypersecretion; • hirsutism and virilization, caused by hypersecretion of androgens.

ACC can be asymptomatic or can present with symptoms of hormone excess or complaints referable to the mass (Brennan 1987; Schulick and Brennan 1999a). Generally ACC present an immature steroidogenesis and almost all of these tumors exhibit hormonal precursor excess but, approximately, 60% of all ACC patients will present with hormone-related signs and symptoms (so-called “functional tumors”) (Schulick and Brennan 1999a; Schulick and Brennan 1999b).

Differential diagnosis between ACA and ACC is of pivotal clinical relevance, as the prognosis and clinical management of benign and malignant ACTs is entirely different. Imaging techniques including computed tomography, magnetic resonance imaging and positron emission tomography with 18F-2-fluoro-2-deoxy-D-glucose (FDG-PET) can be used for assessing malignancy, but none of these techniques are absolutely reliable (Terzolo et al. 2011; Morelli et al. 2013). It is very difficult to establish malignancy in small adrenal tumors and to exclude it in large tumors with the available imaging techniques. Currently used guidelines propose to remove adrenal tumors with a diameter of >6 cm, as they are associated with a risk of malignancy >25% (Aron et al. 2012). Some hormonal features (eg, androgen secretion characteristic for malignant tumors) can also be exploited in diagnosis. Most recent data using urinary steroid hormone metabolomics showed characteristic patterns of steroid secretion and metabolism in ACC samples (Arlt et al. 2011). The histological diagnosis of malignancy is also often difficult (Patalano et al. 2009) and novel markers of

malignancy are intensively searched for using bioinformatics approaches to establish an early and specific differential diagnosis between ACC and ACA.

2.2 Adrenocortical adenoma

It is a benign neoplastic proliferation of adrenocortical cells almost always associated with clinical, histological and instrumental evidences of hyperfunction.

Dimensions are variable depending on the hormone produced:

- adenoma with hyperaldosteronism is usually unilateral and of yellowish color, around 1.5 cm of size and non-enveloped;
- adenoma with hypercortisolism is unilateral, has dimensions of about 4 cm, is yellow-brown and is encapsulated;
- adenoma with virilization is unilateral, has dimensions of about 5 cm, is red-brown and is encapsulated.

In many patients, adrenal adenomas is asymptomatic, except for begning hormones producing tumors. In case of large tumors, patients may have symptoms due to the compression of other organs, such as feeling of abdominal fullness or localized abdominal pain. More frequent with advancing age, adrenocortical adenomas have a peak between 50 and 70 years and the most affected are women (58%) and the right side.

2.3 Adrenocortical carcinoma

People per year with an increased incidence in the first and fourth-fifth decades of life. By gender, females are the most affected (55-60%) (Else et al. 2014). ACC is burdened by a poor prognosis with a mean 5-year survival rate between 16 and 47%, falling to 5-10% in the advanced stages (Barlaskar and Hammer 2007). The cornerstones in the pathogenesis of ACC are considered to be the genetic alterations of the IGF-2, p53 and β-catenin molecular pathways (Barlaskar and Hammer 2007; Ragazzon et al. 2011). Additionally, other genes, such as ZNFR3, identified by a genome-wide study, appear potentially involved in the tumorigenesis of ACC (Assie et al. 2014b). Comparative genomic hybridization (CGH) demonstrated several complex mutations in ACC with chromosomal gains at 4q, 4p16, 5p15, 5q12-13, 9q34, 12q13, 12q24, 19p and losses at 1p, 2q, 11q, 17p, 22p and 22q. Genes within these regions that are potentially involved

in neoplastic transformation include fibroblast growth factor 4 (*FGF4*), cyclin-dependent kinase 4 (*CDK4*), and cyclin E1 (*CCNE1*) (Else et al. 2014). A recent epigenetic study performed on 51 ACCs identified a promoter hypermethylation of the *H19, GOS2, PLAGLI* and *NDRG2* genes (Else et al. 2014). However, it has been recently observed that the dysregulation of some miRNAs, such as the upregulation of miR-483 and the downregulation of miR-195 and miR-335, could play a substantial role in the ACC tumorigenesis (Assie et al. 2014a).

When ACC manifests as a condition of steroid hormone excess, the clinical picture is dominated mainly by hypercortisolism and/or hyperandrogenism, whereas symptoms of estrogen hypersecretion such as gynaecomastia and testicular atrophy are pathognomonic in male patients (Fassnacht et al. 2011). DHEA-S represents a possible hormonal marker of ACC, conversely a decreased serum DHEA-S concentration likely indicates an adrenal adenoma (Fassnacht et al. 2004). Mineralocorticoid excess is a rare event, that occurs with severe hypertension and hypokalemia. Notably however, an excess of glucocorticoids could produce a similar effect (Fassnacht et al. 2011). Although few ACC appear non-secreting, they may produce an excessive amount of adrenal precur- mass spectrometry methods revealed that >95% of ACC patients are able to autonomously secrete steroids or steroid precursors (Arlt et al. 2011). Imaging plays a key role in the diagnosis of primary ACC, in the involvement of surrounding tissues and in its spread to distance sites. Either computerized tomography (CT) or magnetic resonance imaging (MRI) exploiting particular features such as Hounsfield unit (HU) values chemical shift imaging, respectively allow adequate diagnostic accuracy to be achieve (Blake et al. 2006; Terzolo et al. 2011) as suggested by a recent analysis of the German ACC registry showing that the value of 13 HU may be considered as the threshold for benign from malignant adrenal masses (Petersenn et al. 2015). More recently, the fluorine 18 fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) or PET/CT was introduced as a diagnostic tool for ACC (Wong et al. 2011). 11C-metomidate, due to its particular ability to bind 11 β-hydroxylase, has been proposed for the identification of tumors of adrenocortical origin (Hennings et al. 2006). The introduction of [123I]IMTO for single photon emission computed tomography (SPECT) and planar scintigraphy has provided a diagnostic alternative to PET for the discrimination of adrenal masses from non-adrenal tissues (Hahner et al. 2013).The first official TNM classification for ACC was established only in 2004 by the International

Union Against Cancer (UICC) and the World Health Organization (WHO). It was based on the criteria described by MacFarlane (Macfarlane 1958) and later modified by (Sullivan et al. 1978). A significant improvement in the prognostic assessment was due to the adoption of the ENSAT (European Network for the Study of Adrenal Tumors) ACC staging system, which proposes a careful prognostic differentiation among the stages (Fassnacht et al. 2009) (Table I). The use of this system in recent years has greatly improved the diagnostic accuracy and the prediction of survival for stage compared to the criteria previously adopted (Lughezzani et al. 2010).

Stage
1. T1, N0, M0
2. T2, N0, M0
3. T1-T2, N1, M0; T3-T4, N0-N1, M0
4. any T, any N, M1

Table I. Staging system for ACC proposed by the European Network for the Study of Adrenal Tumors (ENSAT).

Classification criteria of the tumor stage according to the TNM system: Stage 1: T1, tumor ≤5 cm; N0, no positive lymph nodes; M0, no distant metastases. Stage 2: T2, tumor >5 cm; N0, no positive lymph nodes; M0, no distant metastases. Stage 3: T1, tumor ≤5 cm - T2, tumor >5 cm; N1, positive lymph node(s); M0, no distant metastases; T3, tumor infiltration into surrounding tissue - T4, tumor invasion into adjacent organs or venous tumor thrombus in vena cava or renal vein. N0, no positive lymph nodes - N1, positive lymph node(s). M0, no distant metastases. Stage 4: T1, tumor ≤5 cm - T2, tumor >5 cm; T3, tumor infiltration into surrounding tissue - T4, tumor invasion into adjacent organs or venous tumor thrombus in vena cava or renal vein; N0, no positive lymph nodes - N1, positive lymph node(s). M1, presence of distant metastasis.

2.3.1 Treatment options

ACC is a neoplastic disease with a poor prognosis. Current studies in this field have indicated the need for a multidisciplinary approach in the management of this tumor (Creemers et al. 2016; Stigliano et al. 2016). Surgery remains the most effective treatment choice for the primary tumor or in for the removal of isolated metastases (Crucitti et al. 1996; Else et al. 2014). The experience that at least one-third of patients show loco-regional recurrence or distant metastases even after a radical surgical excision introduced the concept of adjuvant therapy in these patients (Donatini et al.

2014). Despite an extensive surgical resection, the survival rate of these patients is estimated as ~50% after 5 years (Vaughan 2004). Although these data support the need for an adjuvant cancer therapy, the therapeutic options in ACC currently remain under debate. At present, mitotane represents the only drug approved in Europe and in the United States for ACC treatment; however opinions regarding its use in adjuvant settings are still highly discordant (Huang and Fojo 2008). Currently, chemotherapy is reserved for those cases of advanced disease with evidence of distant metastases unresponsive to mitotane treatment. Many efforts are directed to the development of targeted therapy in ACC. Several strategies have been developed *in vitro* and some clinical trials have been conducted with small molecules, such as inhibitors of tyrosine kinase receptors or serine/threonine kinase receptors and monoclonal antibodies.

2.3.2 Surgery

Surgery is the only truly effective therapy in the treatment of ACC. A complete surgical resection (R0) is the treatment of choice, avoiding tumor spread that is considered an adverse prognostic factor. The achievement of R0 resection status often requires a radical surgery with a wide dissection of the neighboring organs. It represents a predictor of long-term survival (Icard et al. 2001). The choice of an open approach vs laparoscopic approach is debated. Open adrenalectomy is classically the more secure treatment recommended in patients with localized (stage I-II) and locally advanced (stage III) ACC. Comparative data concerning the two surgical techniques are lacking and originate from retrospective data that involved selection bias (Porpiglia et al. 2010; Lombardi et al. 2012). It is likely that laparoscopic surgery might be reserved only for selected cases with masses of small size. However, these statements must be confirmed by prospective trials. Regardless of the surgical option chosen, the surgical team must have proven experience in the oncologic ACC surgery. Although lymphadenectomy has never been considered as a standard procedure in the adrenalectomy, recent studies show that lymph nodes dissection is significantly associated with a reduction of the relapse rate in patients with localized disease (Gaujoux and Brennan 2012; Reibetanz et al. 2012). However, confirmatory data are needed in order to standardize the surgical procedure. The therapeutic option of removing metastases is founded on the observation that their excision is associated with longterm survival (Kemp et al. 2011; Ripley et al. 2011) and the consideration that many ACC are metastatic at the onset (Stojadinovic et al. 2002). Encouraging results from several retrospective studies show that the

metastasectomy correlates with an improvement of progression-free and overall survival (Datrice et al. 2012; Erdogan et al. 2013). Finally, although the objective of debulking surgery is to reduce either the compressive effect exerted by a large size mass, on surrounding organs or the hormonal excess secreted by the tumor, lacking in this surgical approach is an oncological rationale (Crucitti et al. 1996).

2.3.3 Mitotane

Currently, mitotane represents the only therapeutic option approved by the US Food and Drug Administration and European Medicine Executive Agency for the treatment of ACC (Schteingart et al. 2005). Mitotane is a derivative of the insecticide dichlorodiphenyldichloroethane (DDT) with adrenolytic and cytotoxic activity toward the fasciculata and reticularis adrenal areas. It inhibits steroidogenic pathways acting mainly at the level of the cholesterol side chain cleavage enzymes CYP11A1 and CYP11B1 (Lin et al. 2012; Lehmann et al. 2013). Mitotane metabolites (o',p-DDA and o',p- DDE, respectively) are the products of a hydroxylation that occurs in the liver and of which o',p-DDA represents the active form (van Slooten et al. 1984). It has indeed been shown, that o',p-DDA measurements reflect the mitotane response in treated patients (Hermsen et al. 2011). Drug administration is oral, with the aim of reaching the therapeutic target between 14 and 20 mg/dl (Hermsen et al. 2011; Kerkhofs et al. 2013) above which side effects involving the gastrointestinal tract and central nervous system frequently manifest (Fassnacht and Allolio 2009). A recent study showed that blood mitotane concentrations ≥14 mg/l were associated with a prolonged recurrence-free survival (RFS) in patients following macroscopically radical surgery (Terzolo et al. 2013). Furthermore, the measurement of plasma mitotane levels in the management of patients with ACC is mandatory. Different treatment strategies have been proposed to achieve the therapeutic dose even if the high-dose regimen appears to be the most effective for reaching the target concentration more rapidly (Fassnacht and Allolio 2009; Mauclere-Denost et al. 2012). Mitotane has been shown to be an inducer of hepatic cytochrome CYP3A4 and thus able to interfere with the metabolism of other drugs including chemotherapeutic agents (van Erp et al. 2011). This drug property complicates the management of other codition-related treatments, such as antihypertensives, statins, antibiotics and others, that are also being used in ACC patients (Kroiss et al. 2011). Furthermore, future protocols involving mitotane and antineoplastic drugs in combination should consider this particular drug feature. Several

studies regarding mitotane use in the clinical setting of ACC have reported conflicting results. However, the retrospective nature of these studies exposes them to numerous biases, mainly related to the different concentrations used and the lack of mitotane plasma level measurements. In fact, theresponse rate, as evaluated in series in which mitotane treatment was given without considering plasma concentration and those with patients in whose the drug concentration had been assessed, ranged from 25 to 55% respectively (van Slooten et al. 1984; Haak et al. 1994; Baudin et al. 2001; Hermsen et al. 2011). An extensive retrospective case-control study performed on two independent cohorts showed significantly improved RFS compared to untreated control patients ($p<0.0001$). Overall survival (OS) was 110 months in the mitotane group vs 52 and 67 months in the control group ($p=0.01$) (Terzolo et al. 2007). These data have allowed the introduction of the concept of using mitotane in the adjuvant therapy of patients affected by ACC. Currently, an international, multicentric, prospective, randomized trial, called ADIUVO, designed to evaluate the effectiveness of mitotane in adjuvant therapy is ongoing. The recent ESMO guidelines recommend adjuvant mitotane treatment in stage III patients with potential residual disease (R1 or Rx resection status) and Ki-67 >10%. For patients in stages I or II, R0 resection and Ki-67 <10%, adjuvant mitotane therapy is not considered mandatory. Mitotane should be administered progressively to reach the dose of 6 g/day over 4-6 days, adjusting the dosage according to the patient's tolerance and the plasma drug level (Berruti et al. 2012a).

2.3.4 Chemotherapy

Among the different available treatment protocols for advanced ACC, chemotherapy is offered in combination with mitotane. The rationale of this combination is related to the ability of mitotane to overcome the drug-resistance induced by P-glycoprotein which is widely expressed in ACC (Bates et al. 1991). Several chemotherapeutic agents, such as adriamycin, cisplatin, doxorubicin, and others have been used alone or in combination with mitotane in the treatment of advanced ACC (Bukowski et al. 1993; Khan et al. 2000; Schteingart 2000; Berruti et al. 2005). Although variable percentages have been reported, the results from these studies demonstrate that cisplatin alone or in combination with etoposide have a higher effectiveness in advanced ACC (Bukowski et al. 1993; Bonacci et al. 1998; Williamson et al. 2000). The First International Randomized Trial in Locally Advanced and Metastatic Adrenocortical Carcinoma Treatment (FIRM-ACT) clearly confirmed the advantage of the regimen of etoposide-

doxorubicin-cisplatin (EDP) in combination with mitotane (Berruti *et al* protocol) (Berruti et al. 2005) compared to streptozotocin plus mitotane (Khan *et al* protocol) (Khan et al. 2000) (Table II). In Berruti *et al* study, an overall response rate of 48.6% was achieved (Berruti et al. 2005), whereas in the Khan *et al* regimen, the International Consensus Conference on Adrenal Cancer of Ann Arbor recommended the use of these protocols as first-line regimens against metastatic ACC in 2003 (Schteingart et al. 2005). Despite the expectations, no significant differences were found in OS (median 14.8 vs 12 months, respectively). Similarly, quality of life and adverse events were comparable in patients receiving the two treatments, thus confirming the poor prognosis of patients with advanced ACC (Fassnacht et al. 2012). Gemcitabine alone or in combination with mitotane demonstrated a good efficacy *in vitro* and its effectiveness was dependent on the sensitivity of the ACC cells to mitotane (Germano et al. 2014).

Berruti et al protocol (EDP/ M) (53)	*Every 28 days:*	
	Day 1	40 mg/m2 Doxorubicin
	Day 2	100 mg/m2 Etoposide
	Days 3 and 4	100 mg/m2 Etoposide + 40 mg/m2 cisplatin
Khan et al protocol (Sz/ M) (54)	*Every 21 days:*	
	Days 1-5	1 g Streptozotocin
	Subsequently	2 g Streptozotocin
	Daily	Mitotane with a blood level between 14-20 mg/l

Table II. Regimen protocols in the FIRM-ACT study.

2.3.5 Radiotherapy

The effectiveness of radiotherapy in ACC has been extensively debated. A retrospective analysis from the German ACC Registry, demonstrated that adjuvant radiotherapy resulted in a significantly better 5-year RFS, but did not affect OS and disease-free survival (DFS) (Fassnacht et al. 2006). In a recent retrospective study from the United States, radiotherapy was reported to decrease the risk of local failure 4.7 times compared with alone (Sabolch et al. 2011). In contrast another retrospective study did not find a difference between adjuvant radiotherapy and surgery alone (Habra et al. 2013). Some *in vitro* studies support the potential combination of mitotane and radiotherapy. In fact, these studies reported an inhibitory effect of mitotane in association with ionizing radiations on ACC cell lines (Polat et al. 2009; Cerquetti et al. 2010). Considering the current data, radiotherapy is intended for patients with R1 or Rx resection status with a high risk for local recurrence (Polat et al. 2009). However, the potential use of this method alone or in combination with other therapy should be investigated in future prospective clinical trials.

2.3.6 Targeted therapy

The failure of conventional therapies in advanced ACC and recent knowledge regarding the molecular pathways, involving oncosuppressor genes, such as *TP53*, *CDKN1C*, *CDKN2A* and *MEN1*, and oncogenes such as *IGF2*, *CTNNB1* and *RAS* involved in this malignancy have encouraged many efforts in developing new strategies against ACC (Barlaskar and Hammer 2007). *Insulin-like growth factor-2 mTOR pathway*. Overexpression of insulin-like growth factor-2 (IGF-2) is the most important molecular event occurring in >90% of ACCs (Ragazzon et al. 2011). Its hypersecretion induces an uncontrolled activation of the PI3K/Akt/ mTOR pathway by IGF-1R (de Reynies et al. 2009). Preclinical *in vitro* and *in vivo* studies on xenograft models showed that NVP-AEW541, a small molecule inhibitor, and IMC-A12, a human monoclonal antibody, were able to reduce cell proliferation, inhibiting the IGF-2 downstream pathway. The association of both molecules with mitotane sinergistically inhibited tumor growth (Barlaskar et al. 2009; Tacon et al. 2011). Two phase I studies have demonstrated the effectiveness of figitumumab, a monoclonal anti-IGF-1R antibody and linsitinib (OSI-906), a tyrosine kinase inhibitor binding IGF-1R inducing a clinical response in 57 and 33% of patients, respectively (Haluska et al. 2010). Recently, the results of a phase III

study to evaluate the therapeutic potential of OSI-906 were published, which were disappointing (Fassnacht et al. 2015). An association was found between *IGF2* overexpression, mTOR hyper-activation and reduced expression of miR-99a and miR-100 (Doghman et al. 2010). A role of mTOR in normal and adrenal tumors has been demonstrated by several studies (De Martino et al. 2010; Doghman et al. 2010) and its inhibition by everolimus (RAD-001) leads to cell growth reduction both *in vitro* and *in vivo*, confirming the importance of microRNA regulation of the IGF-2/mTOR signalling cascade (Doghman et al. 2010). Based on these data, a phase I trial tested the effects of temsirolimus (CCI-779), another inhibitor of mTOR in combination with cixutumumab, an anti-IGF-R1 monoclonal antibody, demonstrating a positive effect on tumor growth in 4 of 10 patients treated (Naing et al. 2011). A recent trial from the United States investigating the combination of cixutumumab and mitotane as first line treatment in patients with metastatic ACC reported effectiveness in 8/20 patients enrolled (Lerario et al. 2014).

Angiogenesis. Most solid tumors display marked angiogenesis and substantial data highlight the vascular endothelial growth factor (VEGF) overexpression in ACC (70). Despite expectations for the inhibitors of this pathway, the results of clinical trials have been quite disappointing. A monoclonal VEGF antibody in combination with capecitabine administered in a series of 10 patients affected by advanced ACC did not show any positive results (Wortmann et al. 2010). A partial response with capecitabine at a dose of 200 mg/die has been described only in one case, a 40-year-old patient with chemoresistant ACC (Chacon et al. 2005). Both sorafenib and sunitinib, tyrosine kinase inhibitors able to target VEGF, produced poor results also (Tacon et al. 2011). A phase II trial consisting of the administration of sunitinib in 38 patients with unresponsive ACC recorded a progression-free survival ranging from 5.6 to 12.2 months (Kroiss et al. 2012). A phase I trial described a positive response in two patients affected by advanced ACC who received sorafenib in combination with tipifarnib, a farnesyltransferase inhibitor (81). Moreover, only in a single case report, a regression of metastatic ACC with sorafenib administration has been observed (Butler et al. 2010). Recently, a phase II study investigating the combined effect of sorafenib with metronomic paclitaxel did not show any clinical improvement, contradicting the obtained *in vitro* results (Berruti et al. 2012b). A partial response to sunitinib in a patients with metastatic ACC, after chemotherapy treatment, has been described (Lee et al. 2009). Moreover, Gangadhar *et*

al reported a partial response, in a patient with advanced ACC, who received combination treatment with sirolimus, an mTOR inhibitor, and sunitinib (Gangadhar et al. 2011). Finally, a role for heparanase-1 in ACC angiogenesis has been hypothesized thus representing a new selective therapeutic target in ACC (Xu et al. 2011) .

Tyrosine kinase inhibitors. Microarray transcriptome analyses have provided new knowledge regarding the hyperactivated molecular pathways involved in ACC, thereby suggesting new potential target molecules for treatment strategies to address (Assie et al. 2010; Ragazzon et al. 2011). Frequently, these targets are represented by growth factors and therefore the therapeutic concept is based on the inhibition of protein kinases involved in signal transduction, often tyrosine kinase receptors (Tacon et al. 2011). A clinical study performed on 10 patients with advanced disease treated with erlotinib, an EGFR inhibitor, in combination with gemcitabine demonstrated very limited effectiveness (Quinkler et al. 2008). In a phase II study, treatment with imatinib mesilate, a PDGFR inhibitor, was associated with disease progression in 75% of patients with severe side effects (Gross et al. 2006). It is likely that the failure of these therapies is related to the low presence of these receptors in ACC. Interestingly, no mutation of the EGFR gene has been detected (Adam et al. 2010).

MDR/P-glycoprotein. The chemoresistant properties of ACC have been classically related to the overexpression of the multidrug resistance protein MDR-1 (P-glycoprotein, Pgp), a debated drug efflux pump (Bates et al. 1991). Even with results from an *in vitro* study suggesting that mitotane enhances doxorubicin activity by interfering with Pgp (Gagliano et al. 2014), the exact role of MDR-1 protein in ACC needs to be elucidated. Furthermore, a clinical trial using doxorubicin, vincristine, and etoposide in combination with mitotane failed to demonstrated therapeutic effectiveness (Abraham et al. 2002).

PPAR-γ antagonists. Thiazolidinediones effects are somewhat different in the oncologic field (Kahn et al. 2000). The PPAR-γ receptor is widely expressed in normal and in tumor adrenal tissue. It is involved in the IGF-2/IGF-1R signaling pathway, by inhibiting Akt activation (Betz et al. 2005; Ferruzzi et al. 2005). It has been shown that these compounds are able to inhibit cell proliferation in ACC cell models and in xenograft models (Betz et al. 2005; Ferruzzi et al. 2005; Luconi et al. 2010; Cerquetti et al. 2011). In particular, it has been shown that rosiglitazone, a thiazolidinediones class

member, activating both PPAR-γ-dependent and -independent pathways, led to a growth arrest, cell death and reduction of *VEGF* expression involved in adrenocortical angiogenesis (Betz et al. 2005; Cerquetti et al. 2011).

Wnt/β-catenin pathway. The constitutive activation of this pathway is found in 85% of ACC (Tissier et al. 2005). Moreover, nuclear localization of β-catenin represents a worse prognostic factor in ACC (Tissier et al. 2005; Gaujoux et al. 2011). Preclinical *in vitro* studies were performed with PKF115-584, a small molecule inhibitor of the T-cell factor (Tcf)/β-catenin complex, on β-catenin-dependent transcription and proliferation processes in H295R ACC cells, that harbored *CTNNB1* gene mutations. As a results, this treatment was able to inhibit cell growth and induce apoptosis in the H295R adrenocortical cell line but not in HeLa, a human epitheliod cervical carcinoma cell line, confirming that the Wnt/β-catenin pathway is an useful target in ACC (Doghman et al. 2008). Another *in vitro* study demonstrated that β-catenin silencing inhibited cell proliferation and induced apoptosis in the H295R cell line (Gaujoux et al. 2013). A phase I study with CWP232291, a compound recognized for its ability to promote β-catenin degradation with activity in several multiple myeloma cell lines, is currently ongoing in patients with relapsed or refractory acute myeloid leukemia (Demeure et al. 2011). However, no clinical study is currently underway for ACC.

Steroidogenic factor-1. Steroidogenic factor-1 (SF-1) is a nuclear transcription factor involved in the steroidogenic tissue development (Sbiera et al. 2010). It is frequently overexpressed in pediatric ACCs, whereas in the adult population some abnormalities on chromosome 9 have been described. However, a higher nuclear SF-1 expression level has been associated with a worse prognosis in ACC (Sbiera et al. 2010) and is positively correlated with advanced ENSAT stages, and a higher mitotic index and Weiss score (Duregon et al. 2013). An increased SF-1 dosage was observed to stimulate proliferation, decrease apoptosis in adrenocortical cells, and induce tumorigenesis in transgenic mice (Doghman et al. 2007). SF-1 silencing affected TGF-β and Wnt/β-catenin signaling, suggesting crosstalk between these pathways in a study performed on the H295R adrenocortical cell line. Moreover, SF-1 knockdown showed a significant reduction of cell proliferation be interference with S-phase of the cell cycle (Doghman et al. 2007; Ehrlund et al. 2012). Two members of the alkyloxyphenol class, AC-45594 and OOP, the synthetic SF-1 inverse agonists have been shown to inhibit proliferation in both H295R and SW13 adrenocortical cell lines through an SF-1 non-selective

mechanism. In contrast, SID7969543 (IsoQ A) and the compounds numbered 31 and 32, members of the IsoQ class, induced a selective inhibition of cell proliferation when SF-1 was increased strongly suggesting that the IsoQ molecules targeted SF-1-related genes (Doghman et al. 2009).

Gene therapy and immunotherapy. The rationale of gene therapy lies in correcting the gene regulation, reactivating oncosuppressor genes and/or inhibiting oncogenes during tumorigenesis. Systemic therapy with antisense oligonucleotides represents an innovative approach for ACC treatment. A construct, composed of the herpes simplex virus thymidine kinase (HSV-TK) gene driven by the CYP11B1 promoter with a P450scc enhancer element, increased the chemosensitivity in a Y1 mouse ACC cell line (Chuman et al. 2000). Immunotherapy represents another therapeutic approach that relies on the stimulation of the immune system against specific target proteins of neoplastic cells. This approach, using dendritic cells, was effective in stimulating the immune response (Sbiera et al. 2008), inducing antigen-specific Th1 immunity in a study performed on two patients with advanced secreting ACC. However, no clinical benefit has been shown (Papewalis et al. 2006). In the cytokine family, interferon-β (INF-β) showed an inhibitory effect *in vitro* on ACC cell lines and primary ACC human cell cultures. Furthermore, when co-administered INF-β, sensitized ACC cells to mitotane (van Koetsveld et al. 2013). Recently, a phase I clinical study was conducted with interleukin- 13-*Pseudomonas* exotoxin in patients affected by advanced ACC, exploiting the rationale that the α2 receptor of interleukin 13 (IL13Rα2) is more highly expressed in ACC than in normal adrenal tissue. This study showed a low disease stability rate lasting a few months before the ACCs progressed (Liu-Chittenden et al. 2015). *miRNA.* Recent biological advances concerning microRNA dysregulation in all cancers including ACC highlights the hypothetical consideration of these small non-coding RNAs as potential target molecules for anti-cancer treatment (Iorio and Croce 2012). Because miRNAs may function as tumor suppressors, the assumption of replacement miRNA cancer therapy must not disregard the identification of an miRNA deficiency. In a previous analysis of miRNA expression in adrenocortical tumors, miR-7 was the most significantly under-expressed miRNA when compared to normal adrenal tissue (Soon et al. 2009). Glover *et al* provided the first demonstration of the effectiveness of the nanoparticle systemic delivery of miR-7 in the reduction of cell growth in both cell lines and in an ACC xenograft model, respectively. Furthermore,

they demonstrated that miR-7 functions as a tumor suppressor in ACC leading to the repression of several genes involved in the pathogenesis of ACC, including *RAF-1*, *mTOR* and *CDK1* (Glover et al. 2015).

Estrogen pathway. Recent advance confirm an estrogenic pathway in normal adrenal tissue and in adrenal tumors. A differential expression of estrogen receptors (ER) α and β has been demonstrated in ACCs (de Cremoux et al. 2008). Moreover, Barzon *et al* showed an increased aromatase activity in ACC, hypothesizing a paracrine estrogenic effect at the tumor level (Barzon et al. 2008). An *in vitro* study demonstrated that hydroxytamoxifen, increasing the pro-apoptotic factor FasL expression, reduced H295R cell proliferation by ERα downregulation and ERβ upregulation, respectively (Montanaro et al. 2005). ERα activation may occur by an 17-β estradiol (E2)-dependent mechanism or alternatively by IGF-II/ IGF1R in a ligand-independent manner, activating proliferative pathways *in vitro*, such as IGF1R/AKT signaling, in H295R cell lines. Furthermore, in the same study, hydroxytamoxifen, an active metabolite of the estrogen antagonist tamoxifen, reduced IGF1R protein levels and cell proliferation induced by E2 and IGF-II both *in vitro* and in an ACC xenograft model (Sirianni et al. 2012). These data indicate a crucial role of the estrogenic pathway in ACC and support the possibility of using antiestrogens in the treatment of ACC. A recent interesting study elucidated the ability of a non-steroidal G-protein-coupled receptor (GPER) agonist to exert a growth inhibitory effect, mediated by activation of the ERK1/2 pathway, both in the H295R cell line and in xenograft ACC (Chimento et al. 2015). Finally, the compound XCT790, an inverse agonist of the transcription factor estrogen-related receptor α (ERRα) (Giguere et al. 1988), an orphan member of the nuclear hormone receptor superfamily with a similar sequence to ERα involved in cellular metabolism and mitochondrial biogenesis (Deblois and Giguere 2011), was able to reduce cell growth in both the H295R cell line and in an ACC xenograft model, with impaired mitochondrial functioning leading progressively to cell death (Casaburi et al. 2015).

Metomidate. $^{[123I]}$IMTO single-photon emission CT imaging has been recently introduced as a novel tracer for the identification of adrenocortical tumors (Hahner et al. 2013). Treatment options with $^{[131I]}$IMTO depend on the $^{[123I]}$IMTO uptake in the lesion potentially related to ACC (Hahner et al. 2013). A recent experience in 11 patients with advanced ACC receiving ≤20GBq $^{[131I]}$IMTO, showed a low progression-free survival in six responders (Hahner et al. 2012).

Interventional radiology. Minimally invasive procedures such as radiofrequency thermal ablation (RFA), or transarterial chemoembolization (TACE) represent an alternative to surgery in advanced metastatic malignancies. The same approach was adopted also in the treatment of lesions in the liver, kidney, lymph nodes and lung for stage IV ACC patients (Wood et al. 2003). Wood *et al* observed that RFA induced a growth arrest in 8 of 15 lesions after 6 months of follow-up. The procedure was safe and was not associated with any particular side effects (Wood et al. 2003). TACE allows the selective infusion of high doses of cytotoxic drugs in the metastatic lesion reducing the systemic toxicity. In a French study, this technique was associated with a median survival of 11 months in 21 patients with liver metastatic disease (Cazejust et al. 2010). These procedures provide palliative benefits, are safe and inexpensive while implying minimal morbidity and a short recovery, however, none of these methods have been supported by a clinical trial.

2.3.7 Conclusion

ACC in the past was considered an orphan disease for which surgery represented the only feasible therapy. Over the years the focus on this aggressive endocrine malignancy has gradually grown, capturing the interest of many investigators. Despite the enormous progress achieved in the biological knowledge of this tumor, the ACC remains an oncological disease burdened by a high mortality. Surgery is still the first therapeutic option and the only potentially curative treatment. Mitotane has represented the first drug in the treatment of ACC since 1959. Subsequently, knowledge regarding its mechanism of action has increased substantially while its clinical use has become much more controlled and appropriate. Currently, mitotane is the only drug approved by international pharmaceutical agencies for ACC treatment. Although mitotane treatment in the adjuvant setting is still debated, the recent ESMO guidelines recommend its use in adjuvant setting after surgery in patients with incomplete resection status. The optimal chemotherapy regimen is considered to be etoposide-doxorubicin-cisplatin (EDP) in combination with mitotane in patients with advanced metastatic disease, although this regimen is burdened by substantial side effects. The current -OMICs approach has permitted the discover of different molecules belonging to pathways potentially involved in the pathogenesis of the ACC. The therapeutic option described in isolated and metastatic ACC are summarized in Figure 2.1 Future efforts should be made not only to explore new frontiers but also to investigate innovative therapies in the

clinical field. ACC urgently requires new therapeutic strategies. The clinical translation of new research products *in vitro* and in preclinical studies may help improve the standard of care in these patients. Achieving this objective in a rare disease such as ACC will require the carefully selection of the clinical series to be devoted to experimentation and an increasead collaborative network of research centers involved in the study of this malignancy.

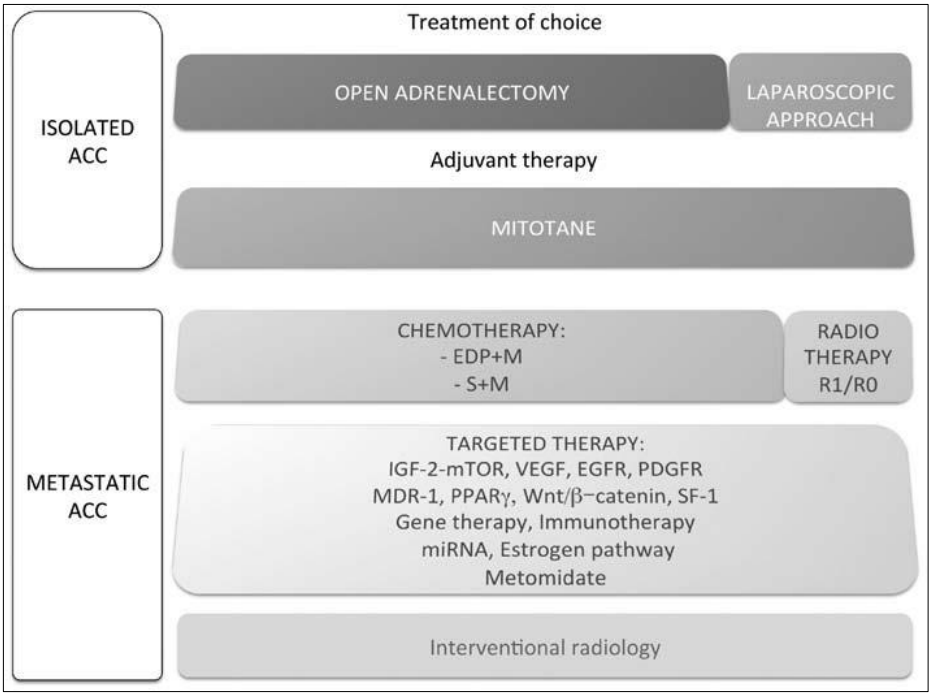


Fig. 2.1 Treatment of choice in isolated and metastatic adrenocortical carcinoma (ACC).

2.4 Cancer cell metabolism and ACC

During carcinogenesis and tumor progression, neoplastic cells may reprogram their metabolism, showing a preference for glycolysis pathway for energy production, even in the presence of oxygen, a phenomenon known as the "Warburg effect" (Figure 2.2).

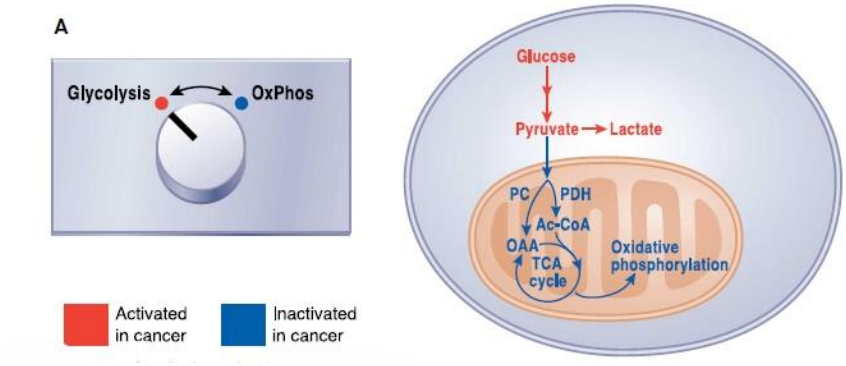


Fig. 2.3 Relationship between glycolysis and oxidative phosphorylation in cancer cells. A common view of cancer cell metabolism invokes aswitch from glucose oxidation in normal tissues toward glycolysis and suppressed oxidative phosphorylation (OxPhos) in cancer.

This aerobic glycolysis implies the conversion of pyruvate to lactic acid, leading to a reduction in intracellular pH (Gatenby and Gillies 2004). To prevent acid-induced apoptosis as well as glycolysis inhibition by accumulation of the end product, cancer cells upregulate proteins related to pH regulation and lactate transport, including the glucose transporter GLUT1, the pH regulator carbonic anhydrase IX (CAIX) (Chiche et al. 2010; Tennant et al. 2010) and monocarboxylate transporters (MCTs) (Chiche et al. 2010). Therefore, many malignancies show a significant increase in the expression of these plasma membrane transporters, with associations with poor patient's prognosis (de Oliveira et al. 2012; Pinheiro et al. 2012; Miranda-Goncalves et al. 2013; Pinheiro et al. 2014). The MCT family has 14 members, being isoforms 1, 2 and 4 the most well studied isoforms responsible for the transport of monocarboxylates, including lactate, coupled with a proton across the plasma membrane. As consequence from their substrate affinities, MCT1 and MCT4 mediate monocarboxylate efflux from cells, while MCT2 is involved in monocarboxylate uptake (Halestrap 2012). Importantly, these transporters require co-expression with chaperones for proper plasma membrane localization and activity. The main chaperone of MCT1 and MCT4 is CD147 (Kirk et al. 2000; Wilson et al. 2005), while MCT2 is mainly associated with gp70 [18]. CD44 has also been recently described as a MCT chaperone (Slomiany et al. 2009); however CD147 and CD44 expressions do not account for all MCT1/4 positive cases, suggesting that an additional MCT chaperone still remains to be identified (Pinheiro et al. 2010).

The metabolic profile of adrenocortical tumors is very little explored (Fenske et al. 2009). However, 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) data suggest that adrenocortical carcinomas show high levels of glucose consumption (Nunes et al. 2010; Deandreis et al. 2011; Bourdeau et al. 2013; Takeuchi et al. 2014), indicating a possible clinical relevance of the glycolytic metabolism for the management of this neoplasia.

In a recent study was evaluated the prognostic value of metabolism-related key proteins in adrenocortical carcinoma. The immunohistochemical expression of MCT1, MCT2, MCT4, CD147, CD44, GLUT1 and CAIX was evaluated in a series of 154 adult

patients with adrenocortical neoplasia and associated with patients' clinicopathological parameters. A significant increase in was found for membranous expression of MCT4, GLUT1 and CAIX in carcinomas, when compared to adenomas. Importantly MCT1, GLUT1 and CAIX expressions were significantly associated with poor prognostic variables, including high nuclear grade, high mitotic index, advanced tumor staging, presence of metastasis, as well as shorter overall and disease free survival. In opposition, MCT2 membranous expression was associated with favorable prognostic parameters. Importantly, cytoplasmic expression of CD147 was identified as an independent predictor of longer overall survival and cytoplasmic expression of CAIX as an independent predictor of longer disease-free survival. They provide evidence for a metabolic reprogramming in adrenocortical malignant tumors towards the hyperglycolytic and acid-resistant phenotype, which was associated with poor prognosis (Pinheiro et al. 2015).

The major findings described so far allowed us to establish 2 h predominant expression profiles, the glycolytic and the oxidative phenotypes (Figure 2.3), which were associated with the clinicopathological parameters.

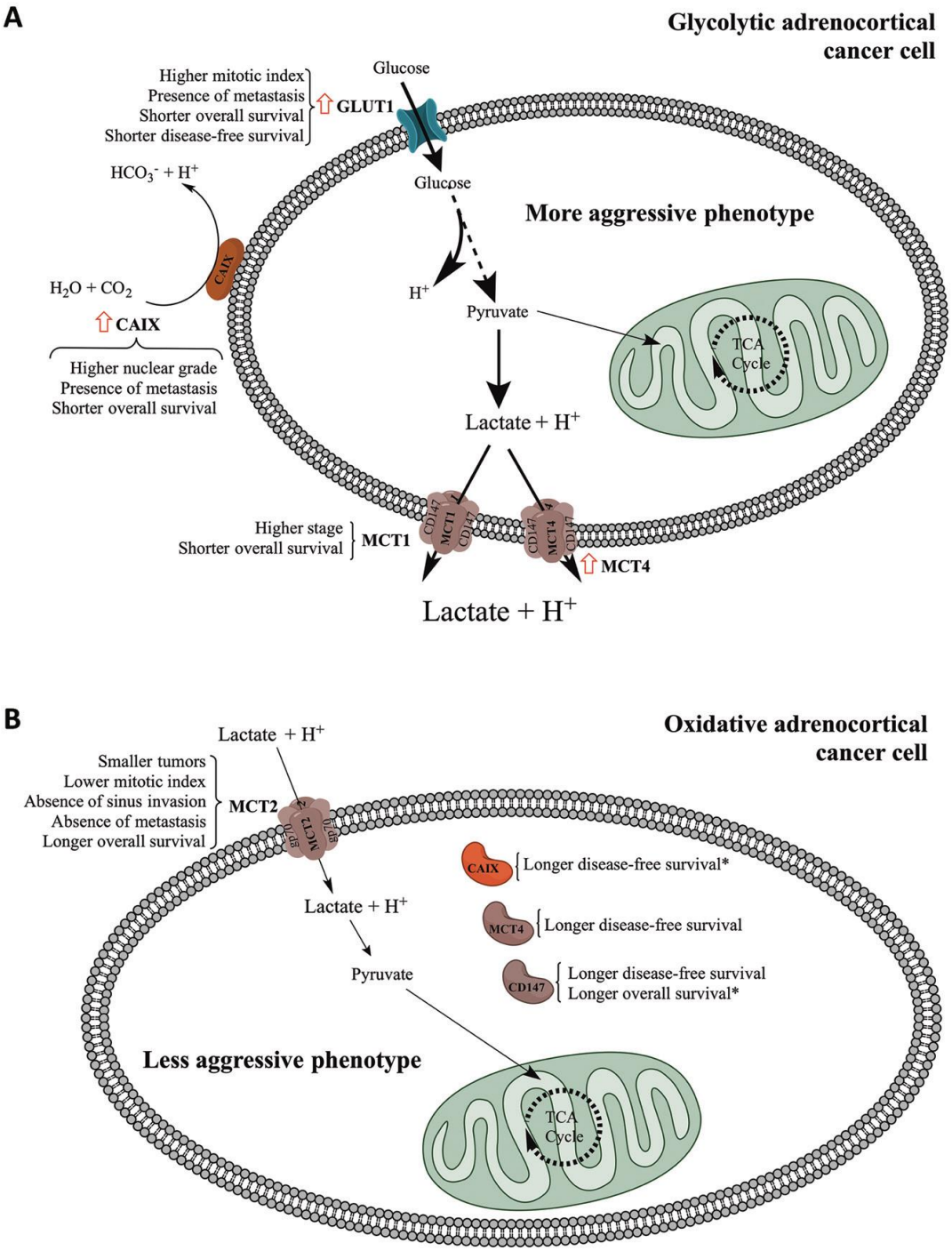


Fig. 2.3 Schematic representation of Glycolytic *versus* oxidative phenotype. The schematic representation distinguishes the major findings for plasma membrane and cytoplasmic expression of the different proteins studied. Red arrow: significant increase in expression frequency when comparing adenomas to carcinomas. * Independent predictors of survival as determined by multivariate analysis.

An important conclusion from recent studies, and from a similar study in mice bearing KRAS-driven tumors (Davidson et al. 2016), is that non–small cell lung tumors demonstrate higher levels of both glycolysis and glucose oxidation relative to adjacent,

benign lung. This finding sharply contrasts with the frequently invoked “switch” from oxidativemetabolism to glycolysis inmalignant tissue, commonly used to explain the Warburg effect (Figure 2.2). Rather, the data support a model in which the amplitude of both pathways is increased simultaneously, perhaps through increased substrate delivery and enzyme expression in tumor cells (Figure 2.4). It is also significant that human tumors exhibit substantial heterogeneity of metabolic phenotypes, both between tumors and even within distinct regions of the same tumor (Hensley et al. 2016).

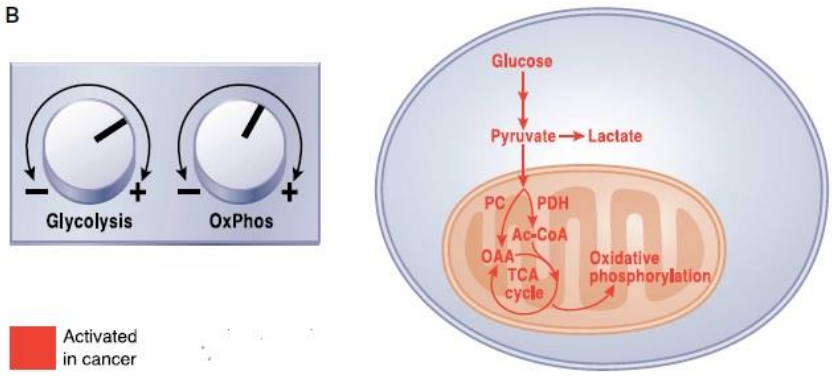


Fig. 2.4 Analysis of metabolic activity in intact tumors from humans ankd mice argues against a switch. Rather, tumors appear to enhance both glycolysis and glucose oxidation simultaneously relative to surrounding tissue.

Awareness that the metabolic phenotype of cells within tumours is heterogeneous — and distinct from that of their normal counterparts — is growing (Figure 2.5). In general, tumour cells metabolize glucose, lactate, pyruvate, hydroxybutyrate, acetate, glutamine, and fatty acids at much higher rates than their nontumour equivalents; however, the metabolic ecology of tumours is complex because they contain multiple metabolic compartments, which are linked by the transfer of these catabolites. This metabolic variability and flexibility enables tumour cells to generate ATP as an energy source, while maintaining the reduction–oxidation (redox) balance and committing resources to biosynthesis — processes that are essential for cell survival, growth, and proliferation. Importantly, experimental evidence indicates that metabolic coupling between cell populations with different, complementary metabolic profiles can induce cancer progression (DeBerardinis and Chandel 2016).

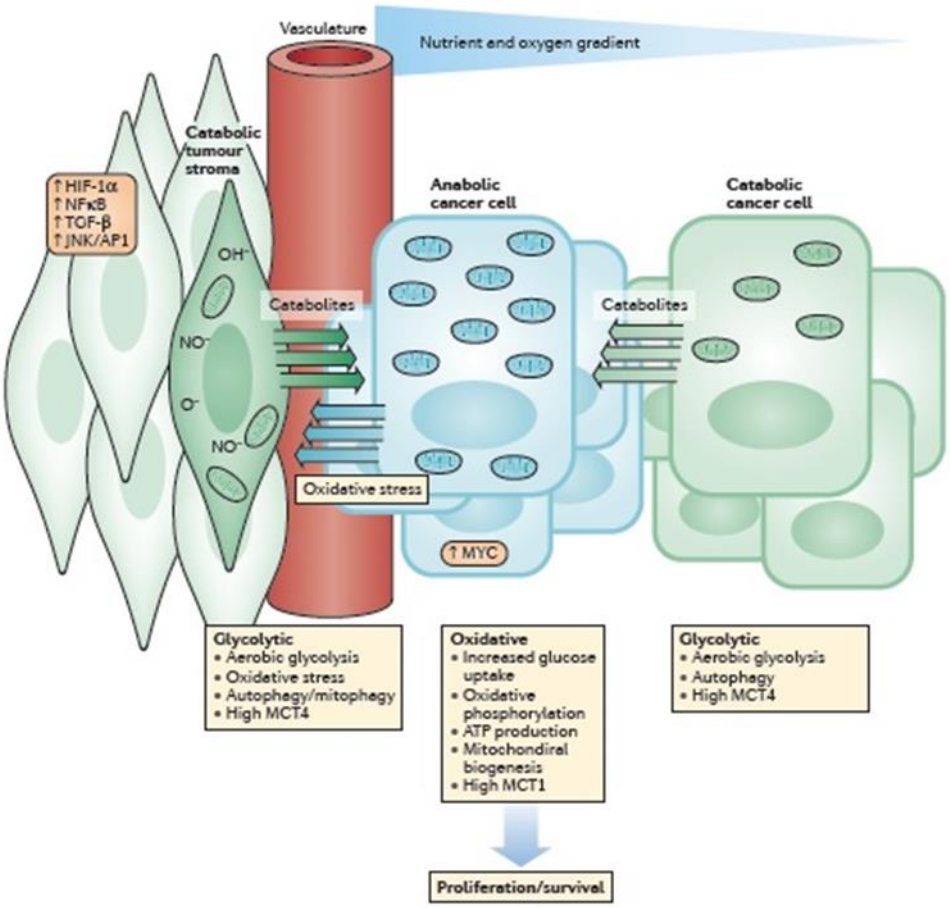


Fig. 2.5 Metabolic heterogeneity in tumours. Within tumours, cancer-cell metabolism can vary depending on influences of the tumour microenvironment and the distance to the vasculature. Cancer cells located closer to the blood supply profit from their access to nutrients and oxygen, and generate ATP aerobically via oxidative phosphorylation and upregulate anabolic pathways, supporting rapid proliferation. The oxidative stress caused by these rapidly proliferating cancer cells induces glycolysis and autophagy in the surrounding stromal cells that generates catabolites, such as lactate or ketones, which in turn are taken up by anabolic cancer cells, and used to fuel mitochondrial metabolism and ATP production (reverse Warburg effect). Similarly, low nutrient availability requires that tumour cells located further from the vasculature and in proximity to anabolic tumour-cell populations commit to alternative catabolic metabolic pathways, such as autophagy, allowing greater adaptability to meet their resources and energy needs. ATP, adenosine triphosphate; HIF-1α, hypoxia-inducible factor 1α; JNK/AP1, c-Jun *N*-terminal kinases/activator protein 1; MCT1, monocarboxylate transporter 1; MCT4, monocarboxylate transporter 4; NFκB, nuclear factor κB; TGF-β, transforming growth factor β.

Indeed, mitochondria remain functional and produce intermediates for the biosynthesis of structural precursors in cancer cells with the oxidative phenotype (DeBerardinis et al. 2008). However, the glycolytic state prevails in tumor cells and reflects a metabolic

profile known to favor tumor angiogenesis, invasion and migration (Pugh and Ratcliffe 2003), events poorly investigated in ACC.

3. Molecular markers involved in adrenocortical tumor cell proliferation and progression: PELP1, EGR-1 and ERRα

3.1 The nuclear receptor coregulator PELP1 (PROLINE-, GLUTAMIC-,AND LEUCINE-RICH PROTEIN 1)

3.1.1 Introduction

Human proline-, glutamic acid-, and Leucine-rich protein 1 (PELP1) map to the chromosomal region 17p13.2 and encodes a protein of 1130 aa. PELP1 gene is highly conserved across species including mouse, rat, dog, cow, and chimpanzee. PELP1 is expressed in a wide variety of tissues; the highest levels of expression are found in the brain, testes, ovaries, and uterus (Vadlamudi et al. 2001; Khan et al. 2005; Pawlak and Beyer 2005; Greger et al. 2006). There are two PELP1 isoforms: a long isoform of 3.8 Kb and a short isoform of 3.4 Kb. The long isoform has an extra intron (435 bp) inframe. The short isoform lacks this intron and is widely expressed inmany cells, including cancer cells (Balasenthil and Vadlamudi 2003). PELP1 expression is developmentally regulated in the mammary glands (Vadlamudi et al. 2001). PELP1 is an estrogen receptor (ESR) target gene. The PELP1 promoter has two estrogen-response element (ERE) half sites and is similarly upregulated by both ESR1 and ESR2 (Mishra et al. 2004). PELP1 contains a central consensus nuclear localization sequence and exhibits both cytoplasmic and nuclear localization depending on the tissue (Vadlamudi et al. 2001). PELP1 is present within several sub-compartments of the nucleus, including the chromatin, nucleoplasm, and nuclear matrix (Nair et al. 2004). In this review, we summarized the emerging biological properties and functions of PELP1 and mostly focused on the functions of short isoform that is commonly expressed in normal and cancer cells.

3.1.2 PELP1 structure

PELP1 protein contains 10 nuclear receptor (NR)-interacting boxes (LXXLL motifs) that facilitate its interactions with nuclear receptors (Vadlamudi et al. 2001). A unique feature of PELP1 is the presence of an unusual stretch of 70 acidic amino acids in the C-

terminus that functions as a histone-binding region (Choi et al. 2004; Nair et al. 2004). PELP1 contains several consensus PXXP motifs that facilitate its interactions with proteins containing Src homology 3 (SH3) domains. The PELP1 sequence further contains several conserved protein–protein interaction motifs that bind to forkhead-associated (FHA), Src homology 2 (SH2), SH3, PDZ, and WW domains. PELP1 also has two nucleolar domains (Nuc 202) that play an important role in PELP1-mediated ribosomal functions (Gonugunta et al. 2011).

3.1.3 PELP1 post-translational modifications

PELP1 is phosphorylated by hormonal and growth factor signals and thus has potential to couple physiological signals to nuclear receptors and transcriptional factors. Epidermal growth factor (EGF) signaling promotes tyrosine as well as serine phosphorylation of PELP1 (Vadlamudi et al. 2005b). Growth factors promote phosphorylation of PELP1 via protein kinase A (PKA) at Ser350, Ser415, and Ser613 (Nagpal et al. 2008). Glycogen synthase kinase 3 β (GSK3β) phosphorylate PELP1 at Thr745 and Ser1059 in the brain and it play a role in its stability (Sareddy et al. 2015). CDKs phosphorylate PELP1 at Ser477 and Ser991 in a cell cycle-dependent manner (Nair et al. 2010a). DNA damage induced kinases (ATM, ATR) phosphorylates PELP1 on Ser1033 (Nair et al. 2014). Phosphorylation of PELP1 seems to be the key regulatory mechanism that controls its localization, modulates its interactions with adaptor proteins, alter its stability depending on the site of phosphorylation and may function as a sensor of the physiologic signals by connecting them to nuclear receptors.

3.1.4 PELP1 interactome

PELP1 interacts and functions as a coregulator of several NRs, including ESR1 (Vadlamudi et al. 2001), ESR2 (Vadlamudi et al. 2004), estrogen-related receptor α (ERRα) (Rajhans et al. 2008) progesterone receptor (PR) (Daniel et al. 2015), glucocorticoid receptor (GR) (Kayahara et al. 2008), androgen receptor (AR) (Nair et al. 2007), retinoid X receptor (RXR) (Singh et al. 2006). PELP1 also functions as a coregulator of several other transcription factors, including activator protein 1 (AP1), specificity protein 1 (SP1), nuclear factor κB (NF-κB) (Choi et al. 2004), signal transducer and activator of transcription (STAT3) (Manavathi et al. 2005) and four and a half LIMdomains 2 (FHL2) (Nair et al. 2007). The LXXLLmotifs present in PELP1 are implicated for its interaction with liganded steroid receptors. However, PELP1 is

also able to interact with unliganded steroid receptors and other transcription factors suggesting additional protein– protein interactions other than LXXLL motifs may also play a role in PELP1 interactions (Figure 3.1).

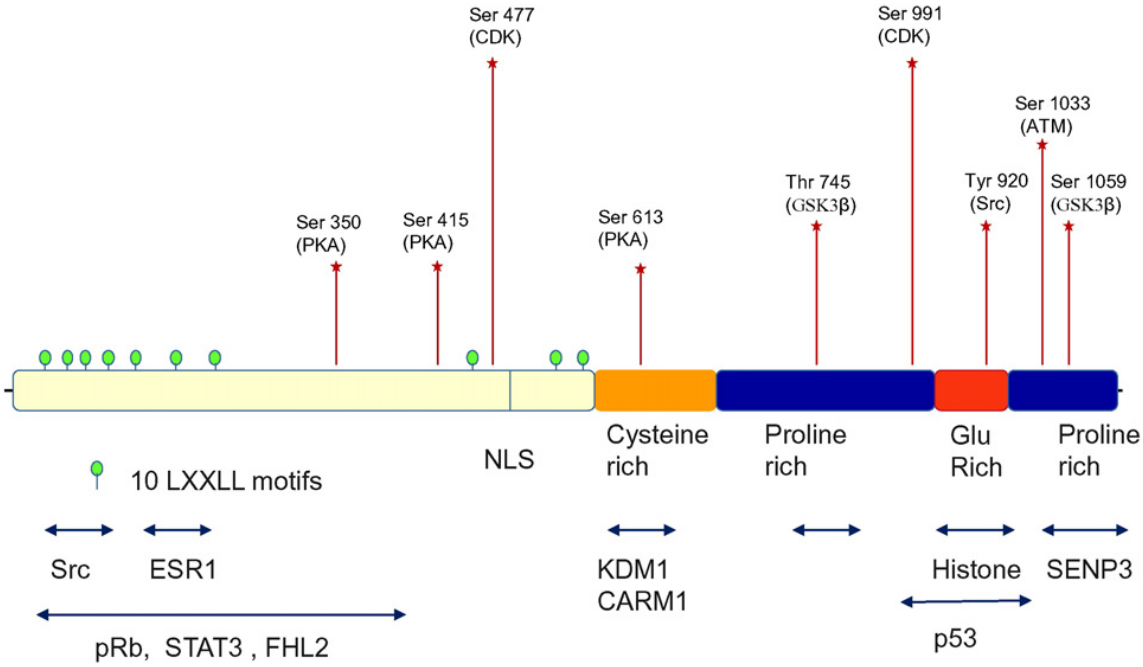


Fig. 3.1 Schematic representation of PELP1 domains that are important for its scaffolding functions. PELP1 contain 10 LXXLL motifs that facilitate its interactions with nuclear receptors (NRs). PELP1 contain multiple SH2, SH3, binding sites that facilitate its interactions with Src and p85 subunit of PI3K. PELP1 is phosphorylated by multiple kinases including CDKs, PKA, GSK3β, Src, EGFR, ATM and their phosphorylation regulate PELP1 oncogenic functions. Glu rich domain contain 70 acidic amino acids that facilitate PELP1 binding to histone. Proline rich domain confer additional specificity to Glu rich region. Bidirectional arrow indicate putative binding region of respective proteins.

In addition to nuclear receptors and transcription factors, PELP1 is shown to interact with several key players of cell cycle progression, including retinoblastoma protein (pRb) (Balasenthil and Vadlamudi 2003), cyclin-dependent kinase 2 (CDK2), and -4 (CDK4) (Nair et al. 2010a), E2F1 (Krishnan et al. 2015) and p53 (Nair et al. 2014; Krishnan et al. 2015). PELP1 also interacts with several kinases including c-Src (Chakravarty et al. 2010a), phosphoinositide 3-kinase (PI3K) (Dimple et al. 2008), epidermal growth factor receptor (EGFR) (Vadlamudi et al. 2005b) integrin-linked kinase (ILK1) (Chakravarty et al. 2010a), mechanistic target of rapamycin (mTOR) (Gonugunta et al. 2014b) and GSK3β (Sareddy et al. 2015). PELP1 interactions with

cell cycle and growth factor signaling components suggest its potential role as a mediator of hormonal signaling cross talk with cell cycle machinery. PELP1 also interacts with several components of chromatin-modifying complexes, including CBP/p300 (Vadlamudi et al. 2001), histone deacetylase 2 (Vadlamudi et al. 2001; Choi et al. 2004), histones (Choi et al. 2004; Nair et al. 2004), Sumo-2, (Rosendorff et al. 2006), lysinespecific demethylase 1A (KDM1A) (Nair et al. 2010b) protein arginine methyltransferases (PRMTs) (Mann et al. 2014), coactivator-associated arginine methyltransferase 1(CARM1) (Mann et al. 2013). Global mass spectrometry studies identified PELP1 as a stable component of the LAS1L, TEX10, and SENP3 complex (Malovannaya et al. 2011) and the Five Friends of Methylated Chtop (5FMC) complex (Fanis et al. 2012). Using proteomics, PELP1 was also identified as a DACH1-binding protein (Popov et al. 2009). Collectively, these emerging findings suggest that PELP1 lacks known enzymatic activity and functions as a scaffolding protein coupling various proteins to transcription factors and nuclear receptors (Figure 3.2).

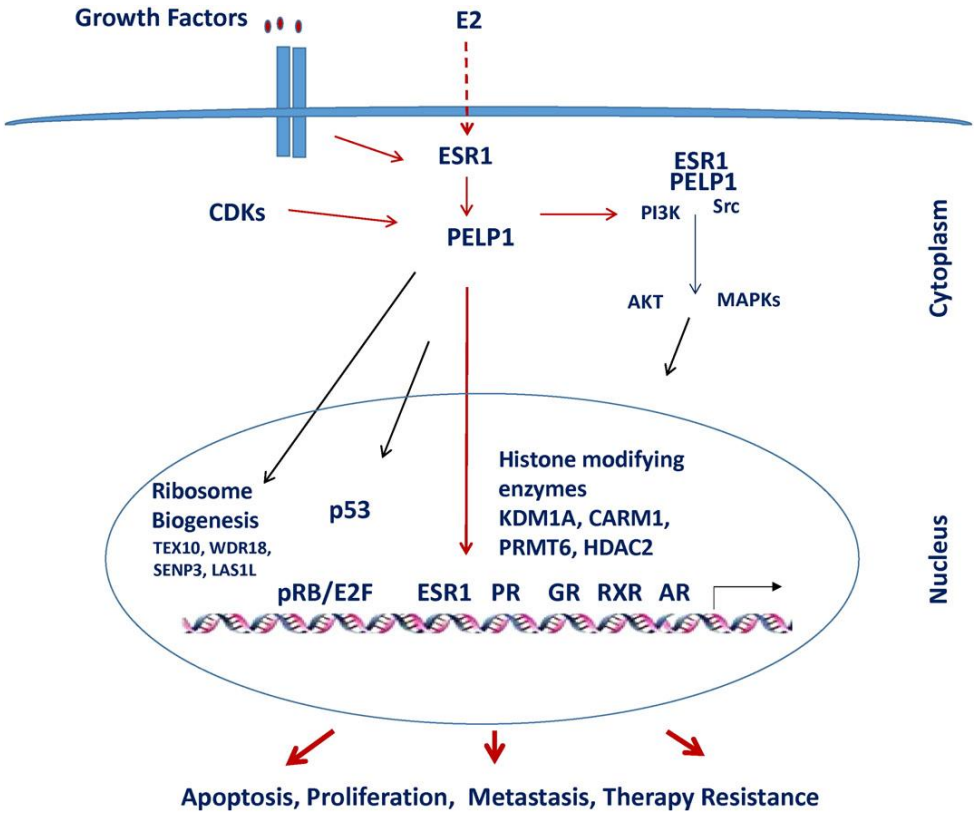


Fig. 3.2 Overview of PELP1 signaling. PELP1 interacts with and function as a coregulator of several nuclear receptors (NRs) including ESR1, PR, GR, AR and RXR. PELP1 facilitates chromatin

modifications via interactions with several enzymes including KDM1A, PRMT, CARM1, and HDAC2. PELP1 interacts with and modulate functions of several key players in cell cycle progression including CDKs, pRb, E2F, and p53. PELP1 participate in NR extra-nuclear signaling by coupling NRs with cytosolic kinases such as Src, PI3K that contribute to activation of pathways such as MAPK and AKT. PELP1 play an active role in the ribosomal biogenesis via its interaction with TEX10-WDR18-SENP3 complex. Deregulation of PELP1 signaling contribute to alterations in tumor cell proliferation, apoptosis, metastasis and therapy resistance.

3.1.5 Biological functions of PELP1

Role in Genomic SR Signaling

Initial characterization revealed an abundance of PELP1 in the mammary epithelium, where ERα is predominant (Vadlamudi et al. 2001). PELP1 was then demonstrated to be a coactivator of estradiol-induced ER-driven gene expression. PELP1 can enhance or repress SR transactivation depending on cellular context and ligand concentration (Kayahara et al. 2008). For example, in A549 cells that express significant endogenous GR, PELP1 inhibited GR transactivation. In contrast, in HEK293 cells that express very low levels of endogenous GR, PELP1 enhanced ligand-dependent GR transactivation. Biphasic behavior of exogenously expressed signaling molecules (ie, often when overexpressed) is indicative of scaffold activity, where stoichiometric concentrations of the interacting components that participate within a protein complex may facilitate maximal signal output, whereas too much of a particular player (ie, in this case, the scaffold itself) may interfere with signal output via sequestration of 1 of more of the interacting components. Suspected scaffold activity may be examined by physiologic manipulation of the levels of 1 protein (ie, altering total PELP1 levels using small interfering RNA (siRNA) or short hairpin RNA knockdown or exogenous PELP1 overexpression) while maintaining the same levels of its interacting partners. PELP1 interaction with SRs is mediated through its multiple LXXLL domains, and the sequences flanking each LXXLL domain mediate its specificity for individual SRs. PELP1 may also interact with SRs through non-LXXLL domains For example, exogenous overexpression of fragments of GR in HEK293 cells revealed that PELP1 interacts with both the amino and the C termini of GR. PELP1 was able to regulate both activation function 1 and activation function 2 transactivation functions of GR through these differential interactions (Kayahara et al. 2008). The role of PELP1 in genomic signaling mediated by multiple SRs has been well characterized. For example, PELP1 cooperates with the orphan SR, estrogen-related receptor-α to increase aromatase gene

expression (Rajhans et al. 2008). Knockdown of PELP1 affects the genomic signaling mediated by several SRs, with multiple mechanisms proposed. PELP1 may enable SR genomic signaling via its interactions with traditional SR-transcriptional coactivators, such as and the closely related CREB binding protein (CBP) (Figure 3.3) (Gururaj et al. 2007). PELP1 may recruit both SRs and their specific coactivators, thereby decreasing the entropy of such interactions within a cell via "forced" proximity. PELP1 may also stabilize SR-coregulator complexes that otherwise interact weakly in the absence of PELP1 (Figure 3.3). PELP1 may also induce chromatin remodeling at ER target gene promoters by displacing histone H1 (Nair et al. 2004). PELP1 mediates methyl modifications of histone H3 at gene promoters via interactions with histone demethylase KDM1 (lysine-specific histone demethylase 1A) (Nair et al. 2010b) or coactivator-associated arginine methyltransferase 1 (CARM1) (Figure 3.3) (Mann et al. 2014). PELP1 may enable SRs to interact with some coregulators, even the SRs and coregulators cannot directly interact. For example, PELP1 aids the AR-coregulator four-and-a-half lens intrinsic membrane protein 2 (FHL2) that lacks motifs for direct interactions with AR by binding both AR and FHL2 at the N-terminal region (1–600 amino acids); the close proximity of these proteins on PELP1 enables the coregulator and AR to "functionally interact" without a direct physical interaction (Nair et al. 2007). PELP1 further aids in FHL2 transactivation function by binding other proteins through its SH3, PDZ, and WW motifs at its C-terminal end (Figure 3.3). Thus, PELP1 may serve as a platform or "node" for the recruitment and organization of transcription complexes.

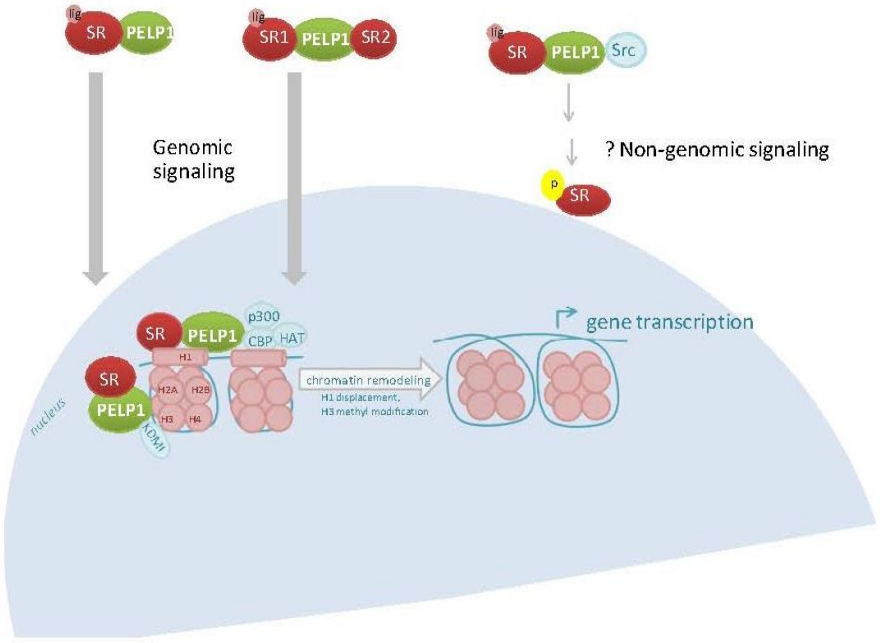


Fig. 3.3 Role of PELP1 in SR signaling. SR bound to PELP1 is translocated into the nucleus, where it binds chromatin. PELP1 facilitates transcription of SR-regulated genes by recruiting coregulators p300, CBP, and HAT to the chromatin that displace histone H1, and by interacting with KDM1 that causes methyl modifications on histone H3. In the absence of ligand, PELP1 may couple an unliganded SR to a liganded SR and activate genomic signaling. In the cytoplasm, PELP1 interacts with both Src and SRs. However, the role of PELP1 in nongenomic signaling needs to be robustly validated.

Role in Nongenomic SR Signaling

Although PELP1 is predominantly localized in the nucleus, its localization to the cytoplasm has been observed in a subset of breast tumors (Vadlamudi et al. 2005b; Rayala et al. 2006b). In the cytoplasm, PELP1 may couple SRs with kinase signaling cascades, as shown for AR, which enables interaction with signaling proteins such as G proteins (Haas et al. 2005) and Src (Unni et al. 2004). Mutations of the PxxP domains on PELP1 resulted in the loss of ER-Src interaction and estrogen-induced MAPK activation (Boonyaratanakornkit 2011). Overexpression of cytoplasmic PELP1 resulted in the rapid induction of MAPK and AKT signaling pathways in a hormone-independent manner, which in turn phosphorylated ER (Figure 3.3) (Vadlamudi et al. 2005b; Rayala et al. 2006a). Complexes of AR, PELP1, and Src have been observed in LNCaP cells; however, their role in Src signaling has not been validated (Unni et al. 2004). The role for PELP1 in nongenomic SR signaling in mammalian cells has been proposed but needs robust validation and remains unproven.

Role in SR Cross Talk

PELP1 functions as a bridge for SRs, enabling the activation of 1 SR to activate another SR, even in the absence of the ligand for the second SR. For example, in prostate cancer cells, in the absence of androgens, PELP1 facilitates estradiol binding to ER to the unliganded AR and activates AR signaling in these cells (Figure 3) (Yang et al. 2012). Knockdown of PELP1 abrogated the coupling of liganded ER signal to unliganded AR (Yang et al. 2012). Similarly, in breast cancer cells, PELP1 facilitated cross talk between ER and PR, in the absence of the PR ligand progestin (Daniel et al. 2015). Gene expression analyses indicated that the interaction between PR, PELP1, and ER enabled the activation of a subset of ER target genes (Daniel et al. 2015). Together, these data indicated that PELP1 might enable a liganded SR to influence the activity of another unliganded SR or vice versa. This finding has significant implications for resistance pathways in endocrine cancers, where the ligands of specific SRs are targeted.

Role in Chromatin Modification

PELP1 participates as a transcriptional coactivator/corepressor of nonnuclear receptors activator protein 1, nuclear factor kB, ternary complex factor/serum response factor (Choi et al. 2004). The glutamic acid-rich domain of PELP1 enables its interaction with the chromatin and interaction with basic histones (Figure 3.4). Although PELP1 does not directly modify the histones, its recruitment of histone modifiers enables histone modification, for example, the leucine-rich N-terminal region of PELP1 recruits histone deacetylase 2 to the chromatin, which, in turn, deacetylateslysine residues on core histones (Choi et al. 2004). PELP1 then binds to the hypoacetylated histones and protects them from acetylation, thereby hindering chromatin remodeling and influencing gene transcription.

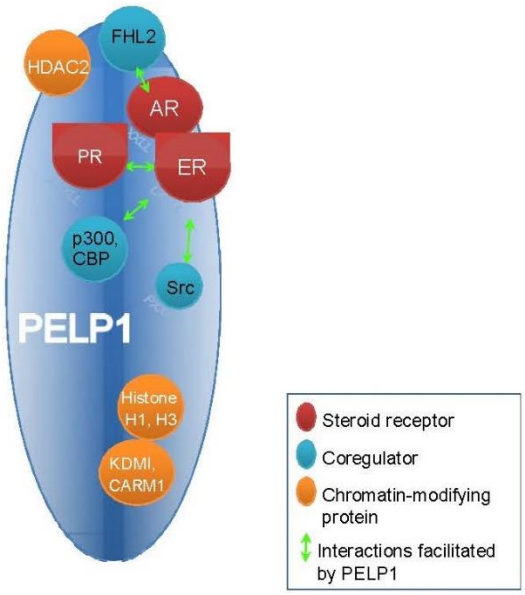


Fig. 3.4 PELP1 as a scaffolding protein. PELP1 interacts with SRs such as AR, ER, and PR through its LXXLL motif, SR coregulator proteins such as FHL2, p300, CBP, and Src, and chromatin-modifying proteins such as KDM1, coactivator-associated arginine methyltransferase 1 (CARM1), and histones through its glutamic acidrich region. The interactome of PELP1 enables cross talk between these proteins and with the DNA.

Extra-nuclear functions

PELP1 acts as a scaffolding protein coupling the ERα with Src kinase, leading to activation of the ESR1–Src–MAPK pathway (Vadlamudi et al. 2005b). PELP1 is required for optimal activation of ESR1 extranuclear actions (Chakravarty et al. 2010a). It facilitates E2 and growth factor-mediated activation of PI3K in the cytosolic compartment (Dimple et al. 2008). Growth factor signals promote PELP1 interactions with STAT3 and PELP1-mediated genomic and non-genomic functions play a role in the growth factor-mediated STAT3 transactivation functions (Manavathi et al. 2005). Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) is a novel PELP1-binding protein that sequesters PELP1 in the cytoplasm, leading to the activation of MAPK (Rayala et al. 2006a). PELP1 modulates the ESR1–Src–ILK1 signaling to promote cytoskeletal rearrangements, motility and metastasis (Chakravarty et al. 2010a). PELP1 also interacts with mTOR and activates its downstream signaling (Gonugunta et al. 2014b).

Cell cycle

Estrogens induce proliferation of ESR1-positive breast epithelial cells by stimulating G1/S transition (Foster et al. 2001). PELP1 is a novel substrate of CDKs, and is sequentially phosphorylated by the CDK4/cyclin D1, CDK2/cyclin E and CDK2/cyclin A complexes (Nair et al. 2010a). PELP1 couples E2 signaling to the E2F axis, and CDK phosphorylation plays a key role in the PELP1 oncogenic functions (Nair et al. 2010b). PELP1 regulates the biologically relevant process of meiosis and participates in maintaining meiotic arrest via interactions with G beta-gamma (Gβγ) and AR (Haas et al. 2005). EGF can promote PELP1 phosphorylation at CDK sites (Olsen et al. 2006). Studies using inducible transgenic mice model revealed that PELP1 regulates expression of a number of known ESR1 target genes involved in cellular proliferation including cyclin D1 and CDKs, and that PELP1 was hyper-phosphorylated at its CDK phosphorylation site, suggesting an autocrine loop involving the CDK–cyclin D1–PELP1 axis in promoting mammary tumorigenesis (Cortez et al. 2014).

Chromatin modifications

PELP1 interacts with and recruits a number of epigenetic modifiers in the target chromatin and facilitates activation of number genes involved in cell proliferation and cancer progression (Nair et al. 2010b). PELP1 interacts with KDM1 and alters the substrate specificity of KDM1 from H3K4 to H3K9. Effective demethylation of dimethyl H3K9 by KDM1 requires a KDM1–ESR1–PELP1 functional complex (Nair et al. 2010b). Since PELP1 expression is deregulated in cancer, PELP1 ability to modulate KDM1 substrate specificity has the potential to alter histone methylation at ESR1 target genes, contributing to hormone-driven tumor progression. PELP1 functionally interacts with the arginine methyltransferase CARM1, and the PELP1–CARM1 interactions synergistically enhance ESR1 transactivation and PELP1 status determines histone H3 arginine methylation code at ESR1 target gene promoters (Mann et al. 2013). PELP1 functions as the core component of 5FMC, and provides a mechanistic link between arginine methylation and (de)sumoylation in the control of transcriptional activity (Fanis et al. 2012). PELP1 interacts with LAS1L and SENP3, components of the MLL1–WDR5 supercomplex involved in chromatin remodeling. Further, PELP1 in pancreatic cancer cells is glutaminated by polyglutamylase TTLL4 and these post

translational modifications play a role in coordination of chromatin remodeling by PELP1 (Kashiwaya et al. 2010).

Reader of histones

PELP1 is a unique NR coregulator that contains a histone binding domain and interacts with histones. Initial studies suggested that PELP1 participates in chromatin remodeling activity via displacement of histone H1 in cancer cells (Nair et al. 2004). Subsequent studies showed that the PELP1 histone binding domain also recognizes the hypoacetylated histones H3 and H4 and prevents them from becoming substrates of histone acetyltransferase. These studies suggested that PELP1 maintains the hypoacetylated state of histones at the target genomic site, and ER binding reverses its role to hyperacetylate histones (Choi et al. 2004). Using histone peptide arrays, it was demonstrated that PELP1 uniquely recognizes histones modified by arginine and lysine dimethylation (Mann et al. 2013). MacroH2A1 is a histone variant that plays a role in transcriptional repression. Recent studies identified PELP1 as a ligand-independent macrodomain-interacting factor and the co-recruitment of macroH2A1 and PELP1 is suggested to cooperatively regulate gene expression outcomes (Hussey et al. 2014).

DNA damage response

p53 is an important transcription factor and tumor suppressor that plays a critical role in DNA damage response (DDR), including cell cycle arrest, repair, or apoptosis. PELP1 is phosphorylated by DDR kinases, and this phosphorylation of PELP1 is important for p53 coacti vation functions. PELP1-depleted p53 (wild-type) breast cancer cells were less sensitive to various genotoxic agents, including etoposide, camptothecin or gamma-radiation (Nair et al. 2014). PELP1 also interacts with MTp53, regulates its recruitment, and alters MTp53 target gene expression. PELP1 knockdown decreased cell survival and increased apoptosis upon genotoxic stress. Mechanistic studies revealed that PELP1 depletion contributes to increased stability of E2F1, a transcription factor that regulates both cell cycle and apoptosis in a context-dependent manner(Krishnan et al. 2015).

RNA splicing

Recent data indicates that PELP1 oncogenic functions involve alternative splicing, leading to the activation of unique pathways that support tumor progression. RNA-seq analysis also revealed that PELP1 regulates the expression of several genes involved in

alternative splicing, and the PELP1-regulated genome includes several uniquely spliced isoforms. PELP1 binds RNA with a preference to poly-C, co-localizes with the splicing factor SC35 at nuclear speckles, and participates in alternative splicing. Further, PELP1 interacts with the arginine methyltransferase PRMT6 and modifies its functions (Mann et al. 2014).

Ribosome biogenesis

PELP1 plays a critical role in ribosomal biogenesis. The SENP3- associated complex comprising PELP1, TEX10 andWDR18 is involved in maturation and nucleolar release of the large ribosomal subunit. The PELP1-associated factor LAS1L is a SENP3-sensitive target of SUMO and SUMO conjugation/deconjugation determines the nucleolar partitioning of PELP1 complex (Finkbeiner et al. 2011). Additional studies confirmed that LAS1L interacts with Rix1 complex (that contain PELP1, TEX10, and WDR18) along with NOL9 and SENP3, and that PELP1 is required for optimal 60S ribosomal subunit synthesis (Castle et al. 2012). PELP1 is localized in the nucleolus and is needed for the active ribosomal RNA transcription. The phosphorylation of PELP1 by CDK also plays an important role in PELP1 nucleolar localization (Gonugunta et al. 2011).

Neuronal functions

PELP1 is widely expressed in many regions of brain, including the hippocampus, hypothalamus, and cerebral cortex (Brann et al. 2008). Subcellular localization studies revealed that PELP1 is highly localized in the cell nucleus of neurons and has some cytoplasm localization (Khan et al. 2006). PELP1 interacts with ESR1, Src, PI3K and GSK3β in the brain. Using forebrain-specific PELP1 knockout mice, it was shown that PELP1 is essential for E2-mediated extranuclear signaling (including activation of ERK and Akt) and anti-apoptotic effects (such as the attenuation of JNK signaling and the increase in phosphorylation of GSK3β following global cerebral ischemia (GCI) (Sareddy et al. 2015).

3.1.6 Functions of PELP1 in cancer

PELP1 is a proto-oncogene (Rajhans et al. 2007) and functions as a critical coregulatory protein that provides cancer cells with a distinct growth and survival advantage (Gonugunta et al. 2014a; Ravindranathan et al. 2015). Overexpression of PELP1 in the

mammary gland using transgenic mice model contributed to mammary gland carcinoma, further supporting its oncogenic potential in vivo (Cortez et al. 2014). PELP1 oncogenic signaling is implicated in progression of several cancers including breast (Krishnan et al. 2015; Zhang et al. 2015), endometrial (Vadlamudi et al. 2004) ovarian (Dimple et al. 2008), salivary (Vadlamudi et al. 2005a), prostate (Yang et al. 2012), lung (Slowikowski et al. 2015), pancreas (Kashiwaya et al. 2010) and colon (Ning et al. 2014).

PELP1 and metastasis

Enhanced expression of PELP1 has been correlated with increased motility and invasion of tumor cells (Chakravarty et al. 2010a; Roy et al. 2014) . In contrast, knockdown of PELP1 results in decreased cell migration via downregulation of the ERa-Src-PELP1-PI3K-ILK1 pathway, which disrupts cytoskeletal organization (Chakravarty et al. 2010a). The overexpression of PELP1 enhances EMT by regulating the expression of genes involved in EMT including MMPs, SNAIL (SNAI1), TWIST (TWIST1), ZEB (ZEB1), MYC, and MTA1 as well as miR-200a and miR-141 in breast cancer patients (Chakravarty et al. 2010a; Roy et al. 2012; Wan and Li 2012; Roy et al. 2014)

PELP1 and therapy resistance

Several studies have indicated that the deregulation of PELP1 contributes to therapy resistance and that the knockdown of PELP1 or blockage of PELP1-mediated extranuclear signaling sensitizes cells to therapy (Vadlamudi et al. 2005b; Nagpal et al. 2008; Kumar et al. 2009; Nair et al. 2011; Vallabhaneni et al. 2011). Interestingly, the subcellular localization of PELP1 is dysregulated in tumors with a cytosolic predominance in a subset of endometrial tumors, which exhibit resistance to tamoxifen anti-hormonal therapy. Patients whose tumors have high levels of cytoplasmic PELP1 respond poorly to tamoxifen therapy compared with patients whose tumors have low levels of cytoplasmic PELP1 (Kumar et al. 2009). These observations are in agreement with results froman experiment in which tamoxifen-susceptible MCF-7 cells engineered to express PELP1 in cytosol (by modification the nuclear localization sequence) were found to exhibit resistance to tamoxifen (Vadlamudi et al. 2005b; Kumar et al. 2009; Gonugunta et al. 2014b). The subcellular localization of PELP1 could be used as a biomarker of hormone sensitivity or vulnerability.

Targeting PELP1 in hormonal cancers

The role of PELP1 in a number of cellular processes and signaling pathways via its various domains makes PELP1 both an attractive and a daunting target for therapeutic modulation. Genetic intervention studies have shown PELP1 knockout to be lethal during embryonic development, which indicates the importance of PELP1 in development (Gonugunta et al. 2014a). Initial attempts targeted blocking PELP1 downstream signaling pathways such as the PELP1–Src axis, PELP1–CDK2 axis, and PELP1–KDM1 axis. The Src inhibitor dasatinib has been found to exhibit therapeutic utility in blocking the PELP1 signaling axis (Vallabhaneni et al. 2011). The CDK inhibitor roscovitine has been found to be effective at reducing the oncogenic processes mediated by PELP1 (Nair et al. 2011). KDM1 and CARM1 inhibitors have also been found to substantially inhibit tumorigenic functions of PELP1 (Cortez et al. 2012; Mann et al. 2013). Even though these studies have established the therapeutic potential of the PELP1 axis in treating hormone-relatedcancer patients, these drugs are not specific to PELP1 and new drugs directly targeting PELP1 interactions with increased specificity are required. One such strategy involves targeting specific functions of PELP1 as a coregulator and scaffolding protein. A recent study has demonstrated the feasibility of targeting the interface between PELP1 and AR interactions with small peptidomimetics that compete for AR binding to PELP1 and effectively disrupt AR–PELP1 interactions (Ravindranathan et al. 2013). Consequently, the peptidomimetics result in decreased AR uptake into the nucleus, effectively blocking ligand-driven AR activation, with reduced expression of canonical AR-regulated genes, decreased cancer cell proliferation, and inhibition of tumor growth in xenograft and ex vivo cultures of primary prostate tumor cells (Ravindranathan et al. 2013). The results of these studies indicate that specific targeting of PELP1 in tumors may serve as a viable therapeutic strategy.

3.2 The Early growth response-1 (EGR1)

3.2.1 Introduction

Early growth response gene-1 (*EGR1*), also known as *NGFI-A*, *krox-24*, *ZIF268* and *TIS8*, is an immediate early gene which encodes a Cys2-His2-type zinc finger transcription factor widely expressed in eukaryotic cells from yeast to humans (Shelly et al. 2000; Ferguson et al. 2013; Wang et al. 2015). It is one of the largest studies of

tumor-specific proteins, which are located in the 5q31 region (Brown et al. 2011; MacKinnon et al. 2011). It has an important role in controlling synaptic plasticity, wound repair, female reproductive capacity, inflammation, growth control, differentiation, apoptosis and tumor progression (Li et al. 2015). Experiments have also proved that acute myeloid leukemia and myelodysplastic syndromes are associated with heterozygous loss of *EGR1* (Volkert et al. 2014).

3.2.2 EGR1's discovery and function

EGR1 was first discovered in the mid-1980s (Yamamoto et al. 2007). The EGR family includes *EGR1*, *EGR2*, *EGR3*, *EGR4* four related members, that can quickly and briefly be up-regulated through a variety of external stimuli, including activation, proliferation and differentiation signals, tissue damage and apoptosis signals (Guilak et al. 2010). *EGR1*, *EGR2*, *EGR3* and *EGR4* share a highly conserved DNA binding domain, composed of three zinc finger motifs that together bind to a 9-bp G/C-rich consensus sequence (GCGGGGGCG) (Chandra et al. 2013). It has been used extensively as a model system for detecting how TFIIIA-like zinc fingers recognize DNA, and how it has served as a basis for engineering some types of artificial DNA-binding proteins (Squires et al. 2015). EGRs are involved in regulating the immune response by means of the induction of differentiation of lymphocyte precursors, and activation of B and T cells (Chen et al. 2015). *EGR1* binds to DNA G/C-rich sequences through 3 zinc-finger motifs in its carboxyl terminal and regulates gene transcription through co-operation with other activating or repressing factors (Charolidi et al. 2015). It may be divided into three zones. The N-terminal portion (amino acids 1-331) is rich in proline (14.2%) and serine (16%) and has 7.9% alanine and 7.9%, threonine. The C-terminal region (residues 417-533) also contains a very high proportion of proline and serine (15.4% and 26.5%, respectively) as well as 10.3% alanine and 11.1% threonine (Shingyochi et al. 2015) (Figure 3.5).

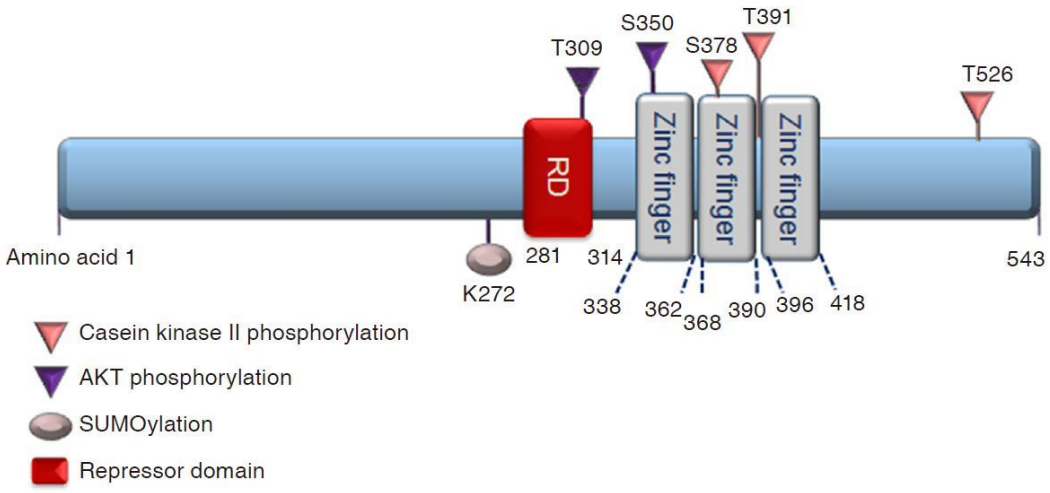


Fig. 3.5 Schematic representation of EGR1 protein structure and post-translational modifications. EGR1 is a 543-amino acid (aa) protein consisting of three Cysteine 2-Histidine 2 (C2H2) zinc fingers DNA-binding domains, approximately 23 aa each. Zinc fingers 2 and 3 (amino acids 361-419) interact with amino acids 315-330 for EGR1 nuclear localization. The T309 and S350 sites are phosphorylated by protein kinase B (PKB, also known as AKT); whereas, S378, T391, and T526 sites are phosphorylated by casein kinase II. EGR1 protein can be SUMOylated by SUMO1 at K272. Transcriptional co-repressors NGFI-A binding protein 1and 2 (NAB1 and NAB2, respectively) inhibit Egr1 transcriptional activity by binding to the repressor domain (RD). EGR1: early growth response 1.

The unstimulated EGR1 level of expression is low in most tissues, except in brain where high expression is observed (Beckmann and Wilce 1997). EGR1 contains a highly conserved DNA-binding domain composed of three zinc fingers that bind to the prototype target GC-rich consensus sequence GCG (G/T) GGGCG (Liu et al. 1998; O'Donovan et al. 1999). In addition, EGR1 binds to the regulatory proteins NAB-1 and NAB-2 (NGF-I A-binding proteins) that repress its transcriptional activity (Gashler et al. 1993; Russo et al. 1993; Svaren et al. 1996).

EGR1 is induced by growth factors, cytokines and stress signals such as radiation, injury or mechanical stress (Gashler and Sukhatme 1995; Liu et al. 1998; O'Donovan et al. 1999). Cloning of the *EGR1* promoter has revealed the presence of response elements for various transcription factors. Specifically, the promoter contains several serum response elements (SREs), an AP-1 binding site, several cAMP regulatory elements (CREs) and Sp1 consensus sequences (Tsai-Morris et al. 1988; Christy and

Nathans 1989; Sakamoto et al. 1991; Schwachtgen et al. 2000). Most often, increased transcription of EGR1 is mediated by the MAP-K signaling pathway (Figure 3.6).

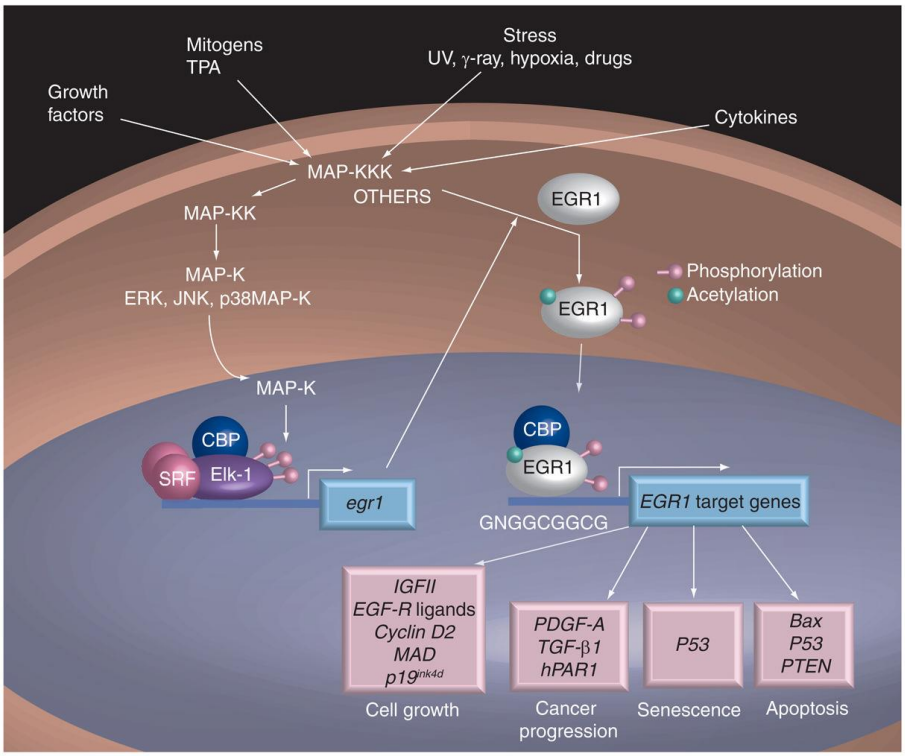


Fig. 3.6 EGR1 mechanism of activation.

Three MAP-K families, known as ERK1/2, JNK and p38MAP-K, are most commonly involved in EGR1 activation. While ERK1/2 mediates EGR1 expression in response to growth factors (Hipskind et al. 1994; Cohen et al. 1996; Harada et al. 1996; Hodge et al. 1998), a combination of ERK1/2, p38MAP-K and/or JNK is required to induce EGR1 in response to stress (Lim et al. 1998; Rolli et al. 1999). The activated MAP-K translocates to the nucleus, phosphorylates and activates transcription factors of the Elk-1/SAP-1/2 family. Elk-1 then associates with the ternary complex factor consisting of CREB-binding protein (CBP) and serum response factor (SRF), and binds to and transactivates the *EGR1* promoter (Karin and Hunter 1995; Silverman and Collins 1999). An alternative pathway for EGR1 activation was recently discovered, in which transcription factor NF-κB mediates EGR1 transcription in response to UV exposure of human skin. This study identified a canonical NF-κB binding site and demonstrated the direct binding of p65 (RelA) to the *EGR1* promoter (Thyss et al. 2005). In another study, two functional nonconsensus binding sites for the tumor suppressor p53 were identified. Binding of p53 to the *EGR1* promoter in response to DNA damage leads to

sustained expression of EGR1 and efficient apoptosis (Yu et al. 2007). Finally, EGR1 binds to its own promoter and represses transcription, thereby initiating a negative feedback loop soon after activation (Cao et al. 1993). EGR1 activity and stability are regulated by post-translational modifications. Acetylation, mediated by the transcriptional co-activator p300/CBP, stabilizes EGR1 and may promote survival, whereas phosphorylation in response to stress may favor cell death (Yu et al. 2004). Sumoylation is mediated by tumor suppressor p19ARF and directs EGR1 to the nucleus (Yu et al. 2009). The shortlived EGR1 is then ubiquitinated on multiple sites and degraded by the proteasome (Bae et al. 2002). We suspect that the full spectrum of post-translational modifications regulating EGR1 activity is far from being fully deciphered.

3.2.3 Biological function and role in tumors

The *EGR1* gene encodes a zinc finger protein and its expression is modulated in diverse biological systems with kinetics resembling those of c-fos (Shingyochi et al. 2015). *EGR1* together with c-fos is crucial for normal myeloid cell differentiation through transcriptional regulation (Saeed et al. 2011). Gene expression analysis revealed that *EGR1* and *c-fos* were down-regulated in hematopoietic primitive cells (Jiang et al. 2015). *C-fos* and *EGR1* represent the key transcription factors that are differentially activated by macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF) to resolve neutrophil versus monocyte cell fate (Hu et al. 2015). However, *EGR1* has more of an advantage than c-fos because of different structure, which increases its expression and decreases sensitivity to stimulation (Lee et al. 2015a). *EGR1* can regulate cell growth, differentiation, growth inhibition, and apoptosis in various kinds of cells (Zhao et al. 2015). Many factors can regulate expression of *EGR1*, including *miR-424*, *miR-146a*, *miR- 181a*, *E2h2*, wilms tumor suppressor 1 (WT1), and Iron (Signorelli and Ghidoni 2005; Guilak et al. 2010; Tanaka et al. 2012; Shen et al. 2013; Cahill et al. 2015; Lee et al. 2015b; Verduci et al. 2015). It's also reported in the literature that *EGR1* can be regulated by erythropoietin (EPO) (Fang et al. 2007; Thiel et al. 2011). *MiR675* upregulates long noncoding RNA H19 through activating *EGR1* in human liver cancer (Fricke 2011). More importantly, *EGR1* can regulate some signaling such as p53, transforming growth factor beta 1 (TGFβ1), phosphatase and tensin homolog deleted on chromosome ten (PTEN), Fibronectin, and enterovirus 71 (EV71) (Baron et al. 2006; Joseph et al. 2015; Liu et al. 2015a; Song et al. 2015). The promoter of the human TGFβ1, p53, and the fibronectin gene contains at

least two EGR1-binding sites, both of which can bind *EGR1* to activate transcription. The proximal promoter of PTEN is GC rich and contains one functional *EGR1*-binding site (Baron et al. 2006). Recent studies indicate that Egr1 is a direct regulator of at least four major suppressors: TGF β 1, PTEN, p53 family members, and fibronectin. These factors have several overlapping functions suggesting that cooperative interactions occur that favor the maintenance of the normal cell phenotype and work to eliminate the emergence of transformed cells. The combined direct regulatory effects of Egr1 and the potential downstream effects are summarized in Figure 3.7.

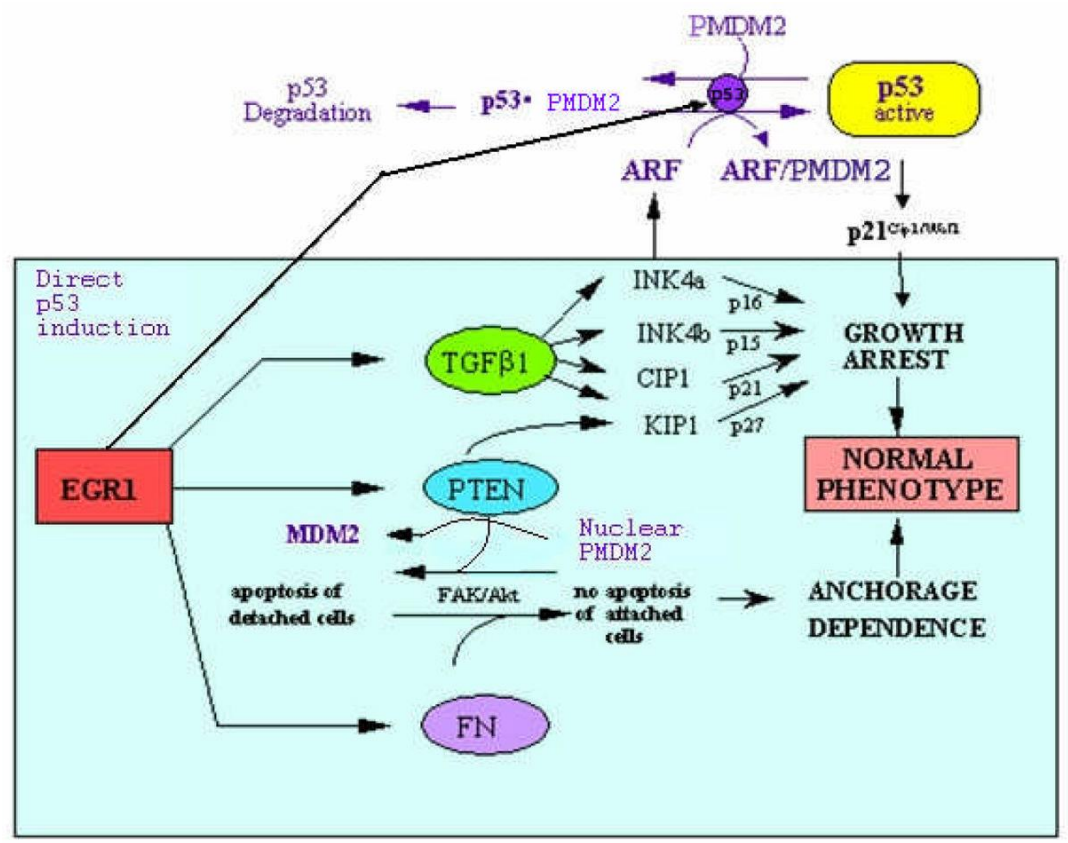


Fig. 3.7 Summary of the overall network of suppression factors that are under the direct control of Egr-1: TGF β 1, PTEN, p53 and fibronectin. The solid arrows from Egr1 indicate regulation of target genes by direct promoter binding known in one or more human cell types to be under the direct regulation by Egr1. The subsequent downstream interactions have been observed in one or more of these systems except the potential roles of the Inks, Akt, and Kip1 which are taken from known interactions in other systems.

Moreover, it plays important roles in decidualization, megakaryocyte differentiation, apoptosis, tendon development, lung injury, liver injury, kidney diseases, chronic obstructive pulmonary disease (COPD), angiogenesis, fibrosis, atherosclerosis, cell

cycle and other biological functions (Tarnawski and Jones 2003; Pritchard and Nagy 2005; Ngiam et al. 2007; Yi et al. 2007; Jaluria et al. 2008; Bhattacharyya et al. 2009; Orbay et al. 2011; Leonardi et al. 2012; Ghosh et al. 2013; Turroni et al. 2013; Liang et al. 2014; Liu et al. 2014; Kushibiki et al. 2015; Liu et al. 2015b; Oh et al. 2015; Petzuch et al. 2015; Tain et al. 2015; Yamamoto et al. 2015; Liao et al. 2016). *EGR1* has a critical role in promoting autophagy and apoptosis in response to cigarette smoke exposure *in vitro* and *in vivo* (Chen et al. 2008). *EGR1* controls metabolism, especially its suppression of lipolysis and promotes fat accumulation by inhibiting the expression of triglyceride lipase (Singh et al. 2015). Although the expression of *EGR1* is low in most tissues, it is high in islets. *EGR1* regulates insulin gene expression by up-regulating Pdx1 (Cheong et al. 2015). *EGR1* gene expression may contribute to the decrease of B-cell proliferation and the consequent cell failure observed in the later stages of type 2 diabetes (Rondinone 2006). The increase of *EGR1* expression in the brain is associated with formation of emotional memory and schizophrenia (Cattane et al. 2015). It has been proved that *EGR1* mutant mice had no changes in short-term memory, but long-term memory was severely damaged (Bozon et al. 2003). Ischemia-induced *EGR1* expression may exaggerate brain injury by reducing brain-derived neurotrophic factor (BDNF) expression (Yang et al. 2015). *EGR1* exhibited a biphasic expression behavior. It was previously described to be down-regulated in many breast carcinoma tissues while it was upregulated in highly invasive inflammatory breast carcinoma. It started to be upregulated 4 h after SNAI1 induction, and was repressed after 24 h (Li et al. 2015). Interestingly, in prostate cancer, kidney cancer and stomach cancer *EGR1* stimulates the growth of tumor cells, and is associated with poor prognosis. In contrast, *EGR1* is a tumor suppressor in fibrosarcoma, glioblastoma, melanoma, esophageal cancer, lung cancer and breast cancer (Abdulkadir 2005; Lu et al. 2010; Sarma et al. 2011; Genet et al. 2015; Yoon et al. 2015).

3.3 The Estrogen-Related Receptor Alpha (ERRα)

3.3.1 Estrogens

Steroid hormones are small hydrophobic molecules that are transported in the blood bound to sex hormone binding globulin and are able to diffuse in and out of cells. Estrogen target tissues include breast, endometrium, bone, brain, liver, and heart.

Derived from cholesterol, estrogens share a common four-ring structure and have important roles in sex determination, fertility, pregnancy, immune response, bone formation, and in the cardiovascular system. Cholesterol is converted into progestins, then into androgens and, finally, estrogens in a series of enzymatic reactions.6 Synthesized predominantly in the ovaries in premenopausal women, the principal site of estrogen synthesis in older women and in men is in peripheral tissues, notably adipose tissue (Westley and May 2013).

There are three estrogens, which are named for the number of hydroxyl groups: estrone, estradiol, and estriol. Estrone and estradiol are produced by aromatization of androstenedione and testosterone, respectively. Estriol is synthesized in the liver and placenta. Estriol is considered the major estrogen in pregnant women, estradiol in premenopausal women, and estrone in postmenopausal women. Concentrations of estrone and estradiol increase with obesity in postmenopausal women and in men (Schneider et al. 1979; Westley and May 2013). The surge in estrogen concentrations at puberty contributes to the development of secondary sexual characteristics including the female breast. Cyclical changes in concentration during the menstrual cycle cause cyclical changes in breast size and tenderness. Reduction in estrogen concentrations on cessation of lactation or menopause lead to breast involution.

3.3.2 The role of the estrogen receptor

The existence of a high-affinity receptor for estrogen was recognized initially by Jensen (Jacobson et al. 1960). Each steroid hormone family has its own protein receptor. These receptors are found in the steroid target cells and are ligand-dependent transcription factors, inactive until bound by their ligand (Figure 3.8A).

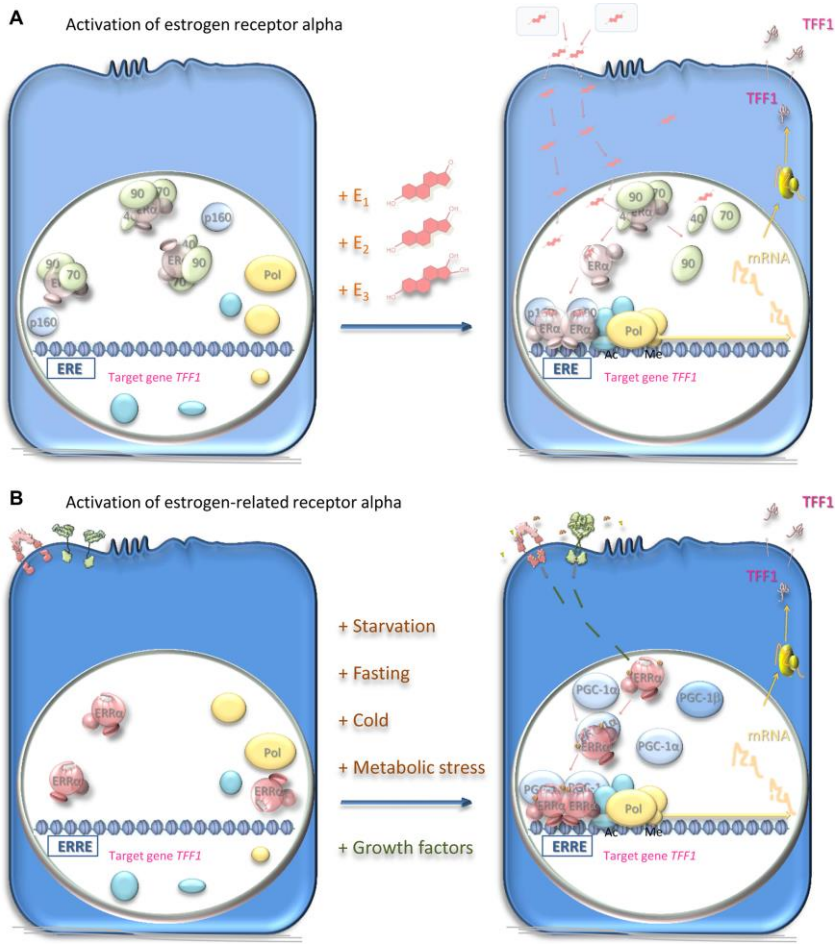


Fig. 3.8 Mechanisms of activation of estrogen receptor alpha and estrogen-related receptor alpha.
Notes: (**A**) Estrogen-responsive cells harbor the estrogen receptor alpha (pale pink), which is a ligand-dependent transcription factor inactive in the absence of ligands. Estrogens: estrone (E1), estradiol (E2), and estriol (E3) are synthesized in the ovaries, in peripheral tissue, or in the placenta and transported in the blood bound to sex hormone-binding globulin (pale blue-gray). Estrogens diffuse through the cellular plasma and nuclear membranes and bind with high affinity to the estrogen receptor. This interaction leads to dissociation of chaperone proteins (pale green), including heat shock protein 90 (90), heat shock protein 70 (70), and cyclophilin 40 (40), from the receptor, which undergoes conformational structural changes and dimerization. The dimerized estrogen receptor ligand complex interacts with specific estrogen response elements (EREs) in the DNA of its responsive genes such as *TFF1*. Interaction with coregulator proteins (pale blue) via the coactivator recruitment surface brings these coregulators into the vicinity of the promoters of the responsive genes. Coactivator proteins interact in turn with proteins, such as histone acetyltransferases or methyltransferases (cyan-blue), that acetylate or methylate the histones to induce local chromatin decondensation and increased accessibility to the promoter elements for the RNA polymerase IIinitiation complex (yellow); transcription of the responsive genes ensues. TFF1 mRNA is translated and the protein secreted from the target cells to exert its effects, which are thought to include cell migration on its target cells (Prest et al. 2002). Alternatively, the receptor interacts with corepressors, and the transcription of other responsive genes is ablated. The estrogen response may be inhibited by prevention of estrogen synthesis with aromatase inhibitors or by competitive inhibition of the interaction

of the ligand with the receptor in target cells. (**B**) The estrogen-related receptor alpha (pink) is present in responsive cells. Being an orphan receptor, its activity is not thought to require the presence of a ligand. Instead, the activity is thought to depend upon the availability of coactivator proteins such as peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α or PGC-1β (pale blue) or corepressor proteins. Induction of the expression of these proteins in response to metabolic stress or starvation allows the estrogen-related receptor alpha to function as a transcription factor and induce or repress the expression of its responsive genes. The dimerized estrogen-related receptor alpha complex interacts with specific response elements (ERREs) in the DNA of its responsive genes such as *TFF1*. The activity of estrogen-related receptor alpha is affected also by phosphorylation (gold) in response to stimuli through the insulin-like growth factor or epidermal growth factor cell surface receptors (pink and green, respectively). The activity of estrogen-related receptor alpha may be inhibited by cessation of synthesis of the receptor or of its activator proteins. Inhibitors of epidermal growth factor or insulin-like growth factor signal transduction may also have utility in responsive cells. Alternatively, inverse agonists prevent its interaction with the coregulators. The activity of estrogen-related receptor alpha may be enhanced by ligands that increase its affinity for the coactivators, possibly by stabilizing the receptor in its active conformation.

Abbreviations: Ac, acetylation; Me, methylation; ERα, estrogen receptor alpha; ERRα, estrogen-related receptor alpha; mRNA, messenger RNA; Pol, RNA polymerase II.

The activated transcription factors coordinate formation of complexes of coactivator or corepressor proteins on the chromatin of their target genes. The composition of the transcription complexes, which depends on the receptor, the availability of coactivator and corepressor proteins, and on the DNA that surrounds the interaction site in the responsive gene, determines whether transcription is activated or repressed. The estrogen receptor is a 66 kDa protein of 595 amino acid residues sequestered in estrogen target cells in its inactive form in a complex with proteins including heat shock proteins 70 and 90, cyclophilin 40, FKBP51, and FKBP52 (Figure 3.8A). Estrogens diffuse into the target cells and bind with high affinity to their receptor, which dissociates from its sequestered complex. Estradiol has the highest affinity for the estrogen receptor, followed by estriol and then estrone (Blair et al. 2000). Interaction with the ligand leads to dimerization of the receptor and stabilizes a conformation that creates a surface on the receptor with which transcriptional coregulators interact. The dimeric receptor binds to estrogen response elements (EREs) in the promoters of estrogen-responsive genes (May and Westley 1988; Wright et al. 2009). Coactivators, for instance, members of the p160 steroid receptor coactivator (SRC) family (SRC-1, SRC-2, and SRC-3), bind through one of three LXXLL motifs that form amphipathic alpha-helices (Xu et al. 2009; Manavathi et al. 2013). The p160 SRC proteins interact, in turn, with the histone

acetyltransferases, cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB)-binding protein (CBP), and p300, which acetylate, and with coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine N-methyltransferase 1 (PRMT1), which methylate, histones within the nucleosomes. An RNA helicase A (RHA), and an ATP-dependent chromatin remodeling complex, SWItch/Sucrose NonFermentable (SW1/SNF), are recruited. The resultant coactivator complex modifies the nucleosomes and alters the surrounding chromatin to allow access to the activating transcription factor proteins, TATA-binding protein, and RNA polymerase II machinery, and transcription ensues. Posttranslational modifications may affect the activity of the estrogen receptor (Le Romancer et al. 2011; Manavathi et al. 2013). Alternatively, corepressors, such as ligand-dependent corepressor (LCoR) and receptor-interacting protein 140 (RIP140), are recruited and attract deacetylases and demethylases to inhibit transcription.

3.3.3 Domain organization of estrogen receptors

Nuclear steroid receptors comprise five separate domains (Figure 3.9A). From the amino-terminus, the hormone-independent transcription activation domain comprises also a nuclear localization signal. It is followed by the DNA-binding domain, which contains a second nuclear localization signal. The DNA-binding domain is separated by a short hinge region from the ligand-binding domain, which is the largest domain and contains a dimerization domain and a transcription repression domain. The second transcription activator domain, which is ligand dependent, is at the carboxy-terminus. There are two estrogen receptors. The first, cloned in 1985 from estrogen-responsive breast cancer cells (Walter et al. 1985; Green et al. 1986) , is expressed in classic estrogen target cells and tissues and is responsible for the standard estrogen responses listed above. It is this receptor that is measured as an important prognostic and predictive biomarker in hormone-dependent breast cancer. The second, which is a 59.2 kDa receptor of 530 amino acids, identified in 1996,56 was called estrogen receptor beta; the former was renamed estrogen receptor alpha at this time to distinguish between the two. Estrogen receptor beta is reported to be expressed more widely than oestrogen receptor alpha and its function is less well understood. Conservation between the two paralogs is variable: low in the domains that interact with transcription activators and in the hinge region, at 25-30%; intermediate in the ligand-binding domain, at 60%; and highest, at 90%, in the DNA-binding domain (Figure 3.9C).

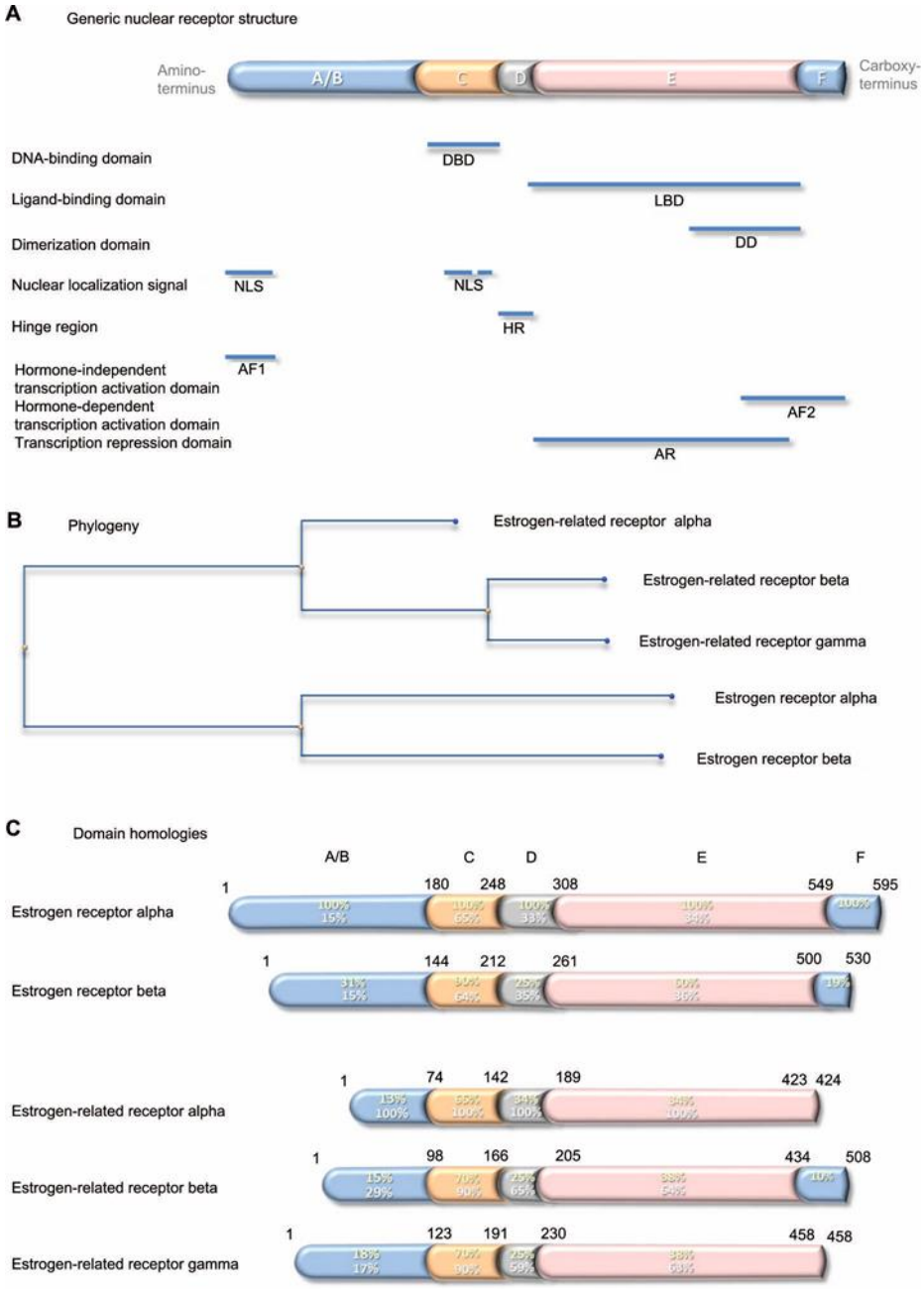


Fig. 3.9 Generic nuclear receptor structure, and estrogen receptor family phylogeny and shared homologies.

Notes: Steroid hormone receptors share a conserved five-domain structure (**A**): domain A/B, which is involved in transcription activation and nuclear localization; domain C, which is the DBD; domain D, which serves as a hinge between the DBD and LBD; domain E, which is the LBD, responsible also for dimerization, transcription activation, and transcription repression; and domain F, which contains part of the second transcription activation domain. Phylogenetic analysis indicates that the genes that encode the five estrogen receptor proteins have evolved from a common precursor gene (**B**). The estrogen-related receptor alpha is the most distant. The percentage conservation between the estrogen receptor alpha (yellow text) and the other four proteins, and between the estrogen-related receptor alpha (white text) and the other four proteins, is shown for each of the five domains (**C**).

Abbreviations: NLS, nuclear localization signal; AF1, hormone-independent transcription activation domain; DBD, DNA-binding domain; HR, hinge region; LBD, ligand-binding domain; DD, dimerization domain; AR, transcription repression domain; AF2, hormone-dependent transcription activation domain.

3.3.4 Estrogen-related receptor alpha

In 1988, 8 years before discovery of estrogen receptor beta, two other members of the estrogen receptor family were discovered by Giguère et al. (Giguere et al. 1988) a 45.5 kDa, 423 amino acid residue protein named estrogen-related receptor alpha; and a 56.2 kDa, 508 amino acid residue protein named estrogen-related receptor beta. Later, the estrogen-related receptor gamma of 51.3 kDa, 458 amino acid residue protein was identified (Schwabe et al. 1993; Chen et al. 1999). Evolutionarily, estrogen-related receptor beta and gamma are closer to each other than to estrogen-related receptor alpha (Figure 3.9 B). Comparison of the primary sequences in the different receptor domains shows relatively strong conservation of around 65% in the DNA-binding domains and less, around 35%, in the ligand-binding domains. Conservation is loweroutwith these domains, and the estrogen-related receptors alpha and gamma lack an F domain (Figure 3.9 C). The estrogen-related receptor alpha interacts with coregulator proteins and binds to specific DNA sequences of its target gene promoters, primarily as a homodimer (Figure 3.8 B). The peroxisome proliferator-activated receptor (PPAR) gamma coactivator (PGC)-1 family (PGC-1α, PGC-1β, and PPRC-1) and the p160 SRC proteins interact with this estrogen-related receptor alpha coactivator surface via LXXLL motifs (Xu et al. 2009). The most notable difference between estrogen-related receptors and estrogen receptors is that the former function as aporeceptors when they are not bound to ligand.

3.3.5 Estrogen receptor family DNA-binding domains

Arguably the domain most central to the specific functions of the estrogen receptor family is the conserved DNA-binding domain that recognizes sequences in the responsive genes and dictates with which genes the receptor will interact. This relatively short sequence of 70 amino acid residues contains two zinc-binding elements. In each, a zinc ion is ligated tetrahedrally by four cysteine residues (Figure 3.10). This class II zinc-binding motif comprises, from the amino-terminus, a zinc finger, an alpha helix, the second zinc finger, and the second alpha helix. The first alpha helix is the recognition helix that fits into the major groove of the double-stranded DNA. The structure of the estrogen receptor alpha DNA-binding domain in complex with DNA

illustrates that the receptor dimer locates primarily on one face of the DNA helix (Figure 3.10C) (Schwabe et al. 1993). The side chains of the residues make specific contacts with four DNA bases. The three residues responsible for the DNA interface are referred to sometimes as the proximal box. One of these residues, alanine in the estrogen-related receptors, is a glycine in the two estrogen receptors. The second region of intermolecular interaction is responsible for the dimerization interface and is referred to sometimes as the distal box. This region contains uniquely in estrogen-related receptor alpha a single conservative substitution of a serine in place of a threonine.

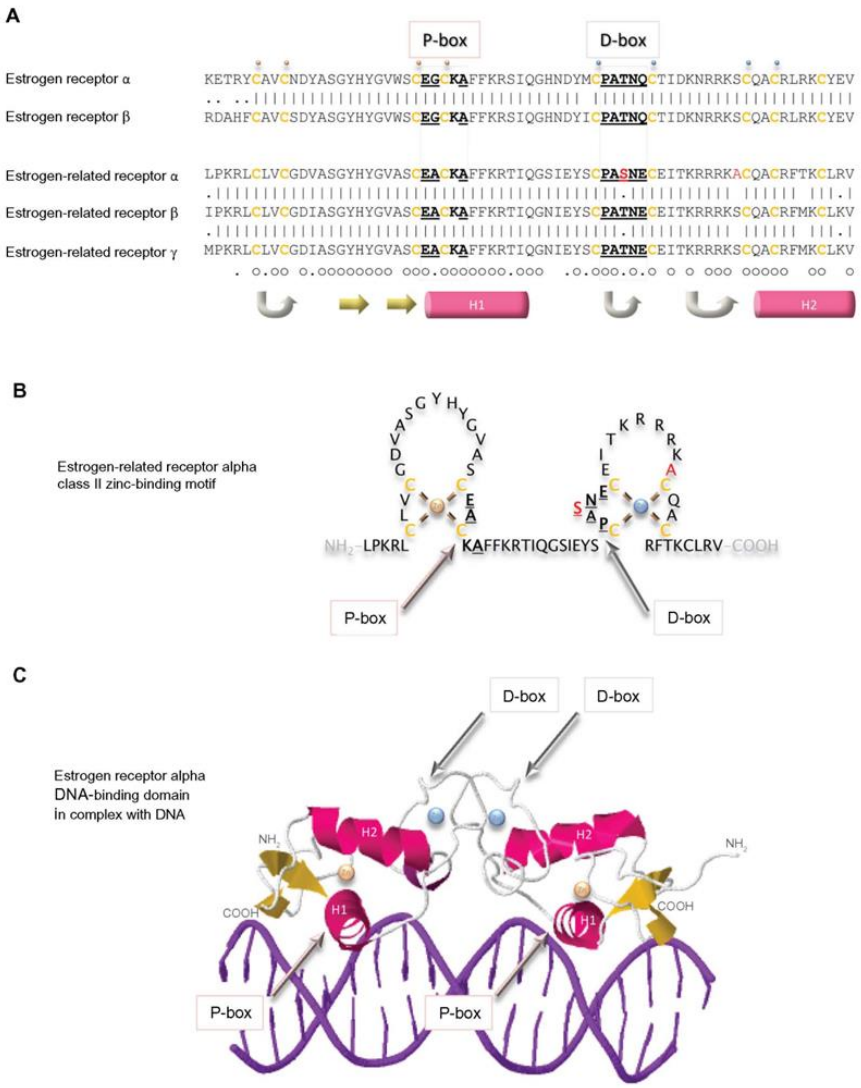


Fig. 3.10 DNA-binding domains of the estrogen receptors.

Notes: The primary sequences of the DNA-binding domains of the five estrogen receptors are aligned (**A**). Vertical lines indicate amino acid residues that are identical in estrogen receptor alpha and beta, or in the three estrogen-related receptors, and dots indicate conserved substitution of residues. Residues that are identical in all five proteins are indicated by a small circle, and those that are substituted conservatively

by a dot underneath the sequences. Cysteine residues are colored ochre and are in bold. The residues in the proximal box (P-box) that are responsible for interaction with DNA and those in the distal box (D-box) that are involved in the dimerization interface are in bold and are underlined. The two residues that are unique to estrogen-related receptor alpha are colored red. A small gold sphere is above the four cysteine residues that bind tetrahedrally to one zinc ion and a small blue sphere is above the cysteine residues that bind tetrahedrally to the second zinc ion. Regions of secondary structure are indicated below the sequences: turn (gray arrow), beta strand (yellow arrow), and alpha helix (pink cylinder). The estrogen-related receptor alpha DNA-binding class IIzinc-binding motif is illustrated graphically (**B**) and a ribbon representation of the structure of the of the estrogen receptor alpha in complex with DNA is shown (**C**) with the same conventions as in (**A**).

Members of the estrogen receptor family bind to specific conserved DNA recognition sequences in the DNA of their responsive genes (Figure 3.11). Often, but not always, located proximal to the promoters, they may be up to 100 kb from the promoter of the responsive gene. The canonical estrogen response element or ERE 5′-AGGTCA-3′ is followed by three bases of indeterminate sequence and then by the inverse sequence 5′-TGACCT-3′ (Klein-Hitpass et al. 1986; Berry et al. 1989; May et al. 1993; May and Westley 1995; Klinge et al. 2001). The palindromic nature of the perfect ERE is predictable given the perfect dimeric structure formed by interaction of the two receptor DNA-binding domains (Figure 3.10C). Reduction in the length of the palindromic sequence by a base on either side reduces the affinity of the interaction with the receptor dimer. The third base pair of the ERE half-site, G–C, provides binding energy, and the fourth base pair, T–A, makes a positive contact with the receptor. Most EREs identified differ from the canonical sequence by at least one base pair (May and Westley 1995).

The estrogen-related receptor alpha affects the transcription of known estrogen-responsive genes, such as those that encode lactoferrin, osteopontin, thyroid receptor alpha, aromatase (*CYP19*), and TFF1(May and Westley 1986; Prest et al. 2002) via interaction with the EREs in these genes (Yang et al. 1996; Vanacker et al. 1998a; Vanacker et al. 1998b; Yang et al. 1998; Lu et al. 2001). Detailed analysis suggests that estrogen-related receptor alpha binds particularly well if the sequence is preceded by 5′-TAA-3′ or 5′-TCA-3′, and it is suggested that the estrogen-related receptor alpha response element (ERRE) is 5′-TA/CA AGGTCA-3′. The presence of a combined ERRE and ERE will ensure regulation of a gene by both receptors. It is noteworthy that the *TFF1* promoter contains an imperfect ERRE and an imperfect ERE (Figure 3.11).

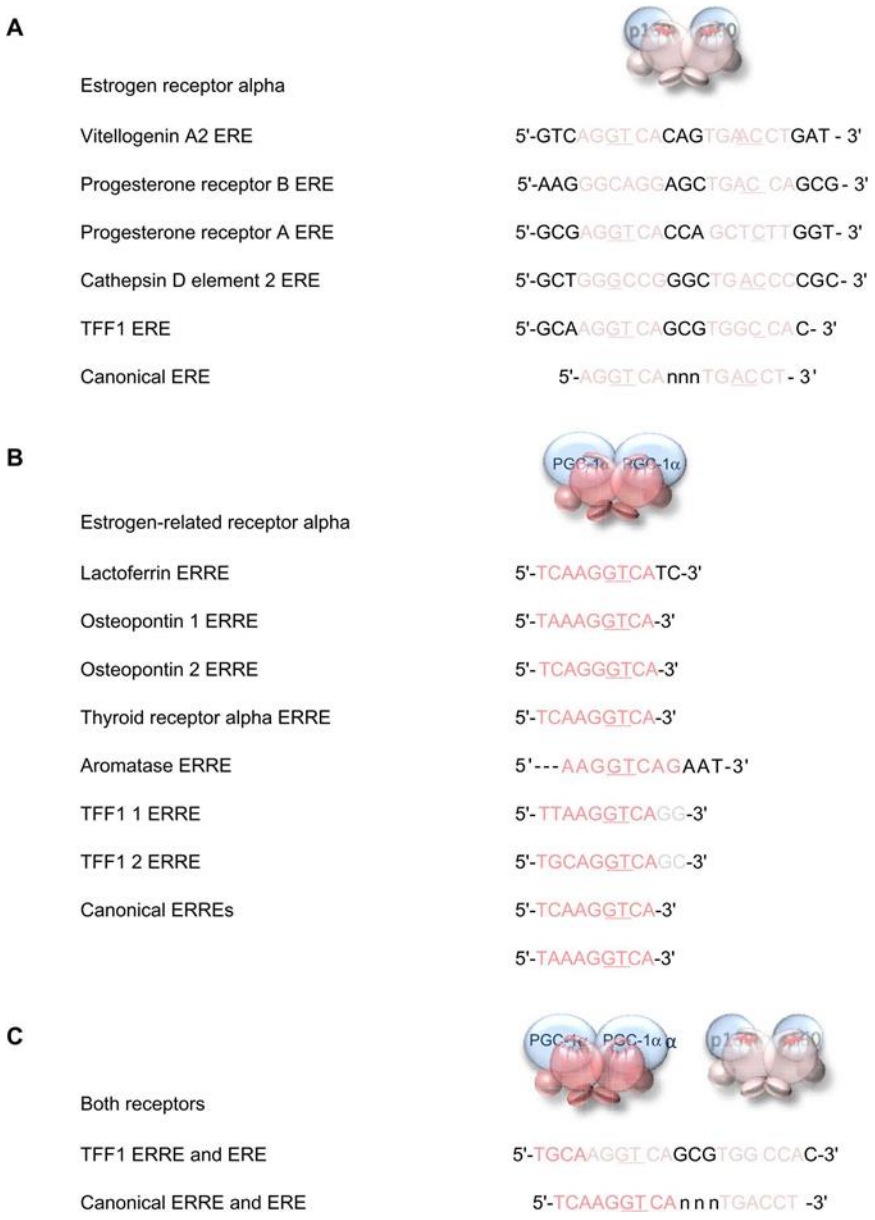


Fig. 3.11 DNA response elements recognized by estrogen receptor alpha and estrogen-related receptor alpha.

Notes: The sequences of the EREs identified in key estrogen responsive genes are aligned above the consensus perfect palindromic sequence (**A**). The sequences of the ERREs identified in its responsive genes are aligned above the canonical sequence (**B**). The dual ERRE and ERE found in the promoter region of the *TFF1* gene is shown above a perfect combined ERRE and ERE (**C**). The important third and fourth base pairs of the EREor ERRE half-sites that provide binding energy and make positive contact with the receptor, respectively, are underlined when they are the conserved canonical sequence in the response elements.

Abbreviations: ERE, estrogen response element; ERRE, estrogen-related receptor alpha response element.

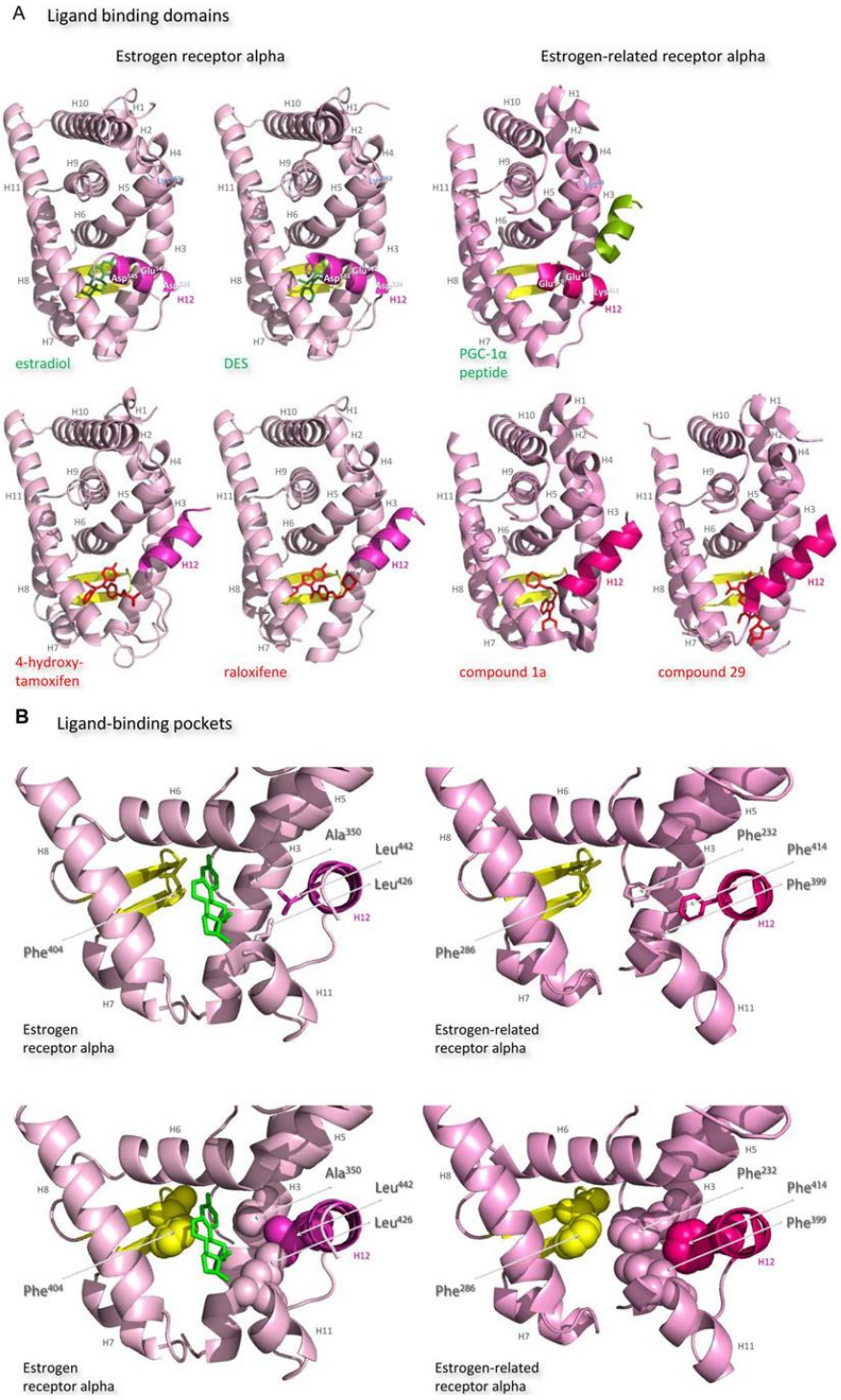
3.3.6 Estrogen receptor family ligand-binding domains

The ligand-binding domains of the estrogen receptor family are composed almost entirely of 12 helices, of which eleven are arranged in three antiparallel layers (Brzozowski et al. 1997). Helices 5, 6, 9, and 10 comprise the central core layer,which is sandwiched between helices 1–4 on one face and helices 7, 8, and 11 on the other (Figure 3.12 A). In the estrogen receptors, this wedge-shaped molecular scaffold creates a sizeable hydrophobic cavity at its narrower end into which estrogens slip and interact with high affinity. The remaining secondary structural elements, a small two-stranded antiparallel β-sheet and helix 12, flank the main three-layered motif on either side of the hydrophobic pocket (Figure 3.12A). After interaction with estrogens, helix 12 is positioned as a lid over the ligand-binding pocket to secure the ligand in position and posit the hydrophobic side chains of helix 12 toward the steroid (Figure 3.12B) (Brzozowski et al. 1997). This conformation creates a surface on the receptor that includes the charged residues, Lys362 at the end of helix 3 and Asp538, Glu542, and Asp545 from helix 12, that were identified by mutation analysis to be important for transcription activation (Figure 3.12A) (Danielian et al. 1992). Subsequent analysis of the structure of the ligand-binding domain of estrogen receptor alpha bound to the synthetic estrogen diethylstilbestrol and a coactivator peptide with an LXXLL motif identified more fully the coactivator recruitment surface (Shiau et al. 1998). The interaction surface comprises a hydrophobic cleft formed with residues from helices 3, 4, 5, and 12 and the turn between helices 3 and 4 (Figure 3.12C). Interaction of the amphipathic alpha-helical coactivator peptide buries approximately 1,000 Å2 of the hydrophobic interaction surface. The majority of the residues involved in the interaction are hydrophobic. In addition, the main chain conformation of the coactivator peptide is stabilized by charged capping interactions at either end of the peptide helix with Lys362 from helix 3 and Glu542 from helix 12 of the receptor (Figure 3.12C).

After interaction of antiestrogens such as the active metabolite of tamoxifen, 4-hydroxytamoxifen, or raloxifene with the ligand-binding domain of estrogen receptor alpha, a portion of the ligand remains outside the ligand-binding pocket (Figure 3.12A) (Brzozowski et al. 1997; Shiau et al. 1998). The extruded ligand prevents alignment of helix 12 over the ligand-binding pocket and hence formation of a complete coactivator recruitment surface. Instead, helix 12 is positioned over the hydrophobic cleft between

helices 3, 4, and 5, in which position it precludes completely interaction of coactivators with this surface of the receptor. The secondary and tertiary structures of the ligand-binding domains of estrogen-related receptors are extremely similar to those of the estrogen receptors, but subtle differences are proposed to allow them to function as aporeceptors and explain the failure to identify their natural ligands. Notably, in the structure of the estrogen-related aporeceptor alpha, helix 12 is positioned across the ligand-binding domain (Figure 3.12A) (Kallen et al. 2004). Thus the four charged residues, Lys244 in helix 3 and Lys412, Glu416, and Glu419 in helix 12, equivalent to those thought originally to be critical for activation of transcription in estrogen receptor alpha, are on the same face of the receptor in the absence of ligand. The crystal structure of estrogen-related receptor alpha includes the coactivator peptide of PGC-1α bound to the receptor coactivator recruitment surface; it is not known if the presence of this peptide facilitates stabilization of the active conformation of estrogen-related receptor alpha in the crystals, or if the structure of the aporeceptor ligand-binding pocket would be more open in the absence of the PGC-1α peptide. The coactivator recruitment surface of estrogen-related receptor alpha formed from helices 3, 4, 5, and 12 is similar to that of estrogen receptor alpha. The PGC-1α peptide is anchored by canonical charge clamp interactions with Lys244 from helix 3 and Glu416 from helix 12, and many of the conserved hydrophobic residues interact with the coactivator peptides in both structures (Figure 3.12C) (Shiau et al. 1998; Kallen et al. 2004). The estrogen-related receptor alpha ligand-binding pocket is delineated by 22 amino acid residues, most of which have hydrophobic side chains. It is occluded by bulky hydrophobic side chains, in particular that of the phenylalanine Phe232 from helix 3, (Kallen et al. 2004) which is an alanine in the other four receptors, to create a cavity of only 100 Å3, which is substantially smaller than those of the estrogen receptor alpha (490 Å3) or beta (390 Å3) (Figure 3.12B). Removal of this hydrophobic side chain abolishes activity of the aporeceptor. It is proposed that the presence in the ligand-binding pocket of Phe232 and other bulky hydrophobic side chains, notably Phe286 from the small beta sheet, Phe399 from helix 11, and Phe414 from helix 12, recapitulates the interactions provided by hydrophobic steroids in the estrogen receptors and allows the aporeceptor to hold an active conformation able to interact with coactivator proteins (Kallen et al. 2004). Disruption of the interactions between Phe232, Phe286, Phe399, and Phe414 destabilizes the active conformation of the estrogen-related aporeceptor alpha.

Interestingly, estrogen-related receptor alpha retains the charged Glu235 and Arg276, of which the equivalent residues in estrogen receptor alpha form a hydrogen bond network with the hydroxyl group on carbon 3 of the A ring of all three estrogens. Estrogen-related receptor alpha retains also the polar His298 that forms a hydrogen bond with the hydroxyl group on carbon 17 of the D ring of estradiol and estriol. It is suggested, however, that insertion of an estrogen into the ligand-binding pocket of estrogen-related receptor alpha would cause such serious steric clashes, notably with the side chains of Phe232 and Phe399, that even the A ring would not be accommodated (Figure 3.12B) (Kallen et al. 2004). The occlusion of the estrogen-related receptor alpha ligand-binding pocket by bulky hydrophobic side chains indicates that the introduction of a molecule with more than four or five carbon atoms would necessitate a conformational change that would displace helix 12 from the coactivator surface (Kallen et al. 2004). The estrogen-related receptor alpha does not bind estrogens or 4-hydroxytamoxifen, but does interact with the synthetic estrogen diethylstilbestrol to prevent the receptor interaction with SRC-1. Antagonist effects of diethylstilboestrol on estrogen-related receptor alpha activity have been reported by some but not all authors (Lu et al. 2001; Suetsugi et al. 2003). Modeling indicates that diethylstilbestrol would only be accommodated in the estrogen-related receptor alpha ligand-binding pocket if the side chains of Phe232 from helix 3 and Phe399 from helix 11 were to assume different conformations, and if Phe414 was removed from the hydrophobic cavity by displacement of helix 12.29,30 That these conformational changes would disrupt the favorable cluster of phenylalanines Phe232, Phe286, Phe399, and Phe414, might indicate that the affinity of diethylstilboestrol for estrogen-related receptor alpha would be weak. Substitution of Phe232 with an alanine residue, which is found in the equivalent position in the other four members of the estrogen receptor family, allows 4-hydroxytamoxifen to bind estrogen-related receptor alpha with a relatively high affinity of 4×10–8 M (Coward et al. 2001). Despite the steric constraints described above, several phytoestrogens: the flavone 6,3′,4′-trihydroxyflavone and the isoflavones genistein, daidzein, and biochanin A, have been reported to be agonists for estrogen-related receptor alpha activity (Suetsugi et al. 2003). Phytoestrogens are produced by plants, have bactericidal and fungicidal activity, and represent the major natural exogenous sources of estrogenic compounds. The results indicate that it is possible for a ligand to interact with estrogen-related receptor alpha to augment its activity.


A Ligand binding domains
Estrogen receptor alpha
Estrogen-related receptor alpha
estradiol
DES
PGC-1α peptide
4-hydroxy-tamoxifen
raloxifene
compound 1a
compound 29
B Ligand-binding pockets
Ala350
Leu442
Leu426
Phe404
Estrogen receptor alpha
Phe232
Phe414
Phe399
Phe286
Estrogen-related receptor alpha

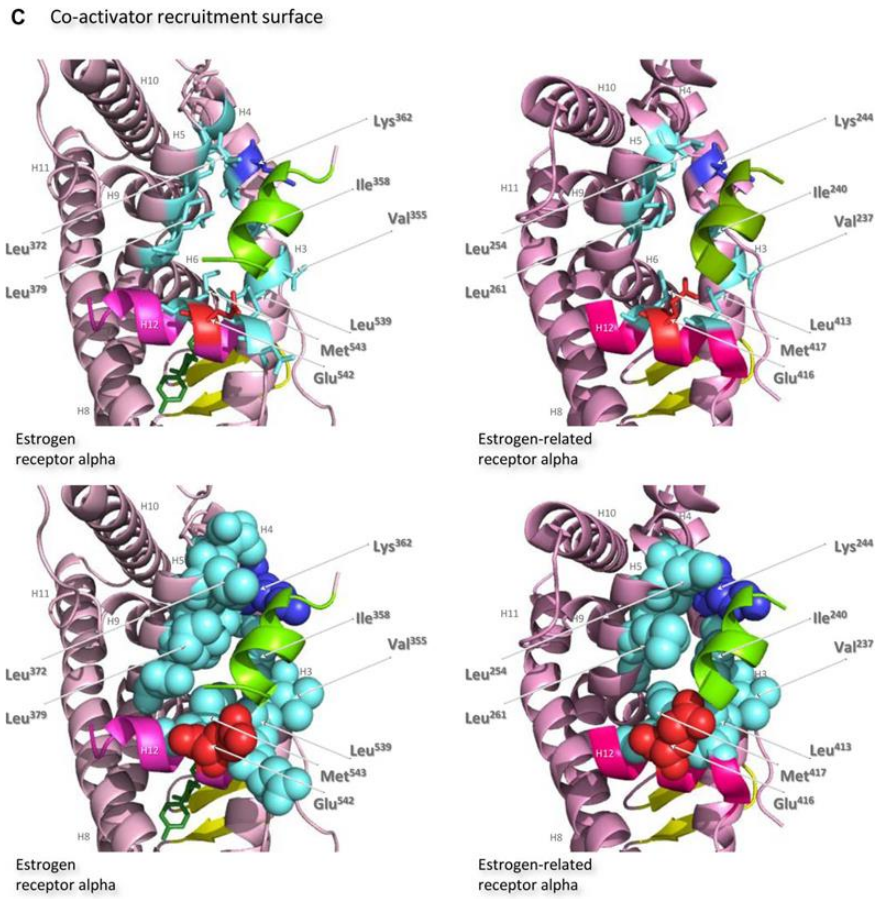


Fig. 3.12 Structures of the ligand-binding domains of estrogen receptor alpha and estrogen-related receptor alpha in complex with their ligands.

Notes: Ribbon representations of the three-dimensional crystal structures of the ligand-binding domains of estrogen receptor alpha in complex with estradiol (a); DES (b); 4-hydroxy-tamoxifen, a high-affinity metabolite of tamoxifen (c); and raloxifene (d), and of estrogen-related receptor alpha in complex with a PGC-1α peptide (e); compound 1a (f); and compound 29 (g) (A). Helices 1–11 of estrogen receptor alpha are colored pale pink and those of estrogen-related receptor alpha pink. The 12th helices are colored darker shades of pink and the short antiparallel beta sheets are colored yellow. The ligands, shown in stick view, and coactivator peptides, shown in a ribbon representation, are colored green if they increase activity of the receptor and red if they inhibit its activity. The helices are numbered and the four charged residues proposed initially to be critical for coregulator interaction are labeled. Ribbon representations of the ligand-binding pockets of estrogen receptor alpha in complex with estradiol and of estrogen-related receptor alpha in complex with a PGC-1α peptide are shown with estradiol shown in stick representation (B). The molecules are rotated to the right compared to the views shown in (A), with helix 11 to the front and helix 12 to the right of the structures. Much of helix 11 has been removed to allow better visualization of the occupancy of the ligand-binding pockets. The side chains of the four phenylalanine residues, Phe232, Phe286, Phe399, and Phe414, that are orientated towards the ligand-binding pocket of estrogen-related receptor alpha and are thought to contribute to stabilization of its active conformation, and the equivalent residues of estrogen receptor alpha, Ala350, Phe404, Leu426, and Leu442, are

indicated and labeled, and their side chains are shown in stick representation (top) or in space-filling mode (bottom). Helices are numbered and colored as in (A). Ribbon representations of the coactivator recruitment surfaces of estrogen receptor alpha in complex with DES and a GRIP1 peptide, and of estrogen-related receptor alpha in complex with a PGC-1α peptide, are shown (C). The molecules are rotated slightly to the left compared to the views shown in (A) to allow better visualization of the hydrophobic cleft formed between helices 3, 4, 5, and 12. The helices are numbered and colored as in (A). The residues involved in the coactivator peptide interaction are shown in stick representation (top) and space-filling representation (bottom). Most have hydrophobic side chains and are colored light blue. The charged Lys and Glu residues that form charged capping interactions at either end of the coactivator peptide are colored blue and red, respectively. Conserved residues that were identified as being involved in interactions with the coactivator peptides in both structures and that are clearly visible in the figure are indicated. All images were created with PyMol Molecular Graphics Software (Schrödinger, Portland, OR, USA).

Abbreviations: DES, diethylstilbestrol; PGC, peroxisome proliferator-activated receptor gamma coactivator.

3.3.7 Physiological functions of estrogen-related receptor alpha

Discovery of estrogen-related receptor alpha immediately prompted questions of its physiological function: whether it overlapped with that of the estrogen receptor and if the receptor had a role in breast cancer. Estrogen-related receptor alpha is expressed in the later stages of embryonic development and is abundant in heart, skeletal muscle, and the nervous system. The physiological role of estrogen-related receptor alpha, and of estrogen-related receptor gamma, is to act as an energy sensor to control cellular adaptation to energy demand and stress. To this end, estrogen-related receptor alpha is expressed at high levels in tissues with high energy demands, such as muscle and brown adipose tissue. Cells that do not express active estrogen-related receptor alpha cannot produce sufficient energy in times of peak demand.

3.3.7.1 Role of estrogen-related receptor alpha in metabolism

In adipose tissue, estrogen-related receptor alpha increases the differentiation of mesenchymal stem cells into adipocytes and hence enhances fat deposition. Further, estrogen-related receptor alpha has a role in the regulation of energy metabolism in adipocytes. It increases lipid uptake, fatty acid beta-oxidation, the tricarboxylic acid cycle, oxidative phosphorylation, and mitochondrial biogenesis and function. Effects of estrogen-related receptor alpha on metabolism extend to other tissues with high energy

requirements, notably cardiomyocytes and macrophages. The importance of estrogen-related receptor alpha in metabolic regulation is emphasized by the demonstration that *esrra*-null mice have impaired fat absorption and metabolism and are relatively resistant to fat-induced obesity (Luo et al. 2003). These lean mice are unable to adapt to cold environments and develop cardiac contractile dysfunction. The cardiac hypertrophy induced by stress in *esrra*-null mice is caused by reduced ATP synthesis and reduced phosphocreatine storage (Huss et al. 2007).

3.3.7.2 Role of estrogen-related receptor alpha in osteogenesis

Estrogen-related receptor alpha influences the differentiation of myocytes, T-cells, intestinal epithelial cells, and osteoblasts. A report indicated that estrogen-related receptor alpha has a role in bone development and metabolism during embryogenesis (Bonnelye et al. 1997). Its messenger RNA is expressed in murine bone cells during bone formation by endochondral and intramembranous ossification and in primary human osteoblasts. Estrogen-related receptor alpha was found to affect transcription of an osteopontin gene promoter; osteopontin is an important constituent of the mineralized extracellular matrix of bones (Bonnelye et al. 1997). In *essra*-null mice, absence of estrogen-related receptor alpha increased modestly osteoblast differentiation and cancellous bone mineral density, as well as mesenchymal cell differentiation into osteoblasts (Delhon et al. 2009). Further, estrogen-related receptor alpha was shown to decrease differentiation of human mesenchymal stem cells into osteoblasts, osteopontin expression, and mineral deposition, but to increase adipocyte differentiation (Delhon et al. 2009). In a different strain of *essra*-null mice, female bones aged less compared to those of wild-type mice even after estrogen depletion and their marrow mesenchymal stem cells showed greater ability to differentiate into osteoblasts ex vivo (Teyssier et al. 2009). Thus estrogen-related receptor alpha has a pivotal role in determination of mesenchymal stem cell fate and is implicated in inhibition of mineralization by osteoblasts (Bonnelye et al. 1997; Delhon et al. 2009; Teyssier et al. 2009).

3.3.7.3 Genes induced by estrogen-related receptor alpha

Genomic studies indicate that estrogen-related receptor alpha regulates large numbers of genes involved in energy metabolism. Estrogen-related receptors interact with the promoters of most mitochondrial and cellular genes that encode enzymes involved in the glycolytic pathway, the tricarboxylic acid cycle, and oxidative phosphorylation, and in

nucleic acid, amino acid, lipid, and pyruvate synthesis. Estrogen-related receptor alpha is involved in the transcriptional regulation of genes required for mitochondrial biogenesis, the tricarboxylic acid cycle, oxidative phosphorylation, fatty acid oxidation, and lipid metabolism (Puigserver et al. 1998; Yoon et al. 2001; Huss et al. 2004). For instance, estrogen-related receptor alpha induces expression of *NRF1*, *GAP*α, and *PPAR*α (Mootha et al. 2004). The nuclear receptor coactivators PGC-1α, PGC-1β, and PPRC-1 are implicated in the regulation of these genes and in the autoregulation of the expression of estrogen-related receptor alpha. It has been suggested that the metabolic effects of estrogen-related receptor alpha are controlled by PGC-1α (Huss et al. 2004). PGC-1α is expressed at low basal levels but is induced by fasting and other metabolic stresses (Puigserver et al. 1998). PGC-1β, a related coactivator, has similar functions, but its expression may not be regulated as acutely by variations in energy demand (Yoon et al. 2001) (Figure 3.13).

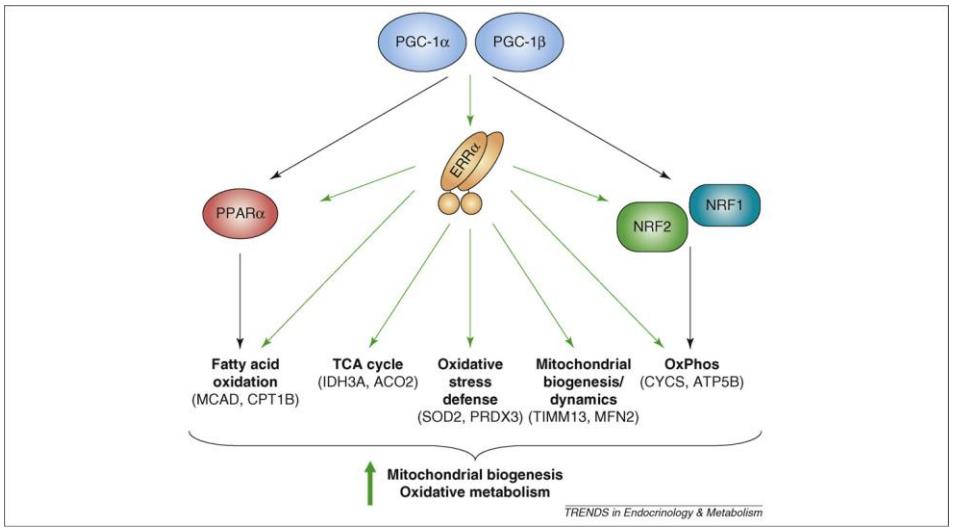


Fig. 3.13 ERRa regulates genes important for mitochondrial biogenesis and oxidative metabolism. ERRa acts as a downstream effector of PGC-1a and PGC-1b in the regulation of expression of genes with roles in fatty acid oxidation, the TCA cycle, oxidative stress responses, mitochondrial biogenesis and dynamics, and oxidative phosphorylation (OxPhos). It acts both directly on genes encoding mitochondrial structural components and enzymes, as well as indirectly by enhancing expression of other transcriptional factors, such as PPARa, NRF1 and NRF2/GABP, that regulate these pathways.

3.3.7.4 Activation of estrogen-related receptor alpha

If the estrogen-related receptor alpha is fully functional in the absence of ligand, does this mean that it is constitutively active? Current thoughts are that its activity is

regulated in two main ways (Figure 3.8). First, its activation is limited by the intracellular concentrations of its coregulators. Rather than being regulated by interaction with a classic ligand with resultant stabilization of an active receptor conformation, the magnitude of estrogen-related receptor alpha activity is thought to be dependent largely on the presence of transcriptional coactivators of transcription such as PGC-1α, PGC-1β and PPARβ, SRC-3 and PPGC183 (Kressler et al. 2002; Lin et al. 2002), or corepressors of transcription such as RIP140 and nuclear receptor corepressor 1 (NCoR1) (Castet et al. 2006; Perez-Schindler et al. 2012). Whether or not the coactivators induce or stabilize the active conformation of estrogen-related receptor alpha is unknown. Certainly, the coregulators are essential for most estrogen-related receptor alpha activity and have been termed surrogate ligands. Induction of the expression of the coregulators by external metabolic stress activates estrogen-related receptor alpha. Secondly, receptor activity is controlled by posttranslational modification (Figure 3B), namely by phosphorylation initiated by the interaction of growth factors such as the IGFs and epidermal growth factors (EGFs) with their cognate receptors and consequent signal transduction. Recruitment of estrogen-related receptor alpha to the *TFF1* promoter and resultant transcription are increased in the presence of EGF, possibly via phosphorylation of the DNA-binding domain (Barry and Giguere 2005). Activation of HER2 increases the transcriptional activity of estrogen-related receptor alpha by phosphorylation at multiple residues, including in the carboxy-terminus (Ariazi et al. 2007). In vitro analyses of the ability of estrogen-related receptor alpha to induce transcription from the *TFF1* gene in breast cancer cells demonstrated that the induction is increased by activation of growth factor receptors including EGF receptor, HER2 and the type I IGF receptor (Chang et al. 2011). In addition, phosphorylation-dependent, amino-terminal SUMOylation reduces transcriptional activity of estrogen-related receptor alpha (Tremblay et al. 2008) and acetylation by p300 coactivator-associated factor (PCAF) of four lysine resides in its DNA-binding domain modulates its activity (Wilson et al. 2010).

3.3.8 ERRα Agonists

The constitutive activity of ERRα does not exclude the existence of a molecule able to modulate its activity enabling the recruitment of cofactors and playing a critical role in the maintenance of energy homeostasis as well as in disease progression. Recently, several synthetic antagonists have been identified (Busch et al. 2004; Willy et al. 2004;

Chisamore et al. 2009a). Moreover, dietary products, including cholesterol, have been reported as potential agonists (Teng et al. 2014; Teng et al. 2017). Suetsugi and collaborators identified agonists through virtual ligand screening on an ERRα ligand binding model based on the crystalline structure of ERRγ-LBD (Suetsugi et al. 2003). Thus, four ligands, increasing the transcriptional activity of ERRα, have been identified: isoflavones (genistein, daidzein, and biochanin A) and a flavone (trihydroxyflavone) (Suetsugi et al. 2003). Later, scientists synthesized the potential molecules able to interact with the ligand-domain, guided by ERRα crystalline structure, but they were not able to demonstrate the activity of the agonists (Hyatt et al. 2007). Moreover, Peng and collaborators synthesized a series of pyrid (1,2-a) pyrimidin-4 in order to produce more powerful ERRα agonists and to confirm the ability to induce the receptor transcriptional activity (Peng et al. 2011). These compounds have improved glucose and fatty acid uptake from muscle cells (Peng et al. 2011) and thus, could have a clinical utility for the treatment of metabolic diseases, including metabolic syndrome and diabetes.

3.3.9 Cholesterol: The First Endogenous ERRα Agonist

Recently, an important study investigated the binding ability of ERRα with endogenous lipids (Wei et al. 2016). To this aim experiments by using chromatography techniques were performed according to previous approaches validated for the study of PPAR with endogenous lipids from the lipidome. The experimental model used is the mouse brain, selected for the high expression of ERRα. The receptor was expressed, purified, and immobilized onto a resin and then incubated with enriching lipidomes. This experimental approach allowed the identification of a single ion that was significantly enriched by the beads bound to ERRL-BD and this ion was identified as cholesterol. Furthermore, to check the specificity of the interaction between ERRα and cholesterol, authors used targeted LC-MS method to increase the detection sensitivity for the lower-abundance sterols (McDonald et al. 2007). The latter results were in agreement with those from lipidomic experiments. Moreover, in order to verify the specificity of ERRα-LBD-cholesterol interaction, a deeper investigation was performed with a competitive binding assay by using diethylstilbestrol (DES), a synthetic ERRα antagonist, that binds to the lipid-binding pocket of ERRα (Tremblay et al. 2001). A further confirmation was obtained by the authors with circular dichroism (CD) spectroscopy tests, where cholesterol, DES and the inverse agonist XCT790, all induced a conformational change in ERRα-LBD, while estradiol did not. These results suggested that Cholesterol-ERRα-

LBD binding is more than a simple hydrophobic interaction. In addition, dye-labeled cholesterol derivatives were used and, after fluorescence polarization assay, the results showed that cholesterol binds the ligand-binding pocket of ERRα through its hydroxyl group. These findings indicate that cholesterol could exert a functional control of the ERRα activity (Wei et al. 2016).

3.3.10 Cholesterol and ERRα in Breast, Prostate, and Adrenocortical Cancer

A new potential therapeutic application in a clinical setting controlling cholesterol levels come out from the observations on the role played by ERRa in breast (BC) and prostate (PC) cancers. In BC, high ERRα expression characterizes tumors with poor prognosis (Ariazi et al. 2002). Moreover, ERRα mRNA is positively correlated with the oncogene ERBB2 and AIB1 (Surowiak et al. 2006) and inversely correlated with that of ERα and progesterone receptor that are good prognostic factors for the anti-hormonal treatment of breast cancer patients. Indeed, depending on the cellular context, ERRα could act promoting or inhibiting transcription (Kraus et al. 2002). Findings suggested that in ER-negative BC, ERRα compensates for the loss of ERα in addition to triggering the expression of ERα-independent genes since it recognizes estrogen response element (ERE) as is the case for vascular endothelial growth factor (VEGF) promoting BC metastasis (Stein et al. 2008; Stein et al. 2009). By contrast, in ERpositive BC cells, ERRα negatively controls ERE transcription by interacting with corepressor such as RIP1. Alternatively, ERRα could promote BC cells growth by enhancing circulating estrogen production. In fact, it has been found that ERRα could activate steroid sulfotransferase (SULT2A1) that works to maintain high level of peripheral dehydroepiandrosteronesulfate (DHEAS), an important metabolite in estrogen synthesis in adrenal tissues. In addition, it has also been evidenced that SULT2A1 inactivates tamoxifen and raloxifene (Apak and Duffel 2004). Thus, high ERRα expression in breast cancer by enhancing SULT2A1 activity could also support breast cancer cell resistance to anti-hormonal therapy (Apak and Duffel 2004). The enhanced expression of ERRα has been found also in prostate cancer (PCa) and PCa cell lines (Cheung et al. 2005). A study indicates a positive correlation between ERRα expression and the Gleason jscore while results from a preclinical study showed that ERRα can promote the hypoxic growth adaptation of prostate cancer cells by interacting with HIF-1α. As above explained, ERRα is also expressed in the bone regulating activity of osteoblasts and osteoclasts, that is implicated into the mixed osteolytic and osteoblastic lesions

observed in advanced prostate cancer patients (Bonnelye and Aubin 2013). An increased cholesterol biosynthesis, regulated by sterol regulatory element-binding protein−2 (SREBP-2), is a key player in the initiation and progression of PCa where an enhanced stem cell population was observed (Li et al. 2016). Moreover, aberrant cholesteryl ester accumulation in lipid droplets exacerbates cancer invasiveness and characterize high-grade PCa with PTEN loss and consequently, constitutive PI3K/Akt activation promotes metabolic dysregulation where ERRα/PGC-1α, as already mentioned, play a central role (Deblois et al. 2013). In addition, the cholesterol metabolite, 27-hydroxyl-cholesterol (27-OHC) is now recognized as selective estrogen receptor modulator (SERM) which promotes tumorigenesis in ER-positive BC (Warner and Gustafsson 2014). Higher levels of 27-OHC have been reported in ERα-positive breast cancers with respect to normal breast tissue, along with an observed reduction in the 27-OHC metabolizing enzyme such as CYP7B1 (Wu et al. 2013). Results from in vivo experiments demonstrated that 27-OHC alone is sufficient to support estrogenic activity in ER-dependent breast cancer cells (Wu et al. 2013). Accordingly, an increased growth and metastasis of ER-positive tumors were observed in a mousemodel of breast cancer fed only with a cholesterol-rich diet (Wu et al. 2013). The function of cholesterol as an ERRα agonist may provide the molecular basis and mechanistic insight into clinical studies suggesting that drugs able to lower cholesterol levels (i.e., statins) can be used to treat or prevent breast and prostate cancer.

Cholesterol could have positive growing effects, over its physiological role, also in the adrenocortical cancer (ACC). The treatment of ACC cell model with XCT-790, to the purpose of reducing ERRa expression, impaired cancer cell growth, both *in vitro* and *in vivo* (Casaburi et al. 2015). These data well correlate with that reporting an increased ERRα expression in ACC compared to normal adrenal and adenoma (Felizola et al. 2013) underling the involvement of this metabolic receptor in ACC biology.

As previously explained, ERRα transcriptional activity in normal cells is directed to modulate cellular metabolism, supporting the growth of rapidly dividing cells and to control metabolic programs required for cellular energy homeostasis in differentiated cells and to satisfy energy request during cell differentiation. The recent identification of cholesterol as an endogenous ERRα agonist evidenced that this sterol enhances the interaction between ERRα and PGC-1β in osteoclasts, promoting osteoclastogenesis and bone resorption. Similarly, cholesterol promotes ERRα interaction with PGC-1α in

myocytes inducing myogenesis and decreasing muscle toxicity. The discovery of this new molecular mechanism has elucidated the genesis of two important phenomena with an unexplained mechanism: the statin-induced muscle toxicity and the bisphosphonate suppression of bone resorption. Moreover, the discovery of cholesterol as an agonist of ERRα demonstrated that this receptor works as a metabolicsensing nuclear receptor distinguishing it from steroid receptors that respond to an acute and steep rise in hormonal levels. Consequently, ERRα is constitutively active because cholesterol is ubiquitous. This new mechanism calls fresh thinking about the role of ERRα in cancer cells keeping in mind the key role played by this receptor as modulator of cancer metabolism. As previously explained, the metabolic alterations of lipids, carbohydrates, and proteins are one of the hallmarks of cancers (Hanahan and Weinberg 2011). In particular, an increase in the glycolytic rate at the expense of oxidative phosphorylation even in the presence of adequate oxygen concentrations (Warburg effect) (Warburg 1956) allows a rapid adaptation of tumor cells to the continuous metabolic changes that, together with the tumor microenvironment, are the driving forces for cancer survival and its evolution. Given the high interconnection between enzymes that regulate the metabolism and the molecular pathways induced by altered oncogenes, research of the key regulators that behave on metabolic adaptations and proliferative, anti-apoptotic, invasive and metastatic responses, could represent elective targets to break down tumors with a single shot. The ERRα could work for this end due to its location at the intersection of dysregulated metabolism and oncogenic pathways. In several cancer cells, the expression and the activity of ERRα, together with its cofactors (PGC-1 α/β), is further influenced by oncogenic signals (IGF1-/IGF1R pathway, estrogen signaling, Wnt/b-cat/TCF, mTOR pathway) (Figure 3.13 A) and can thus be re-directed to induce metabolic programs (Figure 3.23 B) favoring tumor growth and progression. (Figure 3.13 C). In this context, an increased level of cholesterol, through the new molecular mechanism, supports all tumor-related processes. (Figure 3.13 D). Accordingly, high levels of cholesterol are associated with an increased risk of different type of cancers including breast, prostate (Gutierrez-Pajares et al. 2016) and CRC (Wang et al. 2017). Although epidemiological data on the correlation between cholesterol and cancer are conflicting, the preclinical results positively highlight different molecular aspects revealing how oncogenic growth signaling meet the bioenergetics and biosynthetic demands of rapidly proliferating tumor cells. In fact, altered cholesterol pathway in cancer could be reached through different mechanisms. One of the most important is the

constitutive activity of the oncogenic PI3K/AKT/mTOR signaling pathway that enhances intracellular cholesterol levels by: (i) inducing cholesterol synthesis through the activation of the transcription factor SREBP (sterol regulatory element binding proteins); (ii) inducing LDL receptor–mediated cholesterol import; (iii) inhibiting ABCA1-mediated cholesterol export. Moreover, high-energy demanding cancer related process are strictly related to the cross-talk between tumor cells and some key players of the tumor microenvironment (TME), such as macrophages (TAM, tumor-associated macrophage), that in turn, fuels cancer progression through the formation of an inflammatory milieu characterized by the production of different cytokines such as IL-1, IL-6, and IL-8 among others. The latter, as above reported, could be a target of ERRα action (Figure 3.13 E). For most solid tumors, infiltration by inflammatory cells such as macrophages is associated with poor prognosis (Illemann et al. 2014; Mantovani et al. 2017). The links between inflammation and cholesterol are best exemplified by atherosclerosis, but similar mechanisms may also contribute to other metabolic disorders including cancer. It is noteworthy that cholesterol accumulation in TAM triggers the phenotype switch from M1, antitumorigenic, to M2-like macrophage, protumorigenic (Leitinger and Schulman 2013; Rhee 2016). Based on these considerations, the use of therapeutic strategy aimed to reduce cholesterol levels, such as statins (Figure 3.13F) or drugs targeting the SREBP metabolic pathways (Figure 13.13G), could be a promising option to counteract metabolic rewiring in cancer cells where ERRα plays a pivotal role.

In conclusion, identification of cholesterol as an endogenous ERRα agonist has already elucidated the most likely mechanisms underlying the side-effects induced by statins and bisphosphonate, but at the same time, it gives new perspectives to be further investigated in order to explore new therapeutic options for the treatment of ERRα overexpressing tumors. This alternative approach could bring additional benefits to the treatment of tumors that have already adopted successful therapies, but especially for those tumors, such as ACC, which are characterized by a limited or failed therapeutic choice (Casaburi et al. 2018).

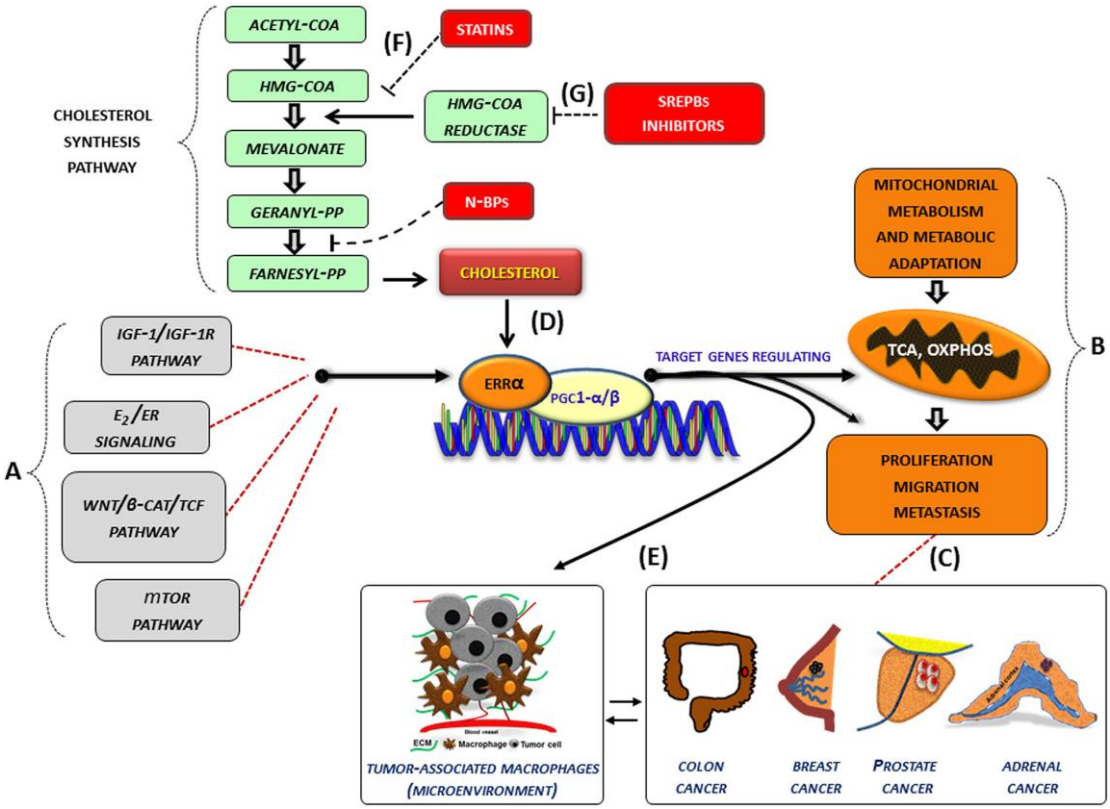


Fig. 3.13 Role of cholesterol as modulator of ERRα action in cancer. (A–G) Schematic representation of how cholesterol, as a new ERRα ligand, can contribute to the complex molecular network consisting in the functional cross-talk between oncogenes and oncogenic pathways (IGF-1/IGF-1R, E2/ER, β-catenin/TCF, mTOR) **(A)** that support the overexpression of ERRa. In turn, ERRa, together with its main cofactors (PGC-1a and PGC-1b) and activators, such as cholesterol **(D)**, affects cancer cell metabolism promoting proliferation, migration and, metastasis **(B)** of different tumor phenotypes (C). All these bioenergy-consuming functions are strictly related to **(E)** the cross-talk between tumor cells and some key players of the tumor microenvironment, such as macrophages (tumor-associate macrophages). The use of drugs [statins, (F), N-bisphosphonates, N-BPs, SREBPs inhibitors, **(G)**] able to reduce cholesterol levels and ERRa transcriptional activity could widen the therapeutic opportunities for the treatment of different ERRa overexpressing tumors. More details are explained within the text. E2, estradiol; ER, estrogen receptor; IGF-1/IGF-1R, insulin-like growth factor-1/insulin-like growth factor-1 receptor; WNT, Wingless-type MMTV integration site family member; TCF, T-cell factor; TOR, mammalian target of rapamycin; N-BPs, nitrogen-containing bisphosphonates.

3.3.11 ERRα in invasion, angiogenesis and metastasis

One of the hallmarks of aggressive tumors is their metastatic ability dictated by their invasion and migration potential as well as their capability to establish tumor vascularization. The glycolytic state that prevails in tumor cells reflects a metabolic profile known to favor tumor angiogenesis, invasion and migration (Pugh and Ratcliffe

2003). Since ERRα could contribute to the metabolic reprogramming of cancer cells, it can be envisioned that it also affects metastatic potential of these cells. In normal tissues, decreased expression of ERRα was shown to affect cell migration during zebrafish gastrulation *in vivo* (Bardet et al. 2005). It also affects breast cancer cell migration *in vitro*, albeit without affecting proliferation rates (Stein et al. 2008). The absence of ERRα is also able to impair tumorigenic potential in aggressive breast cancer cells xenograft in nude mice (Stein et al. 2008). These observations are supported by the finding that ERRα and its coactivator PGC-1α bind to the promoter of and regulate the expression of VEGF, an important factor involved in tumor angiogenesis and invasion (Arany et al. 2008; Stein et al. 2009). The PGC-1/ERR axis has also been shown to be a potent inducer of VEGF53 and to promote angiogenesis (Arany et al. 2008) in muscle. In addition, the kinase suppressor of ras1 (KSR1) is able to regulate ERRα and PGC-1α to promote oncogenic ras-dependent and anchorage-independent growth of cancer cells (Fisher et al. 2011). ERRa has also been shown to regulate the expression of HIF in breast cancer cells and to associate with the HIFα/β heterodimer to promote its transcriptional activity on angiogenic and migratory target genes like VEGF (Ao et al. 2008). ERRα is also thought to cooperate HIF to induce the metabolic reprogramming towards the metastatic-promoting glycolytic state in tumor cells (Ao et al. 2008). The PGC-1/ERR axis also regulates the expression of WNT11, a process that involves ERRα in a transcriptional complex with β-catenin (Dwyer et al. 2010). Ablation of either ERRα or β-catenin expression decreases the migratory capacity of cancer cells of various origins, an observation that provides biological significance to this ERRα/β-catenin/WNT11 signaling pathway. In addition, functional genomics has identified ERRα target genes involved in invasion and migration and in promoting tumor vascularization such as FGF and CXCR4 (Deblois et al. 2010). Studies reviewed herein clearly show that modulation of cellular energy metabolism by the PGC-1/ERR transcriptional axis plays a major role in the process of tumorigenesis. In normal cells, the activity of the PGC-1/ERR axis can be used to increase cellular metabolism, to support the growth of rapidly dividing cells, to direct metabolic programs necessary for cell differentiation and to maintain cellular energy homeostasis in differentiated cells. Indeed, the action of the PGC-1/ERR axis can have both proliferative and anti-proliferative outcomes, which are likely dependent on the composition of the PGC-1/ERR complexes present in these cells. In cancer cells, the activity of the PGC-1/ERR axis is further influenced by oncogenic signals and can thus

be re-directed to induce metabolic programs favoring or attenuating cell growth and proliferation (Figure 3.14) (Deblois et al. 2013).

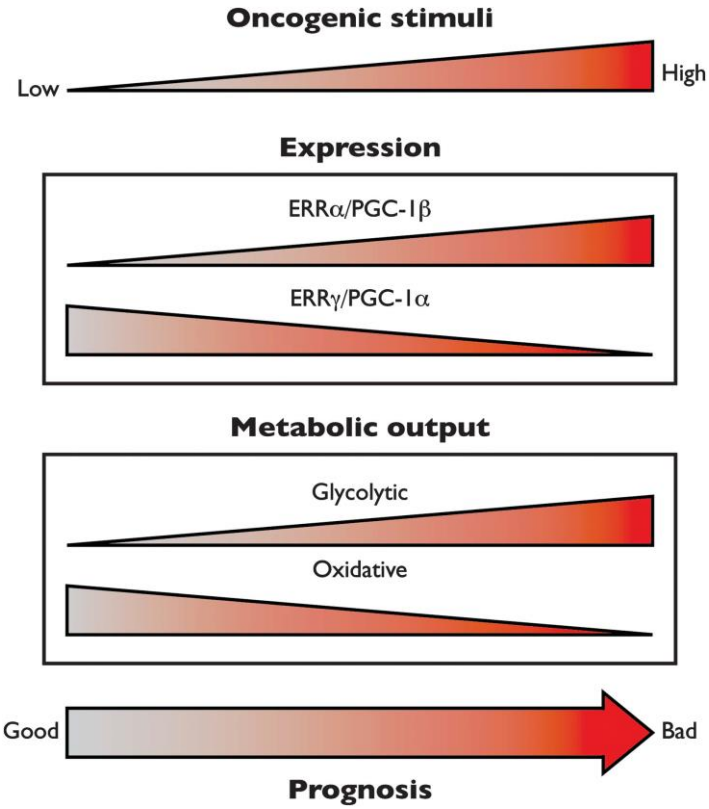


Fig. 3.14 Schematic representation of the potential involvement of the PGC-1/ERR axis and its specific components on metabolic output and its consequences on oncogenic progression.

4. MATERIALS AND METHODS

4.1 Cell culture and tissues

H295R cells were obtained from Dr. Antonio Stigliano (Department of Clinical and Molecular Medicine, Sant'Andrea Hospital, Faculty of Medicine and Psychology, Rome, Italy) (Rainey et al. 1994) and cultured in Dulbecco's modified Eagle's medium/Ham's F12 DMEM/F12 (Sigma-Aldrich, Milano, Italy) supplemented with 1% ITS Liquid Media Supplement (Sigma), 10% fetal bovine serum, 1% glutamine, 2% penicillin/streptomycin (Sigma) (complete medium), at 37 °C in an atmosphere of humidified air containing 5% CO2 (Figure 4.1). Cells were subcultured for 48 h in complete medium on 100 mm dishes for IP assays ($5x10^6$ cells/plate), 60 mm dishes for protein and RNA extraction ($600x10^3$ cells/plate), 6-well culture dishes for Trypan blue exclusion test ($7x10^4$ cells/well) and Wound-Healing assay (90% confluence), 12-well culture dishes for Colony formation ($3x10^3$ cells/well) and 24-well culture dishes for proliferation experiments ($50x10^3$ cells/well). Prior to experiments, cells were starved overnight in DMEM/F-12 medium without phenol red, containing only antibiotics. Cells were treated with 17β-estradiol (E2, 100 nM) (Sigma) and IGF-II (100 ng/mL) (Sigma). Cells were treated with G-1 (1μM) (Tocris Bioscience, Bristol, UK) in DMEM/F-12 containing 2, 5% FBS-DCC (fetal bovine serum dextran-coated charcoal-treated). Inhibitors such as PD98059 (10μM), SB203580 (10μM), SP600125 (10 μM) (Calbiochem, Merck KGaA, Darmstadt, Germany) and ROS scavenge molecule NAC (N-acetyl cysteine, Sigma) (5mM) were used 1h prior to G-1. H295R cells were cultured in complete medium for 48 hours in 60 mm plates ($2x10^6$ cells) and then processed in complete medium for 48 hours with XCT790 (1-5-10 μM) for the extraction of total proteins. The wild type H295R cells and the cells deriving from the different cell lines in which the ERRα gene has been stably silenced (sh ERRα # 1, sh ERRα # 2, sh ERRα # 3, sh ERRα # 5, sh ERRα # 6, sh ERRα # 7, sh ERRα # 8), were grown in complete medium for 48h in 60 mm plates ($1x10^6$ cells). Cells were treated with XCT790 (1-5-10 μM) (Sigma) or Simvastatin (2,5-5-10 μM) (Sigma) in DMEM/F-12 containing 10% FBS (fetal bovine serum).

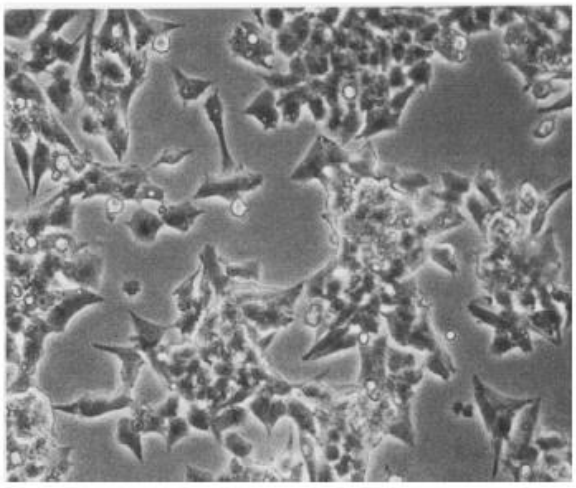


Figure 4.1 H295R cells

Adrenocortical tumors, removed at surgery, and normal adrenal cortex, macroscopically dissected from adrenal glands of kidney donors, were collected at the hospital-based Divisions of the University of Padova (Padova, Italy). Tissue samples were obtained with the approval of local ethics committees and consent from patients (Project Tissue Biobank Resource n. 130) in accordance with the Declaration of Helsinki. Diagnosis of malignancy was performed according to the histopathological criteria proposed by Weiss et al. (Weiss et al. 1989) and modified by Aubert et al. (Aubert et al. 2002). Patients' clinical data related to ACC samples included in this study are shown in Table 4.1 Sample C6 belonged to a patient who quit mitotane treatment after six months due to severe gastrointestinal side effects. Patients C1 and C2 received chemotherapy following EAP protocol (etoposide, doxorubicin, and cisplatin) plus mitotane (Fassnacht et al. 2012).

Sample ID	Age(years)	Gender	Stage at surgery	Syndrome	Weiss score	Size (cm)	Outcome
C1	41	M	IV	Cushing	9	16	Died, 1 year
C2	17	F	IV	Cushing	9	14	Died, 18 months
C3	43	F	III	None	4	9	Died, 8 years
C4	46	M	III	None	3	18	Remission, 7 years
C5	47	M	IV	Cushing	9	14	Died, 1 year
C6	57	M	II	SubclinicalCushing	5	14	Remission, 4 years

Table 4.1 Clinical data of the six ACC patients analyzed in this study.

4.2 Protein extraction and Western Blotting

To obtain cytoplasmic and nuclear proteins cells were lifted in ice-cold PBS, transfered to 15 ml tubes, centrifuged for 5 min at 3000 rpm and resuspended in 200 μl of buffer A (10 mM N-2-hydroxyethylpiperazine N′- 2-ethanesulfonic acid (HEPES), PH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride). After swelling on ice for 10 min, plasma membranes were disrupted by adding 0.1% Nonidet P-40 and vortexing for 10 sec. Lysates were transferred to 1.5 ml tubes. After centrifugation for 10 min at 10000 rpm at 4°C, supernatant contained cytoplasmic proteins while pellet contained nuclei. Nuclei were washed twice with ice cold PBS. Nuclei were incubated for 20 min on ice in buffer C (20 mM HEPES PH 7.9, 1 mM EDTA, 1 mMEGTA, 400 mM NaCl, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride vortexing every 5 min. Samples were centrifuged at 12,000 rpm for 15 min at 4°C to recover nuclear fraction (supernatant). Total protein were prepared using RIPA buffer: 50 mM Tris, pH 8.0, 150 mM sodium chloride; 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate). Fifty μg of protein were subjected to Western blot analysis. Blots were incubated overnight at 4°C with specific antibodies: (a) anti-IGF1Rβ antibody (C-20) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); (b) anti-pERK1/2 antibody (T202/Y204) (1:1000; Cell Signaling Technology, Bervely, MA, USA); (c) anti-ERK1/2 antibody (1:1000; Cell Signaling Technology); (d) anti-CCND1 antibody (3H2043) (1:1000; Santa Cruz Biotechnology); (e) anti-ERα (F-10) antibody (1:1000; Santa Cruz Biotechnology); (f) anti-c-Src antibody (1:500; Santa Cruz Biotechnology); (g) anti-PELP1/NMAR antibody (1:10,000; Bethyl Laboratories Inc. Montgomery, TX, USA); (f) anti-Egr-1 (1:1000); anti-p21Waf1/Cip1 (1:1000); anti-BAX (1:500) (all from Santa Cruz Biotechnology, Santa Cruz CA, USA); (g) anti-ERRα (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA); (h) anti-N-cadherin (1:100; Santa Cruz biotechnology); (i) anti-Vimentin (1:4000; Santa Cruz biotechnology); (l) anti-Snail+Slug (1:125; Abcam); (m) TOM20 (1:1000; Santa Cruz biotechnology); (n) Mitoprofile Total OXPHOS Human WB Antibody Cocktail (1:1000; Bethyl Laboratories). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) and immunoreactive bands were visualized with the ECL Western blotting detection system (Amersham). To assure equal loading of proteins, membranes were stripped and

incubated overnight with anti-Glyceraldehyde 3-Phosphate DeHydrogenase (GAPDH 1:1000); anti-Lamin B (nuclear fraction) antibodies, (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-β-actina (1:10000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

4.3 MTT assay and [3H] thymidine incorporation assay

A3-(4,5-Dimethylthiaoly)-2,5-diphenyltetrazolium bromide (MTT) assay, as well as thymidine incorporation, was performed to detect cell proliferation. Briefly, the wild type H295R cells and the cells deriving from the different cell lines in which the ERRα gene has been stably silenced (sh ERRα # 1, sh ERRα # 2, sh ERRα # 3, sh ERRα # 5, sh ERRα # 6, sh ERRα # 7, sh ERRα # 8) were seeded in complete medium in 12 well multiwells ($1x10^5$) and cultured in a humidified incubator at 37 ° C in an atmospheric pressure of 5% (v/v) of carbon dioxide/air. Respectively after 1, 3, 5 days and at the end of each treatment, fresh MTT (Sigma), resuspended in PBS, was added to each well (final concentration 0.33 mg/mL). After 2 h incubation, cells were lyzed in dimethylsulfoxide (Sigma). Each experiment was performed in triplicate and the OD was measured at 570 nm in a monochromator-based multi-mode microplate reader (Synergy H1 Hybrid Reader, BioTek, Germany, Europe). [3H] thymidine incorporation was performed as described in Casaburi et al. (Casaburi et al. 2012). Briefly, H295R as well as 24 h PELP1 silenced H295R cells were untreated or treated with E2 or IGF-II for an additional 48 h. For the last 6 h, [3H] thymidine (1 μCi/mL) was added to the culture medium. After rinsing with PBS, cells were washed once with 10% and three times with 5% trichloroacetic acid. Cells were lyzed by adding 0.1 N NaOH and then incubated for 30 min at 37°C. Thymidine incorporation was determined by scintillation counting.

4.4 RNA interference

PELP1 small interfering RNA (siRNA) and nontargeting siRNA (scrambled or control siRNA) were purchased from Dharmacon (ON-TARGET plus siRNA Human PELP1 cat # J-004463-06 50 nmol) (Invitrogen, Carlsbad, CA, USA). Cells were plated into 60 mm dishes for protein extraction and into 24-well plates for proliferation assay. siRNAs were transfected to a final concentration of 100 or 200 nM using the DharmaFECT transfection Reagent (cat # T-2001-002), according to the manufacturer's

recommendations (Dharmacon, CO, USA). PELP1 knockdown was checked by Western analysis on proteins extracted from cells transfected for 24 h.

4.5 Immunoprecipitation assay

H295R cells were lyzed in ice-cold radioimmunoprecipitation assay buffer (seeWestern blot analysis). The protein content was determined by the Bradford method and 500 µg of protein lysates were first precleared with 10 µL of protein A agarose beads (Santa Cruz) for 1 h. Then precleared cell lysates were incubated over night with primary anti-PELP1 antibody together with 20 µL of protein A agarose beads at 4 °C in HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.1 mM Na3VO4). Negative control samples were obtained by replacing anti-PELP1 antibody with normal rabbit IgG. Immunoprecipitated proteins were washed three times with HNTG buffer, separated on an 11% polyacrylamide denaturing gel, analyzed by WB and visualized by ECL (Amersham).

4.6 Microarray

RNA from H295R cells untreated (basal) or treated for 24 hour with G-1 (1µM) were hybridized to an Affymetrix human HG-U133plus oligonucleotide two-microarray set containing more than 54,000 probe sets representing over 38,500 independent human genes (Affymetrix, Santa Clara, CA). The arrays were scanned at high resolution using an Affymetrix GeneChip Scanner 3000. Results were analyzed using GeneSpring version 6.1 software (Silicon Genetics, Redwood City, CA). Pure signal values were normalized using a list of 100 normalization control probe sets published by Affymetrix and used to identify genotypic differences between untreated and treated cells. Probe ID for Egr-1: 227404 PM.

4.7 RNA extraction, reverse transcription and real time PCR

TRizol RNA isolation system (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from H295R. Each RNA sample was treated with DNase I (Invitrogen), and purity and integrity of the RNA were confirmed spectroscopically and by gel electrophoresis before use. One microgram of total RNA was reverse transcribed in a final volume of 30 µl using the ImProm-II Reverse transcription system kit (Promega Italia S.r.l., Milano, Italy); cDNA was diluted 1:2 in nuclease-free water, aliquoted, and stored at

−20°C. The nucleotide sequences for Egr-1, p21Waf1/Cip1 and BAX amplification were:

- Egr-1, forward, 5′-CTCTCCAGCCTGCTCGTC-3′, and reverse, 5′-AGCAGCATCATCTCCTCCAG-3′;
- p21Waf1/Cip1, forward, 5‘-CATGACAGATTTCTACCACTCC-3’ and reverse, 5‘-AAGATGTAGAGCGGGCCTTT-3’;
- BAX, forward 5‘-GCTCTGAGCAGATCATGAAGACA-3’ and reverse 5‘-TCGCCCTGCTCGATCCT-3’.
- The nucleotide sequences for 18S amplification were forward, 5’-CGGCGACGACCCATTCGAAC-3’, and reverse, 5’-GAATCGAACCCTGATTCCCCGTC-3’.

PCR reactions were performed in the iCycler iQ Detection System (Bio- Rad Laboratories S.r.l., Milano, Italia) using 0.1 μmol/L of each primer, in a total volume of 30 μl reaction mixture following the manufacturer’s recommendations. SYBR Green Universal PCR Master Mix (Bio-Rad) with the dissociation protocol was used for gene amplification; negative controls contained water instead of first-strand cDNA. Each sample was normalized to its GAPDH content. The relative gene expression levels were normalized to a calibrator (Basal, untreated H295R cells). Final results were expressed as n-fold differences in gene expression relative to GAPDH and calibrator, calculated using the ΔΔCt method as previously published (Chimento et al. 2015).

4.8 Egr-1 gene silencing

Cells were plated with regular growth medium two days before transfection to 50–60% confluence. The day of transfection the medium was changed with SFM without P/S, and cells were transfected with selected validated siRNA for Egr1 (ID: s4538) or control siRNA (scrambled) (AMBION), to a final concentration of 30 pmol/well (6 well/plate) or 15 pmol/well (12 well/plate) using Lipofectamine RNAiMAX Reagent (Invitrogen) as recommended by the manufacturer. After 6 h, the transfection medium was changed with DMEM/F-12 containing 2,5% FBS-DCC in order to avoid Lipofectamine toxicity. 24h post-transfection cells were exposed to vehicle or G-1 for further 24h and then harvested (RT-PCR and Western blotting) or processed for the viability assay.

4.9 ROS detection

ROS formation inside the cells were quantified using 2,7-dichlorodihydrofluorescein diacetate (H2- DCFDA). The acetate group of H2-DCFDA is removed by the intracellular esterase forming 2′,7′-dichlorofluorescin (DCF). In presence of ROS, non-fluorescent DCF formed is converted into a fluorescent product. The increase in fluorescence intensity is proportional to the oxidation of the fluorescent probe. Cells were seeded at a density of 50 000 cells/well in 24 well/plate and cultured for 48h and then treated with G-1 (1μM) for different times. Moreover, to determine the effects of NAC (5mM) on G-1-induced ROS generation, cells were pretreated 1h before adding G-1 for 24h. After incubation, the cells were loaded with H2-DCFDA (5μM/well) and incubated for 30 min in the dark. The wells were washed with PBS to remove excess of probe. The fluorescence was measured using fluorescent microplate reader (Monochromator based multimode microplate reader, BioTeck Sinergy H1 Instrument) with excitation at 495 nm and emission at 530 nm.

4.10 Immunofluorescence and immunohistochemistry

For immunofluorescent analysis (IF), H295R cells were plated on glass coverslips, washed with PBS and fixed with 3.7% formaldehyde in PBS for 20 min at room temperature (RT), followed by permeabilization with 0.2% Triton X-100 in PBS for 3 min at RT. Coverslips were washed with PBS, and nonspecific binding of IgG was blocked with 3% BSA (Sigma) in PBS for 20 min at room temperature. Cells were then incubated overnight in a cold room with an anti-Egr-1 antibody (Santa Cruz Biotechnology,). The following day coverslips were washed three times with PBS, and incubated with fluorescein isothiocyanate-conjugated secondary antibodies (Santa Cruz) for 1h at room temperature. Finally, coverslips were washed three times with PBS and mounted on glass slides with Vectashield mounting medium (Vector Laboratories Inc., CA, USA). Fluorescent images were collected on Olympus fluorescent microscope. From our previous work we had access to 5 mm thick paraffin-embedded sections of H295R xenograft tumors from mice treated with vehicle and G-1 (Chimento et al. 2015). Slides were deparaffinized and dehydrated (seven to eight serial sections). Immunohistochemical (IHC) experiments were performed as previously described (Chimento et al. 2015), using Egr-1 primary antibody at 4°C over-night. Then, a biotinylated goat-anti-mouse IgG was applied for 1h at room temperature, to form the

avidin biotin-horseradish peroxidase complex (Vector Laboratories, CA, USA). Immunoreactivity was visualized by using diaminobenzidine chromogen (Vector Laboratories). The primary antibody was replaced by normal rabbit serum in negative control sections for both IF and IHC experiments.

4.11 Scoring system

The immunostained slides of tumor samples were evaluated as previously described (Casaburi et al. 2015) by using the Allred Score. Briefly, 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.

4.12 Glucose uptake assay

The H295R cells transfected with an empty vector (-) or an expression plasmid for ERRα (pcDNA3.1-ERRα) were deprived of serum for 24h and then incubated with 2-[N- (7-nitrobenz-2-oxa- 1,3-diazol-4-yl) amino] -2-deoxy-D-glucose (2-NBDG), a fluorescent analogue of 2-deoxyglucose2-NBDG, for 30 minutes. The non-metabolized 2-NBDG was removed and the fluorescence intensity (ex.450, em 480) was analyzed using a multi-mode plate reader (Synergy H1, Biotek).

4.13 Stable transfection

The H295R cells were grown in a complete, antibiotic-free medium in 6-well multiwells to reach a confluence of approximately 60%. After 48 h cells were transfected following the protocol indicated by the Santa Cruz. For this purpose two solutions were prepared: A.140 µl of transfection medium with addition of 10 µl of Plasmid Transfection Reagent, B. 130 µl of transfection medium with addition of 20 µl corresponding to 2 µg of plasmid DNA (respectively sh-ERRα and sh-control). The two solutions were incubated 5 minutes at room temperature, then they were combined and the mixture was incubated 30 minutes at room temperature. After the incubation time, the A / B mixture was added to the cells in culture. The cells were then incubated for 24 hours in a

humidified incubator at 37 ° C and 5% CO2. After this period the transfection solution was removed and the normal growth medium was added. For the production of stable clones, 72 hours after transfection, the cells were selected by adding puromycin (Sigma Aldrich) in a concentration of 10 µg/ml. Cells resistant to antibiotics have formed clones that have been isolated and amplified. After about three weeks the concentration of puromycin was decreased to 1µg/ml.

4.14 Trypan blue exclusion test

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as Trypan blue, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Specifically, wild type H295R cells and cells deriving from the different cell lines produced in which the ERRα gene has been stably silenced (sh ERRα # 1, sh ERRα # 2, sh ERRα # 3, sh ERRα # 5, sh ERRα # 6, sh ERRα # 7, sh ERRα # 8) were seeded in 6-well multiwells ($7x10^4$) and cultured in a humidified incubator at 37 ° C in an atmospheric pressure of 5% (v/v) of carbon dioxide /air. Respectively after 1, 3, 5 days the cells were washed 3 times with 1X and PBS and then trypsinized. 20 microliters of Trypan blue solution were added to 20 microliters of cell suspension. Cell count is carried out using a hemocytometer.

4.15 Colony formation assay

This test allows an agent's ability to inhibit cell growth to be evaluated in vitro. The reduction in the number of colonies can derive both from the block of proliferation and from the induction of cell death. Clonal efficiency, or the ability to form colonies, is a parameter capable of measuring even partial damage to the complex enzyme structure that the cell needs to replicate. Specifically, wild type, sh control and sh ERRα # 5 ($3x10^3$cells/well) H295R cells were plated in 12-well plates in complete medium and and allowed to attach. After this period the colonies formed were fixed for 15 minutes with paraformaldehyde (Sigma) and then stained with blue of comaxies (Sigma) for 10

minutes. The medium was changed and surviving cells were allowed to grow colonies of ≥50 cells for 2 weeks, washed, fixed, and stained with Comassie Brilliant Blue, and counted.

The quantitative analysis of the number of colonies (>50 cells) was performed by counting the colored colonies with Olympus CKX53 Inverted Microscope and was indicated as a colony formation ratio, expressed as a percentage of the untreated control.

4.16 Wound-Healing Assay

The wound-healing assay is simple, inexpensive, and one of the earliest developed methods to study directional cell migration *in vitro*. This method mimics cell migration during wound healing *in vivo*. The basic steps involve creating a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rate of the cells.

Wild type, sh control and sh ERRα # 5 H295R cells were plated in 6-well multiwells at 70% confluence. The next day, using a 10 µl tip, we made a cut along each well; subsequently the cells were washed 3 times with PBS 1X and incubated with new culture medium. After 48h the culture medium was aspirated, the cells were washed 3 times with PBS 1x and a solution of Comassie Brilliant Blue was incubated for 10 minutes. After incubation with the dye solution, three washes with water were performed and images were acquired using the Olympus CKX53 Inverted Microscope.

4.17 Boyden chamber assay

The Boyden chamber assay, is based on a chamber of two medium-filled compartments separated by a microporous membrane. Wild type, sh control and sh ERRα # 5 H295R cells (5×10^4 cells/well) were placed in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment. Cells were incubated at 37 ° C with 5% CO2 for 24h. Cells migrated to the lower surface of the filter were fixed and stained with a Comassie Brilliant Blue solution for 5 minutes. The number of cells that have migrated to the lower side of the membrane was determined by ImageJ.

4.18 Spheroids culture

A single cell suspension was prepared using enzymatic (1x Trypsin-EDTA, Sigma Aldrich, #T3924), and manual disaggregation (25 gauge needle) (Shaw et al. 2012). Cells were plated at a density of 500 cells/cm^2 in spherois medium (DMEM-F12/ B27/ EGF (20ng/ml)/ Pen-Strep) in non-adherent conditions, in culture dishes coated with (2-hydroxyethylmethacrylate) (poly-HEMA, Sigma, #P3932). Cells were grown for 5 days and maintained in a humidified incubator at 37°C at an atmospheric pressure in 5% (v/v) carbon dioxide/air. After 5 days for culture, spheres >50 μm were counted using an eye piece graticule, and the percentage of cells plated which formed spheres was calculated and is referred to as percentage spheroids formation, and was normalized to one (1 = 100% TSFE, tumor-spheres formation efficiency) (De Luca et al. 2015). Prolonged generation of 3D spheroids was obtained from a single cell suspension that was prepared using both enzymatic (1x trypsin-EDTA, Sigma Aldrich, # T3924), and the manual disaggregation (21 gauge needle) to create a single cell suspension (Figure 4.2). Cells were plated at the density of 500 cells/cm^2 in the medium for 3D spheroids (DMEM-F12/B27/EGF (20ng/ml)/Pen-Strep) in non-adherent conditions in culture plates coated with (2-hydroxyethyl methacrylate) (poli-HEMA, Sigma, # P3932). Cells were cultured for 5 days and kept in a humidified incubator at 37 ° C in an atmospheric pressure of 5% (v/v) of carbon dioxide/air. This operation was repeated five consecutive times.

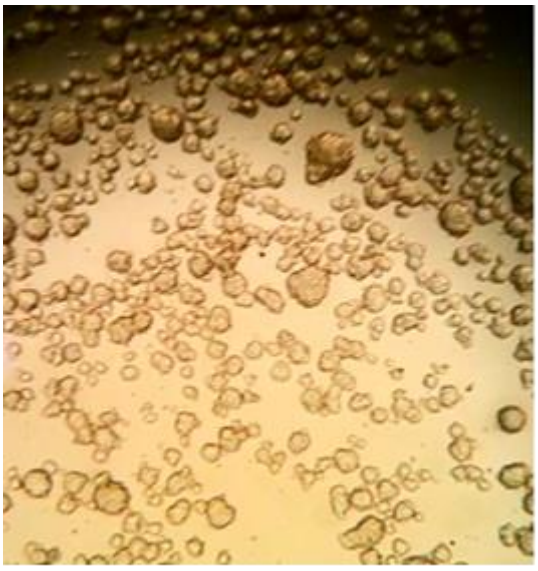


Fig. 4.2 H295R cells grown as 3D-spheroids.

4.19 Seahorse XFe96 metabolic flux analysis

4.19.1 Mitochondrial Stress Analysis

Real-time oxygen consumption rates (OCR) (Figure 4.3) for H295R cells treated with XCT790 or vehicle alone control were determined using the Seahorse Extracellular Flux (XF96) analyzer (Seahorse Bioscience, MA, USA). H295R cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM GlutaMAX, and 1% Pen/Strep. 70.000 cells/well were seeded into XF96-well cell culture plates (Seahorse Bioscience, MA, USA), and incubated overnight at 37°C in a 5% CO2 humidified atmosphere. After 48h, cells were treated with XCT790 (1-5-10 µM) or Simvastatin (2,5-5-10 µM) for 16h. At the end of treatment, cells were washed in warm XF assay media supplemented with 10mM glucose, 1mM Pyruvate, 2mM L-glutamine and adjusted at pH 7.4. Cells were then maintained for 1h in 175 µL/well of XF assay media at 37°C, in a non-CO_2 incubator. During the cell incubation time, 25 µL of a solution of XF assay media containing 10µM oligomycin, 9µM FCCP, 10µM rotenone, 10µM antimycin A ,were loaded into the injection ports of the XFe-96 sensor cartridge. Data set was analyzed by XFe-96 software and GraphPad Prism software, using one-way ANOVA and Student's t-test calculations. All experiments were performed in quadruplicate at least three times.

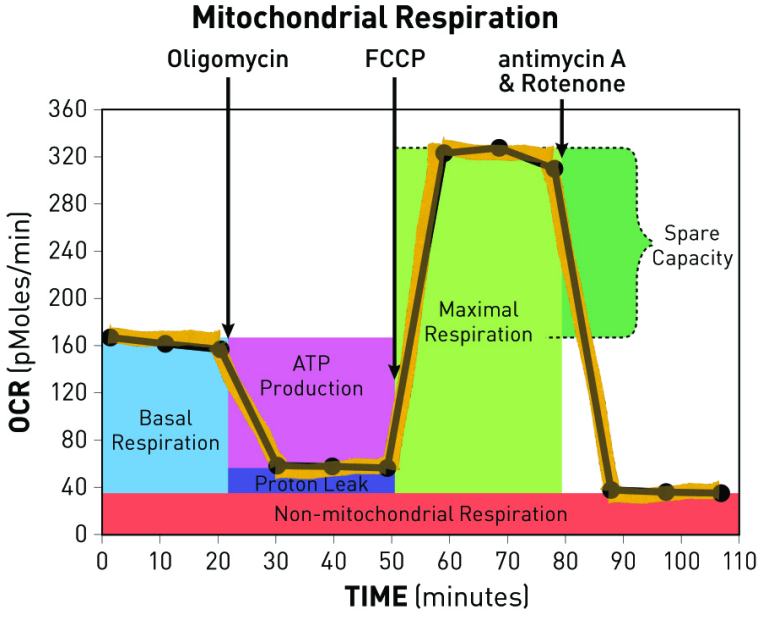


Fig. 4.3 Agilent Seahorse XF Cell Mito Stress Test profile of the key parameters of mitochondrial respiration.

• **Basal respiration:** Oxygen consumption used to meet cellular ATP demand and resulting from mitochondrial proton leak. Shows energetic demand of the cell under baseline conditions.

• **ATP production:** The decrease in oxygen consumption rate upon injection of the ATP synthase inhibitor oligomycin represents the portion of basal respiration that was being used to drive ATP production. Shows ATP produced by the mitochondria that contributes to meeting the energetic needs of the cell.

• **H+ (Proton) leak:** Remaining basal respiration not coupled to ATP production. Proton leak can be a sign of mitochondrial damage or can be used as a mechanism to regulate the mitochondrial ATP production.

• **Maximal respiration:** The maximal oxygen consumption rate attained by adding the uncoupler FCCP. FCCP mimics a physiological "energy demand" by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates (sugars, fats, amino acids) to meet this metabolic challenge. Shows the maximum rate of respiration that the cell can achieve.

• **Spare respiratory capacity:** This measurement indicates the capability of the cell to respond to an energetic demand as well as how closely the cell is to respiring to its theoretical maximum. The cell's ability to respond to demand can be an indicator of cell fitness or flexibility.

• **Nonmitochondrial respiration:** Oxygen consumption that persists due to a subset of cellular enzymes that continue to consume oxygen after rotenone and antimycin A addition. This is important for getting an accurate measure of mitochondrial respiration.

4.19.2 Glycolytic Stress Analysis

The extracellular acidification rate in real time (ECAR) (Figure 4.4), in H295R cells was determined using the Seahorse Extracellular Flux Analyzer (XF96, Seahorse Bioscience, MA, USA). H295R cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM GlutaMAX, and 1% Pen/Strep. 70.000 cells/well were seeded into XF96-well cell culture plates (Seahorse Bioscience, MA, USA), and incubated overnight at 37°C in a 5% CO2 humidified atmosphere. After 48h, cells were treated with XCT790 (1-5-10 μM) or Simvastatin (2,5-5-10 μM) for 16h. At the end of treatment, cells were washed in a specific buffer (XF medium, pH 7.4) for the determination of metabolic flows added with 2 mM of L-glutamine. The cells were then maintained for 1 hour in 175 μl of XF medium at 37 ° C, in an incubator without CO_2. During the incubation time, 25 μl of a XF buffer solution containing glucose (10 mM) oligomycin (1μM), 2-deoxy-D-glucose (50 mM), were placed in specific housings of the analyzer (injection ports). The various ECAR measurements obtained were normalized taking into account the protein concentration within the individual wells.

The data were analyzed using the XFe-96 software and the GraphPad Prism software, using one-way ANOVA and the Student's T-test. All experiments were performed in quadruplicate at least three times.

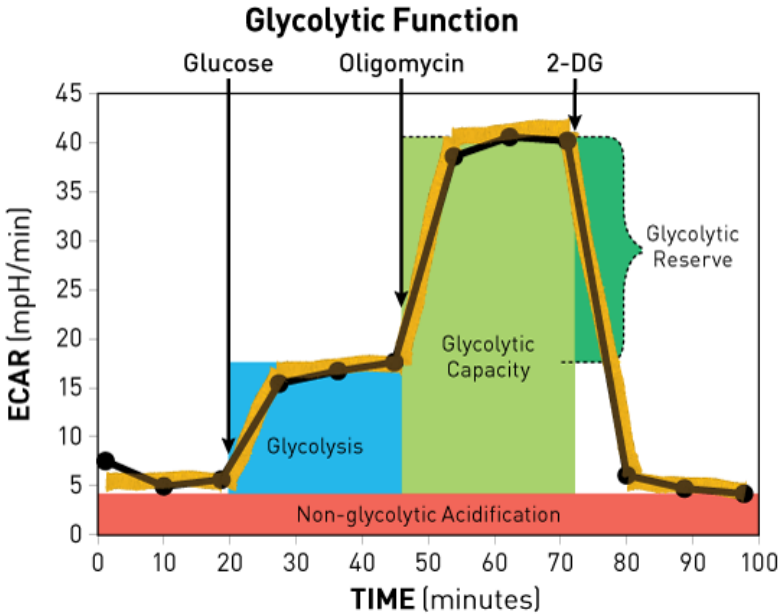


Fig. 4.4 Agilent Seahorse XF Glycolysis Stress Test profile of the key parameters of glycolytic function. Sequential compound injections measure glycolysis, glycolytic capacity, and allow calculation of glycolytic reserve and nonglycolytic acidification.

• **Glycolysis:** The process of converting glucose to pyruvate. The XF Glycolysis Stress Test presents the measure of glycolysis as the ECAR rate reached by a given cell after the addition of saturating amounts of glucose.

• **Glycolytic capacity:** This measurement is the maximum ECAR rate reached by a cell following the addition of oligomycin, effectively shutting down oxidative phosphorylation and driving the cell to use glycolysis to its maximum capacity.

• **Glycolytic reserve:** This measure indicates the capability of a cell to respond to an energetic demand as well as how close the glycolytic function is to the cell's theoretical maximum.

• **Nonglycolytic acidification:** This measures other sources of extracellular acidification that are not attributed to glycolysis.

4.20 Intracellular cholesterol extraction and colorimetric cholesterol assay

Cholesterol was measured using a colorimetric cholesterol assay kit (Cell Biolabs). Intracellular cholesterol was extracted from cells using a mixture of chloroform, isopropanol and NP-40 (7: 11: 0.1). Purified water was then added, and upon centrifugation, the organic, bottom phase was taken and dried by vacuum centrifugation. The resulting lipid pellet was resuspended in 200 µl of 1× cholesterol assay buffer. Then, 50 µL of sample were added to 50 µL of cholesterol reaction reagent

containing cholesterol esterase, cholesterol oxidase, colorimetric probe, and horseradish peroxidase diluted in assay buffer. After an incubation step, absorbance was read at 562 nm.

For the extraction of cholesterol from the tissues sections equal to 1/6 of the tumor mass were used, to which 500 μL of the extraction buffer was added in the Bullet blender storm-EC homogenizer (Diatech Lab Line) before being taken to dryness and processed as indicated for cell cultures. The results were normalized for the weight of each tissue.

4.21 Data analysis and statistical methods

All experiments were performed at least three times. Data were expressed as mean values ± standard deviation (SD), statistical significance between control and treated samples and between H295R wild type, sh control H295R and sh ERRα # 5 H295R, were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA) software. Control and treated groups, and also H295R wild type, sh control H295R and sh ERRα # 5 H295R, were compared using the analysis of variance (ANOVA).

5. RESULTS

5.1 Role of Scaffold Protein Proline-, Glutamic Acid-, and Leucine-Rich Protein 1 (PELP1) in the Modulation of Adrenocortical Cancer Cell Growth

5.1.1 PELP1 is expressed in Human ACC Samples and in H295R cells

We first examined PELP1 expression in normal human adrenal tissue, six different ACC samples, and the H295R cell line. Using Western blot analysis we showed that PELP1 is expressed in normal and ACC samples (Figure 5.1A), as well as in H295R cells (Figure 5.1B) with a similar expression pattern to that of prostate carcinoma cell line LNCaP, which was used as a positive control (Greger et al. 2006). It is worth noting that differences in PELP1 expression levels were not seen among the ACC samples, despite the different associated chemotherapeutic protocols (Table 4.1).

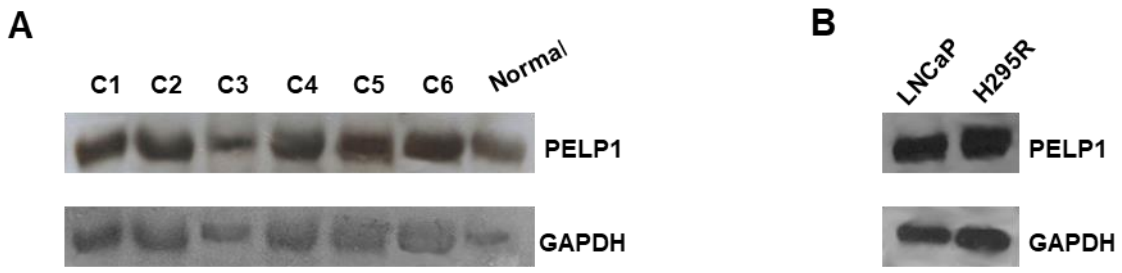


Fig. 5.1 PELP1 expression in human tissues of ACC and H295R cells. (**A**) Western blot analysis of PELP1 was performed on 50 µg of total protein extracted from normal human adrenal tissues (normal) and ACCs (C1–C6); (**B**)Western analysis of PELP1 was performed on 50 µg of total protein extracted from LNCaP and H295R cells. GAPDH was used as a loading control. Results are representative of three different experiments.

5.1.2 PELP1 is recruited to form a multiprotein complex in H295R cells after E2 and IGF-II treatment

In order to establish a role for PELP1 as a scaffold protein able to connect rapid estrogen-dependent and IGF-II-dependent signaling, we used an anti-PELP1 antibody to immunoprecipitate protein lysates from H295R cells treated for 10 min with E2 or IGF-II. We observed that both treatments rapidly induced the formation of a multiprotein complex in which we revealed the interaction of PELP1with IGFIR, ERα and c-Src (Figure 5.2).

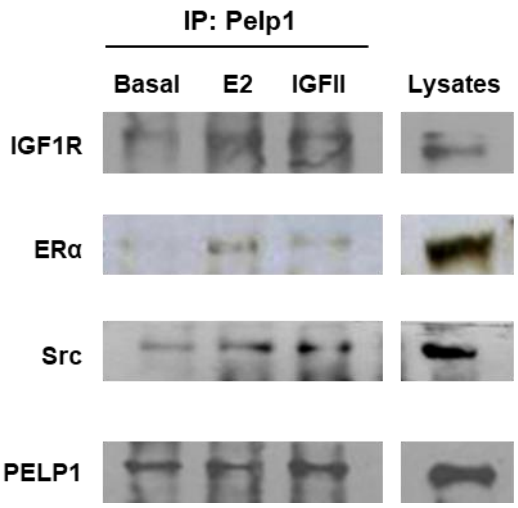


Fig. 5.2 PELP1 is recruited to form a multiprotein complex in H295R cells after treatment with E2 and IGF-II. H295R cells were treated for 10 min with E2 (100 nM) or IGF-II (100 ng/mL). Total protein extract (500 μg) was immunoprecipitated with 1 μg of anti-PELP1 antibody. The samples were immunoblotted for IGF1R, ERα, and c-Src. Protein expression for each sample was normalized to PELP1 content. Results are representative of three independent experiments.

5.1.3 PELP1 knockdown decreases ERK1/2 phosphorylation in H295R cells

The aim of the next set of experiments was to determine if PELP1 plays a role in rapid ERK1/2 activation induced by E2 and IGF-II. First we tested different concentrations (100 and 200 nM) of a specific siRNA and the reduced PELP1 expression was observed by Western blot analysis (Figure 5.3A). On the basis of Western blot results, we chose 200 nM as the best siRNA concentration to reduce PELP1 expression in all subsequent experiments. Next H295R cells were transfected for 24 h with scrambled or siRNA for

PELP1 and then treated for 10 min with E2 or IGF-II. In the presence of scrambled siRNA, E2 and IGF-II retained their ability to increase ERK phosphorylation, while in the presence of a reduced PELP1 protein expression the E2- and IGF-II-dependent ERK1/2 activation was decreased (Figure 5.3B). These data indicate that, in H295R cells, the formation of a multiprotein complex containing PELP1 is required to allow rapid MAPK activation induced by E2 and IGF-II treatment.

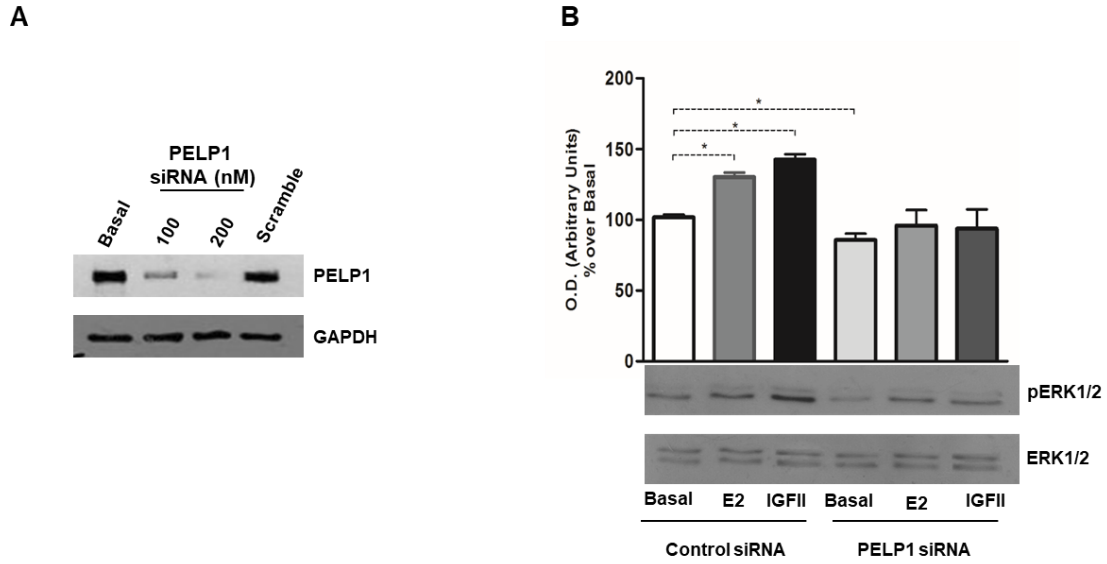


Fig. 5.3 PELP1 knockdown decreases ERK1/2 phosphorylation. (**A**) H295R cells were transfected with PELP1 siRNA (100 nM and 200 nM) or a non-targeting siRNA (control siRNA) for 24 h. Western blot analyses of PELP1 were performed on 50 _g of total protein; (**B**) H295R cells were transfected with control siRNA or PELP1 siRNA. After 24 h cells were treated for 10 min with E2 (100 nM) or IGF-II (100 ng/mL). Western blot analyses of PELP1 were performed on 10 _g of total protein. Results are representative of three independent experiments. GAPDH and ERK1/2 were used as a loading control; upper panel graph represents mean of pERK1/2 optical density (O.D.) from three independent experiments with similar results normalized to ERK1/2 content (* $p < 0.001$ compared to untreated control sample (basal) assumed as 100).

5.1.4 PELP1 knockdown decreases IGFIR expression in H295R cells

Starting from our previous observation that in H295R cells estrogens can modulate IGF1R expression (Sirianni et al. 2012), we wanted to investigate the effect of PELP1 silencing on E2-induced IGF1R expression. We confirmed our previous data on the ability of E2 to increase IGF1R expression and we also demonstrated that PELP1 is required for this event. In fact, the ability of E2 to increase IGF-1R protein expression

in PELP1 silenced H295R cells was no longer detectable. Importantly, PELP1 silencing reduced IGF1R expression, even in basal conditions (Figure 5.4).

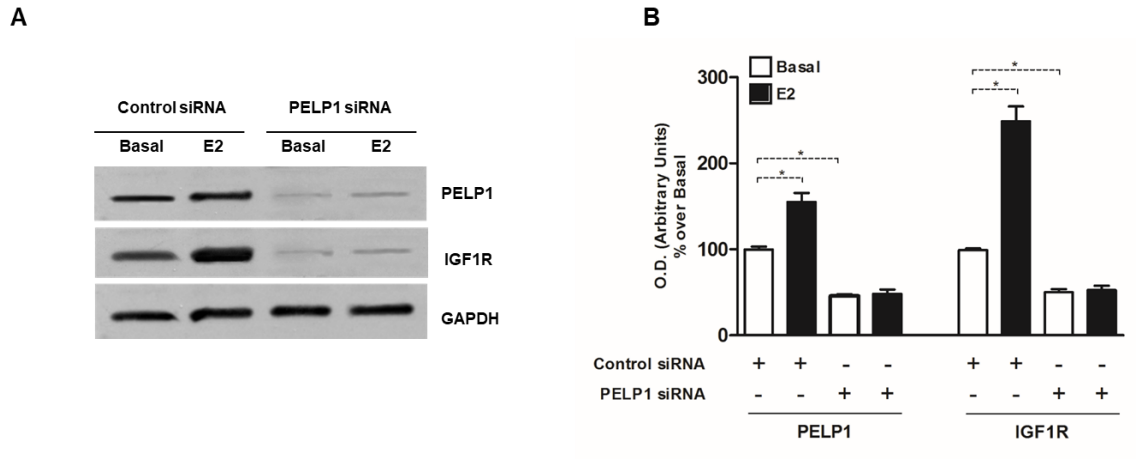


Fig. 5.4 PELP1 knockdown decreases IGF1R expression in H295R cells. (**A**) H295R cells were transfected with PELP1 siRNA or a non-targeting siRNA (control siRNA) for 24 h. After transfection cells were treated for 24 h with E2 (100 nM).Western blot analyses of PELP1 and IGF1R were performed on 50 µg of total protein. Results are representative of three independent experiments. GAPDH was used as a loading control; (**B**) The graph represents mean of PELP1 and IGF1R optical densities (O.D.) from three independent experiments with similar results normalized to GAPDH content (* $p < 0.001$ compared to untreated control sample (Basal) assumed as 100).

5.1.5 PELP1 knockdown decreases Cyclin D1 expression in H295R cells

Our previous study indicated that ACC cell proliferation in response to E2 and IGF-II relies on the activation of Cyclin D1 (Sirianni et al. 2012). For this reason, we evaluated Cyclin D1 expression in H295R cells after PELP1gene silencing. Our experiments showed that in H295R cells the reduced PELP1 expression abrogated the increase in Cyclin D1 expression induced by E2 and IGF-II treatment. It is worth noting that, in basal condition also, the Cyclin D1 expression is lowered in the presence of silenced PELP-1 expression (Figure 5.5).

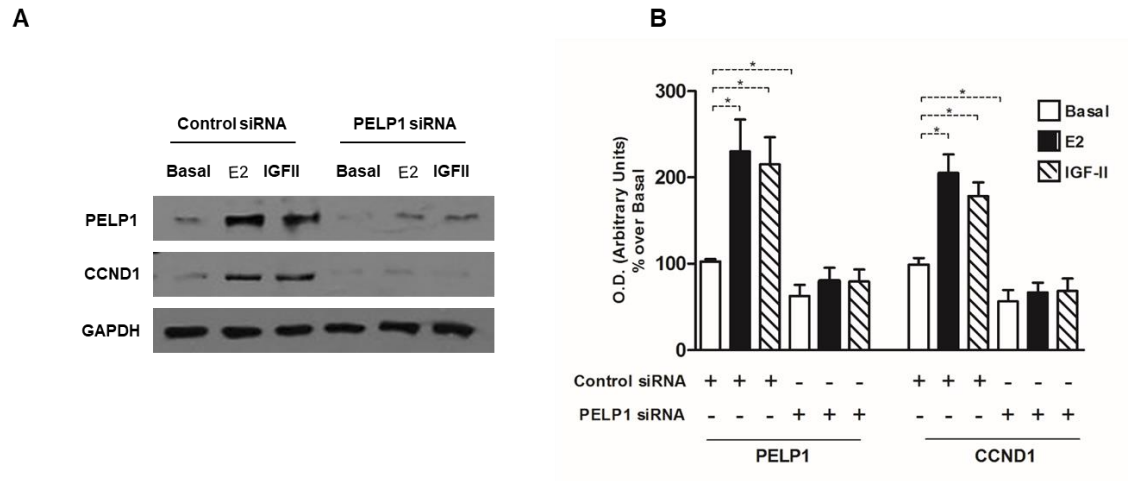


Fig. 5.5 PELP1 knockdown decreases Cyclin D1 expression in H295R cells. Cells were transfected with PELP1 siRNA or a non-targeting siRNA (control siRNA) for 24 h. After transfection cells were treated for 24 h with E2 (100 nM) and IGF-II (100 ng/mL). **(A)** Western blot analyses of Cyclin D1 were performed on 50 µg of total protein. Results are representative of at least three independent experiments. GAPDH was used as a loading control; **(B)** the right panel graph represents mean of Cyclin D1 optical density (O.D.) from three independent experiments with similar results normalized to GAPDH content (* $p < 0.001$ compared to untreated control sample (Basal) assumed as 100).

5.1.6 PELP1 knockdown reduces proliferation of H295R cells

Considering that PELP1 silencing was able to reduce the expression of genes involved in E2- and IGF-II-dependent H295R cell growth, we investigated the effects of PELP1 silencing on H295R cell proliferation. As shown in Figure 5.6A, B, PELP1 silencing in H295R cells significantly reduced the ability of both E2 and IGF-II to induce cell proliferation as assessed by both MTT assay and [3H] thymidine incorporation. Moreover, according to the above results where the expression of IGF-1R and Cyclin D1 was reduced, basal cell proliferation was also affected in untreated PELP1-silenced H295R cells.

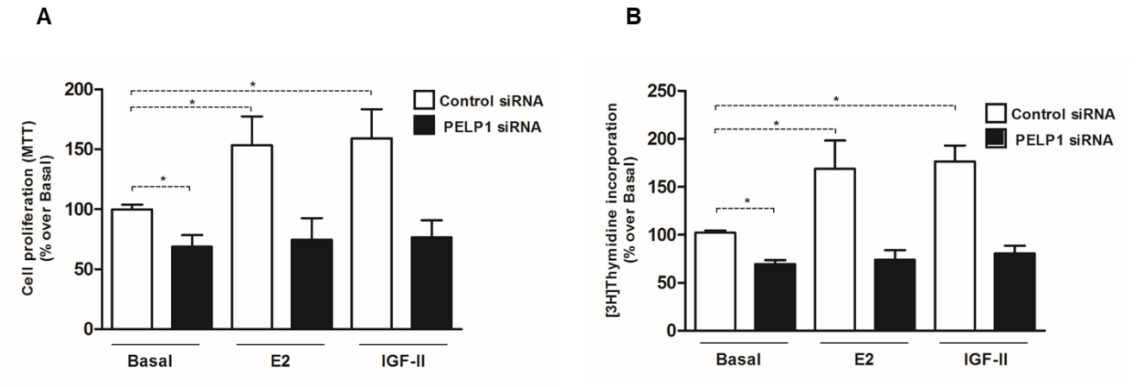


Fig. 5.6 PELP1 knockdown reduces cell proliferation in H295R. (**A**,**B**), H295R cells were transfected with PELP1 siRNA or a non-targeting siRNA (control siRNA). Twenty-four hours after silencing, cells were left untreated (Basal) or treated for an additional 48 h with IGF-II (100 ng/mL) or E2 (100 nM). (* $p < 0.0001$ compared with Basal). At the end of the experiment, cells were incubated with MTT (**A**) or [3H] thymidine (**B**) as described in Materials and Methods.

5.2 GPER-independent inhibition of adrenocortical cancer growth by G-1 involves ROS/Egr-1/BAX pathway

5.2.1 G-1-inducible genes in H295R cells defined by microarray analysis

To identify genes that were induced by G-1 in H295R cells, we cultured cells with or without G-1 (1μM) for 24h. This exposure time point was selected from previous results demonstrating 24h as the first visible signs of G-1-induced apoptosis. Total RNA was extracted and subjected to microarray analysis using Affymetrix human U133 plus 2.0 GeneChips. By using the GeneChips analysis suite, we sorted genes that were either up- or down-regulated by greater than two-fold following G-1 treatment in three independent experiments (Figure 5.7A). Several genes were modulated by G-1 in all three experiments but we focused our attention on Egr- 1, a gene with a role in both cell growth and apoptosis (Pagel and Deindl 2011). Egr-1 was up-regulated by 2.9-fold, a result that was further confirmed in H295R cells at both transcriptional and post-transcriptional level (Figure 5.7B, C) by real-time reverse transcription PCR (qRT-PCR) and Western blot analyses, respectively.

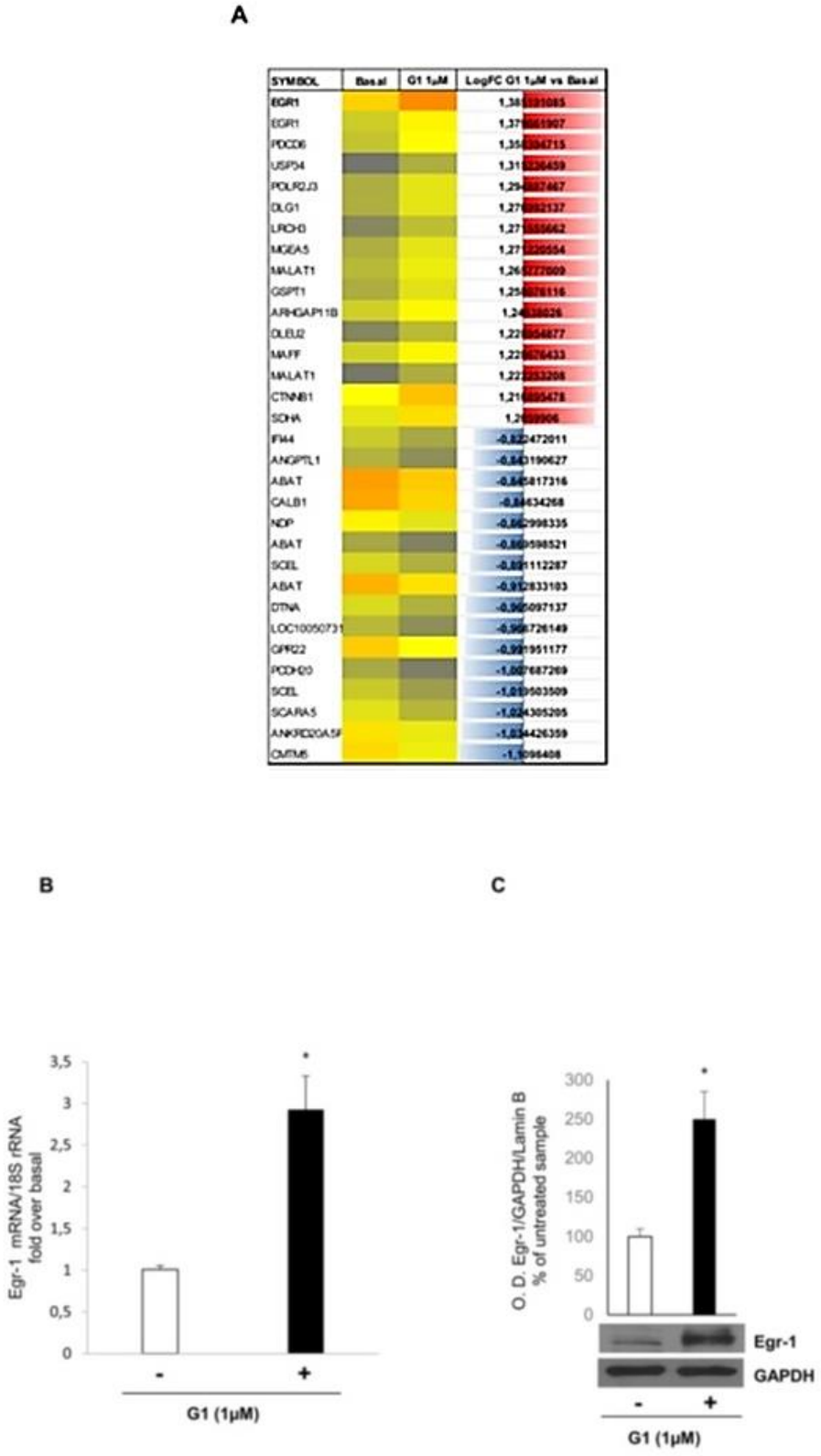


Fig. 5.7 G-1 stimulation induces Egr-1 expression in H295R cells. (A) representative microarray analysis with the most highly up-regulated (blu) and down-regulated (red) genes. **(B)** Egr-1 mRNA

expression in H295R cells treated for 24 h with vehicle (−) or G-1 (1μM) was analyzed by real time RT-PCR. Each sample was normalized to 18S rRNA content. Final results are expressed as n-fold differences of gene expression relative to calibrator. Data represent the mean ± SD of values from at least three separate RNA samples (*$p < 0.001$, versus calibrator). **(C)** Total extracts from H295R cells left untreated (–) or treated with G-1 (1μM) for 24 h were resolved by SDS-PAGE and subjected to immunoblot analysis using specific antibodies against human Egr-1. Blots are representative of three independent experiments with similar results. GAPDH served as loading controls. The upper graph are optical densities (O. D.) ±SD, **$p < 0.01$.

5.2.2 G-1 induces Egr-1 nuclear translocation in H295R cells

Egr-1 is a nuclear transcription factor that represents a point of convergence of many intracellular signaling pathways (Thiel and Cibelli 2002). To verify its nuclear translocation upon G-1 treatment we used different experimental approaches. First, we performed immunofluorescence assay where untreated or G-1-treated cells were fixed and incubated with anti-Egr-1 antibody followed by an incubation with a secondary FITC-conjugated antibody. In Figure 5.8A (upper panel) positive nuclear staining for Egr-1 is clearly visible after 24h treatment with G-1 while in untreated control cells Egr-1 appears as dotted areas within the cytoplasm and around the nucleus. Moreover, immunohistochemistry using tissue slides of H295R xenograft tumors derived from mice treated with vehicle and G-1 showed an increased cytoplasmic and nuclear staining for Egr-1 after G-1 exposure (Figure 5.8A, lower panel), as demonstrated by Allred immunostaining score (Table 5.1) (Chimento et al. 2015). In addition, Western blot analysis of cytoplasmic and nuclear protein clearly showed that Egr-1 accumulates within the nuclei after 24h treatment with G-1 1μM (Figure 5.8B). These results clearly showed that G-1 causes Egr-1 activation in H295R cells.

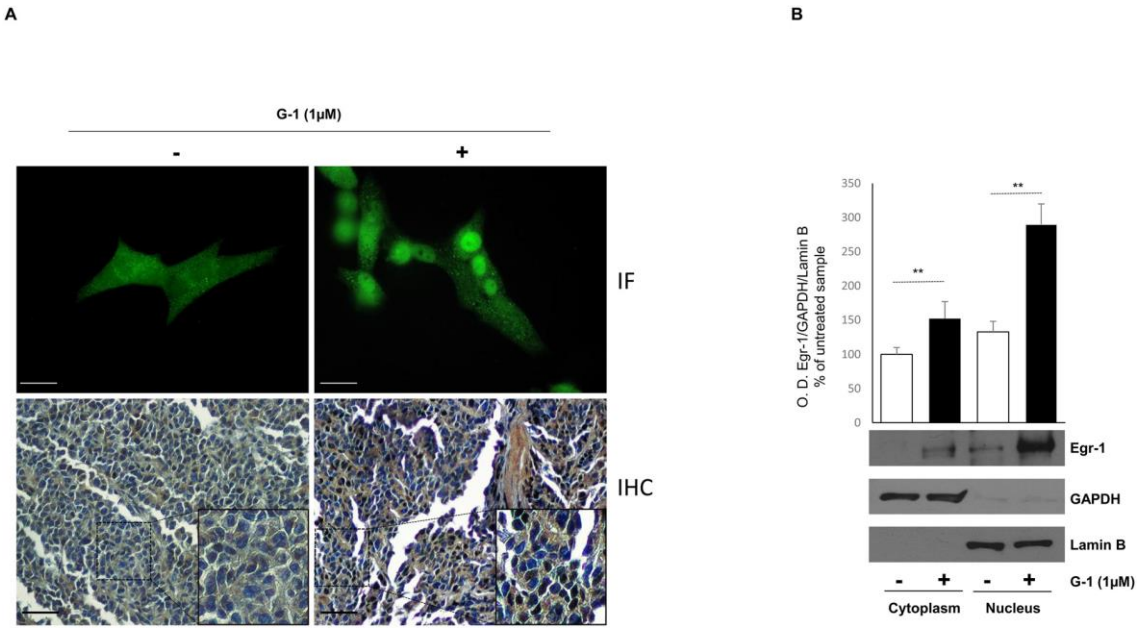


Fig. 5.8 G-1 induces nuclear translocation of Egr-1 in H295R cells. (A) (Upper panel) positive nuclear fluorescent staining for Egr-1 expression in H295R cells treated for 24 with vehicle (−) or G-1 (1μM). (A) (Lower panel) immunohistochemical staining for Egr-1 in untreated and G-1 treated H295R xenograft tumors. Insets are an higher magnification (400x) of the marked area. **(B)** Cytoplasmic and nuclear extracts from H295R cells left untreated (–) or treated with G-1 (1μM) for 24 h were resolved by SDS-PAGE and subjected to immunoblot analysis using specific antibodies against human Egr-1. Blots are representative of three independent experiments with similar results. GAPDH and Lamin B served as loading controls. The upper graph are optical densities obtained from three independent experiments ±SD, **$p < 0.001$.

Table 5.1 Egr-1 immunoreactivity (Allred score) in xenografted H295R cells

	Untreated control cells	G-1 1μM
Cytoplasm	1	3*
Nucleus	3	7*

Total immunostaining score (n: 6-7 serial section for each treatment): Proportion score + Intensity score (range 0–8). Significant difference: *$p < 0.05$ compared to untreated control cells.

5.2.3 G-1 induces ROS-dependent Egr-1 upregulation

Several studies indicate that Egr-1 is induced by a number of extracellular stimuli, including growth factors, mitogens, cytokines and injury-related stimuli as well as many inducers of ROS-mediated signaling and inflammation leading to cell death (Thiel and Cibelli 2002). In our previous work we demonstrated that G-1 inhibits H295R cell growth by activating the mitochondrial apoptotic pathway (Chimento et al. 2015) and

one of the mechanisms able to induce mitochondria-dependent apoptosis is through the generation of ROS. Therefore, we investigated the ability of G-1 to generate intracellular ROS. To this aim, H295R cells were treated for different times with G-1 and then incubated with CM-H2DCFDA. H2DCFDA is rapidly taken up by the cells where, is converted into non-fluorescent CM-H2DCF by esterase action and subsequently oxidized by intracellular oxidants, such as ROS, into highly fluorescent CM-DCF. The fluorescence intensity was monitored using a microplate reader (Ex/Em: ~492–495/517–527 nm). Results illustrated in Figure 5.9A show how G-1 is able to increase ROS production up to 24h, an event that was promptly reversed when cells were pre-treated for 1h with 5mM NAC, a commonly used reactive oxygen intermediate scavenger. Because G-1 generated ROS and Egr-1 expression were maximal between 12 and 24h, (data not shown), potential ROS-induced regulation of Egr-1 expression was investigated. Immunoblotting data indicated that blocking the generation of ROS by pre-treating cells with NAC markedly prevented G-1-induced Egr-1 protein expression (Figure 5.9B). In addition, we already demonstrated that G-1 treatment caused sustained ERK1/2 phosphorylation leading to cell death by apoptosis (Chimento et al. 2015). Here we also showed that pre-treatment of H295R cells with NAC prevented G-1-induced ERK1/2 phosphorylation (Figure 5.9C). These results clearly show the requirement of ROS formation in ERK1/2 activation by G-1.

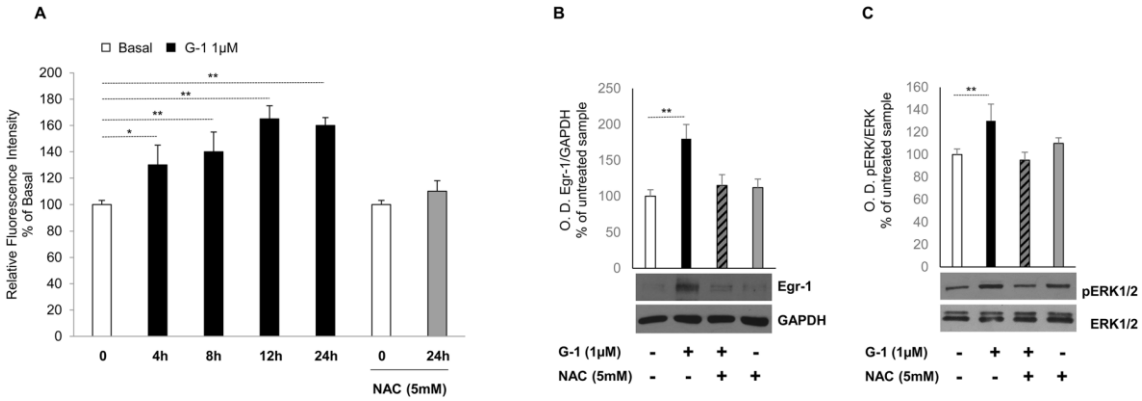


Fig. 5.9 G-1-induced Egr-1 expression is associated with ROS generation in H295R cells. (A) H295R cells were treated for different times with G-1 (1μM) and then incubated with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). Where indicated cells were pre-treated for 1 h with NAC (5mM), and then treated with G-1 (1μM) for 24 h and finally incubated with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). Relative fluorescence intensity was monitored using a microplate reader (Ex/Em: ~492–495/517–527 nm). ROS generation was expressed as relative fluorescence intensity of treated cells versus untreated control cells. Each column represents the mean ± SD of three independent experiments. **(B** and **C)** H295R cells were pre-treated with

NAC (5 mM) for 1h and then treated for 24h with vehicle (−) or G-1 (1μM). Western blot analysis of Egr-1 and GAPDH, used as a loading control (B), or pERK1/2 and total ERK1/2 (C) was performed on equal amounts of total proteins. Blots are representative of three independent experiments with similar results, (*$p < 0.05$, **$p < 0.001$) compared to untreated control sample.

5.2.4 G-1 activates Egr-1/BAX signaling in H295R cells through ERK signaling

The existence of a close association between ROS formation and the activation of MAPK signaling has long been known (McCubrey et al. 2006; Ray et al. 2012). Indeed, in mammalian cells there are three well-defined subgroups of MAPKs: the extracellular signal regulated kinases (ERKs), the c-Jun N-terminal kinases (JNK), and the p38-MAPK. The three subgroups of MAPKs are involved in both cell growth and cell death, and the tight regulation of these pathways is paramount in determining cell fate (Dhillon et al. 2007). To determine whether the activation of ERK, JNK and p38-MAPK participated in G-1-mediated upregulation of Egr-1 expression, the effects of specific inhibitors for ERK (PD98059), JNK (SP600125), and P38 (SB203580) were tested on G-1-treated cells (Figure 5.10A). The results showed that pretreatment with PD98059 did abrogate G-1- induced Egr-1 expression. By contrast, pharmacological inhibition of p38-MAPK or JNK activity failed to suppress G-1-induced Egr-1 expression. Moreover, upregulation of Egr-1 expression was concomitant with an increased expression, at both transcriptional (data not shown) and post-transcriptional (Figure 5.10B and 5.10C) levels, of two known Egr-1 target genes, specifically p21Waf1/Cip1 and BAX. Both proteins have been shown to play a role in G-1-mediated H295R cell cycle arrest and apoptosis (Chimento et al. 2014).

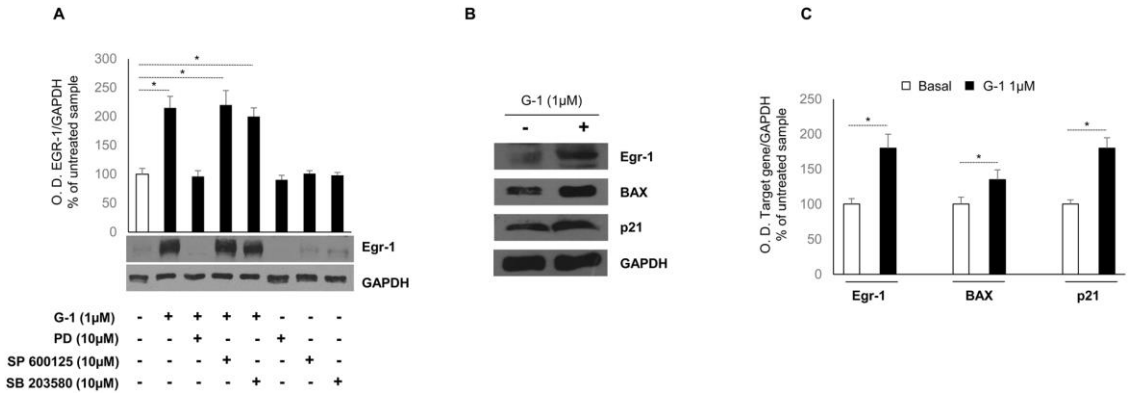


Fig. 5.10 Role of MAPKs and Egr-1/BAX signaling in G-1-treated H295R cells. (A) H295R cells were treated for 24 h with vehicle (−) or G-1 (1μM) alone or combined with PD98059 (10μM), SP600125 (10μM), SB203580 (10μM). Western blot analysis of Egr- 1was performed on equal amounts of total proteins. GAPDH was used as a loading control. Blots are representative of three independent

experiments with similar results. Graph represents mean of Egr-1 optical density (O.D.) from three independent experiments with similar results normalized to GAPDH content (*p < 0.01 compared to untreated control sample assumed as 100). **(B)** Total proteins from H295R cells left untreated (–) or treated with G-1 (1μM) for 24 h were resolved by SDS-PAGE and subjected to immunoblot analysis using specific antibodies against human Egr-1, BAX, p21Waf1/Cip1. Blots are representative of three independent experiments with similar results. GAPDH served as loading control. **(C)** Histograms represent the mean ± SD of band intensities evaluated as optical density (O.D.) arbitrary units and expressed as the percentage of the control assumed as 100%, *p < 0.01 compared with untreated cells.

5.2.5 Egr-1 gene silencing abolishes G-1-mediated effects on H295R cells

To further define the prominent role of Egr-1 in G-1-mediated effects, we decided to silence Egr-1 gene expression. In this experimental condition, we first examined the effect of gene silencing on the ability of G-1 to upregulate Egr-1 target genes such as p21Waf1/Cip1 and BAX. As show in Figure 5.11, silencing of Egr-1 gene expression (Figure 5.11A) abrogated the transcription of both p21Waf1/Cip1 (Figure 5.11B) and BAX (Figure 5.11C) genes following G-1 treatment. Considering that these two genes are responsible for the inhibitory effects exerted by G-1 on H295R cell growth (Chimento et al. 2014), we also investigated the impact of Egr-1 gene silencing on G-1-mediated inhibition of cell viability. Results showed in Figure 5.11D clearly demonstrated that G-1 was unable to reduce the viability in cells with an impaired expression of Egr-1 (Figure 5.11E and 5.11F).

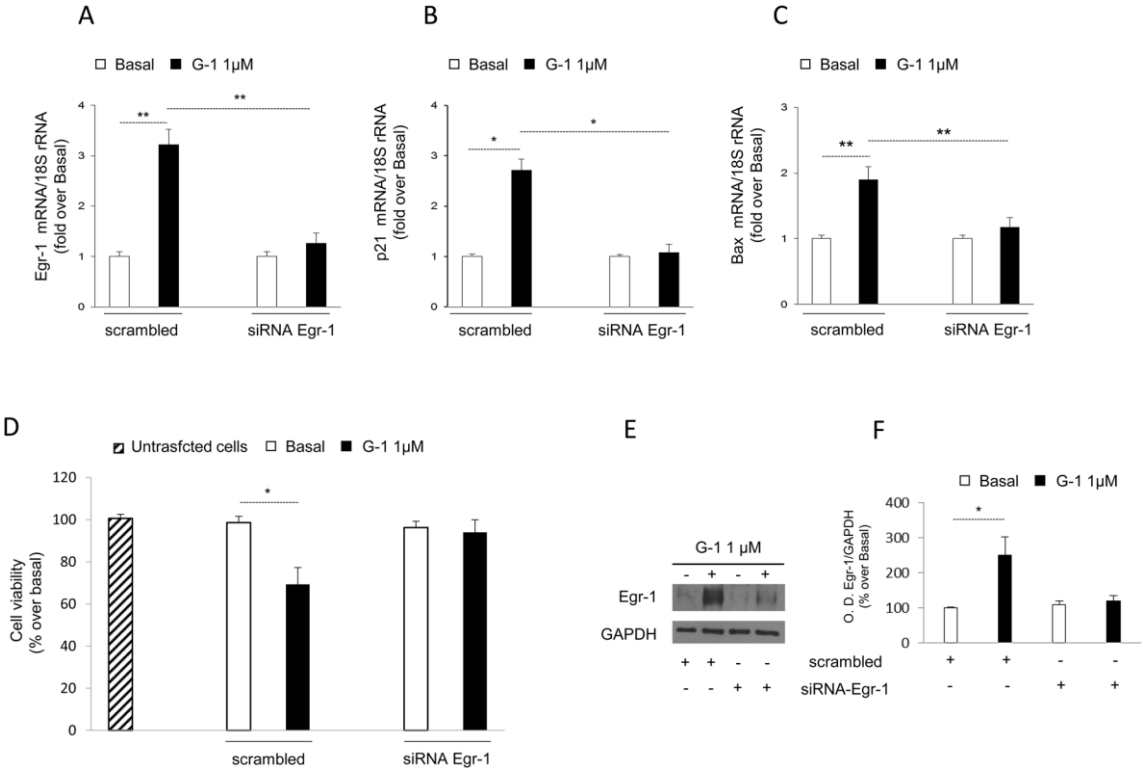


Fig. 5.11 Egr-1 gene silencing reversed G-1-induced effects on H295R. (A-C) Q-PCR analysis was performed in H295R cells to evaluate the expression of Egr-1 or p21Waf1/Cip1 or BAX mRNA in the absence (-) or presence of control siRNA (scramble) or siRNA specific for Egr-1. Ribosomal 18S subunit mRNA was used as internal control. **(D)** MTT assay was performed on H295R cells untransfected, transfected with specific siRNA for Egr-1 or scrambled siRNA for 24h, as indicated in Materials and Methods, and then left untreated (-) or treated with G-1 for additional 24h. **(E)** To assess reduced Egr-1 protein expression in cells used for viability assay, random wells from untreated or G-1 treated cells were used for protein extraction and Western blotting analysis. GAPDH protein expression was used as loading control. **(F)** Histograms represent the mean ± SD of band intensities evaluated as optical density (O.D.) arbitrary units and expressed as the percentage of the control assumed as 100%. Data represent the mean ± SD of three separate experiments each in triplicate. *$p < 0.05$, **$p < 0.001$, compared with untreated cells.

5.3 Estrogen Related Receptor α (ERRα) a key metabolic factor as target to prevent Adrenocortical Cancer progression

5.3.1 Serum total cholesterol levels in ACC patients

In a recent work (Wei et al. 2016) cholesterol (Chol) has been identified as the first natural ERRα ligand able to increase the recruitment of PGC-1s to ERRα up-regulating its transcriptional activity. The adrenal cortex is a tissue of excess in terms of both Chol metabolism and Chol exchange with the circulation. Exceptionally high levels of lipoprotein receptors (particularly HDL receptor scavenger receptor class B type I; SR-BI) in this highly vascularized tissue provide ready access to dietary Chol, allowing the adrenocortical cells to maintain impressive stores of cytoplasmic cholesterol ester (CE) droplets. Tightly packed among the CE droplets are specialized mitochondria, carrying in their inner membranes high levels of the cytochrome P450scc (CYP11A1) responsible for the first step of steroidogenesis consuming Chol to produce pregnenolone, the precursor of cortisol and all other steroids (Jefcoate 2002). An increase in serum Chol levels has been found in a study performed on 152 ACC patients (Miller et al. 2012). Most of these patients were treated with mitotane, known to induce hepatotoxicity and dysregulation of lipid metabolism, events that could determine the increase of Chol. Moreover, our data on the serum of 28 ACC patients treated with mitotane show Chol levels higher than normal physiological values (248±11 mg/dl,

mean ±se). For 5 of these patients Chol levels before mitotane treatment were available (Figure 5.12). Analysis of these samples showed that on-therapy total Chol levels were significantly higher than those before therapy (from 167±21 to 229 ±25 mg/dl).

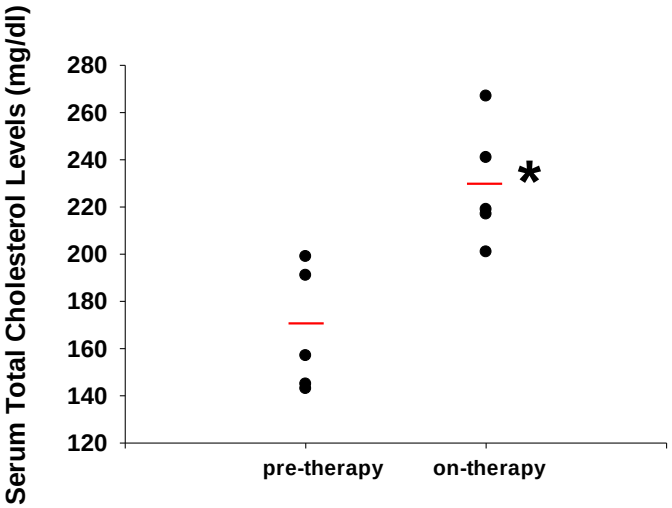


Fig. 5.12 Serum total cholesterol levels in 5 ACC patients before (pre-therapy) and during (on-therapy) mitotane treatment are reported in mg/dl. The bar indicates average values for these patients. (*p< 0.05 vs pre-therapy**)**

5.3.2 ERRα increases H295R cell viability in the presence of cholesterol

Our *in vitro* data show that ERRα overespression in an adrenocortical tumor cell model (H295RERRα) is capable of increasing cell proliferation only when serum contained lipoproteins (Figure 5.13). These data suggest that ERRα requires Chol as agonist to be functional in ACC cell. Based on these data, we can speculate that long term treatment with mitotane, increasing Chol levels, favors ERRα activation selecting a more invasive and metastatic ACC phenotypes. Consequently, any attempt to decrease Chol availability will be a valid therapeutic approach to interfere with ACC progression.

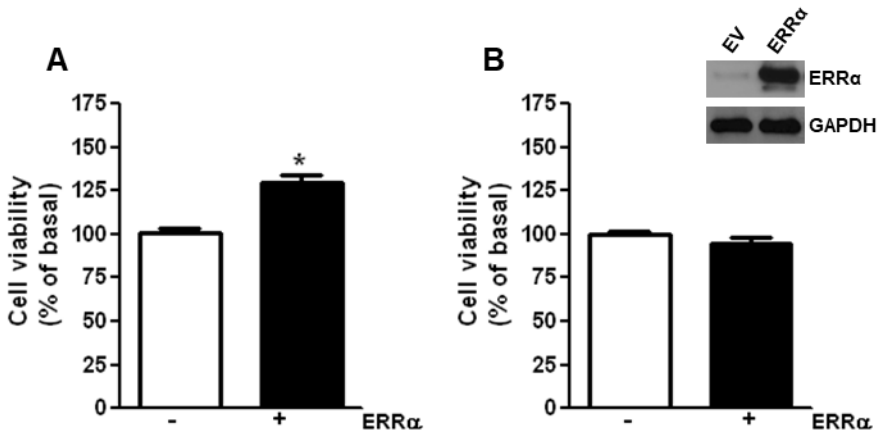


Fig. 5.13 ERRα increases H295R cell viability in the presence of cholesterol. H295R were transfected with an empty vector (-) or an expression plasmid for ERRα for 48h. Cells were mainatained in 5% FBS (A) or 5% lipoprotein-free serum (LFS) (B). Cell viability was evaluated by MTT assay. (*p< 0.05 vs basal). **insert B**) WB for ERRα in H295R transfected with an empty vector (EV) or an expression plasmid for ERRα.

5.3.3 ERRα overexpression increases in vitro glucose uptake in H295R

Studies related to ERRα in adrenal and ACC are very limited. ERRα is expressed in normal adult adrenal and regulates the expression of enzymes involved in steroidogenesis (Seely et al. 2005). Moreover, ERRα seems to be more expressed in ACC compared to normal adrenal and adenoma (Taylor et al. 2016). In our recent study (Casaburi et al. 2015) we tested the effects of ERRα inverse agonist, XCT790, on H295R. XCT790 treatment (1-10 μM) decreased ERRα protein levels in a dose dependent manner causing also a dose-dependent inhibition of cell growth after 48-72h treatment. We also investigated the *in vivo* effects of XCT790 on H295R xenografts. Mice treated with XCT790 (2.5 mg/Kg) displayed a significant tumor growth reduction compared to the vehicle treated control group. These data combined with our preliminary results shown in Fig. 5.13 suggest that Chol/ERRα/PCG1α complex is an eligible target to control ACC growth. We hypothesize that ERRα could be a "master regulator" of reprogramming metabolism in ACC. In fact, ERRα depletion in H295R cells caused a reduction of mitochondrial mass and function (Casaburi et al. 2015). Furthermore, our results showing that ERRα overexpression dramatically increases glucose uptake in H295R cells (Figure 5.14), support the hypothesis that ERRα is involved in ACC metabolic adaptation.

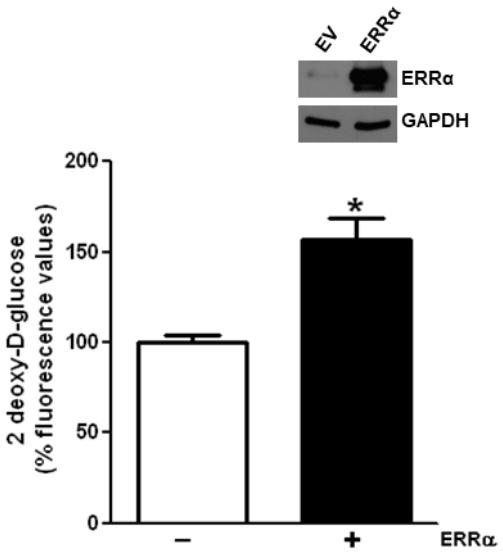


Fig. 5.14 ERRα overexpression increases *in vitro* glucose uptake in H295R. H295R cells transfected with an empty vector (EV) or pERRα plasmid (pcDNA3.1) were serum starved for 24h and then incubated with 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), a fluorescent analogue of 2-deoxyglucose2-NBDG, for 30 minutes. Nonmetabolized 2-NBDG was washed out and fluorescence intensity (ex.450, em 480) was analyzed by microplate-reader (Synergy H1, Biotek). **Insert)** WB for ERRα in H295R transfected with an empty vector (EV) or an expression plasmid for ERRα.

5.3.4 ERRα modulates H295R cells motility and EMT markers expression

Tumor metastasis involves a series of interrelated events including angiogenesis, epithelialmesenchymal transition (EMT) and invasion. The ability of cancer cells to switch from a predominantly oxidative metabolism to glycolysis, even when oxygen is plentiful, causes lactate production and consequently an acidic environment (Gatenby and Gawlinski 2003). This leads to extracellular matrix degradation by proteolytic enzymes (Lardner 2001) and normal cell death (Williams et al. 1999) enhancing cancer cell migration and invasion. Experimental observations support the hypothesis for a role of ERRα in invasion, angiogenesis and metastasis in several tumors (Deblois et al. 2013). The absence of ERRα is able to impair tumorigenic potential in aggressive xenografted breast cancer cells (Stein et al. 2009). In addition, ERRα/PGC-1α complex binds to the promoter of VEGF regulating its expression, promoting tumor angiogenesis and invasion (Stein et al. 2009). Our results show that treatment of H295R cells with XCT790 for 24 hours (a time shorter than what required to cause cell death), significantly reduces cell migration; on the contrary, ERRα overexpression increases this process (Figure 5.15A). Accordingly, ectopic expression of ERRα increases EMT

marker (N-Cadherin, Vimentin, Slug) levels, while XCT790 blocks their up-regulation (Figure 5.15B).

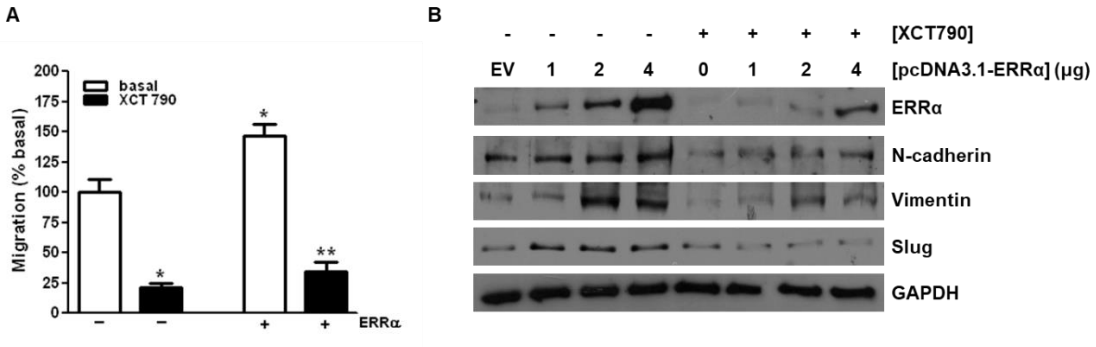


Fig. 5.15 ERRα modulates H295R cells motility and EMT markers expression. H295R were transfected with an empty vector (EV) or an expression plasmid for ERRα (pcDNA3.1-ERRα) for 48h. **A)** Boyden chamber motility assay was performed by filling the lower chamber with 10% FBS to attract cells. 5×10^5cells/well cells were re-suspended in DMEM-F12 containing 0,5%FBS and 0 or 10 μM XCT790 and carefully transferred into the upper chambers. The Boyden chamber was incubated at 37°C with 5% CO_2 for 24 h. At the end of the experiment, cells were removed from upper side of the membrane using a cotton swab, whereas the cells that migrated to the lower wells were fixed and stained in a Coomassie Blue solution for 5 min. Migrated cells were randomly photographed and counted with ImageJ. *p <0.05 versus basal (-); **p <0.05 versus basal (+). **B)** After transfection cells were treated for 24h with XCT790 (10 μM). Total proteins were analyzed by WB using antibodies against ERRα, N-cadherin, Vimentin, Slug. GAPDH was used as loading control.

5.3.5 ERRα overexpression increases resistance to anoikis in H295R cells.

Another interesting observation is the increase in anoikisresistant cells in the presence of ERRα overexpression (Figure 5.16A). Anoikis is a programmed cell death induced upon cell detachment from extracellular matrix, behaving as a critical mechanism in preventing adherent-independent cell growth, thus avoiding colonizing of distant organs. Consequently, ERRα overexpression in H295R increases the growth in adherent-independent conditions, allows formation and increases the number of 3D-spheroids (H295RSph) (Figure 5.16B).

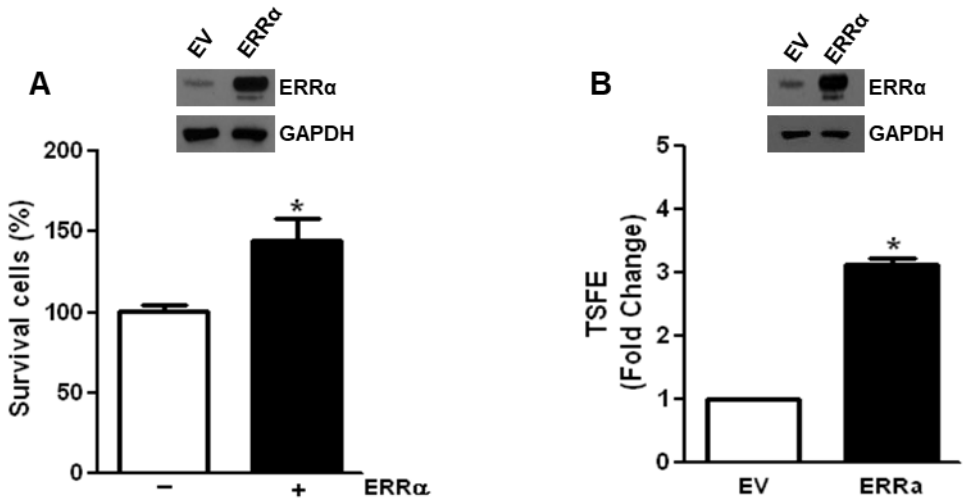


Fig. 5.16 ERRα overexpression increases resistance to anoikis in H295R cells. A) Untrasfected (-) or ERRα transfected H295R cells (+) were plated (5×10^5 cells/0.5 ml/well) in triplicate in poly-HEMA coated 12-well plates using regular culture medium to prohibit attachment. After 48-h incubation in standard culture conditions, cell viability was measured using trypan blue exclusion assay. **insert**) WB for ERRα in H295R transfected with an empty vector (EV) or an expression plasmid for ERRα at the time of spheroid counts. **B**) H295R were transfected with an empty vector (EV) or an expression plasmid for ERRα for 48h and then grown as 3D-spheroids for 5 days. Spheroids were counted under the Olympus CKX53 Inverted Microscope and results were expressed as fold induction over control ±SD (TSFE, tumor spheroids formation efficiency); *p <0.05. **insert B**) WB for ERRα in H295R transfected with an empty vector (EV) or an expression plasmid for ERRα at the time of spheroid counts.

5.3.6 Long serial 3D-spheroid culture of H295R cells enhances EMT markers expression

In addition, we grew H295R as spheroids for 5 days, dissociated and reseeded weekly in spheroid media for 5 weeks (H295RSph-5), before testing cells for motility. Migration of these cells was higher than adherent cells (Fig. 5.17A). H295RSph-5 have a higher expression of mesenchymal markers such as Vimentin, N-cadherin and Slug when compared with H295R cultured in adhesion (Figure 5.17B).

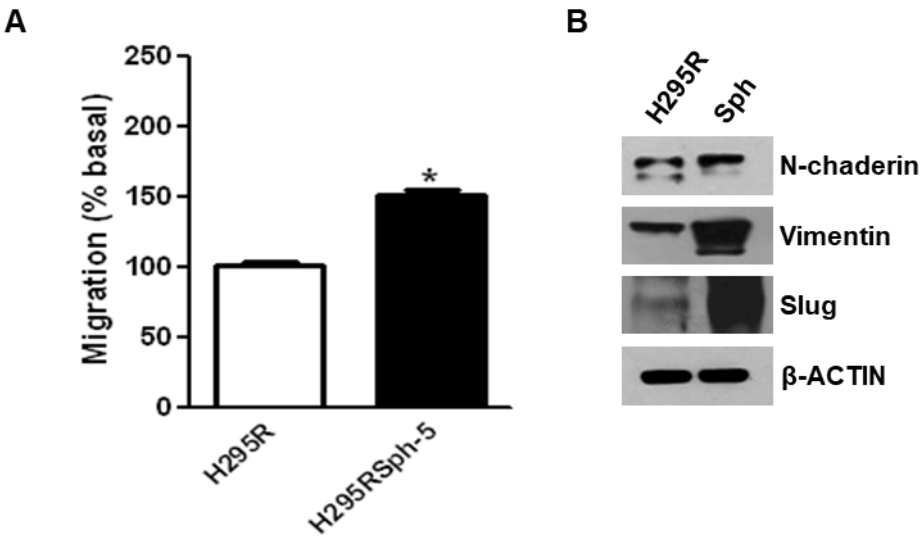


Fig. 5.17 Long serial 3D-spheroid culture of H295R cells enhances EMT markers expression. **A)** H295R spheroids were allowed to grow for 5 days and then trypsinized and reseeded weekly in spheroid media for 5 weeks. Boyden chamber motility assay was performed by filling the lower chamber with 10% FBS to attract cells. 5×10^5cells/well cells were re-suspended in DMEM-F12 containing 0,5% FBS and carefully transferred into the upper chambers. The Boyden chamber was incubated at 37°C with 5% CO_2 for 24 h. At the end of the experiment, cells were removed from upper side of the membrane using a cotton swab, whereas the cells that migrated to the lower wells were fixed and stained in a Comassie Brilliant Blue solution for 5 min. Migrated cells were randomly photographed and counted with ImageJ. *p <0.05 versus H295R cells. **B)** H295R spheroids were allowed to grow for 5 days and then trypsinized and reseeded weekly in spheroid media for 5 weeks. Total protein extracts from H295R cells and H295R grown as spheroids (Sph) for 5 weeks (5), were analyzed by WB using antibodies against N-chaderin, Vimentin and Slug. β-actin was used as loading control.

Then, H295RSph-5 could be a useful experimental model of a more aggressive ACC phenotype. Taking into account our data on the effects of ERRα depletion on mitochondrial activity (Casaburi et al. 2015), our data on glucose uptake (Figure 5.14) and on H295R migration (Figure. 5.15-5.17), we propose to investigate if the metabolic changes driven by ERRα, could also promote an invasive and metastatic phenotype in ACC.

5.3.7 Stable suppression of endogenous ERRα gene expression in H295R cancer cells

In order to clarify and define the functions and the relative molecular mechanism subtended by the ERRα receptor in adrenocortical carcinoma, we investigated the effects associated with growth and cell migration following stable silencing of the gene

encoding the ERRα receptor. We produced a cell line of adrenocortical carcinoma in which the ERRα gene was permanently silenced. Once cellular clones were obtained following clonal operated selection using the scalar doses of the antibiotic puromycin we used the Western blotting technique to compare the expression levels of seven different H295R cell lines generated by the same number of clones. Our results showed a significant reduction of ERRα receptor expression in cell lines deriving from clone 3 and clone 5 compared with wild type H295R (WT) and H295R cells stably transfected with a plasmid vector containing control sequences (sh-control) (Figure 5.18) that is, not able to silence any gene.

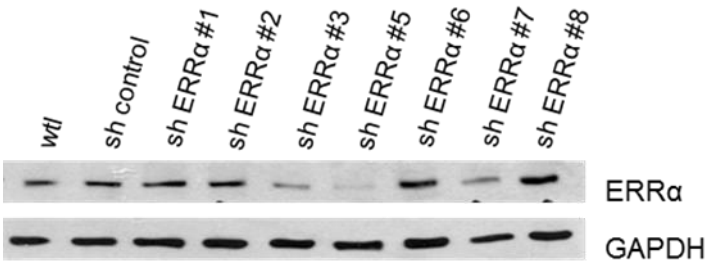


Fig. 5.18 Evaluation of ERRα expression after stable transfection in H295R cells. The total protein extracts derived from wild-type H295R cells and from cells derived from the different clones in which the ERRα gene was stably silenced were analyzed by western blotting using a specific antibody against ERRα. The GAPDH protein was used as a loading control. The blot presented in the figure represents the image of an experiment performed for at least three times and with similar results.

5.3.8 ERRα silencing reduces H295R cell growth

Numerous experimental evidences have shown that in different types of tumors the ERRα receptor is able to regulate cell growth. (Giguere 2008).

In particular, in breast cancer the increase in ERRα expression has been associated with a high risk of recurrence and a poor prognosis (Suzuki et al. 2004; Deblois et al. 2013). Using MTT assay and by cell counting, we investigated and analyzed the effects on cell growth associated with reduced expression of ERRα. In particular, these experiment were conducted only on cell lines in which there was an evident reduction of the

expression of ERRα, therefore in the cell lines deriving from clone3, clone 5 and clone 7. The results obtained (Figure 5.19 A, B) showed, in agreement with Western blotting data, a reduction in cell viability and growth in both H295R wild type (WT) and H295R cells transfected with a control shRNA plasmid. Furthermore, as shown in the graphs in Figure 5.19A, B, both the methods show a significant slowdown in growth, especially when evaluating the last day of analysis corresponding to the 5th after cell seeding.

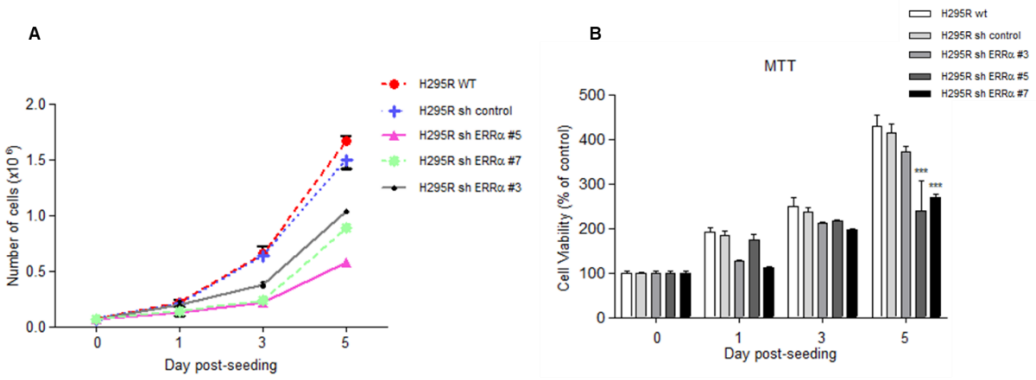


Fig. 5.19 ERRα depletion decreases growth of H295R cell lines. (A) Cell proliferation was evaluated by the MTT assay after 1, 3 and 5 days. MTT (2 mg / ml) was added for 1 h and the absorbance was measured at 570 nm. The results were expressed as mean ± SE of three independent experiments, each performed in triple (***, P <0.001 compared to H295R wt). **(B)** The H295RWT cells, the H295R sh-control cells and those in which ERRα was stably silenced were seeded in six-well plates in complete medium.

5.3.9 ERRα knockdown causes a defect in colony formation

We decided to use only the cell line deriving from clone 5 as the only experimental reference model for stable silencing of ERRα. In particular, we evaluated what were the effects of ERRα silencing on the independent anchorage growth of H295R cells. Anoikis is a programmed cell death induced by the detachment of cells from the extracellular matrix (ECM). It therefore represents a fundamental mechanism in preventing the growth and survival of cells in conditions independent of binding to the proteins of the cellular matrix, thus avoiding the survival in the circulation and the colonization of distant organs. In our study we demonstrated that ERRα knockdown causes a defect in colony formation (Figure 5.20A, B).

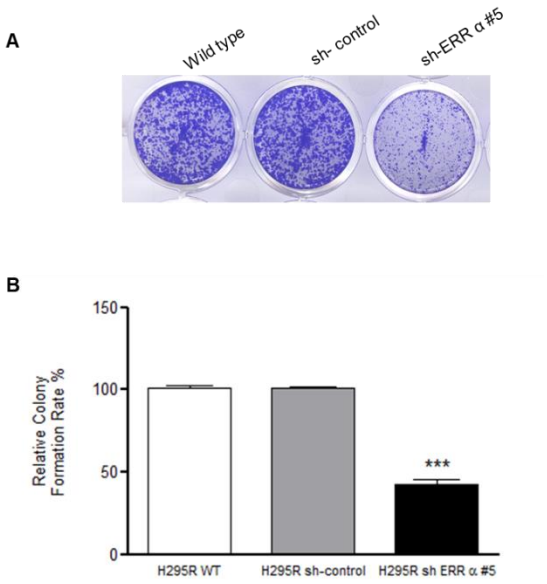


Fig. 5.20 Effects of ERRα silencing on colony formation in H295R cells. The H295R wt, H295R sh-control and H295R sh-ERRα # 5 cells (3000 cells / well) were fixed and stained with a blue solution of Comassie. **(A)** Representative image of colony formation. **(B)** The quantitative analysis of the number of colonies counted with the Olympus CKX53 Inverted Microscope is indicated as colony formation rate expressed as a percentage of the control (H295R wt). The results are expressed as a mean ± SD of three independent experiments each performed in triplicate. (***, P <0.001 compared with H295R wt).

5.3.10 ERRα knockdown suppress cell migration

Several observations support the hypothesis for a key role of the ERRα receptor in processes such as invasiveness, angiogenesis and metastasis in different tumor phenotypes (Deblois et al. 2013).

For this reason, in this work we wanted to investigate the effects of ERRα receptor silencing on H295R cell motility by testing the migration of the Boyden chamber and wound-healing (also known as scratch test). The latter is an essay conventionally used to evaluate cell migration by simulating the tissue repair mechanism. Our results, according to different data in the literature, confirm, also in our experimental model of adrenocortical carcinoma, the crucial role played by ERRα in cell migration processes. Indeed, as shown in Figure 5.21A, the reduction in ERRα expression results in decreased migratory ability of H295R cells. Similar results were obtained with the wound-healing test. The results shown in Figure 5.21B show how in H295R cells, in which ERRα expression has been stably silenced, a considerable distance is observed between the edges of the "wound" compared to wild-type cells indicating a slowing

down or lack of of the migratory capacity of cells following the reduced expression of ERRα.

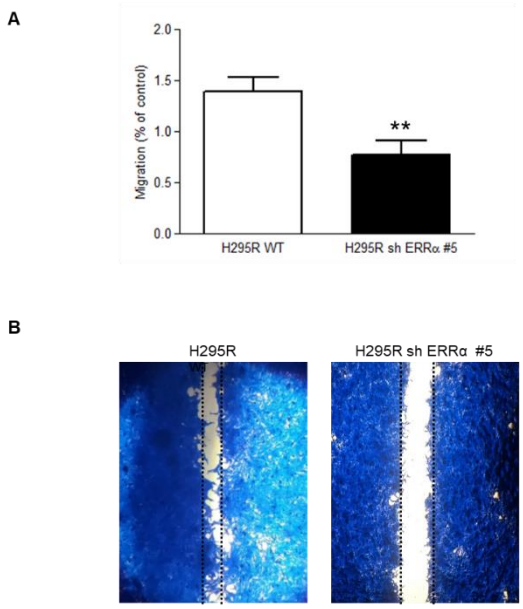


Fig. 5.21 ERRα silencing reduces H295R cell motility (A) H295R WT cells and H295R sh-ERRα # 5 cells (5 × 10^5cells/well) were resuspended in the culture medium and transferred accurately to the chamber higher. The Boyden chamber was incubated at 37 ° C with 5% CO2 for 24h. At the end of the experiment, cells were removed from upper side of the membrane using a cotton swab, whereas the cells that migrated to the lower wells were fixed and stained in a Comassie Brilliant Blue solution for 5 min. Migrated cells were randomly photographed and counted with ImageJ. *p <0.05 versus H295R cells. The experiments were repeated three times * p <0.05. **(B)** The H295R wt cells and H295R sh-ERRα # 5 cells were seeded in six-well plates, at a confluence of 70%, in complete medium. After scratching, the migration was monitored for 48h, the cells were fixed and stained with a solution of Blue Coomassie for 5 minutes. The images were acquired using a 10x magnification Olympus CKX53 Inverted Microscope.

5.3.11 ERRα knockdown decreases EMT markers

Epithelial-mesenchymal transition (EMT) is a process in which, following a chronic stimulus, epithelial cells with basal-apical polarity lose their phenotype and acquire the characteristics of non-polarized and migrating mesenchymal cells (Yang and Weinberg 2008). Literature data have shown that ERRα regulates the processes of tumor cell progression by acting distinctly on the mesenchymal epithelial transition. Our study demonstrated the effects of ERRα depletion in H295R cells. Specifically, we found an inversion in the epithelial-mesenchymal transition process. In fact, the results of

Western blotting analysis have shown a reduction in the expression of mesenchymal proteins such as Vimentin and N-Cadherin in cells permanently transfected with respect to H295R WT (Figure 5.22).

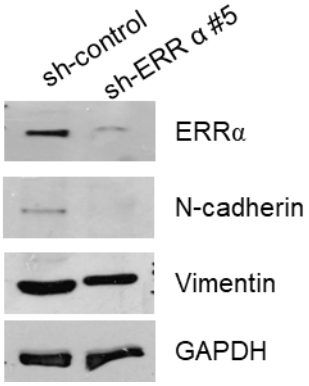


Fig. 5.22 The silencing of ERRα alters the mesenchymal transition-epithelium in H295R cells. Total protein extracts derived from H295R WT cells and H295R sh-ERRα # 5 cells were analyzed by WB using antibodies against ERRα, N-cadherin, Vimentin. The GAPDH was used as a loading control. Blots represent three independent experiments with similar results.

5.3.12 Effect of ERRα gene silencing on mitochondrial activity in H295R cells

Given the results of Western blot experiments and tests on cell viability and proliferation, we decided to use only the cell line derived from clone 5 in the subsequent tests as the only experimental reference model of stable ERRα silencing. in particular we evaluated what were the effects of ERRα silencing on the mitochondrial activity of

H295R cells. To this end, the total H295R cell proteins were extracted and by Western blot analysis we evaluated ERRα expression levels. ERRα silencing leads to the reduction of ERRα expression in H295R cells (Figure 5.23A). We used the OX-Phos antibody cocktail which is a mix of antibodies directed against respiratory complexes and ATP synthase, which allowed us to highlight a reduction of complex II, complex III and complex IV of the respiratory chain (Figure 5.23B). These data suggest that mitochondrial respiration reduction occurs in cells in which ERRα has been silenced.

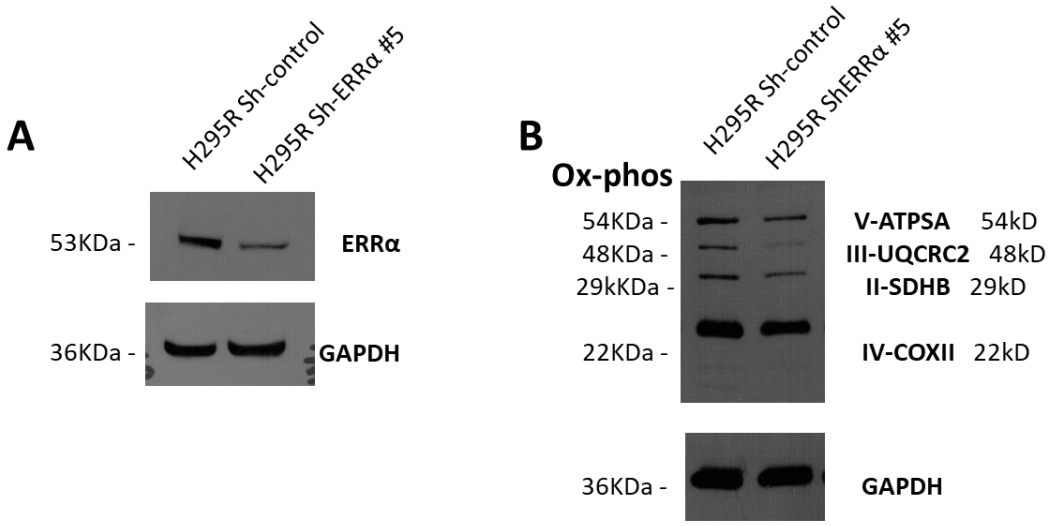


Fig. 5.23 Effects of ERRα silencing on the expression of ERRα and OX-phos in the ACC. Total protein extracts derived from H295R Sh-control cells and H295R sh-ERRα # 5 cells were analyzed by WB using antibodies against ERRα **(A)** and OX-phos **(B)**. The GAPDH was used as a loading control. Blots represent three independent experiments with similar results.

5.3.13 Depletion of ERRα reduces mitochondrial mass

We also used immunoblotting to monitor the amount of the exception marker of the mitochondrial mass, TOM20, in response to ERRα silencing. We have shown how stably silenced cells show a reduced expression of TOM20 (Figure 5.24).

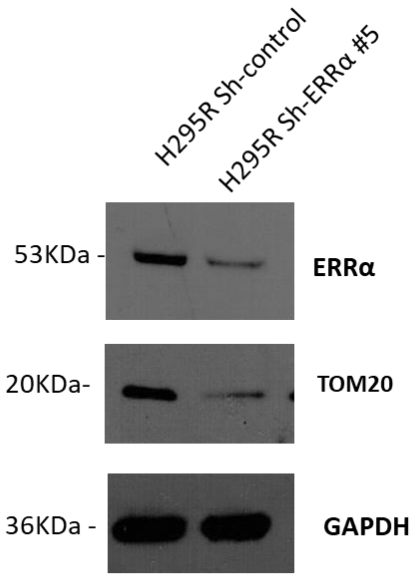


Fig. 5.24 ERRα silencing reduces TOM20 expression in H295R cells. Total protein extracts derived from H295R Sh-control cells and H295R sh-ERRα # 5 cells were analyzed by WB using anti-ERRα and TOM20 antibodies. The GAPDH was used as a loading control. Blots represent three independent experiments with similar results.

5.3.14 ERRα is crucial for H295R cells 3D-spheroid formation and propagation

We also observed that in the H295R cell line deriving from clone 5, growth in anchoring-independence conditions is significantly reduced, causing a drastic decrease in the 3D spheroid formation process (Figure 5.25). Tumor-initiating stem-like cells (TICs) are characterized by the ability to survive and expand in conditions of anchorage-independent growth in the form of clones known as spheroids. Furthermore, TICs exhibit greater mitochondrial dependence for their growth compared to a more differentiated population of tumor cells characterized by a glycolytic-type metabolism (Warburg effect). It must be emphasized that the ERRα receptor, together with its cofactors, is directly involved both in the metabolic pathways that govern glycolysis and in those that govern the oxidative-mitochondrial process. The result obtained underlines the importance of an adequate expression of the ERRα receptor able to sustain, in terms of an adequate energy intake, the growth of TICs.

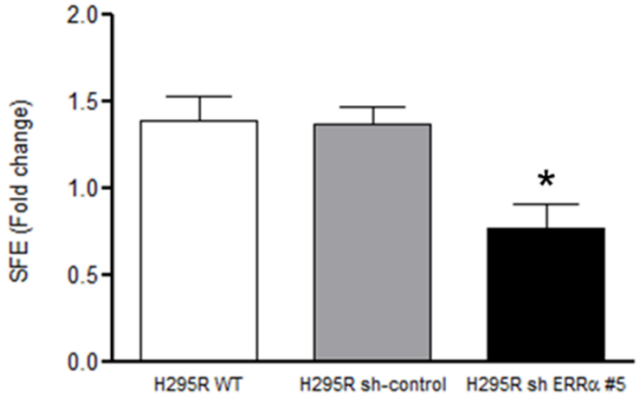


Fig. 5.25 Effects of ERRα silencing on the formation of 3D spheroids in H295R cells. The H295R wt cells, the H295R sh-control cells and those in which ERRα was stably silenced were cultivated as 3D-spheroids for 5 days. The spheroids were counted under the Olympus CKX53 Inverted Microscope and the results were expressed as induction folds with respect to the control ± SE, * $p < 0.05$.

5.3.15 Treatment with the inverse agonist, XCT790, reduces mitochondrial respiration activity in H295R cells

In a recent study it was shown that the reduction of expression of ERRα by a specific inverse agonist (XCT790) causes in the adrenocortical carcinoma H295R cell line, the inhibition of cell growth in vitro and in vivo, and the reduction of mass and mitochondrial function (Casaburi et al. 2015). Furthermore, ERRα has been shown to be a master regulator of mitochondrial metabolic regulation (Huss et al. 2004).

Recently, it has been shown that metabolic reprogramming is a characteristic of neoplasms (Hanahan and Weinberg 2011) and, since tumor cells can reprogram their metabolism, increasing glycolysis or oxidative phosphorylation (OXPHOS) (Martinez-Outschoorn et al. 2017) we wanted to evaluate the effect of ERRα reduction on the expression of some proteins that play a fundamental role at the level of the mitochondrial respiratory chain (CRM).

To this end, the total H295R cell proteins were extracted and by Western blot analysis we verified whether XCT790 treatment had actually induced a reduction in ERRα expression levels. Indeed, treatment for 48 hours with XCT790 results in a dose-dependent reduction in ERRα expression in H295R cells (Figure 5.26A). We then used on the same extracts the antibody which is a mix of antibodies called "OX-Phos" directed against respiratory complexes and ATP synthase, which allowed us to highlight

a reduction of all the respiratory chain complexes (Figure 5.26B). This data suggests that XCT790 by reducing expression of ERRα induces a significant reduction in mitochondrial respiration in H295R cells.

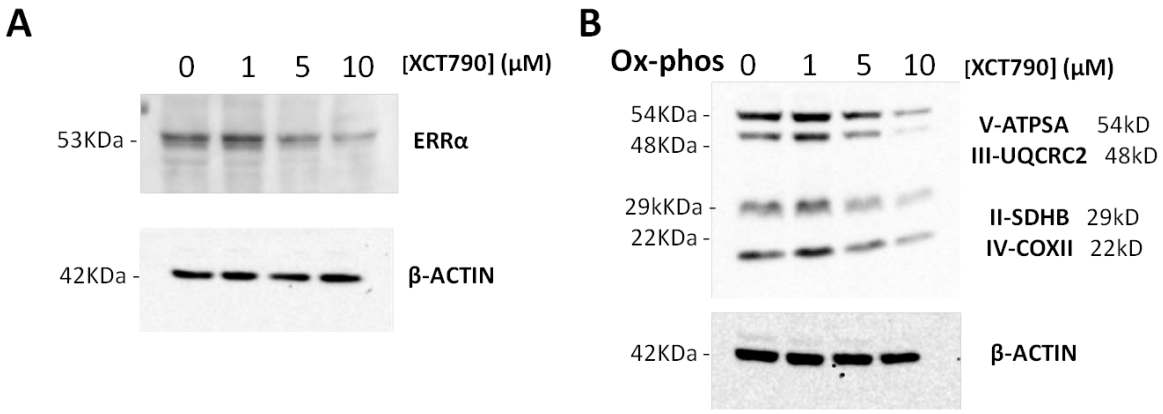


Fig. 5.26 Effects of XCT790 on the expression of ERRα and OX-phos in the ACC. H295R cells were treated for 48 hours with XCT790 (1, 5, 10μM) or vehicle (DMSO). Western blot analysis was used to evaluate the effect of treatment on ERRα **(A)**, and protein expression levels involved in the OX-phos process **(B)**. β-Actin was used as a loading control. Blots represent three independent experiments with similar results**.**

5.3.16 Treatment with the inverse agonist, XCT790, reduces the mitochondrial mass

We also used immunoblotting to monitor the amount of an exception mitochondrial mass marker, TOM20, in response to treatment with XCT790. We have shown how cells treated with XCT790 show a reduced expression of TOM20 (Figure 5.27). This data suggests that the reduction of ERRα also induces a reduction of the mitochondrial mass in H295R cells.

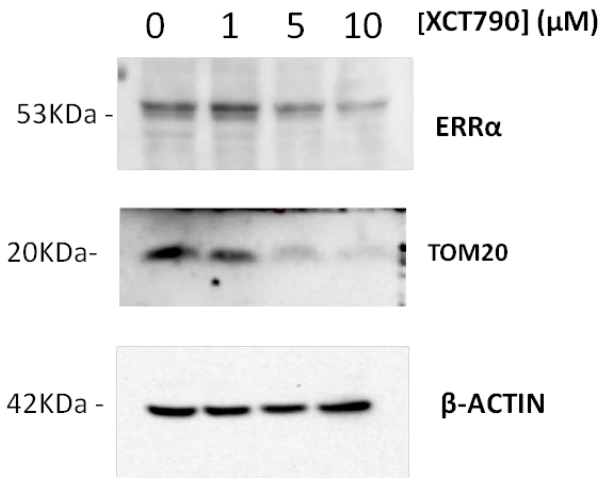
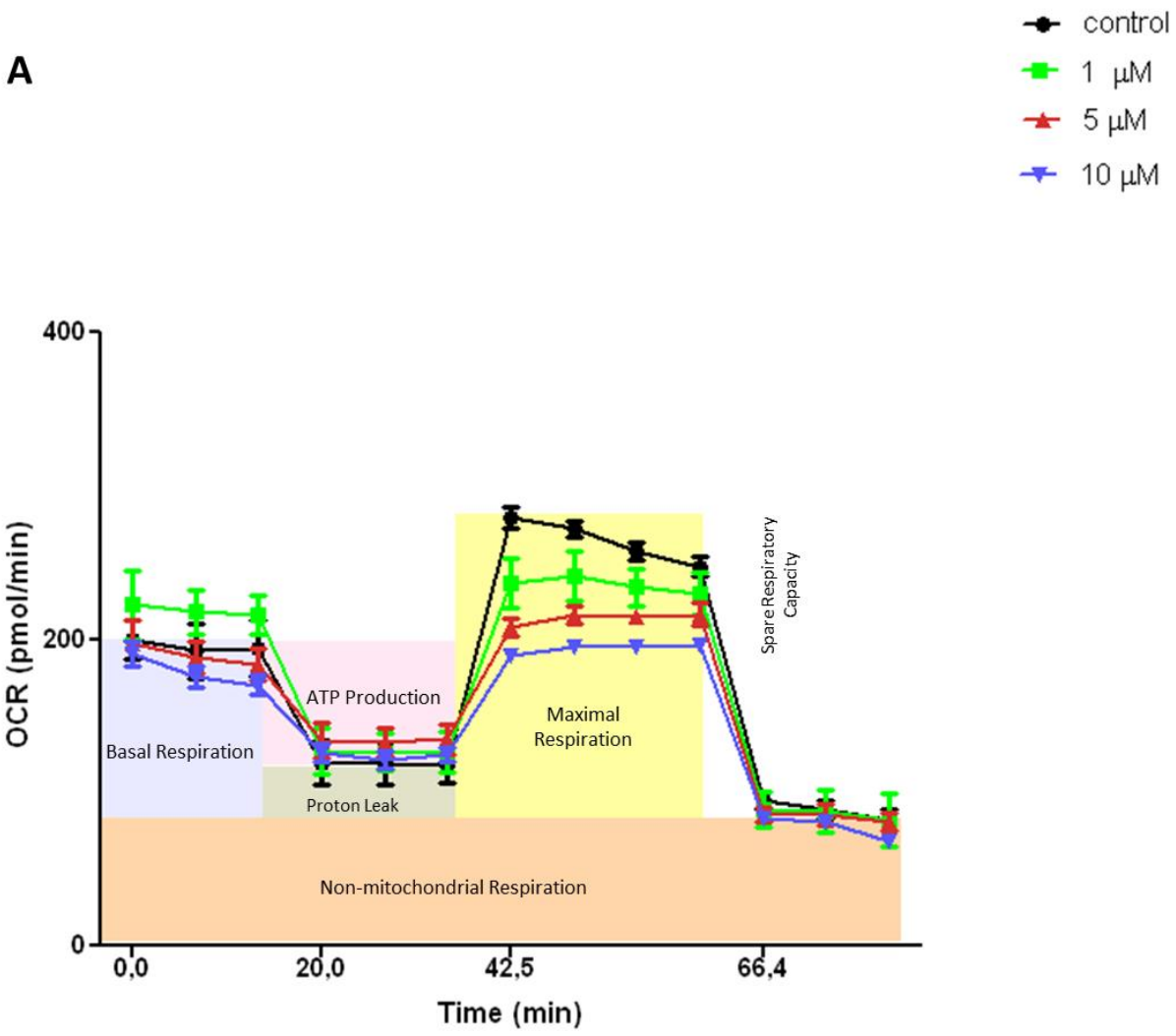


Fig. 5.27 Effects of the XCT790 on the expression of TOM20 in the ACC. H295R cells were treated for 48 hours with XCT790 (1, 5, 10μM) or vehicle (DMSO). Western blot analysis was used to evaluate the effect of treatment on TOM20 expression levels. β-Actin was used as a loading control. Blots represent three independent experiments with similar results.

5.3.17 Metabolic effects induced by XCT790 treatment

In this series of experiments the effects induced by the treatment with the inverse ERRa receptor agonist, XCT790, on mitochondrial respiration of H295R cells were evaluated. Specifically, the cells were incubated with vehicle or with different doses of XCT790 (1, 5 and 10 μM) for 16 hours and the oxygen consumption rate (OCR) was evaluated using the XF96 Flux Analyzer (XF96 flow analyzer). The values derived from the analysis show that treatment with XCT790 is able to reduce oxygen consumption in a dose-dependent manner (Figure 5.28A). Furthermore, treatment with XCT790 profoundly influences the oxidative metabolism of H295R cells. In fact, after 16 hours of treatment, there is a clear dose-dependent reduction of both basal respiration (Figure 5.28B) and maximal respiration (Figure 5.28C). The calculations applied to the different areas subtended by the curves obtained through the metabolic analysis (see Materials and Methods) also show a reduction in the turnover of the ATP (Figure 5.28D) and of the Spare capacity (Figure 5.28E). The values obtained were compared with those relative to the control cell population (untreated H295R cells). The effects on the Proton leak (Figure 5.28F), are observable only at concentrations of XCT-790 equal to 10μM.


A
control
1 µM
5 µM
10 µM
400
200
0
OCR (pmol/min)
Basal Respiration
ATP Production
Proton Leak
Maximal Respiration
Spare Respiratory Capacity
Non-mitochondrial Respiration
0,0
20,0
42,5
66,4
Time (min)

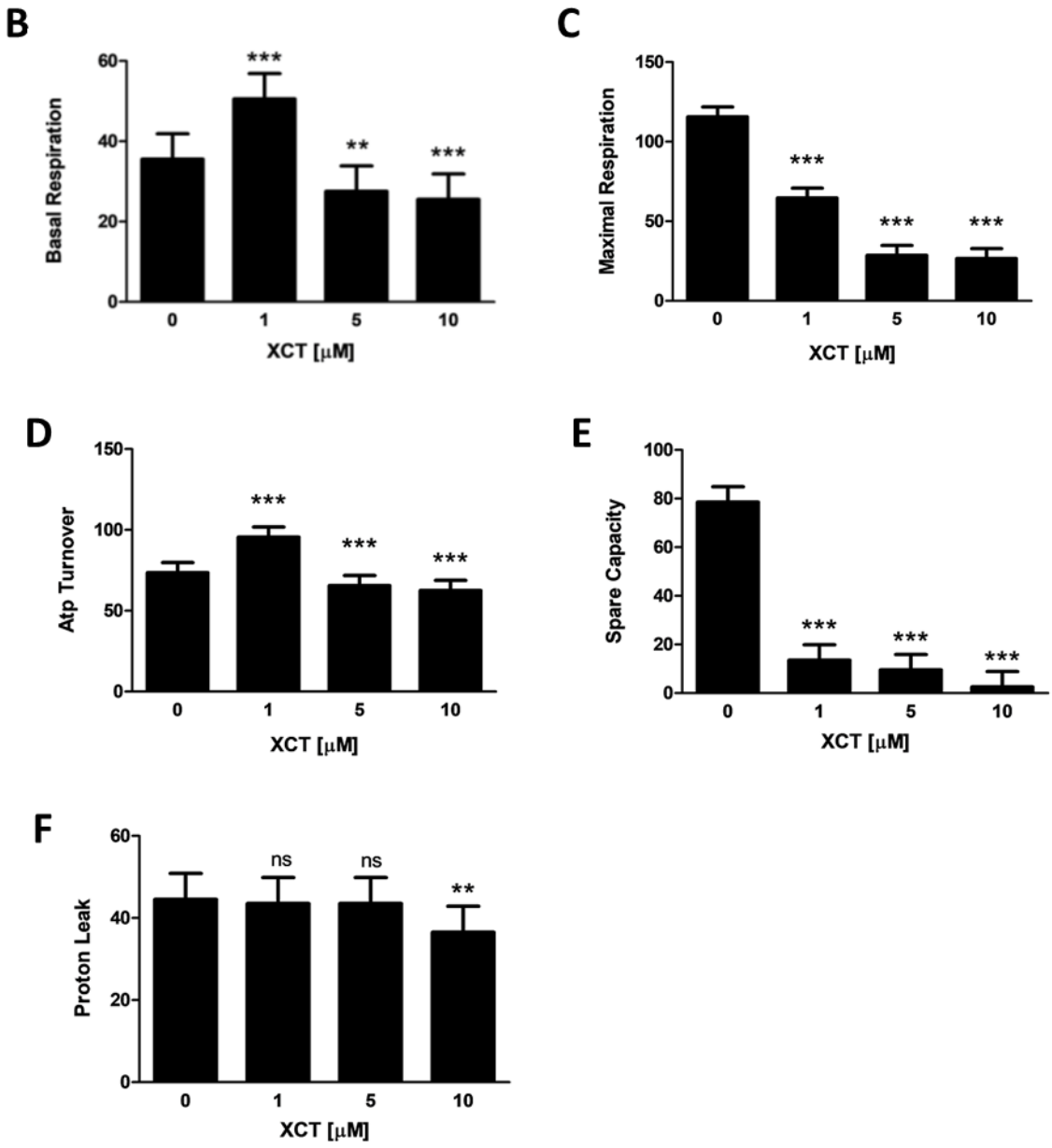
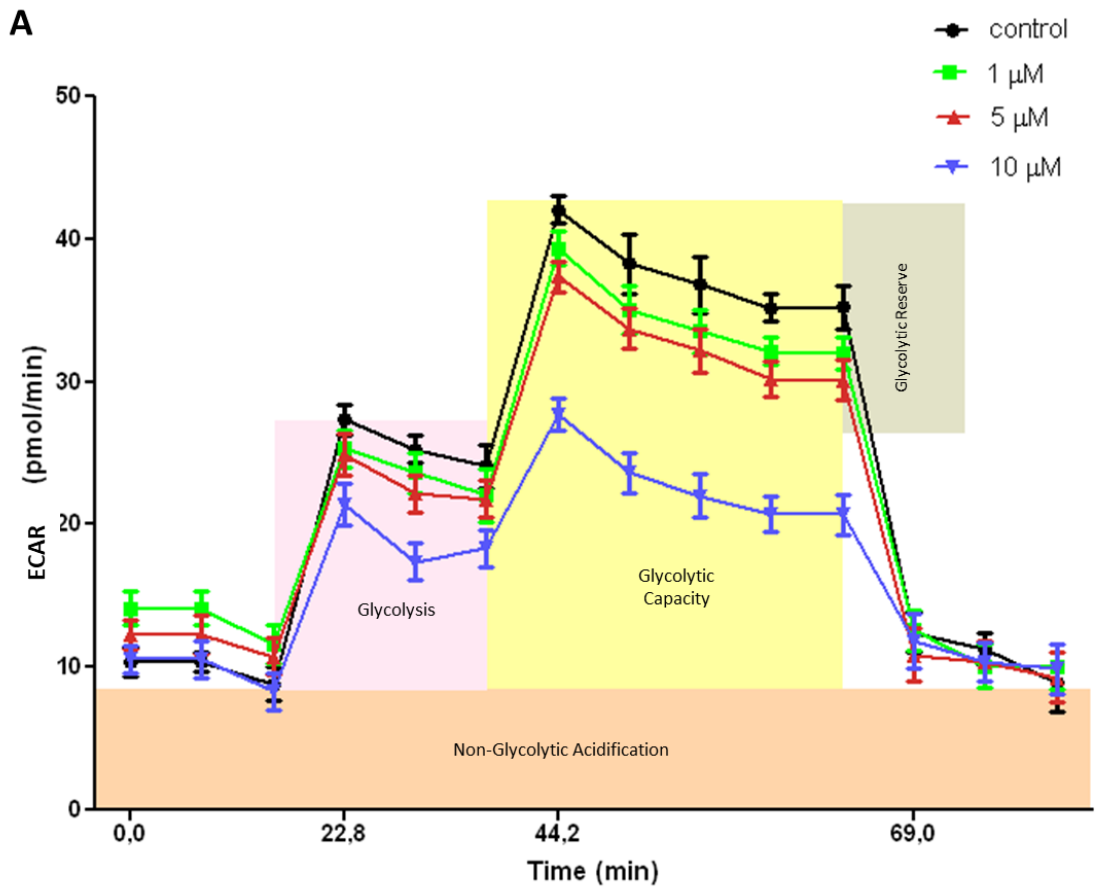


Fig. 5.28 XCT790 reduces the oxidative metabolism of H295R cells. The H295R cells grown in a monolayer were treated with XCT790 (1, 5 and 10 μM) for 16 hours. **(A)** Real-time oxygen consumption (OCR) rates were assessed using Seahorse XF96. The linear graph shows the same time course, but with three different injections to evaluate the oxygen consumption rate: 1-before the injection of oligomycin, 2- after the injection of carbonyl cyanide- (triphoromethoxy) phenylhydazone (FCCP) , 3- after the injection of rotenone antimicin. . **(B)** Basal respiration. **(C)** Maximal respiration. **(D)** ATP turnover. **(E)** Spare capacity. **(F)** Proton leak.

5.3.18 Effects on the rate of extracellular acidification induced by treatment with XCT790

In this series of experiments the effects induced by the treatment with the inverse ERRα receptor agonist, XCT790, on the glycolysis of H295R cells were evaluated. Specifically, the cells were incubated with vehicle or with different doses of XCT790 (1, 5 and 10 μM) for 16 hours and the rate of extracellular acidification (ECAR) was evaluated using the XF96 Flux Analyzer (analyzer of XF96 flow). The values resulting from the analysis show that treatment with XCT790 is able to reduce the rate of extracellular acidification in a dose-dependent manner (Figure 5.29A). Furthermore, treatment with XCT790 profoundly influences the glycolytic metabolism of H295R cells. In fact, after 16 hours of treatment, there is a clear dose-dependent reduction of both glycolysis (Fig. 5.29B) and the glycolytic reserve (Fig. 5.29C).



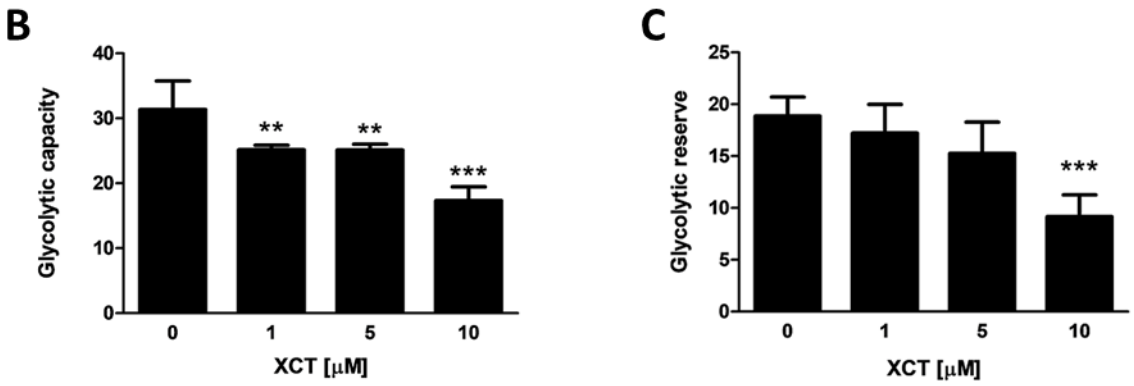
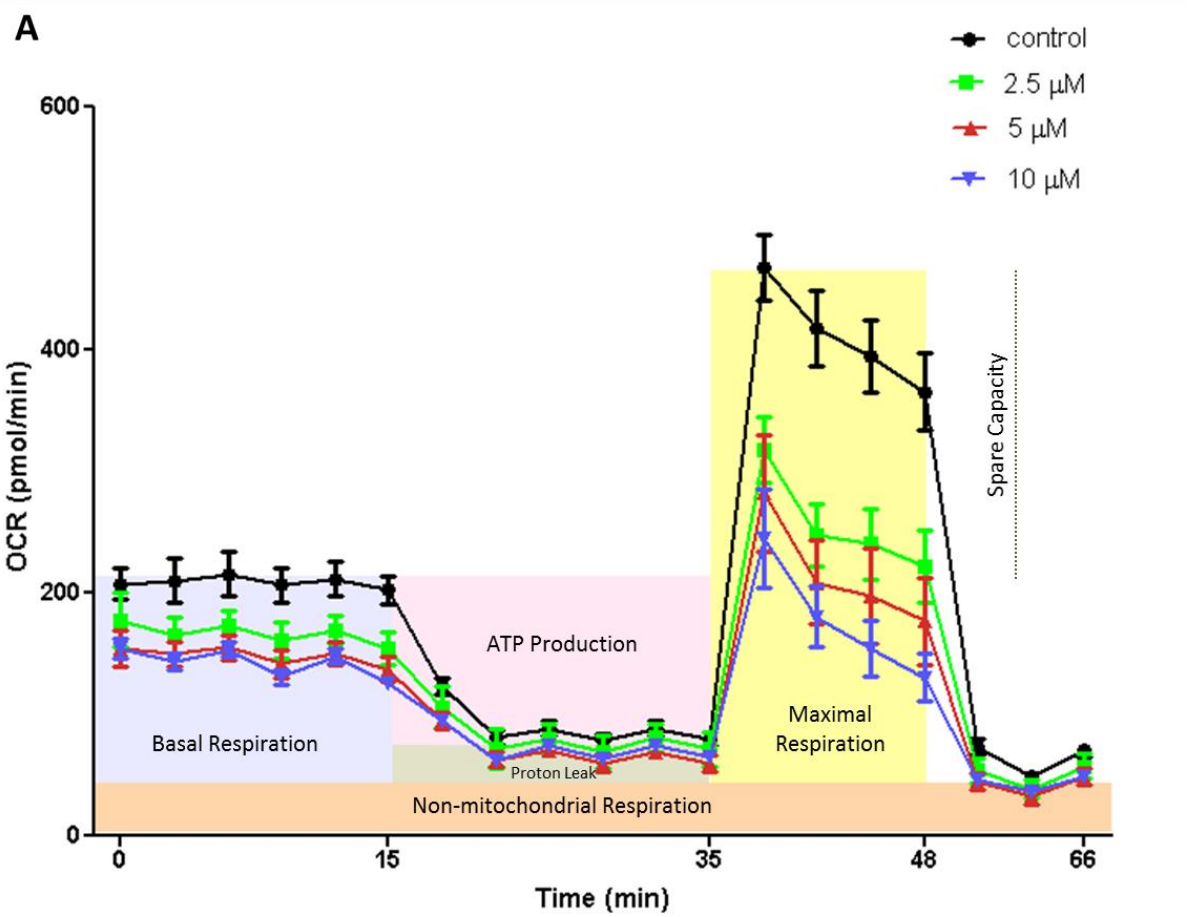


Fig. 5.29 The XCT790 reduces the glycolytic metabolism of H295R cells. The H295R cells grown in a monolayer were treated with XCT790 (1, 5 and 10 μM) for 16 hours**. (A)** Real-time extracellular acidification (ECAR) rates were assessed by Seahorse XF96. The linear graph shows the same time course, but with three different injections to evaluate the extracellular acidification rate: 1-before the glucose injection, 2-after the injection of carbonyl oligomycin, 3-after the injection of 2-deoxy -D-glucose. . **(B)** Glycolytic capacity. **(C)** Glycilitic reserve.

5.3.19 Metabolic effects induced by Simvastatin treatment

In this series of experiments the effects induced by the treatment with the Simvastatin, on mitochondrial respiration of H295R cells were evaluated. Specifically, the cells were incubated with vehicle or with different doses of Simvastatin (2,5, 5 and 10 μM) for 16 hours and the oxygen consumption rate (OCR) was evaluated using the XF96 Flux Analyzer (XF96 flow analyzer). The values derived from the analysis show that treatment with Simvastatin is able to reduce oxygen consumption in a dose-dependent manner (Figure 5.30A). Furthermore, treatment with Simvastatin profoundly influences the oxidative metabolism of H295R cells. In fact, after 16 hours of treatment, there is a clear dose-dependent reduction of both basal respiration (Figure 5.30B) and maximal respiration (Figure 5.30C). The calculations applied to the different areas subtended by the curves obtained through the metabolic analysis (see Materials and Methods) also show a reduction in the turnover of the ATP (Figure 5.30D) and of the Spare capacity (Figure 5.30E). The values obtained were compared with those relative to the control cell population (untreated H295R cells). The effects on the Proton leak (Figure 5.30F), are not observable.


A
control
2.5 µM
5 µM
10 µM
OCR (pmol/min)
600
400
200
0
Spare Capacity
ATP Production
Basal Respiration
Maximal
Respiration
Proton Leak
Non-mitochondrial Respiration
0
15
35
48
66
Time (min)

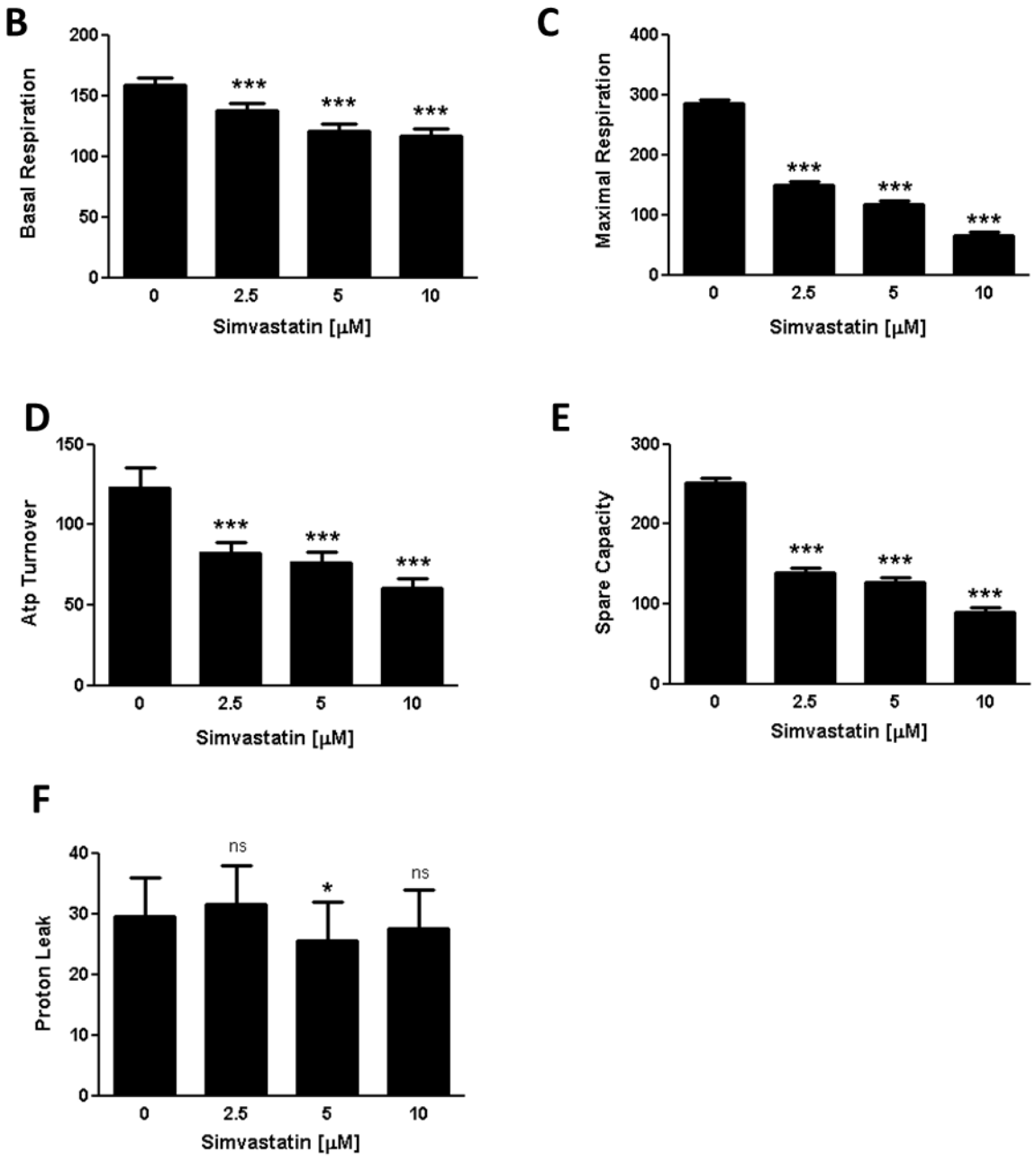
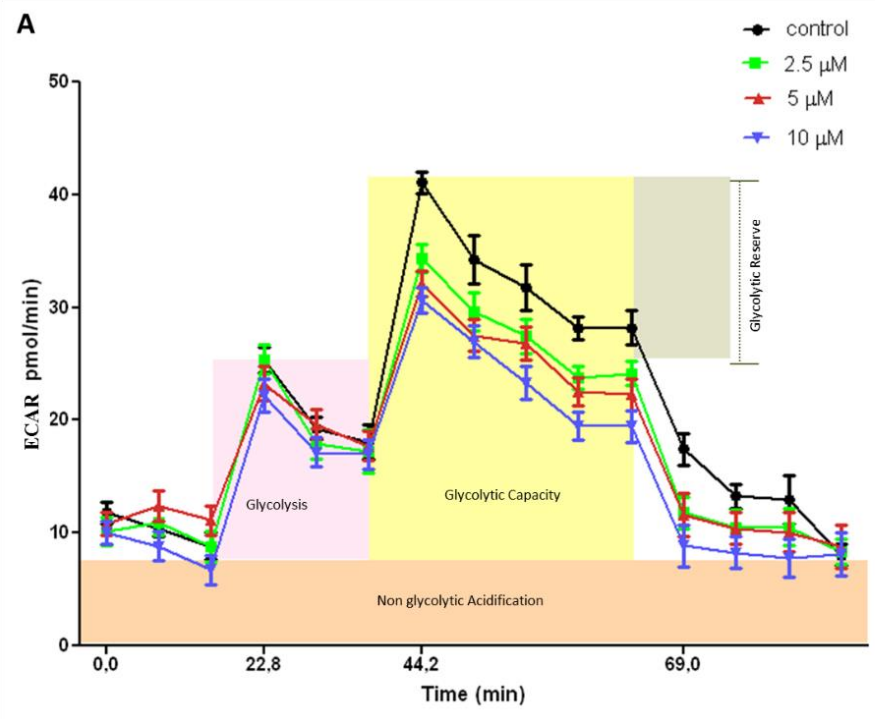


Fig. 5.30 Simvastatin reduces the oxidative metabolism of H295R cells. The H295R cells grown in a monolayer were treated with Simvatstin (2,5, 5 and 10 μM) for 16 hours. **(A)** Real-time oxygen consumption (OCR) rates were assessed using Seahorse XF96. The linear graph shows the same time course, but with three different injections to evaluate the oxygen consumption rate: 1-before the injection of oligomycin, 2- after the injection of carbonyl cyanide- (triphoromethoxy) phenylhydazone (FCCP) , 3- after the injection of rotenone antimicin. . **(B)** Basal respiration. **(C)** Maximal respiration. **(D)** ATP turnover. **(E)** Spare capacity. **(F)** Proton leak.

5.3.20 Effects on the rate of extracellular acidification induced by treatment with Simvastatin

In this series of experiments the effects induced by the treatment with the Simvastatin, on the glycolysis of H295R cells were evaluated. Specifically, the cells were incubated with vehicle or with different doses of Simvatstin (2,5, 5 and 10 μM) for 16 hours and the rate of extracellular acidification (ECAR) was evaluated using the XF96 Flux Analyzer (analyzer of XF96 flow). The values resulting from the analysis show that treatment with Simvatatin is able to reduce the rate of extracellular acidification in a dose-dependent manner (Figure 5.31A). Furthermore, treatment with Simvastatin profoundly influences the glycolytic metabolism of H295R cells. In fact, after 16 hours of treatment, there is a clear dose-dependent reduction of both glycolysis (Fig. 5.31B) and the glycolytic reserve (Fig. 5.31C).



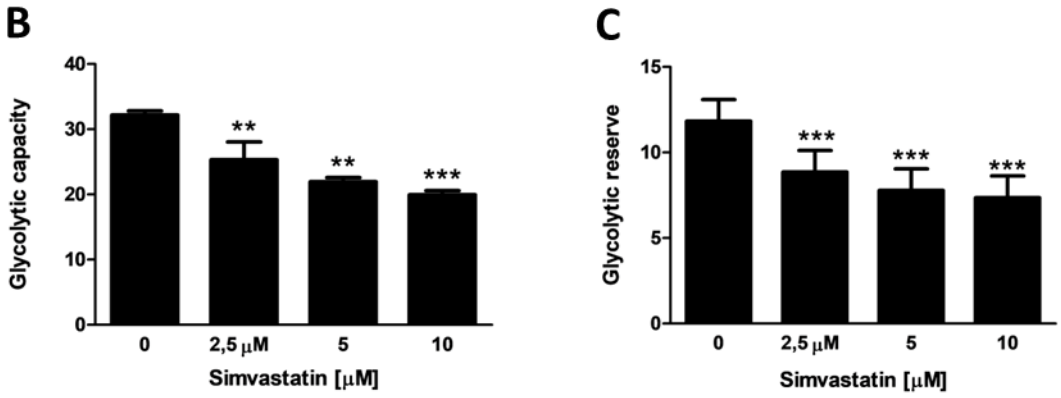


Fig. 5.31 Simvastatin reduces the glycolytic metabolism of H295R cells. The H295R cells grown in a monolayer were treated with Simvastatin (2,5, 5 and 10 μM) for 16 hours**. (A)** Real-time extracellular acidification (ECAR) rates were assessed by Seahorse XF96. The linear graph shows the same time course, but with three different injections to evaluate the extracellular acidification rate: 1-before the glucose injection, 2-after the injection of carbonyl oligomycin, 3-after the injection of 2-deoxy -D-glucose. . **(B)** Glycolytic capacity. **(C)** Glycilitic reserve.

6. DISCUSSION

It is known that the pathogenesis of adrenal carcinoma is due to an abnormal expression of different genes that can deregulate several cellular pathways involved in the modulation of tumor cell growth. The identification of factors that drives the regulatory events of these cellular pathways can help to clarify the molecular mechanisms responsible for the oncogenic alterations and to identify new potential therapeutic targets.

In this thesis work it has been investigated the role of three molecules involved in IGF and estrogen dependent pathways in the onset and progression of adrenal carcinoma: PELP1, EGR-1 and ERRα.

The aim of this study was to determine if PELP1 is expressed in ACC and whether or not it may play a role in promoting the interaction between ERα and IGF1R, allowing the activation of signaling pathways controlling ACC cell growth. First, we showed that PELP1 is expressed in H295R cells, as well as in normal adrenal and ACC tissues. We only aimed to assess if PELP1 was expressed in normal and tumor adrenal samples without indicating any difference in the expression levels since a limited number of samples were available. However, the data obtained support the idea of investigating PELP1 expression in a larger number of ACC samples in order to better determine if PELP1 gene expression is altered in ACCs. Of note, PELP1 expression was found to be deregulated in several cancers (Vadlamudi and Kumar 2007), and can be upregulated by estrogens and differentially regulated by selective estrogen receptor modulators (Mishra et al. 2004). It has been demonstrated that PELP1 participates in ER cytoplasmic and membrane mediated signaling by coupling ER with several cytosolic kinases acting as a scaffolding protein and facilitating the activation of ER-mediated non-genomic signaling (Vadlamudi et al. 2004). PELP1 modulates the interaction of ERs with c-Src, stimulating c-Src enzymatic activity, leading to the activation of the MAPK pathway (Cheskis et al. 2008). Moreover, growth factors promote tyrosine and serine phosphorylation of PELP1 (Vadlamudi et al. 2005b) which can directly interact with several growth factor receptors particularly EGFR and HER2 (Migliaccio et al. 2005; Vadlamudi et al. 2005b; Manavathi and Kumar 2006). Such regulatory interactions of PELP1 have important functional implications in the cross-talk of estrogen receptor and growth factor signaling (Manavathi and Kumar 2006). In H295R cells, we demonstrated that a short time treatment with E2 or IGF-II allowed the formation of a multiprotein

complex consisting of PELP1, IGF1R, ERα, and c-Src. Moreover, while E2 and IGF-II were able to induce rapid ERK1/2 activation, this effect was lost after PELP1 silencing. These data indicate PELP1 as a key player in both E2- and IGF-II-dependent ERK1/2 phosphorylation in H295R cells. Similar mechanisms have been discovered in breast cancer cells where PELP1 has been shown to be a critical mediator of estrogen-induced MAPK activation via c-Src. In fact, upon E2 stimulation, ERα induces rapid activation of MAPK in a c-Src dependent manner (Boonyaratanakornkit 2011). Mutation of the PELP1 PXXP c-Src interaction site abolished estrogen-induced MAPK activation and ER transcriptional activity. ER is also able to interact with c-Src, further increasing signaling efficiency to MAPK (Barletta et al. 2004). The PELP1/ER/c-Src signaling axis has also been shown to include growth factor receptors (EGFR, HER2) and the stimulation of PI3K/AKT and integrin-linked kinase 1 (ILK1) pathways (Manavathi and Kumar 2006; Chakravarty et al. 2010a; Vallabhaneni et al. 2011; Girard et al. 2014). Here, we demonstrated that PELP1 physically interacts with the IGF1R and this interaction is a required molecular event for IGF-II-mediated ERK1/2 phosphorylation. Mechanistic studies demonstrated that overexpression of a mutated form of PELP1 that lacks the nuclear localization signal was able to drive MAPK signaling and constitutive AKT activation in unstimulated breast cancer cells, resulting in increased phosphorylation of ERα at Serine 118 and Serine 167 (Vadlamudi et al. 2005b). Starting from these observations, we hypothesized that PELP1 could also have a role in IGF-II-dependent ERα phosphorylation that we previously demonstrated in H295R cells (Sirianni et al. 2012). On the other hand, E2 is able to modulate IGF1R expression as a consequence of increased cAMP-responsive element binding (CREB) protein activation and binding to the IGF1R promoter (Sirianni et al. 2012). Here, we demonstrated that PELP1 silencing is able to inhibit both basal and E2-induced IGF1R protein expression. A further demonstration of PELP1 involvement in pathways regulating ACC cell growth came from the observation that PELP1 gene silencing was able to decrease basal and abrogate E2- and IGF-II-dependent expression of Cyclin D1. Accordingly, reduced H295R cell growth was also detected after PELP1 gene silencing. Importantly, silencing of PELP1 using a liposomal formulation to vehicle PELP1 siRNA in vivo was shown to be significantly effective in reducing the growth of an orthotopic model of ER positive breast cancer (Cortez et al. 2012), suggesting that targeting this protein is feasible *in vivo* and could have promising therapeutic effects. Further studies will be required to define if PELP1 has any genomic effect in ACC cells. So far, our data suggest that the

main role played by PELP1 in controlling ACC cell growth is by coupling ERα with IGF1R and c-Src, working as a scaffolding protein and facilitating the activation of ER-nongenomic signaling and IGF1R-dependent pathways.
The important role played by the PELP1/ER/IGF1R/c-Src signaling axis in promoting both E2- and IGF-II-dependent ACC cell proliferation suggests that further studies are needed to consider PELP1 as a new target for the therapy of ACCs.

A previous study in Prof. Pezzi's lab showed that micromolar concentrations of G-1 significantly suppressed H295R cell proliferation both *in vitro* and *in vivo* by the activation of the intrinsic mitochondria-mediated apoptotic pathway and the associated molecular mechanism involve the long and sustained phosphorylation of ERK1/2 (Chimento et al. 2015).
G-1 has been reported to be a selective GPER agonist. For this reason we expected that GPER gene silencing in H295R cells would have completely abrogate cell growth inhibition by G-1. Surprisingly, when we effectively knocked-down GPER expression by siRNA, we found that the inhibitory effects of G-1 were only partially reversed. This finding suggested that G-1 might also suppress H295R cell proliferation in a GPER-independent manner. This hypothesis was supported by others describing the inhibitory role of G-1 regardless of GPER expression in both breast and ovarian cancer cell lines (Wang et al. 2012), as well as in human ovarian endometriosis stromal (Mori et al. 2015) and vascular smooth muscle cells (Gui et al. 2015). The aim of the this part of the study was to clarify the potential intracellular targets and the molecular mechanisms activated by G-1 able to inhibit ACC cell proliferation and to induce apoptosis. For these reasons, we started our study with a microarray analysis of H295R cells treated with G-1. Of several genes that were either up-regulated or down-regulated by G-1 treatment, we focused our attention on upregulated Egr-1 gene for two main reasons. First, Egr-1 is involved in G-1 induced signaling in different tumor cells (Vivacqua et al. 2012); second, Egr-1 has a dichotomic function since it can work as an oncogene but also as tumor suppressor (Thiel and Cibelli 2002).
Our results clearly showed that G-1 significantly upregulated Egr-1 expression at both transcriptional and post-transcriptional level. Moreover, its nuclear translocation, as evidenced by immunofluorescence and immunohistochemistry assays, highlighted G-1 ability to increase and activate Egr-1 protein.

In addition to its regulation by multiple extracellular stimuli (Thiel and Cibelli 2002), Egr-1 can be considered a redox-regulated gene because it is activated by all inducers of ROS-mediated signaling and inflammation (Yan et al. 1999; Jones and Agani 2003; Shin et al. 2009; Aggeli et al. 2010; Shin et al. 2013). This feature is due to the presence of oxidative stress-responsive DNA sequence within its promoter region (Datta et al. 1993), and most inducers of ROS-mediated signaling pathways increase the levels of Egr-1 (Yan et al. 1999; Jones and Agani 2003; Shin et al. 2009; Aggeli et al. 2010; Shin et al. 2013). Here we determined that G-1 induces ROS production in a GPER-independent manner. It is worth noting that other tumor types respond to G-1 treatment by enhancing ROS production (Cabas et al. 2013; Wei et al. 2014; De Giusti et al. 2015). However, in contrast to our observation on a GPER-independent mechanism, those studies claim the involvement of G-protein receptor. Indeed, we previously demonstrated that doses of G-1 1μM and onward elicit GPER-independent effects as supported by RNA interference experiments (Chimento et al. 2015). In agreement with previous observations on the biological effects exerted by G-1, we proved that G-1 activated the intrinsic apoptotic pathway where mitochondria play a key role as target of different stress stimuli including, above all, ROS (DeBerardinis and Chandel 2016). Therefore, we demonstrated that treatment of ACC cells with G-1 results in a significant increase in ROS production that was no longer detectable in the presence of the antioxidant NAC. The same observation was recently reported for colon cancer (Liu et al. 2017). Taking into account the critical role of Egr-1 in coordinating cellular events following oxidative stress (Pagel and Deindl 2012; Han et al. 2013), we showed that the presence of NAC was also able to reverse G-1- mediated up-regulation of Egr-1 expression. The scavenger effect of NAC on the G-1-dependent ROS increase has also been shown with regard to the activation of ERK1/2, one of the major ROS targets that is responsible for the up-regulation of the pro-apoptotic factor BAX in ACC cells (Chimento et al. 2015). Other important targets of ROS are represented by JNK and p38MAPK. Therefore, using specific inhibitors of these pathways, the results clearly demonstrated a selective involvement of ERK pathway in mediating the G-1 induced Egr-1 expression. By contrast, no role was observed for both JNK and p38. These findings are in agreement with those of others revealing that MAPKs regulate the expression of Egr-1 under stress conditions and elevated ROS levels (Hartney et al. 2011; Iyoda et al. 2012).

Finally, after silencing Egr-1 we confirmed its involvement in cell cycle arrest and mitochondrial apoptotic process. In fact, the upregulation of $p21^{Waf1/Cip1}$ and BAX expression seen under G-1 treatment, and consequently, the inhibitory effect on cell viability, were promptly reversed by silencing Egr-1 gene expression. These last results underlie the prominent role of Egr-1 in the inhibitory effects exerted by G-1 on ACC cells.

In conclusion, despite several reports indicate G-1 ability to increase ROS production and then cell apoptosis, a clear mechanism has not been defined. We can only confirm that mitochondrial dysfunctions and mitochondrial-mediated apoptotic pathways are involved in the inhibitory effects of G-1.

The identification of ROS/MAPK/Egr-1/BAX pathway as specific target activated by micromolar concentration of G-1 gives indication for new pharmacological approaches addressed to ACC therapy.

A study (De Martino et al. 2013) investigating a large cohort of advanced ACC confirmed the presence different gene alterations (TP53, CTNNB1, IGF-II) that could represent potential targetable molecules. This study suggest also that ACC is a disease extremely heterogeneous and that ACC pathogenesis involves integration of signals and the interplay of downstream pathways. Consequently, one useful strategy to develop an effective therapy for ACC will be to identify downstream target of multiple pathways. A good target could be the Estrogen Related Receptor α (ERRα). ERRα is an orphan member of the superfamily of hormone nuclear receptors and it is expressed in several tissues requiring a high energy demand. In addition to its control of energy metabolism and mitochondrial biogenesis, ERRα has recently been associated with cancer progression in which it requires an elevated cell metabolism (Chang and McDonnell 2012). Notably, increased expression of ERRα has been shown in several cancerous tissues, including breast, ovary and colon (Bernatchez et al. 2013). An association between elevated expression of ERRα and a poor clinical outcome in both breast and ovarian tumors was observed in several independent studies (Ariazi et al. 2002; Fujimoto et al. 2007). Several studies suggested that peroxisome proliferator-activated receptor γ coactivator-1 α and β (PGC-1α or PGC-1β) expression level and/or activity could regulate the transcriptional activity of ERRα. The ERRα/PGC-1 complex is a downstream target of multiple signaling pathways in cancer. Several signaling pathways relevant to cancer pathogenesis have been shown to converge upon and regulate the

expression and activity of PGC-1α and β. It has been shown recently that activation of HER2 and IGF1R signaling pathways increase the expression of PGC-1β through the induction of c-MYC gene. Similarly, activation of TOR/YY-1 pathway secondary to phosphoinositide 3-kinase (PI3K)/AKT activation can induce the expression of PGC-1α. The resulting ERRα/PGC-1 complex induces the expression of genes mainly involved in cell metabolism such as glycolysis, TCA cycle and oxidative phosphorylation (OXPHOS). ERRα has also been shown to interact with the β-cat/TCF complex and with HIF-1 and reciprocally modulate each other's transcriptional activities to affect cell motility and angiogenesis (Chang and McDonnell 2012). In a recent work (Wei et al. 2016) Chol has been identified as the first endogenous ERRα ligand able to increase the recruitment of PGC-1s to ERRα up-regulating its transcriptional activity. This new scenario needs to be investigated particularly in the adrenal gland, a high Chol demanding tissue for steroidogenesis. Moreover, this aspect is more interesting since we have shown that mitotane therapy can increase serum total cholesterol level in ACC patients (Figure 5.12). Moreover, it has been demonstrated in breast cancer that ERRα expression can be regulated by estrogen through ERα and it contributes to regulate aromatase expression (Rajhans et al. 2008). ERRα shares significant sequence homology and structural similarity to ER (Giguere et al. 1988) and recognizes the same responsive elements (Johnston et al. 1997). It was initially considered, therefore, that ERRα might exhibit similar activities as ER and that it would play a role in breast cancer. However, a comprehensive evaluation of the impact of ERRα activation on ERα-dependent transcriptional regulation in MCF-7 breast cancer cells revealed surprisingly few genes that were coregulated by these receptors (Stein et al. 2008).

We have shown *in vitro* that ERRα overexpression in H295R cells ($H295R^{ERR\alpha}$) is able to increase cell proliferation only in serum containing lipoproteins (Figure 5.13). These data suggest that ERRα requires Chol as an agonist to be functional also in ACC.

Several studies have reported that ERRα inverse agonist, XCT790, can induce cell growth arrest in different tumors cell line (Chisamore et al. 2009b; Wang et al. 2010). Unfortunately, there are few data on the role of ERRα in the ACC. ERRα is expressed in normal adult adrenal and regulates the expression of enzymes involved in steroidogenesis (Seely et al. 2005). Moreover, ERRα seems to be more expressed in ACC respect to normal adrenal and adenoma (Felizola et al. 2013). In a Pezzi's lab recent study (Casaburi et al. 2015) we showed that a decreased ERRα protein level by

XCT790 caused also a dose-dependent inhibition of cell growth. We also investigated the *in vivo* effects of XCT790 on H295R xenografts. Mice treated with XCT790 revealed a significant tumor growth reduction. These data suggest that Chol/ERRα/PCG1α complex is an eligible target to control ACC growth. We hypothesize that ERRα could be a "master regulator"of reprogramming metabolism in ACC. In fact, ERRα depletion in H295R cells caused a reduction of mitochondrial mass and function (Casaburi et al. 2015).

In addition, we also demonstrated that ERRα overexpression significantly increases glucose uptake in H295R cells (Figure 5.14), supporting the hypothesis that ERRα is involved in metabolic adaptation and reprogramming in ACC.

Tumor metastasis involves a series of interrelated events including angiogenesis, EMT and invasion. The ability of cancer cells to switch from a predominantly oxidative metabolism to glycolysis, promotes an acidic environment by an increased production and secretion of lactate (Gatenby and Gawlinski 2003). This leads to extracellular matrix degradation by proteolytic enzymes (Lardner 2001) and normal cell death (Williams et al. 1999) enhancing cancer cell migration and invasion. Experimental observations support the hypothesis for a role of ERRα in invasion, angiogenesis and metastasis in several tumors (Deblois et al. 2013). The absence of ERRα is able to impair tumorigenic potential in aggressive xenografted breast cancer cells (Stein et al. 2009). In addition, ERRα/PGC-1α complex binds to the promoter of VEGF regulating its expression, promoting tumor angiogenesis and invasion (Stein et al. 2009).

Our results demonstrate that adrenocortical H295R tumor cells, treated with XCT790 for 24h, displayed a reduced cell migration, supporting the hypothesis that the metabolic role played by ERRα could also favor invasiveness and the metastatic potential in ACC cells (Figure. 5.15). We also showed that XCT790 is able to inhibit H295R cell motility even in ERRα overexpressing cells . Accordingly, EMT markers are decreased after treatment with XCT790. We also demonstrated that ERRα overexpression is able to inhibit *anoikis* resistance in H295R cells (Figure. 5.16A).

As a barrier to metastasis, cells normally undergo an apoptotic process known as "anoikis," a form of cell death due to loss of contact with the extracellular matrix or neighboring cells. Cell resistant to anoikis are also able to growth in non-adherent culture condition. The latter phenomenon promotes the formation of spheroids or

tumor-initiating stem-like cells (TICs) in which, under appropriate culture conditions, it is possible to characterize cancer stem cells. ERRα overexpression increased the spheroids formation (Figure. 5.16B).

Furthermore, TICs exhibit greater mitochondrial dependence for their growth compared to a more differentiated population of tumor cells characterized by a glycolytic-type metabolism (Warburg effect). We therefore evaluated the involvement of the ERRα receptor on the ability to increase the formation and the number of 3D-spheroids, a model simulating TICs, starting from H295R cells. We demonstrated that XCT790 blocks mitochondrial biogenesis, survival and 3D-spheroid growth. Furthermore, we demonstrated that the over-expression of ERRα in H295R cells promotes the formation of TICs. Indeed, our results demonstrate that after prolonged culture represented by 3D spheroids, the expression of mesenchymal markers, such as Vimentin, N-cadherin and Slug is greater in 3D-H295R-derived spheroids compared to H295R monolayer cultures and cells displayed an increased migratory ability (Figure. 5.17).

In the last part of the study, we evaluated the effects of a stable reduced expression of ERRα in H295R cells (Figure. 5.18). As already showed for XCT790, H295R cells with stable silenced ERRα gene displayed a reduced cell proliferation and growth (Figure 5.19-5.20), as well as cell motility and migration (Figure 5.21). At the molecular level, $H295R^{ERR-}$ clone have a reduced expression of EMT markers (Figure 5.22) and a reduced mitochondrial metabolism (OXPHOS) (Figure 5.23) and mass (TOM20) (Figure 5.24). The $H295R^{ERR\alpha-}$ showed also a reduced 3D-spheroids formation (Figure 5.25). These results highlight a key role of ERRα in cellular metabolism related to a more motile phenotype.

Moreover, we assessed the effects of XCT790 in H295R cells and we showed a reduction of all the respiratory chain complexes (Figure 5.26) as well as mithocondrial mass (Figure 5.27). Accordingly, the functional analysis performed by Seahorse analyzer showed that H295R cells treated with XCT790 have a decreased OCR and ECAR values (Figure 5.28-5.29).

Recently it was demonstrated that ERRα transcriptional activity is enhanced by Chol and can be suppressed by statins. This mechanism have been revealed studing the effects of cholesterol on osteoclasts, macrophages, and myocytes, where ERRα is an

indirect target of the pharmacological effects of statins and bisphosphonates on bone resorption and skeletal remodeling.

In general, mitochondria appear to be an appealing target for the treatment of cancer (Ralph and Neuzil 2009). Similar to the metabolic effects induced by XCT790, we observed that H295R cells treated with simvastatin have a reduced mitochondrial function as demonstrated by the reduction of OCR and ECAR values (Figure 5.30-5.31).
Remain to demonstrate if this inhibitory effect is only due to the cholesterol reduction or to other pleiotropic effects of statins.

In conclusion, we have identified three potential targets for new therapies against adrenocortical carcinoma:

1. PELP1, that is expressed in ACC and may play a role in promoting the interaction between ERα and IGF1R allowing the activation of pathways important for ACC cell growth;
2. Egr-1, because the identified ROS/MAPK/Egr-1/BAX pathway as a potential off-target effect of the G-1 could be useful in implementing the pharmacological approach for ACC therapy;
3. ERRα, that is a master regulator of reprogramming cellular metabolism associated with ACC cell motility, invasion, angiogenesis and metastases. Therefore, the use of drugs that interfere with ERRα activity could be a valid therapeutic strategy to contrast ACC progression.

Future *in vivo* studies are necessary to investigate if targeting Chol (statins) and/or ERRα (XCT790) is a good therapeutic approach to prevent ACC metastases**.**

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