Estudio nutrigenómico de compuestos polifenólicos del cacao y del café en células tumorales humanas

Carlota Oleaga Sancho

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UNIVERSITAT DE BARCELONA

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ESTUDIO NUTRIGENÓMICO DE COMPUESTOS POLIFENÓLICOS DEL CACAO Y DEL CAFÉ EN CÉLULAS TUMORALES HUMANAS

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ESTUDIO NUTRIGENÓMICO DE COMPUESTOS POLIFENÓLICOS DEL CACAO Y DEL CAFÉ EN CÉLULAS TUMORALES HUMANAS

Memoria presentada por Carlota Oleaga para optar al título de doctor por la Universidad de Barcelona

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$$[\ pH = pKa + \log ([A^-]/[AH])\ ]$$

Llegó el final de esta etapa, un precioso inicio de mi carrera profesional. Obviamente, no hubiera sido igual sin todas aquellas personas que en algún momento de este proyecto me han podido enseñar, ayudar, animar, soportar, alegrar, regañar, acompañar, estimular, emocionar y generar pasión por la investigación.

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Sara, Sarita! Cuanto te he echado de menos… tu paso de la anguila fue un antes y un después.
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Imagination is more important than knowledge...
Albert Einstein

-To the RNAse Free concept belivers -

ABREVIATURAS

^{14}C	*Carbon-14 isotope* (Isótopo 14 del carbono)
17β-HSD	*17-β Hydroxysteroid dehydrogenase* (17-β hidroxiesteroide deshidrogenasa)
3-HFA	*3-hydroxyphenylacetic acid* (Ácido 3-hidroxifenilacético)
3-M-EPI	*3'-O-methylepicatechin* (3'-O-metil-epicatequina)
3,4-HFA	*3,4-hydroxyphenylacetic acid* (Ácido 3,4-hidroxifenilacético)
^{32}P	*Phosphorus-32 isotope* (Isótopo 32 del fósforo)
^{3}H	*Tritium or Hydrogen-3 isotope* (Tritio o Isótopo 3 del hidrógeno)
4-M-EPI	*4'-O-methylepicatechin* (4'-O-metilepicatequina)
A	*Adenine* (Adenina)
AHH	*Aryl hydrocarbon hydroxylase* (Aril Hidrocarburo hidroxilasa)
AhR	*Aryl hydrocarbon receptor* (Receptor de Aril hidrocarburo)
Akt	*v-akt murine thymoma viral oncogene homolog 1* (Homologo a oncogén viral del timoma murino v-akt)
AMP	*Adenosine Monophosphate* (Monofosfato de adenosina)
ANOVA	*Analysis of variance* (Análisis de la varianza)
AP-1	*Activator protein 1* (Proteína activadora 1)
ApoAI	*Apolipoprotein AI* (Apolipoproteína AI)
APRT	*Adenine Phosphoribosyltransferase* (Adenina fosforribosiltransferasa)
Arnt	*Aryl hydrocarbon receptor nuclear translocator* (Translocador Nuclear del Receptor de Aril hidrocarburo)
ATF	*Activating Transcription Factor* (Factor Activador de la transcripción)
ATP	*Adenosine Triphosphate* (Trifosfato de adenosina)
ATR	*Atorvastatin* (Atorvastatina)
AURKA	*Aurora A kinase* (Quinasa Aurora A)
B.LUC	*Site B luciferase construct* (Construcción luciferasa con Site B)
BAN	*Biological association network* (Red de asociación biológica)
Bcl	*B-cell lymphoma gene* (Gen del linfoma de células B)
bp	*Base pairs* (Pares de bases)
BSA	*Bovine serum albumin* (Albúmina de suero bovino)
C	*Cytosine* (Citosina)
c-Myc	*Myelocytomatosis viral oncogene homolog* (Homologo a oncogén viral de la mielocitomatosis)
CA	*Caffeic acid* (Ácido cafeico)
CAT	*Catalase gene* (Gen de la catalasa)
cDNA	*Complementary DNA* (DNA complementario)
cFLIP	*Cellular FLICE-like inhibitory protein. Also known as Caspase 8* (Proteína inhibitoria de FLICE y similares. También llamada Caspasa 8)
CHX	*Cycloheximide* (Cicloheximida)
CI	*Combination Index* (Índice de combinación)
Ci, µCi	*Curie, Microcurie* (Curio, Microcurio)

CMV	Cytomegalovirus (Citomegalovirus)
CNT	*Control* (Control)
CRE	*cAMP responsive element* (Elemento de respuesta de AMPc)
Ct	*Threshold cycle* (Ciclo umbral)
CVD	*Cardiovascular disease* (Enfermedades o eventos cardiovasculates)
CYP1A1	*Cytochrome P450 family 1 subfamily A polypeptide 1* (Citocromo p450 familia 1 subfamilia A polipéptido 1)
D	*Dose* (Dosis)
DEPC	*Diethyl pyrocarbonate* (Dietil pirocarbonato)
DIM	*3,30- Diindolylmethane* (3,30-Diindolimetano)
Dm	*Median-effect dose* (Dosis del efecto medio que expresa la fuerza)
DMSO	*Dimethyl sulfoxide* (Dimetil sulfóxido)
DNA	*Deoxyribonucleic acid* (Ácido desoxirribonucleico)
dNTPs	*Deoxyribonucleotides triphosphate* (Desoxirribonucleótidos trifosfato)
DTT	*1,4-dithiothreitol* (1,4-ditiotreitol)
E1	*Estrone* (Estrona)
E2	*Estradiol* (Estradiol)
E3	*Estriol* (Estriol)
EGCG	*Epigallocatechin-3-gallate* (Epigalocatequina-3-galato)
EGF, EGFR	*Epidermal growth factor, EGF Receptor* (Factor de crecimiento epidérmico, Receptor del EGF)
ELISA	*Enzyme-linked immunosorbent assay* (Ensayo por inmunoabsorción ligado a enzimas)
EMSA	*Electrophoretic mobility shift assay* (Ensayo de retardación de la movilidad electroforética)
EPI	*Epicatechin* (Epicatequina)
EPI-S, EPI-3-S	*Epicatechin-sulphate, Epicatechin-3-sulphate* (Epicatequina-sulfato, Epicatequina-3-sulfato)
ER	*Estrogen receptor* (Receptor de estrógeno)
ERE	*Estrogen receptor element* (Elemento de respuesta para el receptor de estrógeno)
fa	*Fraction affected by dose* (Fracción afectada por la dosis)
FBS	*Fetal bovine serum* (Suero bovino fetal)
FMD	*Flow mediated dilation* (Dilatación mediada por el flujo)
FMO	*Flavin-containing monooxygenase* (Flavina monooxigenasa)
fu	*Fraction unaffected* (Fracción inalterada)
FW	*Forward primer* (Cebador sentido)
G	*Guanine* (Guanina)
G0, G1	*Cell cycle phase G* (Fase del ciclo celular G)
GADD	*Growth arrest and DNA-damage-inducible gene* (Gen que puede ser inducido por daño en el DNA y parada del crecimiento)
GDF	*Growth differentiation factor* (Factor regulador del crecimiento y diferenciación)
GEO	*Gene Expression Omnibus*
GHDC	*GH3 domain containing gene* (Gen que contiene el dominio GH3)
GM-CSF	*Granulocyte-macrophage colony-stimulating factor* (Factor estimulante de colonias de granulocitos y macrófagos)
GPX	*Glutathione peroxidise* (Glutatión peroxidasa)
GR	*Glucocorticoid receptor* (Receptor de glucocorticoides)

GS	*Gel shift*
GTP	*Guanosine-triphosphate* (Guanosín trifosfato)
HDL	*High density lipoproteína* (Lipoproteína de elevada densidad)
HER2	*Human Epidermal Growth Factor Receptor 2* (Receptor del factor de crecimiento epidérmico 2)
HGP	*Human Genome Project* (Proyecto del genoma humano)
HNF	*Hepatocyte nuclear factor* (Factor nuclear hepático)
HPLC	*High-performance liquid chromatography* (Cromatografía líquida de alta eficacia)
Hsp	*Heat shock protein* (Proteína de choque térmico)
ICC	*Instant caffeinated coffee* (Café soluble)
IDL	*Intermediate-density lipoproteins* (Lipoproteínas de densidad intermedia)
IFN	*Interferon* (Interferón)
IL	*Interleukin* (Interleucina)
JNK	*c-Jun N-terminal kinase* (Quinasa c-Jun N-terminal)
Kb	*Kilobase* (Kilobase)
KDa	*Kilodalton* (Kilodalton)
Kras	*Kirsten rat sarcoma viral oncogene homolog* (Homólogo del oncogén viral del sarcoma de rata Kirsten)
LDL	*Low-density lipoproteins* (Lipoproteínas de baja densidad)
LTA	*Lymphotoxin alpha* (Linfotoxina alfa)
LUC	*Luciferase reporter vector* (Vector reportero portador del gen *luciferasa*)
m	*Coefficient signifying the shape of the dose-effect relationship* (Coeficiente que expresa la forma de la curva dosis-efecto)
M, mM, μM, nM	*Molar, Milimolar, Micromolar, Nanomolar* (Molar, Milimolar, Micromolar, Nanomolar)
MAP, MAPK, MEKK, MEK1	*Mitogen-activated protein, MAP kinase, MAP kinase kinase* (Proteína activada por mitógenos, MAP quinasa, MAP quinasa quinasa)
MCDF	*6-methyl-1,3,8-trichlorodibenzofuran* (6-metil-1,3,8-triclorodibenzofurano)
Mcl-1	*Myeloid cell leukemia sequence 1* (Gen secuencia de células de leucemia mieloide-1)
MGF	*Mammary gland factor* (Factor de la glándula mamaria)
miRNA	*Short ribonucleic acid* (Ácido ribonucleico de cadena corta)
MMP	*Matrix metalloproteinase* (Metaloproteinasa de matriz)
MMTV	*Mouse mammary tumor virus* (Virus del tumor mamario de ratón)
mQ	*MiliQ water* (Agua miliQ)
mRNA	*Messenger ribonucleic* acid (Ácido ribonucleico mensajero)
MTHFR	*5,10-methylenetetrahydrofolate reductase* (5,10-metilenotetrahidrofolato reductasa)
MTT	*(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (Bromuro de (3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolio)
NAPD+, NADPH	*Nicotinamide adenine dinucleotide phosphate* (Nicotinamida adenina dinucleótido fosfato)
NE	*Nuclear extract* (Extracto Nuclear)
NF-κβ	*Nuclear factor kappa-light-chain-enhancer of activated B cells* (Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas)

NFY	*Nuclear Factor Y* (Factor Nuclear Y)
NOS	*Nitrogen oxigen species* (Especies de oxígeno y nitrógeno)
nt	*Nucleotide* (Nucleótido)
OVN	*Overnight*
p21, p38, p53	*Tumor protein p* (Proteína tumoral p)
PBS	*Phosphate buffered saline* (Tampón fosfato salino)
PCE	*Polyphenolic cocoa extract* (Extracto polifenólico de cacao)
PCR	*Polymerase chain reaction* (Reacción en cadena de la polimerasa)
PI	*Propidium iodide* (Ioduro de propidio)
PI3K	*Phosphoinositide 3-kinase* (Fosfoinositol 3-quinasa)
PKA	*Protein kinase, cAMP-dependent, catalytic, alpha* (Proteína quinasa A subunidad catalítica alfa)
PKC	*Proline-rich transmembrane protein 2* (Proteína transmembrana rica en prolinas 2)
PMSF	*Phenylmethanesulfonylfluoride* (Fluoruro de fenilmetilsulfonilo)
PPi	*Pyrophosphate* (Pirofosfato)
PR	*Progesteron receptor* (Receptor de progesterona)
PTGS1	*Prostaglandin-endoperoxide synthase 1* (Sintasa 1 de prostaglandina endoperoxidasas)
PUFA	*Polyunsaturated fatty acids* (Ácidos grasos poliinsaturados)
PVDF	*Polyvinylidene fluoride* (Fluoruro de polivinilideno)
RAD23A	*RAD23 homolog A* (Homólogo A de RAD23)
RAR	*Retinoic acid receptor* (Receptor del ácido retinoico)
Reg	*Regenerating islet-derived gene* (Gen regenerador de los islotes de Langerhans)
RLU	*Relative luminiscence units* (Unidades relativas de luminiscencia)
RNA	*Ribonucleic* acid (Ácido ribonucleico)
RNAsa	*Ribonuclease* (Ribonucleasa)
ROS	*Reactive oxygen species* (Especies reactivas de oxígeno)
RT	*Room Temperature* (Temperatura ambiente)
RT-PCR	*Reverse transcription-polymerase chain reaction* (Reacción de la transcriptasa inversa)
RV	*Reverse primer* (Cebador antisentido)
RXRα	*Retinoid X receptor, alpha* (Receptor X retinoide alfa)
S	*Cell cycle phase S* (Fase del ciclo celular S)
SDS	*Sodium dodecyl sulfate* (Dodecilsulfato sódico)
SE	*Standard error* (Error estándar)
SERPINE1	*Serpin peptidase inhibitor, clade E* (Gen inhibidor de la proteasa de serina, tipo E)
siRNA	*Small interfering ribonucleic* acid (Ácido ribonucleico pequeño de interferencia)
SNPs	*Single nucleotide polymorphisms* (Polimorfismos de nucleótido simple)
Sp	*Specific protein* (Proteína específica)
SS	*Supershift*
STAT	*Signal Transducer and Transcription Factor* (Transductor de señales y activador transcripcional)
STP	*Staurosporine* (Estaurosporina)
T	*Thymine* (Timina)
TAM	*Tamoxifen* (Tamoxifeno)
TBE	*Tris/Borate/EDTA buffer* (Tampón de Tris/Borato/EDTA)

TBS	*Tris-buffered saline* (Solución salina de Tris)
TCDD	*2,3,7,8-Tetrachlorodibenzo-p-dioxin* (2,3,7,8-Tetraclorodibenzo-p-dioxina)
TE	*Tris-EDTA buffer* (Tampón Tris-EDTA)
TF	*Transcription factor* (Factor de transcripción)
TKR	*Tyrosin kinase receptor* (Receptor de la tirosina quinasa)
TNF-α	*Tumor necrosis factor* (Factor de necrosis tumoral)
TNM	*Tumor, node, metastasis* (Tumor, nodo y metástasis)
TP53	*Tumor protein p* (Proteína tumoral p)
UDP	*Uridine diphosphate* (Uridina difosfato)
VA	*Vanillic acid* (Ácido vanílico)
VEGF	*Vascular endotelial growth factor* (Factor de crecimiento del endotelio vascular)
VLDL	*Very-low-density lipoproteins* (Lipoproteínas de muy baja densidad)
XIAP	*X-linked inhibitor of apoptosis protein* (Proteína inhibidora de la apoptosis ligada al cromosoma X)
XRCC2	*X-ray repair cross-complementing protein* (Proteína Complementado cruzado de reparación de los rayos X)
XRE	*Xenobiotic Response Element* (Elemento de respuesta a Xenobióticos)

ÍNDICE

PRESENTACIÓN

La investigación en genómica nutricional combina las áreas de conocimiento de biología molecular, genética y nutrición. Se trata de una rama científica relativamente nueva que avanza rápidamente y cuyo objetivo es conocer cómo la dieta, o componentes de la misma, afectan el equilibrio entre salud y enfermedad, estudiando las variaciones en el perfil genético de un individuo y la expresión de los genes.

Los factores de riesgo que pueden desencadenar un desequilibrio en la salud se diferencian entre factores no modificables (sexo, edad y herencia genética) y factores modificables (inactividad física, dietas inadecuadas y tabaquismo, entre otros). La alimentación tiene un papel crucial en la regulación de la homeostasis del organismo; por ello, las dos ciencias englobadas en la genómica nutricional, la **nutrigenética** y la **nutrigenómica** están generando grandes expectativas de cara a ayudar a establecer recomendaciones dietéticas personalizadas, tanto a nivel del público en general como para colectivos con patologías particulares.

Los compuestos bioactivos de la dieta son aquellos con potencial para modificar un elevado número de procesos asociados al balance entre salud y enfermedad. Los compuestos fenólicos, principalmente los flavonoides y los ácidos fenólicos, son un ejemplo de compuestos bioactivos y forman parte de nuestra dieta a través de alimentos y bebidas de origen vegetal, como frutas (manzanas, naranjas, frutos rojos, etc.), verduras (pimientos, zanahorias, etc.), cacao y derivados, te o café. Son numerosos los estudios científicos que demuestran la capacidad antioxidante, antiinflamatoria y anticancerígena, entre otras, de estos compuestos.

En la presente tesis se han desarrollado tres estudios nutrigenómicos con compuestos polifenólicos del cacao y del café, con el objetivo de profundizar en el conocimiento de los mecanismos de acción por los que ejercen su actividad. Su dirección ha sido llevada a cabo por las Dras. Verónica Noé y Maria Izquierdo-Pulido. En el desarrollo del proyecto también ha colaborado el Dr. Carlos J. Ciudad como miembro del grupo de genómica nutricional formado por estos tres profesores de la Universidad de Barcelona.

1.INTRODUCCIÓN

1.1 Inicios de la genómica nutricional

La dieta es un proceso vital para todo mamífero, y como tal, tiene una gran repercusión en nuestro organismo. Jean Anthelme Brillant-Savarin fue quien dijo: *"Dis-moi ce que tu manges, je te dirai ce que tu es."* [Dime lo que comes y te diré quien eres], que posteriormente derivó en: *"You are what you eat"* [Eres lo que comes] (Brillat-Savarin, et al., 1800).

La investigación en el campo de la nutrición se inicia durante la revolución química gracias al químico Antoine Lavoisier y al médico François Magendie, pioneros en el estudio del impacto que tiene la alimentación en la salud (Ordovas and Mooser, 2004, van Ommen, et al., 2008). Las primeras observaciones describieron la capacidad que tenía el organismo de regularse ante un déficit alimenticio gracias al proceso de homeostasis. No obstante, si se producía un desequilibrio en la homeostasis, se iniciaba un proceso patológico (van Ommen, et al., 2008). La edad de oro o "Era de la Vitamina" (1912-1940) trae consigo nuevos avances, se enumeran y se describen todos los nutrientes, ampliando así los conocimientos de los requerimientos mínimos nutricionales o enfermedades relacionadas por la falta de éstos (Ordovas and Mooser, 2004).

A principios de 1941, dos investigadores, George Beadle y Earl Tatum, abrieron las puertas a la Genómica Nutricional, gracias al concepto de que un "gen codifica para una proteína". Mediante mutaciones aleatorias inducidas en un modelo eucariota haploide (*Neurospora crassal*) estos dos investigadores analizaron la supervivencia de estos organismos en medios de cultivo selectivos, carentes de algún elemento nutricional (no esenciales). Cuando la mutación afectaba a un gen del metabolismo, el enzima resultante de su traducción no era viable para catalizar correctamente su reacción dentro de la vía metabólica. Más adelante, A.E. Garrod, haciendo uso de los conocimientos de la herencia genética de Gregor Mendel (1865), así como de los mutantes generados por Beadle y Tatum, introdujo el concepto de errores congénitos del metabolismo como un defecto enzimático que resulta de un error puntual de alguna vía metabólica. Su modelo fue la alcaptonuria, un defecto en la enzima homogentisato-1,2-dioxigenasa que provoca la acumulación de ácido homogenístico en el organismo, que al ser eliminado confiere un aspecto negruzco a la orina debido a su oxidación por el aire. Las mutaciones genéticas fueron también utilizadas para estudios posteriores sobre cómo factores ambientales podían regular la expresión génica. François Jacob y Jacques Monod comenzaron a especular cómo algunos factores ambientales, como los nutrientes, podían estar interaccionando con el material genético para activar o silenciar la expresión de algunos genes. La presencia de lactosa en el medio de cultivo

de *E. coli* comporta la sobreexpresión de genes encargados de transportar la lactosa dentro de la célula y poder asimilarla para obtener energía. También la síntesis del aminoácido L-Triptófano se ve silenciada cuando el medio de cultivo es rico en este aminoácido. Las células tienen sistemas para regular el aporte de nutrientes dependiendo de las fuentes que encuentre en el ambiente (DeBusk, 2010).

Actualmente la ciencia se encuentra en la "era post-genoma", gracias al *Human Genome Project (HGP)*, que ha descifrado todo el genoma humano de aproximadamente 25.000 genes (HGP, Spielbauer and Stahl, 2005), y a los avances tecnológicos, que permiten los estudios de cribado de muchos genes con un alto rendimiento. Actualmente ya se han descrito más de 1.000 genes humanos causantes de enfermedades monogenéticas. Aún y así, la mayoría de enfermedades, las enfermedades crónicas, son de origen poligenético, originadas por complejas interacciones entre genes y factores ambientales (Kaput and Rodriguez, 2004). Puesto que los nutrientes de la dieta forman una parte importante de los factores ambientales capaces de interaccionar con los genes, la Genómica Nutricional se dedica al estudio de esta interacción con el objetivo de mejorar la salud pública.

1.2 Genómica Nutricional

La Genómica Nutricional pretende descifrar los mecanismos a través de los cuales la dieta o determinados compuestos de la misma pueden prevenir el riesgo a desarrollar determinadas enfermedades crónicas como obesidad, enfermedades cardiovasculares, diabetes o cáncer. Para llevar a cabo este propósito se emplean las tecnologías y conocimientos de la genómica funcional (combinación de la biología molecular y la genética) y de la nutrición, que ayudan a ampliar el conocimiento de la interrelación entre genes, productos de genes y hábitos dietéticos (Afman and Muller, 2006, Getz, et al., 2010).

El conocimiento de la genómica es clave en la Genómica Nutricional para poder entender como diferentes componentes de la dieta pueden (Kaput and Rodriguez, 2004, Trujillo, et al., 2006):

- Modular los procesos de iniciación, promoción, progresión y severidad de una enfermedades con fenotipo letal.
- Alterar la expresión génica mediante la interacción directa o indirecta con el genoma humano.
- Llegar a compensar o resaltar un determinado polimorfismo genético.

La Genómica Nutricional engloba dos especialidades, la Nutrigenómica y la Nutrigenética. A pesar de tener nombres muy parecidos, sus aproximaciones son diferentes. La figura I.1 representa la interacción gen-nutriente, y enmarca las dos especialidades de la genómica nutricional dependiendo del sentido en que se estudie la interacción. La rama de la **Nutrigenómica** se centra en el efecto que pueden tener determinados nutrientes sobre los genes analizando cambios en la transcripción y traducción de los genes afectados. Es un estudio prospectivo y enmarcado dentro de la investigación básica de la biología de sistemas.

La **Nutrigenética** busca y determina polimorfismos genéticos (SNPs) claves que diferencien la asimilación y utilización de nutrientes u otros componentes, de un individuo a otro. Su objetivo es poder establecer recomendaciones dietéticas individuales, por lo que es una investigación más aplicada y retrospectiva (Castle, et al., 2007, DeBusk, 2010, DeBusk, et al., 2005, Oleaga, C, et al., 2012, Ordovas and Mooser, 2004). Un ejemplo claro de la aproximación nutrigenética mejor descrita es el estudio del polimorfismo que afecta al gen que codifica para la 5,10-metilenotetrahidrofolato reductasa (MTHFR) (Schneider, et al., 1998), una flavoproteína que controla el paso de homocisteína a metionina. El polimorfismo que afecta al nucleótido 677 de este gen se traduce en una proteína termolábil (C677T) con una actividad reducida en comparación con la proteína salvaje. Los heterocigotos (CT) u homocigotos para la forma salvaje (CC) tendrán un funcionamiento normal del enzima. Los homocigotos para la forma termolábil (TT) tendrán un rendimiento inferior en el funcionamiento del enzima, pudiendo llegar a acumular homocisteína en sangre y presentar falta de metionina. Este desequilibrio puede traducirse en un riesgo de enfermedad vascular o un deterioro cognitivo prematuro. La correcta ingesta de folato y riboflavina ayuda a normalizar los niveles de homocisteína y mejorar la actividad MTHFR (Jacques, et al., 2002).

Ambas ramas englobadas en la Genómica Nutricional tienen el mismo objetivo final, llegar a formular recomendaciones dietéticas tanto a nivel colectivo o individual para mejorar la salud pública (Fenech, et al., 2011).

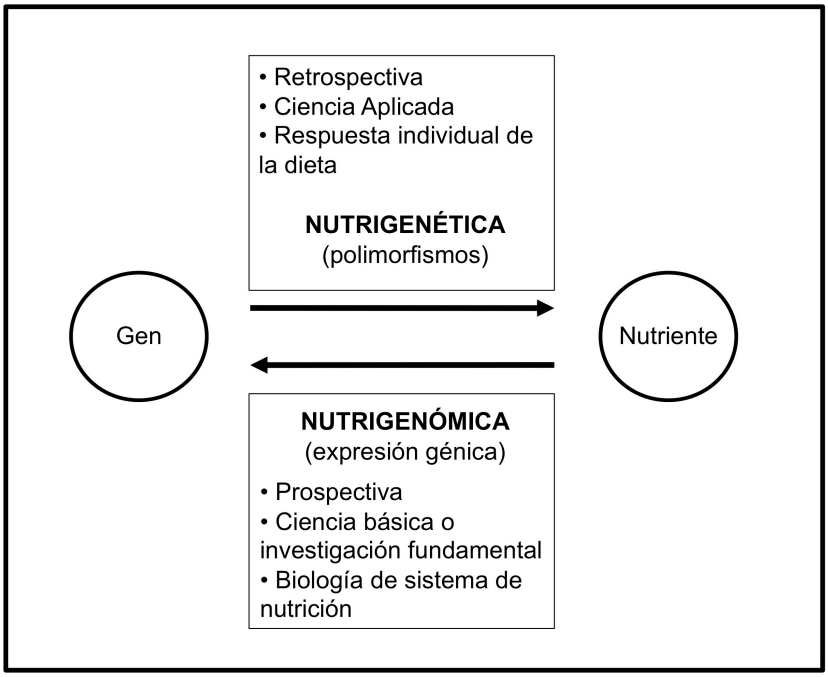

Figura I.1. La Genómica Nutricional y la interacción Nutriente(N)-Gen(G). La Genómica Nutricional estudia la interacción Nutriente-Gen en dos direcciones creando dos grandes áreas de investigación; (i) La Nutrigenómica (N→G) analiza como un determinado nutriente puede afectar la expresión génica. (ii) La Nutrigenética (G→N) estudia como un polimorfismo puede afectar a la asimilación de un nutriente. (Figura adaptada de Gillies, 2003 (Gillies, 2003)).

1.2.1 Nutrigenómica

El nombre de Nutrigenómica se originó hace diez años para describir una rama de la nutrición y de la ciencia de los alimentos que introducía técnicas de genómica y de biología molecular con el objetivo de definir los perfiles transcriptómicos, proteómicos y metabolómicos de las interacciones genoma-nutriente. Se trata de una ciencia todavía joven e incipiente, y será necesario más tiempo para dar los frutos esperados (Afman and Muller, 2006, Trujillo, et al., 2006, Wittwer, et al., 2011).

La Nutrigenómica considera como “moléculas señalizadoras de la dieta” a cualquier estilo de dieta, componentes importantes de ésta o los nutrientes contenidos que sean capaces de ser detectados por “sensores celulares”. Estos sensores serán los que provoquen o induzcan un cambio en el funcionamiento celular. Las competencias que llevan a cabo, por un lado la nutrición con el estudio de las moléculas señalizadoras, y por otro la biología molecular en el ámbito celular, convergen para el entendimiento de esta ciencia (Davis and Hord, 2005, Muller and Kersten, 2003). La figura I.2 es un esquema adaptado de Muller y Kersten, 2003 (Muller and Kersten, 2003) que representa, de manera visual, la

repercusión que tienen las “moléculas señalizadoras de la dieta” sobre los “sensores celulares”.

La combinación de la nutrición, la biología molecular y la genómica hace de la Nutrigenómica un campo de investigación multidisciplinar pudiendo determinar (Fenech, et al., 2011, Garcia-Canas, et al., 2010):

- La estabilidad del genoma: estudio de daño en el DNA o a nivel de cromosoma.
- Las alteraciones en la metilación del DNA y/o modificación de las histonas (conocido como nutriepigenómica)
- Los cambios en los niveles de mRNA y/o miRNA debido a regulaciones transcripcionales, procesamiento, estabilidad y/o transporte de éstos.
- Los cambios en los niveles de proteína, o fosforilación de las proteínas que afecten a vías de señalización, metabolismo o transporte.
- Las variaciones en los metabolitos, de forma individual o en conjunto, para desvelar qué comporta la interacción nutriente-gen

Los estudios en Nutrigenómica pueden desarrollarse haciendo uso de las herramientas genómicas, mediante dos aproximaciones que dependen del conocimiento molecular previo. (i) La **primera aproximación** se aplica cuando no se conocen los sensores celulares que pueden ser afectados por una/as molécula/as señalizadora/as de la dieta. Se le denomina análisis masivo o de amplio alcance porque detecta todos los cambios que puede estar sufriendo el genoma (o un conjunto de genes pertenecientes a una misma categoría) ante la interacción con ese nutriente. Estos análisis se efectúan gracias a las tecnologías de “*high throughput*” o de alto rendimiento (Garcia-Canas, et al., 2010) capaces de generar gran cantidad de datos que posteriormente serán procesados y analizados por potentes herramientas que facilitan la interpretación de los resultados y almacenan la información resultante. (ii) La **segunda aproximación** se emplea una vez se conocen los sensores celulares (dianas) afectados por la/as molécula/as señalizadora/as de la dieta. Se profundiza el estudio de una diana, el mecanismo de acción por el que está siendo alterada, o su selección como molécula biomarcadora del estado de la homeostasis. Las técnicas experimentales aplicadas en esta segunda aproximación serán técnicas que se concentren en una (o unas pocas) diana de estudio (Afacan, et al., 2012, Fenech, et al., 2011, Kaput and Rodriguez, 2004).

Las investigaciones realizadas en esta tesis se enmarcan en el campo de la Nutrigenómica y dependiendo del estudio realizado se han llevado a cabo

aproximaciones de amplio espectro, como son los análisis de transcriptómica por medio de plataformas de PCR Arrays (analiza un número amplio de genes relacionados dentro de una misma temática) o bien de Microarrays (analiza cambios en todo el genoma humano) para poder encontrar los "sensores celulares" afectados por un nutriente, así como estudios más detallados de los "sensores celulares" hallados en las aproximaciones previas u otros ya conocidos en la literatura.

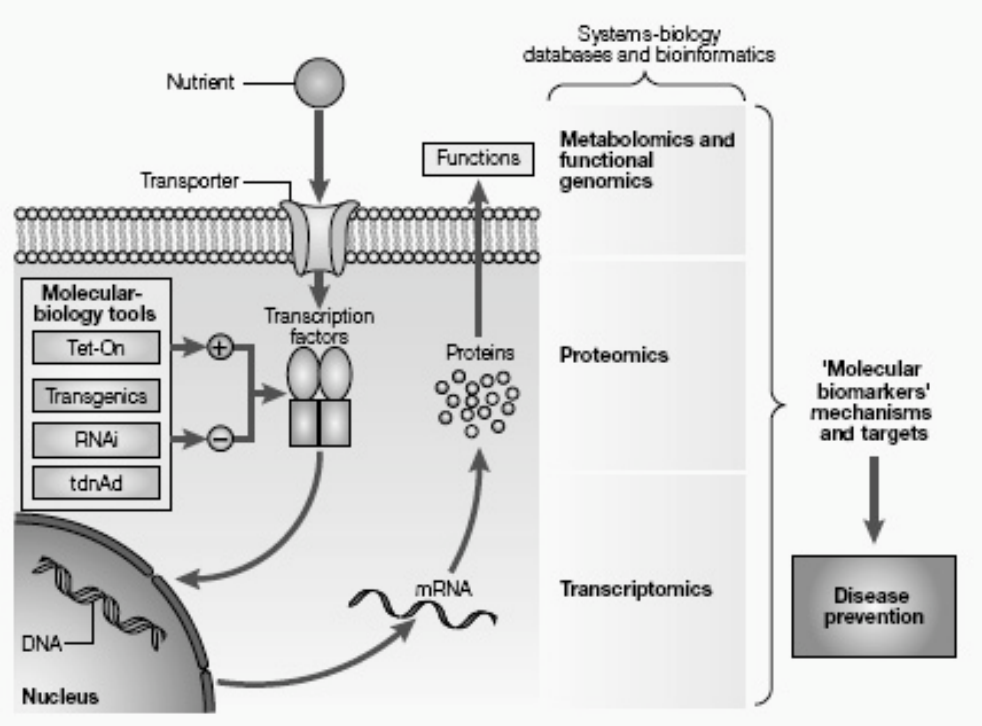

Figura I.2. Esquema de la repercusión de las "moléculas señalizadoras de la dieta" sobre los "sensores celulares". La Nutrigenómica se dedica al estudio de los cambios inducidos por componentes de la dieta a nivel de expresión, proteína y metabolitos celulares. (Figura adaptada de Muller y Kersten, 2003 (Muller and Kersten, 2003)).

1.3 Compuestos Bioactivos

Las rutas metabólicas y biosintéticas de las plantas pueden originar, a parte de compuestos vitales para su desarrollo, otros metabolitos secundarios con funciones no vitales pero también importantes. Los metabolitos secundarios capaces de ejercer efectos farmacológicos o toxicológicos en humanos y animales son los que se denominan, desde del punto de vista de la alimentación, **compuestos bioactivos** (Bernhoft, 2010). Desde los años noventa, se están realizando multitud de estudios cuyo objetivo es conocer y establecer los efectos beneficiosos a nivel fisiológico y celular de estos compuestos que forman parte, de manera natural, de nuestra dieta.

Estos compuestos presentes en la dieta no se consideran esenciales para la vida por lo que no se pueden catalogar como nutrientes (Kris-Etherton, et al., 2004).

Se han descrito hasta la fecha un gran número de compuestos bioactivos, con efectos potencialmente beneficiosos para la salud y su número va en aumento. Actualmente, los compuestos bioactivos se agrupan en doce familias en función de sus estructuras químicas. En la Tabla I.1 se recogen las doce familias así como las principales fuentes vegetales que los contienen.

Familias de compuestos bioactivos	Fuentes de la dieta
Carotenoides	Frutas y verduras verdes, naranjas, rojas y amarillas
Flavonoides y proantocianidinas (polímeros de flavonoides)	Frutas y verduras, productos de la soja, té y cacao
Glucosinolatos e isotiocinatos	Verduras de la familia de las crucíferas como el brócoli o el berro
Lignanos	Centeno, semilla de lino y el aceite de la semilla de lino
Alcoholes monofenólicos	Aceite de oliva y vino
Monoterpenos	Aceites esenciales de cítricos, cerezas, menta y otras hierbas
Compuestos organosulfurados	Ajo, cebolla y puerro
Ácidos fenólicos	Cereales, café, frutas y verduras
Esteroles vegetales	Aceites de arroz, soja y pino (tall oil)
Saponinas	Productos de soja
Estilbenos	Uvas, vino tinto y cacahuetes
Taninos hidrolizables	Frutas y verduras

Tabla I.1. Familias de compuestos bioactivos según su estructura química y las principales fuentes de la alimentación que los contienen (Tabla adaptada de Kris-Etherton y col., 2004 (Kris-Etherton, et al., 2004)).

Los efectos de estos compuestos en el organismo son diversos, como por ejemplo, aportar actividad antioxidante, inhibir o inducir una actividad enzimática, afectar la expresión génica y/o alterar vías de señalización celular. Para cumplir con la condición de compuestos bioactivos, además de ejercer estas actividades, deben proporcionar un efecto beneficioso para la salud. A diferencia de los nutrientes que suelen tener funciones específicas, los compuestos bioactivos pueden ejercer varias funciones simultáneamente, como por ejemplo ejercer como antioxidantes y generar diferencias en la expresión de un gen.

1.4 Polifenoles

Los polifenoles son el grupo más extenso de compuestos estudiados de origen natural. Se caracterizan por presentar grupos fenólicos en su estructura química básica y por ser potentes agentes reductores (Scalbert and Williamson, 2000). Los polifenoles están presentes en la dieta y numerosos estudios avalan su carácter antioxidante *in vitro* equilibrando el balance de electrones. De esta manera previenen el estrés oxidativo que generan las especies reactivas de oxígeno (ROS) capaces de provocar daños significativos en las estructuras celulares (Tsao, 2010). Esta capacidad antioxidante ayuda a evitar el daño oxidativo que se produce en el DNA, en las proteínas y en los lípidos a lo largo de la vida y que con el tiempo acaban desencadenando enfermedades degenerativas (Scalbert, et al., 2005).

Las propiedades biológicas de los polifenoles dependerán de su biodisponibilidad en el organismo. Son diversos los estudios que han demostrado la absorción de polifenoles a través de la medida del aumento de la actividad en plasma. También se han determinado concentraciones de compuestos polifenólicos, o metabolitos de éstos, en plasma y en orina. La estructura química de estos compuestos será la que determine el *ratio* de absorción o eliminación por heces. La absorción puede darse a nivel del intestino delgado, cuando son monómeros, o a través del intestino grueso, cuando los compuestos están glicosilados, acetilados, en forma de ésteres o bien formando polímeros. A nivel intestinal se pueden dar procesos de digestión enzimática, de hidrólisis y de conjugación (por enzimas de los mismos alimentos, de los enterocitos o de la microflora) que favorezcan la absorción. Además de su metabolización a nivel intestinal, los polifenoles pueden sufrir metabolización por conjugación de grupos metilo (catecol-O-metil transferasa), sulfato (fenol sulfotransferasa) o glucurónidos (UDP glucuronosil transferasa) a nivel hepático (Scalbert and Williamson, 2000).

Una vez absorbidos, estos compuestos y sus metabolitos pueden ejercer funciones en los tejidos diana antes de ser eliminados por orina o bilis. La figura I.3 es un esquema de las diferentes rutas por las que pueden distribuirse los polifenoles una vez ingeridos. La eliminación marcará la semivida del compuesto que puede variar entre 1 y 24 h, dependiendo de la cantidad ingerida, del grado de metabolización y de la afinidad por proteínas del torrente sanguíneo como la albúmina.

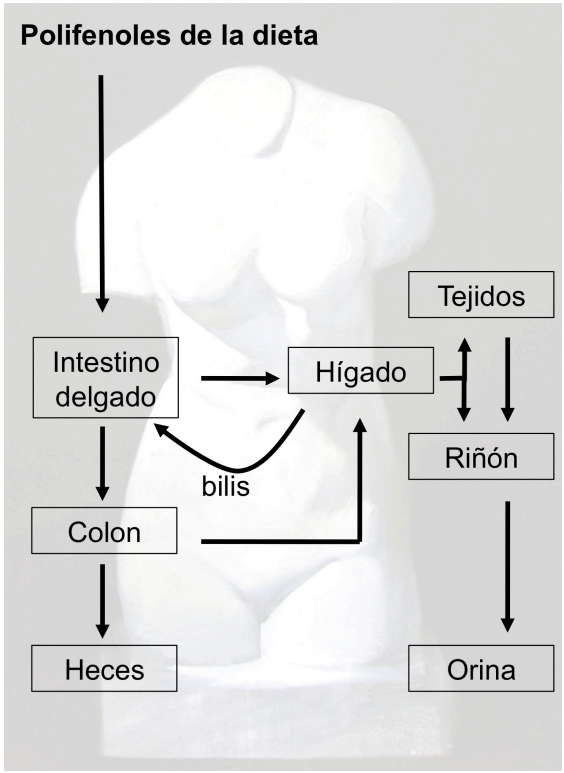

Figura I.3. Posibles rutas para la absorción y eliminación de los polifenoles de la dieta (Figura adaptada de Scalbert y Williamson, 2000 (Scalbert and Williamson, 2000)).

Diferentes estudios han demostrado efectos beneficiosos de los polifenoles en patologías como el cáncer, enfermedades neurodegenerativas, enfermedades cardiovasculares, el síndrome metabólico, la diabetes, y ciertas patologías relacionadas con inflamación (Asensi, et al., 2011). También se han descrito efectos beneficiosos sobre el proceso del envejecimiento. Sin embargo, el poder reductor de los polifenoles demostrado *in vitro* no es suficiente para explicar todos estos efectos. Por ello se están realizando estudios *in vivo* para analizar, con mayor detalle, el mecanismo por el cual estas moléculas ejercen un efecto beneficioso a nivel celular en los tejidos diana.

1.4.1. Cacao

Las semillas de *Theobroma cacao* L. son una fuente rica de polifenoles, constituyendo el 10% de su peso seco. Los derivados del cacao, en particular el cacao y el chocolate negro, son unas de las principales fuentes de antioxidantes de la dieta (Rusconi and Conti, 2010). Los efectos beneficiosos del cacao ya eran conocidos por las culturas precolombinas que consideraban el cacao como la

bebida de los dioses (Corti, et al., 2009). Waterhouse y col. (Waterhouse, et al., 1996) fueron los primeros en sugerir el gran aporte de antioxidantes que representa el chocolate en la dieta. Posteriores estudios han destacado las propiedades principales del cacao y sus derivados por ser antioxidantes, protectores cardiovasculares y antitumorales en humanos (Rusconi and Conti, 2010).

Los polifenoles presentes en las semillas de cacao se agrupan principalmente en flavan-3-oles y procianidinas. El grupo de los flavan-3-oles lo forman los monómeros catequina y epicatequina, siendo (-)-epicatequina el isómero más abundante. Las procianidinas las forman polímeros de catequina y epicatequina (Noe, et al., 2004). Otros flavonoides presentes en el cacao son la quercetina (flavanol) y la cianidina (antocianina). Además también se han detectado ácidos fenólicos como cafeico, clorogénico, cumárico y telúrico (Rusconi and Conti, 2010).

El contenido de polifenoles de las semillas del cacao puede llegar a disminuir en un 90% en el producto final después de los procesos de elaboración (Rusconi and Conti, 2010) y su biodisponibilidad en el organismo variará según el tipo de polifenoles presentes. El monómero epicatequina se absorbe rápidamente (Rein, et al., 2000) y su presencia en sangre es dosis dependiente, de la misma manera se han detectado en sangre procianidinas (dímeros y trímeros) de catequina y epicatequina (Holt, et al., 2002).

La verificación de los efectos observados a nivel de tejido específico se ha demostrado mediante la detección de polifenoles en diferentes tejidos en ratón y rata por medio de ensayos de marcaje de radioactividad con ^{14}C o ^{3}H o por análisis de HPLC. Se han detectado trazas de polifenoles en cerebro, células endoteliales, corazón, riñón, hígado, bazo, páncreas, próstata, útero, ovario, glándula mamaria, testículos, vejiga, huesos y piel (Abd El Mohsen, et al., 2002, Chang, et al., 2000, Datla, et al., 2001, Kim, et al., 2000, Suganuma, et al., 1998, Ueno, et al., 1983).

El estudio de los polifenoles del cacao ha ido confirmando progresivamente una acción beneficiosa en la prevención de enfermedades crónicas, hasta ahora atribuible, principalmente, a su poder antioxidante ya que bloquearía los efectos nocivos de las especies radicales de oxígeno. Algunas de las enfermedades crónicas donde el consumo de cacao ha demostrado ejercer un papel protector son:

I. Neoplasias: El cáncer engloba un número elevado de enfermedades originadas por diferentes factores etiológicos, que pueden asociarse al estilo de

vida (consumo de tabaco, alcohol, determinados hábitos alimentarios, polución, etc.) pudiendo llegan a comportarse como agentes genotóxicos porque son capaces de iniciar, promover o acelerar procesos que modifican el DNA. El poder antioxidante de los compuestos polifenólicos del cacao, así como el de otros alimentos ricos en polifenoles, ejerce un papel protector en procesos de neoplasias gracias a que reducen el efecto de las especies reactivas de oxígeno, tal como se ha observado en estudios epidemiológicos e *in vitro* (Tarka, et al., 1991, Weisburger, 2001). Además de la actividad antioxidante, los polifenoles ejercen actividades quimiopreventivas que afectan diferentes mecanismos moleculares como, por ejemplo, el crecimiento celular, la regulación de proteínas quinasas, la secreción de MMP, la invasión de células tumorales, la adhesión y propagación celular, la apoptosis y la angiogénesis (Kandaswami, et al., 2005).

Son varios los grupos de investigación que han asociado diferentes mecanismos de acción inducidos por el tratamiento con polifenoles de cacao con sus propiedades quimiopreventivas. Algunos ejemplos de mecanismos de acción se citan a continuación:

- Bloqueo de la iniciación y proliferación tumoral a través de la inhibición de la actividad de la MAP quinasa MEKK (Kang, et al., 2008).
- Bloqueo de la sobreexpresión de VEGF inducida por TNF-α a través de la inhibición de la actividad de PI3K y MEK1 (Kim, et al., 2010).
- Bloqueo de la proliferación de células malignas inducidas por K-ras a través de la disminución de la fosforilación de Akt, la actividad transcripcional de NF-κβ y la unión de GTP a Ras. Inhibición del crecimiento de xenógrafos originados por células tumorales inducidas por K-ras (Siddique, et al., 2011).
- Inducción de la apoptosis a través del co-tratamiento con el fármaco tamoxifeno (Oleaga, C., et al., 2012).

Otras dianas donde los polifenoles de cacao ejercen su capacidad antineoplásica están representadas en la Figura I.4.

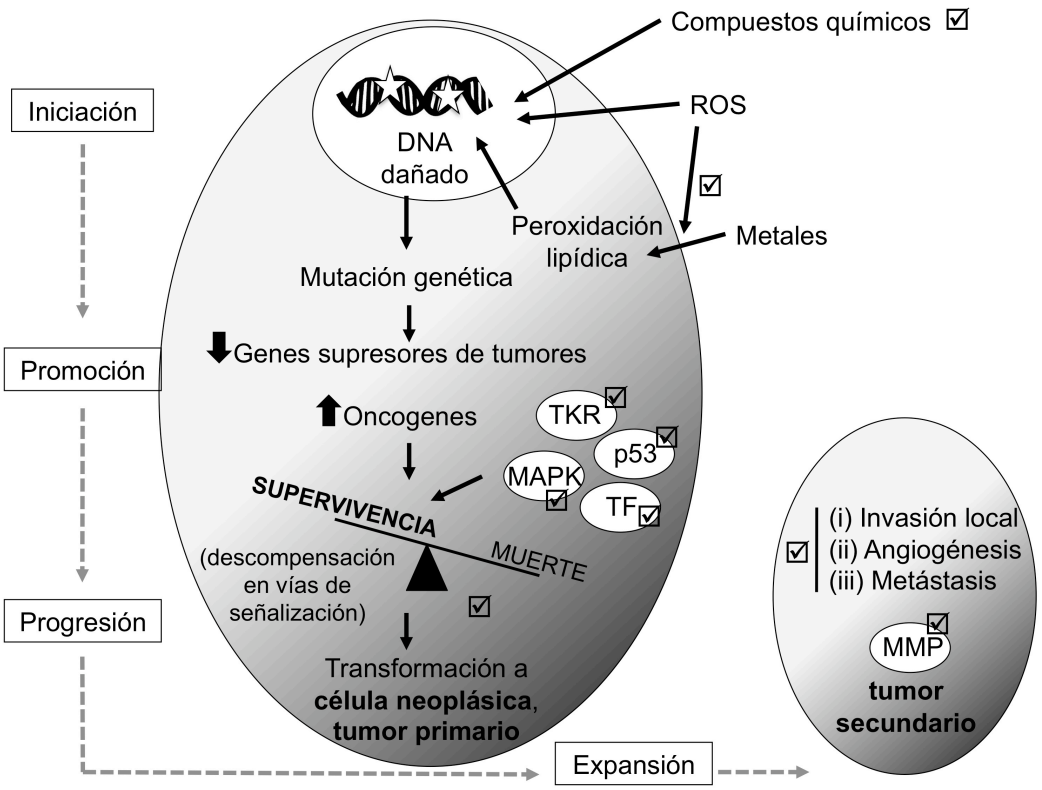

Figura I.4. Esquema de los procesos de carcinogénesis y diseminación celular dónde se ha demostrado la intervención de los polifenoles del cacao (☑). (Figura adaptada de Pérez-Cano y col. 2010 (Pérez-Cano, et al., 2010)).

II. Procesos cardiovasculares: Los primeros indicios que demostraban que el cacao podía prevenir enfermedades cardiovasculares proceden del estudio de la población nativa de los Kuna en la costa de Panamá. Se relacionó un elevado consumo de cacao con niveles de presión arterial más reducidos, con respecto a las otras poblaciones de la zona, dado a que tenían una menor incidencia de mortalidad derivada de problemas cardiovasculares (Corti, et al., 2009). De entre los diferentes efectos beneficiosos que ejercen los polifenoles del cacao a nivel cardiovascular destacan;

(i) la protección del endotelio vascular, elevando los niveles de NOS (*Nitrogen oxigen species*) en sangre e incrementando la respuesta vasodilatadora (FMD; *Flow mediated dilation*) (Heiss, et al., 2006, Schroeter, et al., 2006, Sudano, et al., 2006),

(ii) la actividad antioxidante (Mathur, et al., 2002, Spencer, et al., 2001),

(iii) la reducción de la agregación plaquetaria (Murphy, et al., 2003, Rein, et al., 2000),

(iv) un efecto antihipertensivo y

(v) la prevención de procesos aterogénicos (Corti, et al., 2009) a través de la modulación de los niveles de estados oxidativos, de la respuesta inmunitaria a nivel vascular (Steinberg, et al., 2003) y de las lipoproteínas en plasma (Corti, et al., 2009, Schewe, et al., 2008).

1.4.2 Café

El café es una de las bebidas más consumida en todo el mundo, aproximadamente se consumen anualmente 500 billones de tazas de café (Butt and Sultan, 2011). La planta del café se cultivó por primera vez en una región de Kaffa en Etiopía y, posteriormente, fue introducida en Yemen, Arabia y Egipto. Hoy en día está presente en casi todos los hogares y se consume a diario. Las dos especies más consumidas de café son *Coffea robusta* y *Coffea arabica*. Para el consumo, los granos de café pasan por un proceso de tueste a diferentes temperaturas dependiendo del aroma que se quiera conseguir (Wang and Ho, 2009).

Los ácidos hidroxicinámicos conforman la mayor clase de polifenoles presentes en casi todas las plantas (Scalbert, et al., 2005) y su principal representante es el ácido cafeico (CA), que suele estar esterificado con el ácido quínico formando el ácido clorogénico. La principal fuente de ácido clorogénico en la dieta es precisamente el café. Los individuos que consumen café a diario pueden ingerir entre 0,5 y 1 g/día, mientras que los no consumidores normalmente no superan los 100 mg/día. La biodisponibilidad de estos polifenoles es casi total en el caso del ácido cafeico, que se absorbe en un 95% a nivel intestinal, mientras que el ácido clorogénico se absorbe en un 33% (Olthof, et al., 2001). Aproximadamente dos tercios del ácido clorogénico ingerido llega al intestino y es metabolizado a CA (Olthof, et al., 2003). Los datos referentes a la biodisponibilidad sugieren que los efectos biológicos del ácido clorogénico son los que pueda ejercer una vez metabolizado a CA, por esta razón deberían potenciarse los estudios con este ácido.

Ambos ácidos fenólicos tienen propiedades antioxidantes *in vitro* (Rice-Evans, et al., 1996) e inhiben la formación de compuestos N-nitroso mutagénicos y carcinogénicos ya que pueden detener la reacción de N-nitrosación *in vivo* (Kono, et al., 1995). El ácido clorogénico puede inhibir el daño en el DNA *in vivo* (Shibata, et al., 1999) al evitar la peroxidación lipídica, inductora de la formación de aductos en el DNA (Kasai, et al., 2000). Este compuesto fenólico también inhibe la

activación de los mediadores de especies reactivas de oxígeno AP-1, NF-κβ y MAPK y activa enzimas antioxidantes endógenas (Feng, et al., 2005).

Se ha señalado que los polifenoles del café podrían proteger frente al daño en el DNA, enfermedades cardiovasculares, Diabetes mellitus, Parkinson o Alzheimer (Conney, et al., 2007). De la misma manera que el cacao, los polifenoles del café parecen estar ejerciendo efectos beneficiosos a través de mecanismos moleculares donde intervenga una interacción molécula-diana, además de por su actividad antioxidante.

Los beneficios del consumo de café han sido descritos en varios meta-análisis, donde se ha establecido una relación entre su consumo y un menor riesgo a desarrollar diversos tipos de cáncer como colorrectal, de hígado, de riñón, de ovario, de páncreas, de esófago, de endometrio y de faringe (Butt and Sultan, 2011). Naganuma y col. señalaron una asociación inversa significativa entre el consumo de café y el riesgo a desarrollar cáncer colorrectal (Naganuma, et al., 2007). Otros investigadores (George, et al., 2008, Huber, et al., 2008, Je, et al., 2009, Oba, et al., 2006, Tao, et al., 2008) también sugieren una función como agentes quimiopreventivos de los compuestos fenólicos del café, si bien son necesarios más estudios para establecer sus mecanismos de acción.

Hoy en día, el objetivo de muchos estudios es ampliar el conocimiento de los efectos de estos polifenoles para aprovechar su posible potencial como compuestos beneficiosos y, de esta manera, poder desarrollar estrategias para mejorar un estado de la homeostasis en desequilibrio, o simplemente preservar el estado de equilibrio, o de salud en el individuo.

La búsqueda de perturbaciones entre células normales y neoplásicas, a nivel genómico, además de desvelar nuevos biomarcadores moleculares, también servirá para elaborar estrategias de intervención dietéticas que reduzcan el riesgo a desarrollar un proceso tumoral, o controlar el proceso de una neoplasia ya desarrollada (Milner, 2003).

2.OBJETIVOS

La tesis plantea los siguientes objetivos que se han desarrollado utilizando una aproximación nutrigenómica:

2.1 Estudiar la posible implicación de compuestos polifenólicos de cacao en la prevención y el tratamiento del cáncer de mama.

2.1.1 Determinar el efecto de un extracto polifenólico de cacao (PCE) en dos modelos de cáncer de mama mediante un análisis genómico funcional.

2.1.2 Validar las principales dianas afectadas y profundizar en su regulación transcripcional y traduccional influida por el tratamiento de PCE.

2.1.3 Desarrollar un modelo de terapia combinada con PCE para el tratamiento de cáncer de mama.

2.2 Estudiar la posible modulación de la Apolipoproteína AI (ApoAI) mediada por metabolitos de cacao en un modelo hepático.

2.2.1 Analizar el efecto de la epicatequina (EPI) sobre la expresión y la regulación transcripcional de ApoAI en células hepáticas humanas.

2.2.2 Comparar el efecto del tratamiento con EPI al de sus metabolitos generados en el organismo humano.

2.2.3 Estudiar la implicación de los metabolitos del cacao en la regulación de ApoAI.

2.3 Estudiar el efecto del café soluble cafeinado (ICC) y del ácido cafeico (CA) en células de cáncer de colon y mama.

2.3.1 Analizar los genes diferencialmente expresados en respuesta al ICC y al CA, mediante un estudio genómico funcional.

2.3.2 Generar una red de asociación biológica (BAN) a partir del perfil de expresión común a ambos tratamientos para establecer los genes “nodo”.

2.3.3 Realizar la validación de los genes “nodo” y profundizar en el estudio de las vías de señalización donde intervengan los genes “nodo” modulados por el tratamiento con los compuestos fenólicos de café estudiados.

3.MATERIALES Y MÉTODOS

La metodología utilizada en los diferentes estudios que componen esta Tesis, están referenciadas en los artículos incluidos en el apartado de Resultados de la memoria. No obstante, la descripción de los métodos más innovadores, que se han puesto a punto en nuestro grupo de investigación para este proyecto, se incluye a continuación. Asimismo, se especifican con más detalle los materiales utilizados.

3.1 MATERIALES

3.1.1 Líneas celulares

Las líneas celulares utilizadas en los diferentes ensayos han sido las que se detallan en la Tabla M&M.1:

HEK-293T	*Human Embyonic Kidney 293 cells (immortalized with adenovirus 5 DNA and SV40 Large T-antigen)*. ATCC; CRL-1573
HeLa	*Human Cervix Adenocarcinoma Cell Line*. ATCC; CCL-2
HepG2	*Human Hepatocellular Carcinoma Cell Line* (IGF II). ATCC; HB-8065
HT29	*Human Colorectal Adenocarcinoma Cell Line*. ATCC; HTB-38
MCF-7	*Human Breast Adenocarcinoma Cell Line (positive for ERα).* ATCC; HTB-22
SKBR3	*Human Breast Adenocarcinoma Cell Line (positive for Her2/c-erb-2)*. ATCC; HTB-30

Tabla M&M.1. Líneas celulares humanas utilizadas (columna izquierda). Número de catálogo según ATCC y origen del linaje correspondientes a cada línea celular (columna derecha) (ATCC, 2012).

Todas las líneas celulares han sido mantenidas en medio Ham's F12 (Ref 21700-018, Gibco) suplementado con 14mM de bicarbonato sódico (AppliChem), 100U/mL de penicilina G sódica (Sigma), 100mg/L estreptomicina (Sigma) y un 7% (V/V) de suero bovino fetal (FBS, Gibco), en condiciones de 37ºC de temperatura, atmósfera de 5% de CO_2 y humedad controlada. La expansión del cultivo al llegar a confluencia se realizaba mediante tripsinización con 0,05% Tripsina y 0,02% EDTA (Sigma) en PBS 1X (154mM NaCl, 3,88mM H_2NaPO_4, 6,1mM $HNaPO_4$, pH 7,4) para desprender las células adheridas. La preparación y esterilización mecánica por filtro de 0,2µM (diámetro poro) (Schleicher & Schuell) de ambas soluciones se realizaba en el propio laboratorio.

3.1.2 Compuestos bioactivos

El origen de los compuestos polifenólicos utilizados en los diferentes experimentos difiere según el método de obtención y purificación. Se puede utilizar un extracto, obtenido de un alimento (en nuestro caso cacao o café) que contendrá un concentrado heterogéneo de moléculas con características químicas semejantes. Existen técnicas muy diversas de extracción de compuestos polifenólicos que evolucionan con el tiempo aumentando positivamente en el rendimiento y pureza del extracto (Ajila, et al., 2011). Sin embargo, a pesar de que un análisis pueda desvelar las propiedades de un extracto de un alimento bioactivo, no será lo suficientemente preciso como para asignar esas propiedades a una molécula en concreto. Por esta razón, otra opción es trabajar con moléculas sintetizadas ya descritas como componentes del alimento a estudio, sin olvidar que esta segunda opción no sería posible sin la primera.

Finalmente, para mayor reproducibilidad de la interacción molécula-diana, existe una tercera opción que será el uso de los metabolitos resultantes del proceso metabólico en el organismo. Estas serán las moléculas que finalmente interaccionen con la diana molecular. En los diferentes ensayos desarrollados en esta tesis, se han utilizado distintas aproximaciones que se detallan a continuación.

3.1.2.1 Extracto polifenólico de cacao

Se utilizó un extracto polifenólico de cacao (*Polyphenolic cocoa extract, PCE*), obtenido a partir de una extracción fenólica de 10 g de cacao natural Forastero de Malasia en polvo (Andrés-Lacueva, 2000). Se determinó su contenido fenólico expresado en mg/mL de equivalentes de catequina mediante el procedimiento de Folin-Ciocalteu (Swain, 1969) y también la concentración de los diferentes compuestos fenólicos presentes en el extracto mediante cromatografía líquida de alta precisión (Tabla M&M.2). La solución de trabajo para incubar los cultivos celulares se preparó en un 20% de DMSO para mejorar su solubilidad, siendo el porcentaje de DMSO en el medio celular siempre inferior al 0,4% (V/V).

PCE (compuestos)	mg/mL	µg totales en dosis de 250 ng/µL PCE[1]
(-)-epicatequina	7,20	81,36
(+)-catequina	2,50	28,25
Procianidina B2	1,90	21,47
Isoquercetina	0,10	1,13
Quercetina-3-arabinósido	0,03	0,34
Quercetina	0,02	0,23

Tabla M&M.2. Concentración de los diferentes flavonoides presentes en el PCE. [1]Cantidad de cada componente en la concentración experimental usada en los cultivos celulares.

3.1.2.2 Metabolitos de cacao

Los metabolitos utilizados (Tabla M&M.3) son algunos de los generados en el organismo, a través de la metabolización fase I y II, tras el consumo de cacao soluble (Monagas, et al., 2010, Okushio, et al., 1999, Urpi-Sarda, et al., 2009). Las moléculas seleccionadas fueron disueltas en DMSO a la concentración de 100mM.

Símbolo	Nombre del metabolito
EPI-S	*Epicatechin sulphate* (Epicatequina sulfato)
EPI-3-S	*Epicatechin-3-sulphate* (Epicatequina-3-sulfato)
3-M-EPI	*3'-O-methylepicatechin* (3'-O-metil-epicatequina)
4-M-EPI	*4'-O-methylepicatechin* (4'-O-metilepicatequina)
3-HFA	*3-hydroxyphenylacetic acid* (Ácido 3-hidroxifenilacético)
3,4-HFA	*3,4-hydroxyphenylacetic acid* (Ácido 3,4-hidroxifenilacético)
VA	*Vanillic acid* (Ácido vanílico)

Tabla M&M.3. Metabolitos de cacao. La tabla describe los nombres de los metabolitos utilizados y sus abreviaciones.

3.1.2.3 Café Soluble Cafeinado y Ácido Cafeico

Para el proyecto del café se utilizaron tanto una infusión de café, que contenía una mezcla heterogénea de compuestos polifenólicos, como también una molécula química específica que está contenida en la propia infusión de café. Los efectos observados por ambos tratamientos han ayudado para diferenciar las

propiedades de la mezcla, debidas a la suma de todos sus compuestos, de las de la molécula química *per se*.

Se utilizó un café soluble comercial (*Instant Caffeinated Coffee, ICC*) que contenía una relación en ácidos fenólicos de 12,8 mg de ácido cafeico y 220 mg de ácido clorogénico por cada 100 g de ICC (el análisis se realizó por cromatografía líquida micelar). El ICC se diluyó en agua mQ y posteriormente fue esterilizado por filtración. El ácido cafeico (*Caffeic Acid, CA*) (Sigma) se diluyó en DMSO. Las concentraciones experimentales empleadas fueron de 7 µg/mL para el ICC y de 1,68 µg/mL para el CA, se tuvo en cuenta el consumo de una taza de café y su distribución en el contenido de agua del organismo (75%) (Beyer and Melzig, 2003).

3.1.3 Vectores plasmídicos

Los vectores portadores del gen *LUC* (Promega) permiten el estudio de secuencias reguladoras de la transcripción eucariota. En concreto, se determinan los niveles de actividad luciferasa, enzima oxidativa, de la luciérnaga *Photinus pyralis*.

Los vectores que se han utilizado son:

- pGL3-Basic (Promega): vector que carece de promotor o de secuencia potenciadora (*enhancer*) y que permite el estudio de diferentes promotores situándolos por delante del inicio de traducción del gen luciferasa. Este vector presenta resistencia a ampicilina.
- CYP1A1.LUC (pGudLuc 6.1): construcción generada a partir de la subclonación del fragmento HindIII de 1810 bp procedente del plásmido pGudLuc1.1 (Garrison, et al., 1996) en el vector pGL3-Basic. El fragmento de 1810 pb está formado por un fragmento de 1330 bp del *mouse mammary tumor virus (MMTV) long-terminal repeat* y su secuencia promotora que responde a glucocorticoides ha sido sustituida por un fragmento de 482 pb de la región promotora del gen citocromo P4501A1 (CYP1A1) de ratón. El fragmento correspondiente al promotor de CYP1A1 contiene cuatro cajas XRE que responden a TCDD (Rushing and Denison, 2002). Este vector reportero ha sido cedido generosamente por el Dr. M. Deninson.
- Vector de expresión de ERα (pSG5-ERalpha plasmid): construcción generada a partir de la clonación de un cDNA (λOR8) obtenido por hibridación de sondas que codifica para una proteína de 65KDa

correspondiente a un RNA de 2Kb aproximadamente (Walter, et al., 1985). Este vector ha sido cedido amablemente por el Dr. P. Chambon.

- B.LUC (-169/-146B.LUC): construcción generada a partir del vector pGL3-Basic digerido con Xho I y Mlu I y la posterior inserción unidireccional del fragmento Site B (-169/-146) de la secuencia potenciadora del promotor del gen *ApoAI*.

3.2 MÉTODOS

3.2.1 Plataformas de análisis genómico funcional

La transcriptómica, como análisis genómico funcional, determina y compara los niveles de RNA mensajero (mRNA) de genes específicos entre dos condiciones o muestras biológicas. El estudio del transcriptoma es muy utilizado actualmente y, a pesar de que no se trabaja con el producto final del gen, la información de la transcripción suministra datos sobre su regulación y una predicción de su funcionalidad.

En esta Tesis, se ha empleado esta tecnología tanto para análisis puntuales por técnicas de PCR a tiempo real como para estudios a mayor escala, englobando un número mayor de genes en un mismo análisis. Las plataformas utilizadas para los análisis a gran escala han sido:

3.3.1.1 PCR Arrays

La tecnología de PCR Arrays es una plataforma que permite el análisis genómico funcional de un conjunto amplio de genes, agrupados en una misma categoría biológica, en una sola reacción de PCR haciendo uso de la tecnología de PCR a tiempo real y un sistema operativo de *Excel* para el análisis de datos.

Para el cribado de los genes diferencialmente expresados por el extracto polifenólico de cacao (PCE) en células de cáncer de mama, se utilizó la plataforma PCR Array de *Stress & Toxicity PathwayFinder™, RT2Profiler™* de SuperArray. Esta plataforma consiste en una placa de 96 pocillos con cebadores para amplificar genes relacionados con los procesos de estrés y toxicidad. De los 96 genes, 84 corresponden a genes relevantes en la categoría biológica, cinco a genes *housekeeping* (endógenos) y dos son controles negativos. La figura M&M.1 muestra una representación de la placa de 96 pocillos de PCR Array de *Stress & Toxicity PathwayFinder™*. Cada celda representa un pocillo de la placa que corresponderá a un gen de estudio específico.

El proceso experimental consiste en la extracción del RNA total de las células control y de las tratadas, seguida de una posterior reacción de la transcriptasa inversa para obtener los DNAs complementarios (cDNAs). Los cDNAs de una muestra se usan como producto de partida para el análisis de los 84 genes en la reacción en cadena de la polimerasa a tiempo real (*PCR-Real time*) que se lleva a cabo en un equipo ABI Prism 7000 Sequence Detection System de Applied

Biosystems. La cuantificación de la síntesis de cadenas de DNA se determina a lo largo del tiempo mediante el aumento de la señal de fluorescencia del fluorocromo SYBR Green que va intercalándose en las nuevas cadenas de DNA.

La evaluación del nivel de expresión (cantidad de mRNA) se calcula usando el método estándar de $\Delta\Delta C_T$:

$$\Delta Ct \text{ (control)} = Ct \text{ del gen especifico} - Ct \text{ del gen control}$$

$$\Delta Ct \text{ (tratamiento)} = Ct \text{ del gen especifico} - Ct \text{ del gen control}$$

$$\Delta\Delta Ct = \Delta Ct \text{ (tratamiento)} - \Delta Ct \text{ (control)}$$

$$\text{Fold-change} = 2^{-\Delta\Delta^{Ct}}$$

Este cálculo da un valor de *fold change* respecto al control (veces de cambio) que se obtiene después de normalizar los valores (con los genes endógenos).

El cribado se hará seleccionando los genes que se diferencien, tantas veces en el valor de *fold change* respecto al control. La exigencia del análisis dependerá del punto de corte (*cut off*) y del valor de *p* que se seleccionen.

A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
ANXA5	**ATM**	**BAX**	**BCL2L1**	**CASP1**	**CASP10**	**CASP8**	**CAT**	**CCL21**	**CCL3**	**CCL4**	**CCNC**
B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
CCND1	**CCNG1**	**CDKN1A**	**CHEK2**	**CRYAB**	**CSF2**	**CXCL10**	**CYP1A1**	**CYP2E1**	**CYP7A1**	**DDB1**	**DDIT3**
C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
DNAJA1	**DNAJB4**	**E2F1**	**EGR1**	**EPHX2**	**ERCC1**	**ERCC3**	**FASLG**	**FMO1**	**FMO5**	**GADD45A**	**GDF15**
D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
GPX1	**GSR**	**GSTM3**	**HMOX1**	**HSF1**	**HSPA1A**	**HSPA1L**	**HSPA2**	**HSPA4**	**HSPA5**	**HSPA6**	**HSPA8**
E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
HSPB1	**HSPCA**	**HSPCB**	**HSPD1**	**HSPE1**	**HSPH1**	**IGFBP6**	**IL18**	**IL1A**	**IL1B**	**IL6**	**LTA**
F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
MDM2	**MIF**	**MT2A**	**NFKB1**	**NFKBIA**	**NOS2A**	**PCNA**	**POR**	**PRDX1**	**PRDX2**	**PTGS1**	**RAD23A**
G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
RAD50	**SERPINE1**	**SOD1**	**SOD2**	**TNF**	**TNFRSF1A**	**TNFSF10**	**TP53**	**UGT1A4**	**UNG**	**XRCC1**	**XRCC2**
H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
18SrRNA	**HPRT1**	**RPL13A**	**GAPDH**	**ACTB**	**ACTB**	**ACTB**	**ACTB**	**ACTB**	**ACTB**	**ACTB**	**ACTB**

Figura M&M.1. Diseño de la placa de Stress & Toxicity PathwayFinder™, RT2Profiler™ de PCR Array.

3.3.1.2 Microarray, análisis de datos, construcción de BAN y depósito en la base de datos *Gene Expression Omnibus* (GEO)

El soporte de los microarrays permite realizar un análisis genómico funcional de tipo transcripcional con alto rendimiento y puede llegar a abarca el genoma humano en su totalidad. Así, es posible detectar cualquier cambio en la expresión celular tras un tratamiento con CA o con ICC, por ejemplo.

En el estudio de los polifenoles del café, se llevaron a cabo experimentos de microarrays, cuyo desarrollo experimental y posterior análisis siguieron la

metodología desarrollada por Selga y col. 2009 (Selga, et al., 2009), a excepción del criterio de selección de *fold change* ≥1,3 y algunas modificaciones que se mencionan a continuación, debidas al propio avance tecnológico:

i. La plataforma de Microarray utilizada fue el GeneChip® Human Genome U133A plus 2.0 de Affymetrix. Este chip es capaz de analizar todo el genoma humano transcrito en un solo array, ya que determina la expresión de 47.000 transcritos (y variantes) del genoma gracias a que incorpora, dividido en 1,300.000 localizaciones (*features*), más de 54.000 sondas (*probe sets*) capaces de hibridarse a todos los genes transcritos descritos del genoma humano (Affymetrix technology 2011, GeneChip Human Genome U133 Plus 2.0 Array 2012).

ii. El análisis de datos se procesó con el software GeneSpring GX v.11.5.1 (Agilent Technologies), una versión más actual que, a diferencia de la anterior, es capaz de integrar los datos de múltiples plataformas generando un análisis de "MultiOMICS". La nueva versión tiene una mayor información en su base de datos e incorpora el software del programa Pathway Architect para construir las redes de asociación biológica (BAN).

3.2.2 Actividad enzimática de CYP1A1

Se ha utilizado el kit de la casa comercial Promega, p450-Glo™ CYP1A1 Assay para medir la actividad de CYP1A1. Este ensayo utiliza un método de luminiscencia para detectar la actividad enzimática de CYP1A1. El proceso consta de dos reacciones acopladas, la primera la cataliza el enzima CYP1A1, que transforma el sustrato Luciferina-CEE (Luciferina Cloroetil Éter, precursor de la luciferina incorporado exógenamente) en D-luciferina. La segunda reacción la cataliza la enzima luciferasa (incorporada exógenamente) utilizando como sustrato la D-luciferina, generando luz como producto final de la reacción (Cali, et al., 2006, Jurica and Sulcova, 2012). La figura M&M.2 representa la doble reacción acoplada que se realiza en esta determinación.

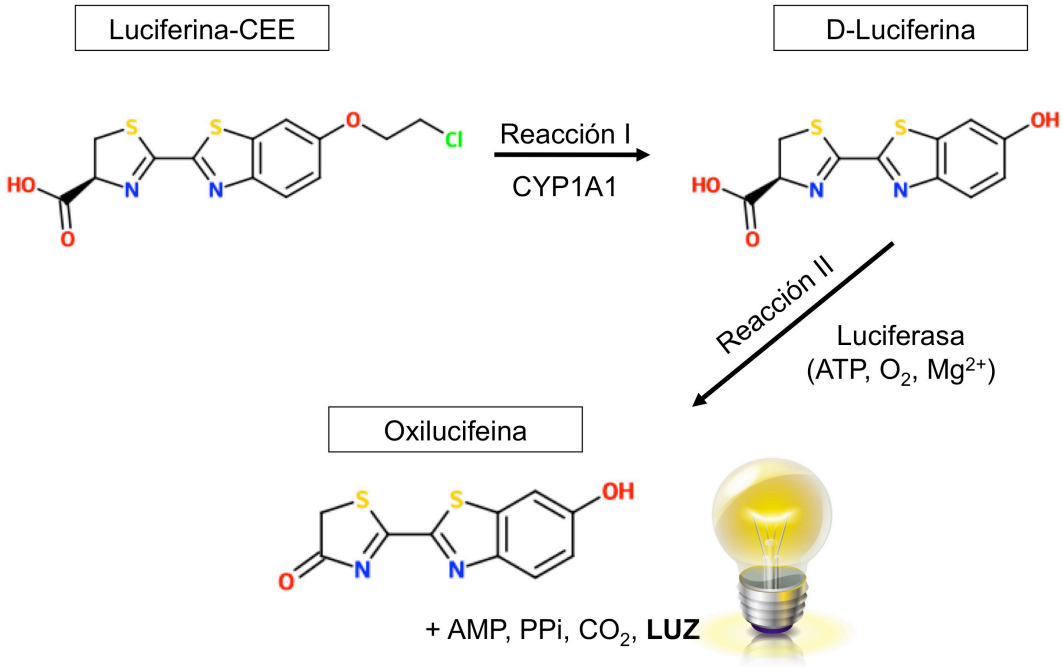

Figura M&M.2. Esquema de la reacción acoplada del ensayo de actividad enzimática de CYP1A1.

El ensayo de actividad enzimática se llevó a cabo sembrando 90.000 células (MCF-7 o SKBR3) en placas de 96 pocillos. La incubación con Luciferina-CEE (100µM) se realizó 8 h (MCF-7) o 2 h (SKBR3) a 37ºC antes de acabar el periodo de incubación con PCE (48 o 72 h), previa renovación del medio de cultivo. Posteriormente se bloqueo la actividad enzimática CYP1A1 añadiendo el agente para la detección de luciferina (solución de lisis que contiene Luciferasa, ATP, O_2, Mg^{2+}) y se inició la reacción catalizada por la luciferasa a temperatura ambiente durante 15 min. Los lisados celulares fueron transferidos, finalmente, a una placa de 96 pocillos opaca para la lectura de luminiscencia en el luminómetro Modulus Microplate (Tuner Biosystems technology).

3.2.3 Análisis de sinergismo entre tamoxifeno y PCE

La acción combinada de varias sustancias químicas que produce un efecto total superior al efecto de cada sustancia química por separado, se define como sinergismo. Para poder analizar la acción combinada de dos o más compuestos se aplican modelos matemáticos que cuantifican la combinación en ensayos de dosis-efecto, basados en la ley de acción de masas; la velocidad de reacción es proporcional a la masa activa de las sustancias reaccionantes, descrita por Guldberg y Waage (1879) (Bothamley, 2002).

El efecto combinatorio de citotoxicidad de TAM y PCE ha sido analizado mediante un software informático, CalcuSyn V2 de Biosoft (Chou, 2006), basado en la ecuación del índice de combinación (CI; *combination index*);

$$CI = ((D)_1 / ((D_m)_1) + ((D)_2 / ((D_m)_2)$$

que derivaron Chou y Talalay de la ecuación del efecto medio desarrollada por Chou;

Ecuación de dosis-efecto (curva) $f_a / f_u = (D / D_m)^m$

Ecuación de dosis-efecto linear $\log (f_a / f_u) = m \log (D) - m \log (D_m)$

f_a: fracción afectada por la dosis

f_u: fracción inalterada, $f_u = 1 - f_a$

D: Dosis del compuesto

D_m: la dosis del efecto medio que expresa la fuerza

m: coeficiente que expresa la forma de la curva dosis-efecto

La unidad experimental Fa-CI se obtiene al confrontar la fracción (Fa) *versus* el índice combinatorio (CI). Esta unidad experimental indica el sinergismo, la adición, o el antagonismo del efecto de la mezcla de dos compuestos. El efecto podrá ir variando según la combinación de concentraciones utilizadas de los compuestos (ver figura M&M.3). Niveles diferentes del efecto producen normalmente grados diferentes de sinergismo, adición, o antagonismo. Los valores de CI; CI<1 indican sinergismo; CI >1 antagonismo, y los valores CI que son iguales a uno indican adición.

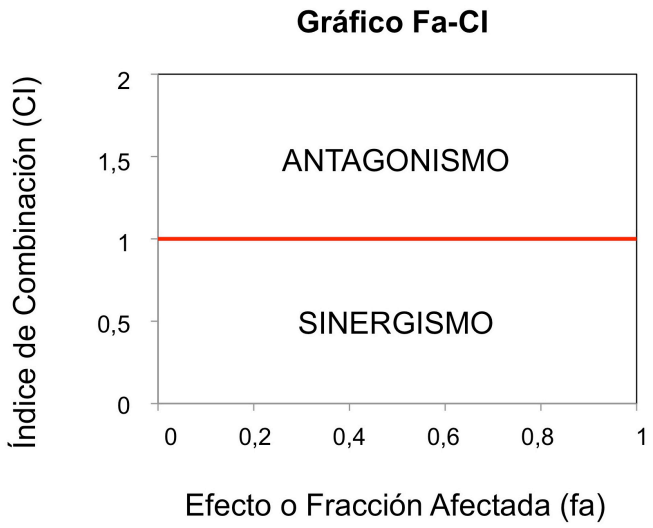

Figura M&M.3. Gráfico representativo de la unidad experimental Fa-CI (índice de combinación vs. fracción afectada)**.** Se reflejan las áreas de antagonismo (CI>1) y sinergismo (CI<1) que caracterizarán el tipo de efecto de la combinación de compuestos.

3.2.4 Inmunotransferencia. Sistema de bloqueo dirigido por vacío

Para la detección de los niveles de proteína se utilizó el sistema de detección patentado por Millipore llamado SNAP i.d.®. A diferencia del sistema tradicional de agregado de anticuerpos en la membrana adsorbente de PVDF por contacto, el SNAP i.d.® logra que las soluciones de incubación pasen a través de la membrana aplicando vacío. De esta manera consigue reducir el tiempo experimental del bloqueo e incubación con anticuerpos primario y secundario a 30 minutos (suma de los dos tiempos). El sistema lo forman el soporte SNAP i.d.® que sujeta y sella los casetes que fijan las membranas en posición horizontal y una bomba de vacío que se conecta al soporte. Entre el soporte y la bomba se coloca un matraz de kitasato que recupera los líquidos de lavado. La Figura M&M.4 muestra el conjunto del sistema.

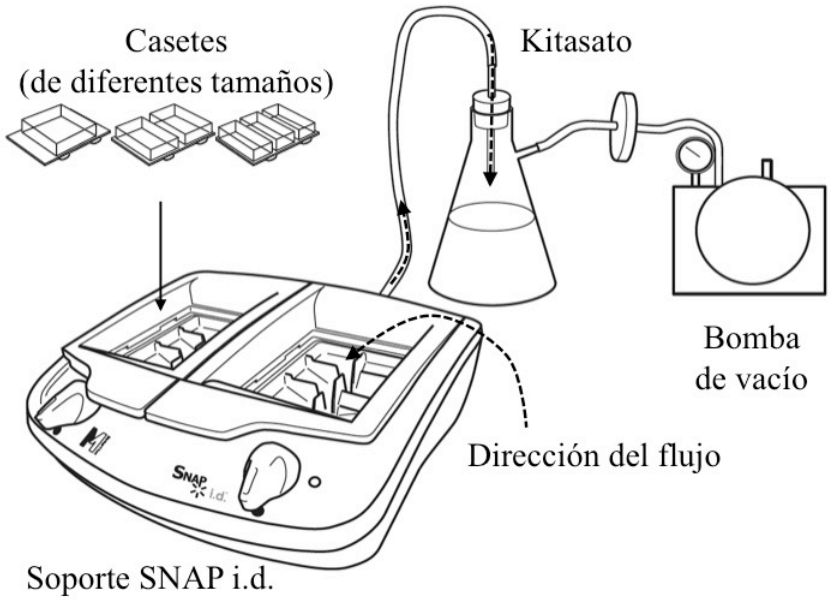

Figura M&M.4. Sistema de detección de proteínas por vacío. Una bomba de vacío se conecta al soporte SNAP i.d. El soporte fija los casetes que contienen las membranas de la inmunotransferencia. Entre la bomba de vacío y el soporte se conecta un matraz de kitasato para almacenar las soluciones de lavado que han pasado a través de las membranas (Merck-Millipore, 2012).

El proceso experimental que se llevó a cabo fue el especificado por la casa comercial, representado paso a paso mediante un esquema en la figura M&M.5, a excepción de pequeñas adaptaciones que se realizaron para mejorar los resultados y que se detallan a continuación:

- Colocar el soporte SNAP i.d.® sobre un agitador en todo el proceso, ya que así se evita la concentración de las soluciones en un punto fijo, a veces debido a la concavidad en el casete generada por el vacío.
- Mantener la presión de succión a 40 cmHg, ya que así el casete permanece en posición horizontal, evitándose su deformación y a la vez se consigue una rápida filtración de las soluciones.
- Realizar el bloqueo de la membrana con una solución de leche semidesnatada (*Blotto*) al 0,05% en TBS-Tween (50mM Tris, 150mM NaCl a pH 7,5 y un 0,1% Tween 20), así evita la saturación o bloqueo del propio casete.
- Realizar las diluciones de los anticuerpos en la solución de bloqueo al 0,05% (*Blotto*-TBS-Tween). Es posible reutilizar las diluciones si se conservan a 4ºC.

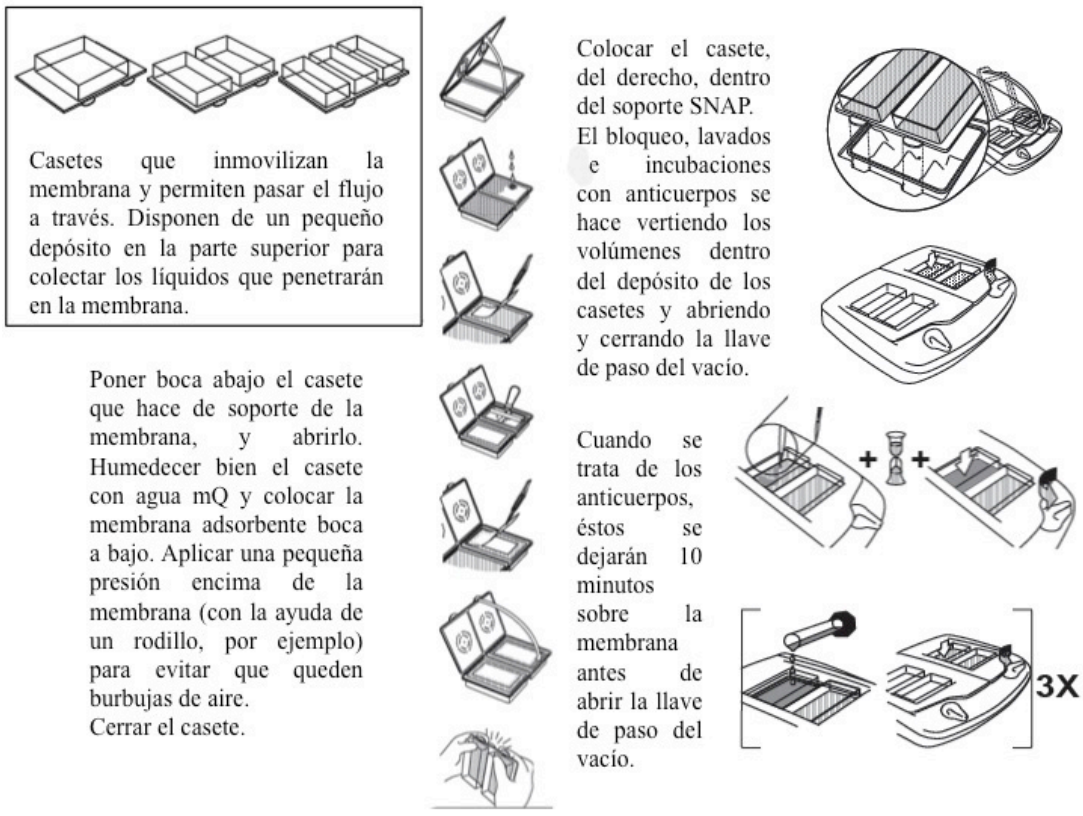

Figura M&M.5. Esquema del procedimiento del sistema de bloqueo dirigido por vacío (Merck-Millipore, 2012).

4.RESULTADOS

4.1 ARTÍCULO I:

CYP1A1 IS OVEREXPRESSED UPON INCUBATION OF BREAST CANCER CELLS WITH A POLYPHENOLIC COCOA EXTRACT.

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European Journal of Nutrition 2012, *51*, 465-476. *(Índice de impacto: 2,750. Posición 24/72 en la categoría de Nutrición y Dietética según índice de impacto).*

Antecedentes: La capacidad de inducir citotoxicidad en células de cáncer de mama, tanto dependientes de estrógenos (ERα-positivas), como no dependientes (ERα-negativas), por compuestos polifenólicos del té, como la Epigalocatequina-3-galato (EGCG; *epigallocatechin-3-gallate*), ya había estado demostrada anteriormente. El cacao, como el té, es otra fuente rica de polifenoles y estudios previos destacan las propiedades como antioxidantes, protectores cardiovasculares y antitumorales del cacao y sus derivados en humanos.

Objetivo: Realizar un análisis funcional específico para 84 genes relacionados con estrés y toxicidad (PCR Array), para establecer el efecto que tiene un extracto polifenólico de cacao (PCE) en dos modelos de cáncer de mama.

Resultados: El perfil de expresión génica en las dos líneas celulares se modificaba tras la incubación con PCE durante 24 horas. Los polifenoles del cacao inducían la sobreexpresión de 7 genes y la infraexpresión de 1 en la línea celular MCF-7, mientras que en las células SKBR3, el PCE causaba la sobreexpresión de 9 genes. De los genes modificados comunes en ambas líneas, el *Citocromo p450 familia 1, subfamilia A, polipéptido 1 (CYP1A1*) es el que aparecía más inducido. Para continuar el estudio de esta diana, se validaron los datos obtenidos mediante PCR Arrays (analizando niveles de mRNA, proteína y actividad) y, posteriormente se estudió su regulación transcripcional a través de la vía de señalización del receptor Aril y su interacción con el receptor de estrógeno.

Los estudios sobre la regulación transcripcional de CYP1A1 mostraron que el incremento de CYP1A1, mediado por PCE, se caracteriza por el aumento de la unión del factor de transcripción Receptor de Aril Hidrocarburo (AhR) a los elementos de respuesta a xenobióticos (XRE) en el promotor de *CYP1A1*. La variación inversa de los niveles del receptor de estrógeno (ERα) en las dos líneas

celulares a consecuencia del tratamiento con PCE sugiere su participación en la vía de señalización de AhR. Tanto en las células MCF-7 como SKBR3, existía una interacción entre AhR y ERα, que se mantenía con el tratamiento con PCE, lo que indica que ERα a nivel nuclear podría interactuar con el complejo AhR/Arnt.

Puesto que una de las funciones de CYP1A1 es metabolizar estrógenos, se desarrolló una estrategia de terapia combinada con un antagonista del receptor de estrógeno, el tamoxifeno (TAM) y un inductor de la metabolización de estrógenos, el PCE, con la finalidad de incrementar la citotoxicidad. El efecto citotóxico de la combinación resultó ser sinérgico en ambos modelos, MCF-7 y SKBR3, y específico para células tumorales puesto que no causaba citotoxicidad en la línea celular no tumorogénica HEK293T.

Conclusiones: La interacción entre ERα y AhR modulada por la incubación con PCE participa en la inducción de CYP1A1 en células de cáncer de mama. El sinergismo obtenido de la combinación de PCE y Tamoxifeno (a concentraciones no citotóxicas) abre la posibilidad a un estudio de terapias combinadas entre el agente quimioterápico y polifenoles del cacao que mejoren su efecto.

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ORIGINAL CONTRIBUTION

CYP1A1 is overexpressed upon incubation of breast cancer cells with a polyphenolic cocoa extract

Carlota Oleaga · Miriam García · Anna Solé · Carlos J. Ciudad · Maria Izquierdo-Pulido · Véronique Noé

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Abstract
Purpose To evaluate the effect of cocoa flavonoids in breast cancer cells at the molecular level, a functional genomic analysis was performed using a polyphenolic cocoa extract (PCE) in MCF-7 and SKBR3 cell lines.
Methods The expression profile of 84 genes included in the Stress & Toxicity PathwayFinder™ PCR Array was analyzed after PCE incubation for 24 h. mRNA and protein levels were analyzed by RT-PCR and western blot, respectively. Gel shift assays were used to evaluate DNA–protein complexes**.** Protein complexes were identified by co-immunoprecipitation. Cell viability was evaluated by MTT assays.
Results Upon PCE incubation, 7 genes were overexpressed and 1 underexpressed in MCF-7 cells, whereas 9 genes were overexpressed in SKBR3 cells. Among the differentially expressed genes in both cell lines, cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) was chosen for further study. CYP1A1 mRNA and protein levels and enzymatic activity increased upon PCE incubation. CYP1A1 transcriptional activation by PCE was mediated through AhR binding to XRE elements within the CYP1A1 promoter in MCF-7 cells. A protein complex including AhR and ERα was detected. The combination of PCE with tamoxifen caused a synergistic cytotoxicity in both cell lines and was due to an increase in apoptosis in MCF-7 cells.
Conclusions The interaction between ERα and AhR upon incubation with PCE leads to CYP1A1 induction in breast cancer cells. The synergy between PCE and non-cytotoxic tamoxifen concentrations opens the possibility for a combination therapy based on polyphenols from cocoa that increased tamoxifen efficacy.

Keywords AhR · Cocoa · CYP1A1 · ERα · Tamoxifen · Breast cancer

Abbreviations

AhR	Aryl hydrocarbon receptor,
Arnt	Aryl hydrocarbon receptor nuclear translocator
BSA	Bovine serum albumin
CHX	Cycloheximide
CI	Combination index
CYP1A1	Cytochrome P450 family 1 subfamily A polypeptide 1
DEPC	Diethyl pyrocarbonate
EGCG	Epigallocatechin-3-gallate
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
GS	Gel shift
NE	Nuclear extract
OVN	Overnight
PCE	Polyphenolic cocoa extract
PI	Propidium iodide
RT-PCR	Reverse transcription-polymerase chain reaction
SE	Standard error
STP	Staurosporine
TAM	Tamoxifen
XRE	Xenobiotic response element

Electronic supplementary material The online version of this article (doi:10.1007/s00394-011-0231-2) contains supplementary material, which is available to authorized users.

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Introduction

Worldwide, more than a million women are diagnosed with breast cancer every year, accounting for a tenth of all new cancers and 23% of all female cases of cancer. Breast cancer incidence rates vary considerably, with the highest rates in the developed world and the lowest rates in Africa and Asia [1]. Around 430,000 new cases of breast cancer occur each year in Europe and an estimated 212,920 cases in the USA [2].

Estrogens are implicated in the initiation and promotion stages of breast cancer, and lifetime estrogen exposure is a major risk factor for breast cancer [3, 4]. Estrogens exert their carcinogenic effects by both estrogen receptor (ER)-dependent and independent mechanisms [3, 5, 6]. The ER-dependent mechanism underlying mammary carcinogenesis involves the activation of the ER by estrogens, leading to the expression of estrogen-responsive genes and stimulation of cell proliferation [3, 5–7]. The ER-independent pathway involves the synthesis of toxic estrogen metabolites that are highly reactive and damage DNA, protein, and lipids [3, 5, 6, 8, 9]. Most human breast cancers are initially positive for ER, and their growth can be stimulated by estrogens and inhibited by antiestrogens such as tamoxifen [10]. Tamoxifen is a selective ER modulator, which acts as an antiestrogen in breast, but as an estrogen in the uterus, the cardiovascular system, and bone. Its antitumor effects are directly related to ER levels in breast tumors [11], although it can also be used as therapy in ER-negative tumors to prevent a second tumor development [12].

Dietary or therapeutic agents interfering with receptor-mediated pathways or reducing the production of genotoxic estrogen metabolites could be effective in modulating estrogen-induced breast carcinogenesis. In this direction, green tea contains polyphenolic compounds, known as catechins, such as epigallocatechin-3-gallate (EGCG) with proven anticarcinogenic effect [13]. EGCG has shown cytotoxic activity in both ERα+ and ERα− breast cancer cells [14, 15], and induction of apoptosis is one of its mechanisms of action [15, 16]. Cocoa is rich in polyphenols, similar to those found in green tea. In fact, cocoa has the highest flavanol contents of all foods on a per-weight basis and is a significant contributor to the total dietary intake of flavonoids [17]. The main subclasses of flavonoids found in cocoa are flavanols, particularly the flavanol monomers catechin and epicatechin, and their oligomers, also known as procyanidins [18]. Many examples of the health benefits of cocoa consumption can be found in the literature [18–21].

The aim of our study was to determine the effect of nontoxic concentrations of cocoa polyphenols at the molecular level using as a model two human breast cancer cell lines, MCF-7 and SKBR3, that show different ERα status. Additionally, we sought to evaluate a possible synergism between cocoa polyphenols and tamoxifen, a drug widely used for breast cancer treatment, similar to that described for EGCG.

Materials and methods

Cocoa powder phenolic extract

Natural Forastero cocoa powder from Malaysia was employed for this study. Ten grams of cocoa was subjected to an extraction of phenols [22]. The total phenolic content in the extract was determined according to the Folin-Ciocalteu method [23] and expressed in mg per mL of catechin equivalents. The phenolic composition of PCE is indicated in Online Resource 1. A stock solution was prepared in 20% DMSO.

Cell culture

Human breast cancer cell lines MCF-7 and SKBR3, and human embryonic kidney immortalized cell line HEK293T were used. Cells were grown in F-12 medium (Gibco) supplemented with 7% (v/v) fetal bovine serum (Gibco), sodium penicillin G, and streptomycin. The concentrations of PCE (150–250 ng/μL) used in cell incubations were not cytotoxic (Online Resource 1). MG-132 (25 μM, Calbiochem) was incubated overnight either alone or in combination with PCE. Cycloheximide (CHX, 50 μg/mL, Sigma) was incubated for 48 h either alone or in combination with PCE. Tamoxifen citrate (TAM, Sigma) was incubated for 48 h at concentrations ranging from 10^{-6} to 10^{-3} M, either alone or in combination with PCE. Staurosporine (STP, 10^{-7}–10^{-6} M, Sigma) was incubated for 24 h. The amount of DMSO in these incubations was always less than 0.4%.

PCR arrays

Total RNA was prepared from 3×10^6 cells following the procedure recommended by Qiagen. Gene expression was analyzed using specific PCR arrays (Stress & Toxicity PathwayFinder™, RT2Profiler™ PCR Array, SuperArray), containing gene-specific primer sets for 84 relevant genes and 5 housekeeping genes, whose Ct values were used as a normalization factor. Fold changes in gene expression were calculated using the standard ΔΔCt method. The expression of each gene was reported as the fold change obtained after each treatment relative to control after normalization of the data. A cutoff of 2-fold was chosen since small changes in gene expression may represent important changes downstream those differentially

expressed genes. Lists of differentially expressed genes, with a p value <0.05, were generated from three independent experiments.

RT-PCR

Total RNA was extracted using Ultraspec (Biotex) in accordance with the manufacturer's instructions. cDNA was synthesized in a total volume of 20 µL from RNA samples as described in Selga et al. [24]. CYP1A1 and ERα mRNA levels were determined in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 3 µL of the cDNA reaction and the assays-on-demand Hs00153120_m1 for CYP1A1, Hs01045818_m1 for ERα and Hs00356991_m1 for APRT (all from Applied Biosystems). APRT mRNA was used as an endogenous control. Fold changes in gene expression were calculated using the standard ΔΔCt method.

Western blot

Whole extracts were obtained from 3×10^6 control or PCE-treated cells according to Selga et al. [25]. Total extracts (50–150 µg) were resolved on SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon P, Millipore) using a semidry electroblotter. The membranes were probed with CYP1A1 antibody H-70 (sc-20772), ERα antibody G-20 (sc-544), or AhR antibody H-211 (sc-5579) (all from Santa Cruz Biotechnology Inc). Signals were detected with secondary horseradish peroxidase–conjugated antibody and enhanced chemiluminescence, as recommended by the manufacturer (Amersham, Millipore). Blots were reprobed with antibodies against β-actin (A2066, Sigma) or tubulin (Cp06, Calbiochem) to normalize the results.

CYPA1 enzymatic activity

The p450-Glo Assay (Promega) luminescent method was used to measure CYP1A1 enzymatic activity. Cells (9×10^3) were seeded in 96-well dishes and incubated with PCE (250 ng/µL) for 48 or 72 h. The medium was renewed either 8 h (MCF-7 cells) or 2 h (SKBR3 cells) before the end of the incubation with PCE, and 100 µM of Luciferin 6′ chloroethyl ether per well was added. At the end of the incubation period at 37 °C, the luciferin detection reagent was added, and the resulting lysates were transferred to an opaque white 96-well dish. Luminescence was read in a Modulus Microplate luminometer (Tuner Biosystems technology).

Co-immunoprecipitations

Total extracts were obtained from 3×10^6 control or PCE-treated cells following Tapias et al. [26]. Co-immunoprecipitations were performed by using either 5 µg of ERα antibody or unspecific IgGs (I-5006, Sigma). The specific detection of the co-immunoprecipitated AhR protein was performed by western blot using 1:200 dilution of the AhR antibody.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from control cells or cells treated with PCE (250 ng/µL) were prepared according to Noé et al. [27]. Binding reactions were performed as described in Tapias et al. [26] using a [γ-^{32}P]-labeled specific XRE probe that was generated by PCR using a pair of CYP1A1-specific primers and HeLa genomic DNA as template. The amplified fragment of 327 bp contained four XRE binding sites and two putative Sp1 binding sites (Fig. 2a).

CYP1A1-FOR 5′ tcaagtcaggctagcCATGCCAAATGGC ACTGGGGC 3′

CYP1A1-REV 5′ cagtgctgcctcgagGTTGCGTGAGAAG GAACCGGAG 3′

The protein–DNA complexes were resolved on a 5% polyacrylamide-glycerol gel using 0.5× TBE buffer (89 mM Tris boric acid, 2.5 mM EDTA, pH 8.0). The shifted bands were quantified by Phosphorimaging using the ImageQuant software v 5.2 (Molecular Dynamics). For competition experiments, the reaction mixture was incubated with different amounts of the unlabeled XRE fragment for 15 min prior to the addition of the radiolabeled probe. In the supershift experiments, 4 µg of the antibody against AhR or unspecific IgGs (Sigma) was incubated OVN at 4 °C with the nuclear extracts before performing the binding reactions.

Cell viability assay

Cells (30,000) were plated in 2 mL of F-12 medium and incubated with increasing concentrations of PCE (50–250 ng/µL), or increasing concentrations of TAM (10^{-7}–10^{-3} M), either alone or in combination with PCE (250 ng/µL). MTT assay was performed 24 h or 72 h later after the addition of PCE [28].

Analysis of synergism

The synergism between TAM and PCE was assessed following the Chou-Talalay method, which combines the median effect equation of Chou where dose–effect curves (from the drugs either alone or in combination) are transformed into linear equations, with the combination index (CI) equation and plot of Chou-Talalay [29]. The analysis was performed using the CalcuSyn V2 software (Biosoft).

Apoptosis assay

The levels of apoptosis in cells treated with STP, PCE, and/or TAM were determined as described in Blasco et al. [30].

Statistical methods

For the RT-PCR and western blot analyses, values are expressed as the mean ± SE. Data were evaluated by unpaired Student's *t* test when analyzing the difference between two conditions, control and treated. One-way ANOVA followed by Bonferroni post hoc multiple range test was used for different conditions that differ in one parameter (i.e. time). Both analyses were performed using the PASW Statistics v 18.0.0. software. Differences with p values <0.05 were considered significant.

Results

Differential gene expression analysis using PCR arrays

The expression profile of the 84 genes included in the Stress & Toxicity PathwayFinder^{TM} PCR Array was analyzed in MCF-7 and SKBR3 cells, both control and treated with a polyphenolic cocoa extract (PCE) for 24 h. Treatment with PCE decreased the expression of *serpine 1* and up-regulated the expression of the *CYP1A1*, *GADD45A*, *GDF15*, *GPX1*, *RAD23A*, *TP53,* and *XRCC2* genes in MCF-7 cells (Online Resource 2). Upon incubation with PCE, 9 genes were overexpressed in SKBR3 cells: *CAT*, *CYP1A1*, *FMO5*, *GADD45A*, *GDF15*, *HSPA5*, *IL18*, *LTA,* and *PTGS1* (Online Resource 3). All changes were statistically significant ($p < 0.05$). The *CYP1A1* gene was chosen from the three differentially expressed genes in common between both cell lines for further validation because CYP1A1 mRNA showed the highest increase upon incubation with PCE, 17.5-fold in MCF-7 cells and 155-fold in SKBR3 cells, respectively.

CYP1A1 mRNA and protein levels and enzymatic activity upon incubation with cocoa extract

The differential expression of CYP1A1 mRNA in control versus treated cells was validated by RT-PCR to confirm the changes obtained in the screening with the PCR arrays. It is worth mentioning that the basal levels of CYP1A1 mRNA in SKBR3 cells were 18-fold higher than in MCF-7 cells (Fig. 1a). Upon incubation with PCE for 24 h, mRNA levels for CYP1A1 were increased by 32-fold compared to control in MCF-7 cells. In SKBR3 cells treated with PCE, CYP1A1 mRNA was increased by 969-fold compared to the MCF-7 control and 54-fold compared to SKBR3 control (Fig. 1a).

Next, we investigated whether the changes at the RNA level were translated into protein. PCE treatment for 24 h led to a very modest increase in CYP1A1 protein levels (1.2-fold). A time course incubation during 24, 48, 72 and 96 h led to an increase in CYP1A1 protein in MCF-7 cells of 3.9-fold after 48 h (Fig. 1b), whereas in SKBR3 cells, CYP1A1 protein was increased 17.7-fold after 72 h (Fig. 1c). The difference between mRNA levels and the corresponding protein levels may indicate that many of the mRNA molecules do nor reach the translational machinery, probably because the translation mechanism is saturated in these conditions.

Finally, CYP1A1 activity was determined upon incubation with PCE. An increase in CYP1A1 activity in good correlation with the observed increased in CYP1A1 protein levels was determined for both cell lines (Fig. 1d).

Effect of cocoa extract on the aryl hydrocarbon receptor pathway

Xenobiotic-responsive element (XRE)-binding protein, a heterodimer of the aryl hydrocarbon receptor (AhR) and its nuclear translocator, regulates the transcription of CYP1A1 through XRE in response to xenobiotic inducers [31, 32]. SKBR3 and MCF-7 cells were treated with PCE for 24 h, and their nuclear extracts were subjected to EMSA. Binding to a probe corresponding to four XREs present in the human CYP1A1 promoter produced a pattern of three bands (Fig. 2b) probably due to AhR binding to XRE, as previously described [33]. The identity of AhR binding was confirmed by competition with increasing amounts of the unlabeled probe that completely abolished the binding pattern observed (Fig. 2d) and by supershift experiments with an AhR antibody that revealed that AhR contributed to this binding pattern (Fig. 2e). Incubation with PCE caused an increase in the DNA-binding capacity of nuclear AhR in MCF-7 cells, whereas in SKBR3 cells, binding to XRE was decreased, although its binding in basal conditions was higher than in MCF-7 cells (Fig. 2b, c).

Effect of PCE on AhR and ERα protein levels

AhR and ERα protein levels were analyzed from total extracts from MCF-7 and SKBR3 cells by western blot. In MCF-7 cells, the highest induction of CYP1A1 at 48 h of incubation with PCE took place simultaneously with the down-regulation of ERα and an increase in AhR levels (Fig. 3a). Levels of ERα in SKBR3 cells were lower than in MCF-7 cells, in accordance with their respective ER statuses, and increased in a time-dependent manner upon incubation with PCE. In these cells, overexpression of

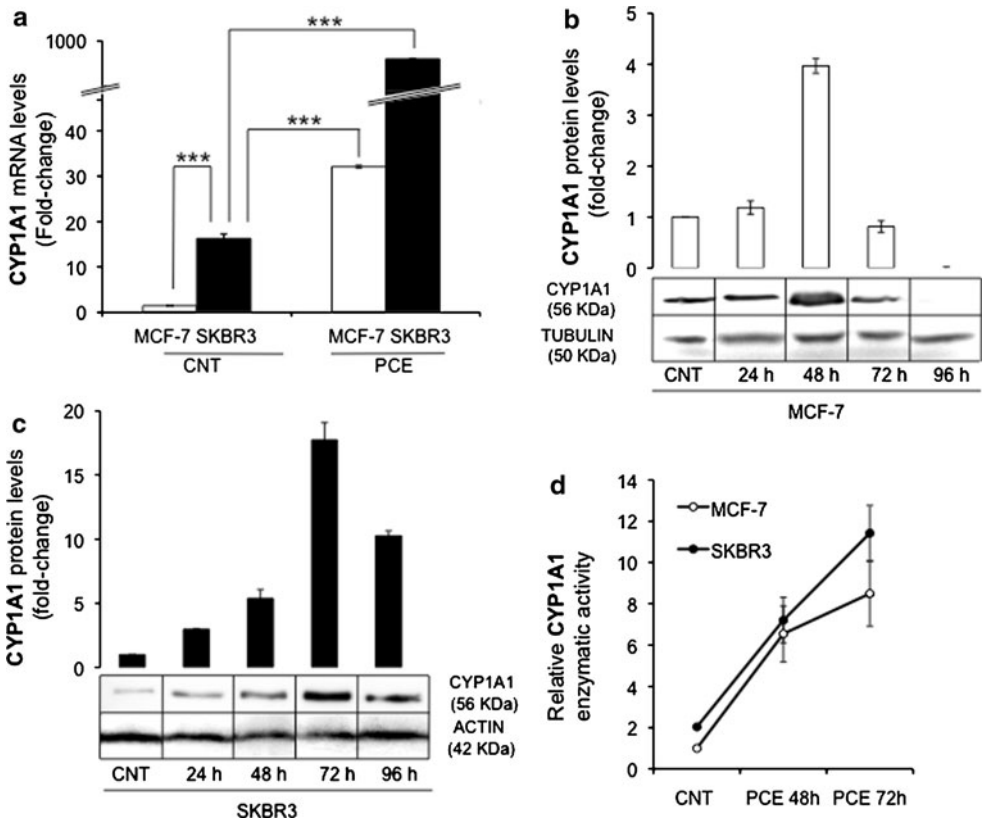

Fig. 1 CYP1A1 overexpression in cells treated with PCE. **a** Determination of CYP1A1 mRNA levels. *Empty bars* indicate CYP1A1 mRNA levels in MCF-7 cells, *filled bars* correspond to the mRNA levels in SKBR3 cells, either control (0.12% of DMSO) or treated with PCE (250 ng/μL) for 24 h for both cell lines. Results are expressed in fold changes compared to MCF-7 control and are the mean ± SE of 3 different experiments. ***$p < 0.001$ compared with the corresponding control situation. Determination of CYP1A1 protein levels. *Empty bars* indicate CYP1A1 protein levels in MCF-7 cells (**b**), *filled bars* correspond to the protein levels in SKBR3 cells (**c**), either control (0.12% of DMSO) or treated with PCE (250 ng/μL) for the indicated times. Results represent the mean ± SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA plus post hoc Bonferroni comparison. **d** Determination of CYP1A1 activity. *Empty circles* indicate CYP1A1 enzymatic activity in MCF-7 cells, whereas *filled circles* correspond to SKBR3 cells, either control (0.12% of DMSO) or treated with PCE (250 ng/μL) for the indicated times. Results are expressed relative to the activity of the control and represent the mean ± SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA, plus post hoc Bonferroni comparison

CYP1A1 protein was maximal at 72 h and correlated with an increase in both ERα and AhR levels (Fig. 3b). We next analyzed whether AhR and ERα were able to interact at the protein level. Co-immunoprecipitations were performed with a specific antibody against ERα, and the presence of AhR was analyzed in the resulting co-immunoprecipitate. As can be seen in Fig. 3c, d, AhR and ERα were part of a protein complex in both cell lines, either control or PCE-treated.

We analyzed the mechanism by which PCE triggered ERα protein levels in SKBR3 cells. This increase in protein was not due to transcriptional activation as ERα mRNA levels remained unchanged (Fig. 4a). CHX, an inhibitor of protein synthesis, was able to block ERα induction in response to PCE, indicating that the increase in ERα protein levels was the result of an increase in protein synthesis (Fig. 4b). Furthermore, the increase in ERα levels upon PCE incubation was dependent on proteasome processing as it was blocked in the presence of MG132, a proteasome inhibitor (Fig. 4b). In addition, PCE also appeared to modify ERα stability. A time course of ERα degradation was performed, using CHX to inhibit new protein synthesis and examining the decay of the remaining ERα protein in control and PCE-treated SKBR3 cells. ERα protein half-life in control cells was about 11.7 h, whereas in cells treated with PCE, it was decreased down to 2.1 h (Fig. 4c).

Synergistic effect between PCE and tamoxifen

A synergistic effect for the combination of tamoxifen with EGCG in breast cancer cells has been described [34–36]. In this direction, we wanted to test whether cocoa polyphenols would also exert a synergistic effect in combination with tamoxifen (TAM). MCF-7 and SKBR3 cells were incubated with increasing concentrations of TAM (10^{-6}–10^{-3} M) either alone or in combination with PCE

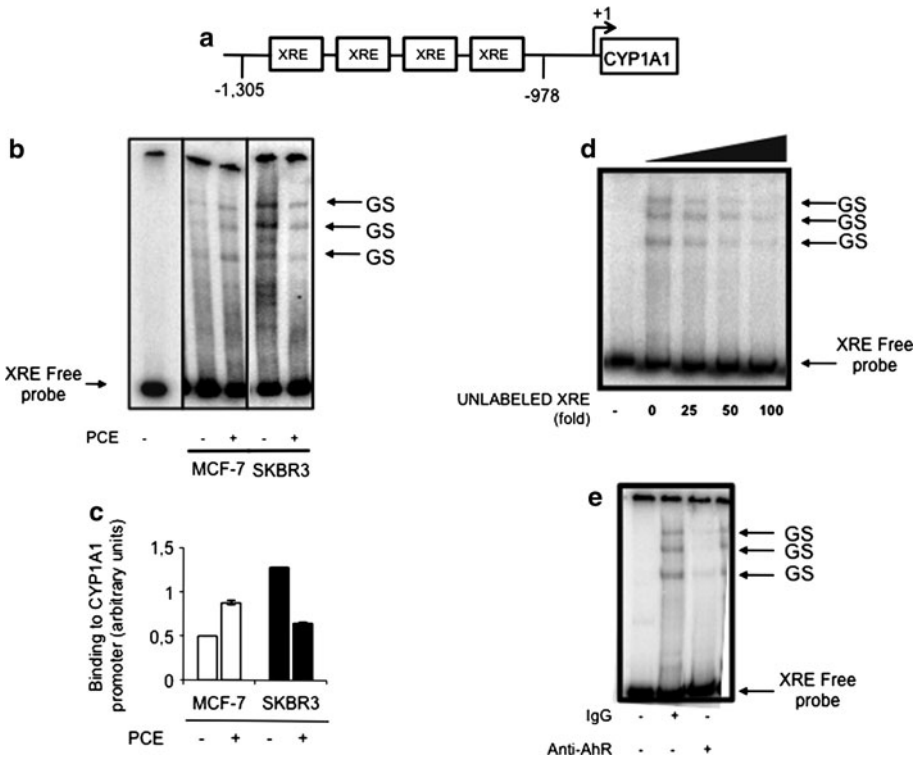

Fig. 2 Binding to the XRE probe. **a** Diagram corresponding to the 327-bp probe used for the binding assays generated by PCR from the CYP1A1 promoter and containing four XRE *boxes*. **b** Gel shift produced by the binding of nuclear extracts (NE) prepared from MCF-7 and SKBR3 cells cultured in the absence or in the presence of PCE (250 ng/μL for 24 h) to an XRE probe. *Arrows* indicate the shifted bands (GS). **c** The global quantification of the shifted bands in control versus PCE-treated cells was performed using the ImageQuant software v 5.2 (Molecular Dynamics) and was plotted as absolute values (arbitrary units). **d** Competition assays. Increasing amounts of the unlabeled XRE probe were added to the binding reaction 15 min before the addition of the labeled probe. **e** Supershift experiment. The AhR antibody was added to the nuclear extracts and incubated OVN at 4 °C before the addition of the probe. IgGs were added in parallel as a control

(250 ng/μL), and cell viability was determined after 48 h. The presence of PCE, which did not cause significant cell death by itself (Online Resource 1), increased the cytotoxic effect of TAM in both cell lines (Fig 5a, b). The reduction in cell viability was more evident in MCF-7 cells, reaching an increase of 44% when combined with 10^{-6} M TAM. Synergism (CI < 1) was confirmed for the combination of 10^{-6} M TAM with PCE in both cell lines and for 10^{-5} M TAM with PCE in SKBR3 cells as well (Online resource 4). As a control, we used HEK293 cells, in which the cytotoxic effect of tamoxifen alone was reduced by 20% when added in combination with PCE (Fig. 5c).

Levels of apoptosis upon incubation with PCE and tamoxifen

To get further insight into the mechanism by which the combination of PCE and TAM increased cytotoxicity, the levels of apoptosis were determined. The level of basal apoptosis in SKBR3 cells was twice than in MCF-7 cells. Incubation with PCE at 150 or 250 ng/μL showed no induction of apoptosis in MCF-7 cells, whereas in SKBR3, it did increase apoptosis around 2-fold over the control. TAM at 10^{-6} M did not induce apoptosis by itself in any cell line, but when combined with 150 ng/μL of PCE, it was able to increase the levels of apoptosis in MCF-7 cells (Fig. 5d). The combination of PCE with TAM in SKBR3 cells (with higher basal apoptotic levels) did not cause an increase in the apoptosis levels, as compared to each condition alone (Fig. 5e).

Discussion

In this work, we analyzed the gene expression profile of human breast cancer cells treated with purified cocoa polyphenolic extract, used as representative of the wide flavonoid spectrum (monomers and oligomers) present in cocoa. Using PCR arrays, we described the differential expression of several genes involved in stress and toxicity pathways from which the CYP1A1 gene was chosen for further study for several reasons: (1) it was the most overexpressed gene in both cell lines analyzed upon incubation with PCE, (2) its overexpression in response to

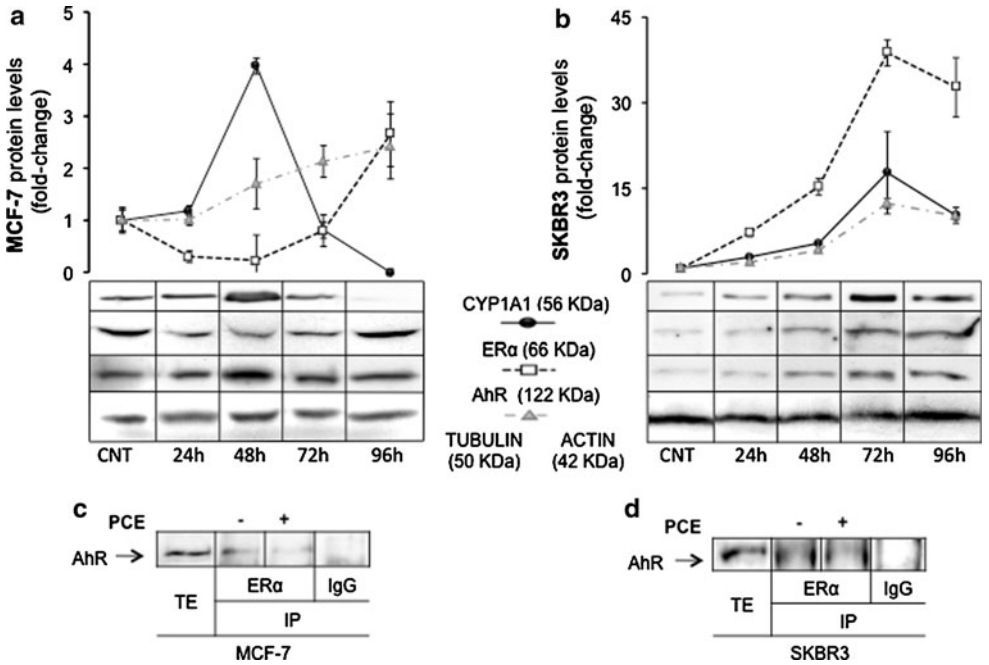

Fig. 3 Protein levels of AhR, ERα, and CYP1A1 in cells extracts.**a** and **b** Levels of AhR, ERα, and CYP1A. Cells were treated with PCE (250 ng/μL) for the indicated periods of time, and the levels of CYP1A1 (*circles*), AhR (*triangles*), and ERα (*squares*) were determined by western blot from total cell extracts from either control (0.12% of DMSO) or PCE-treated MCF-7 (**a**) and SKBR3 (**b**) cells. Representative blots and the plots corresponding to the quantification of the results after normalization are shown. Results represent the mean ± SE of 3 different experiments. Significant differences for all time points were established by ANOVA, and post hoc Bonferroni confirmed significant differences for CYP1A1, as well as ERα for SKBR3. **c** and **d** Co-immunoprecipitations. MCF-7 (**c**) and SKBR3 (**d**) cells were treated with PCE (250 ng/μL). Total extracts were immunoprecipitated with either ERα antibody or IgGs. The presence of the AhR protein in the immunoprecipitates was assessed by western blot. Total extracts were used as a positive control

polyphenols had already been described, and (3) it plays an important role in the oxidative metabolism of estrogens.

CYP1A1 is a candidate gene for low-penetrance breast cancer susceptibility because it plays an important role in the metabolism of xenobiotics or carcinogens as well as in the oxidative metabolism of estrogens [37]. CYP1A1 encodes aryl hydrocarbon hydroxylase (AHH) that catalyzes a hydroxylation reaction in Phase I metabolism as a first step to increase the polarity of different molecules. Some of these metabolites can be more active than the initial molecules and behave as electrophilic compounds, thus initiating or promoting tumorigenic processes. Additionally, other metabolites may behave as chemoprotectors, such as the result of 2-hydroxylation in E1 and E2 metabolism [38]. CYP1A1 expression occurs predominantly in extrahepatic tissues; its mRNA has been detected in normal and cancerous breast tissue and can be induced in human breast-derived cell lines [39]. In humans, CYP1A1 is under the regulatory control of the aryl hydrocarbon receptor (AhR) [31, 40, 41]. Previous studies reported that quercetin induces time- and dose-dependent increases in both CYP1A1 mRNA levels and enzyme activity in MCF-7 cells [32]. The ability of quercetin to modulate CYP1A1 expression is probably mediated by its AhR-binding activity [32, 42].

In our model, treatment with cocoa extract induces CYP1A1 expression in breast cancer cells. The involvement of AhR signaling pathway in CYP1A1 induction by PCE was evaluated by gel shift assays using an XRE probe. In MCF-7 cells, the accumulation of CYP1A1 mRNA following PCE treatment for 24 h was paralleled by the ability of nuclear extracts to increase the form of AhR/DNA complexes, suggesting that some polyphenols present in the cocoa extract may act as activators of the AhR signaling pathway. However, in SKBR3 cells, XRE binding was decreased in the presence of PCE, indicating a different signaling pathway, probably XRE-independent, leading to CYP1A1 overexpression. The existence of a protein complex including AhR and ERα in both cell lines suggests that both proteins could be contributing to the transcriptional activation of the CYP1A1 promoter. In ER-(−) cells (SKBR3), PCE behaves as an ER antagonist, such as TAM in MCF-7 cells, that increases ERα protein levels [43], whereas in ER-(+) cells (MCF-7), PCE incubation decreases ERα levels, acting as an ER agonist. Cocoa polyphenols are inducing ERα protein synthesis and processing by the proteasome in SKBR3 cells. The ERα precursor needs to be activated by a chymotrypsin-like activity of the proteasome [44]. Additionally, ERα half-life in

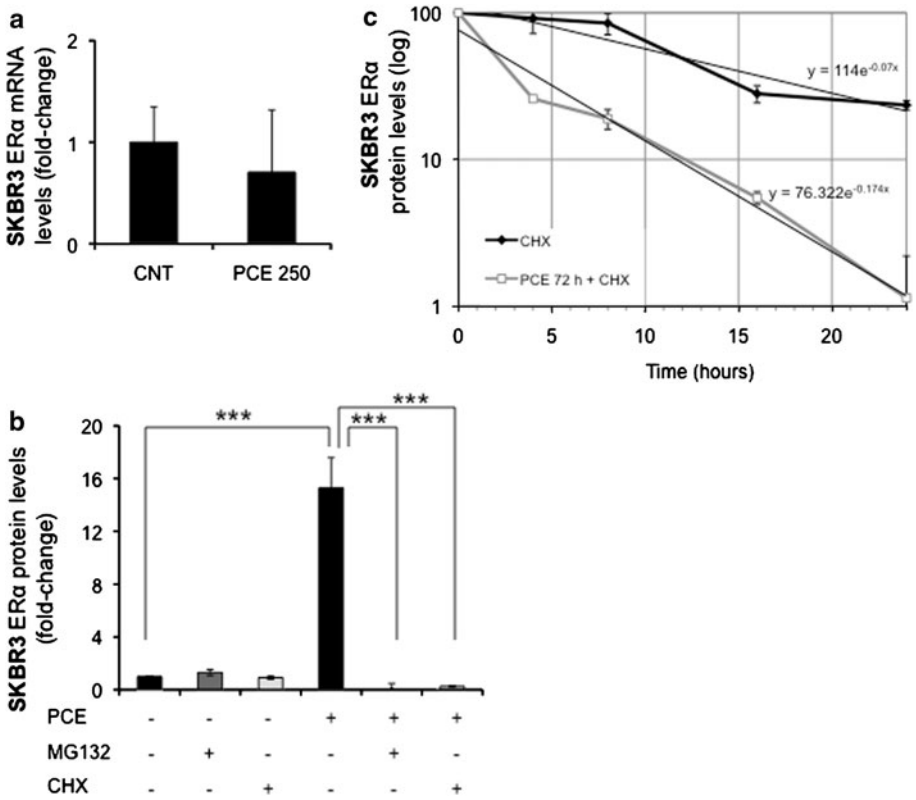

Fig. 4 Mechanism of ERα induction in SKBR3 cells. **a** Determination of ERα mRNA levels. Cells were treated with PCE (250 ng/μL) for 48 h, total RNA was extracted, and ERα mRNA levels were determined by RT-PCR as described. Results represent the mean ± SE of 3 different experiments. **b** Effects of CHX and MG132. SKBR3 cells were treated with PCE (250 ng/μL) for 48 h alone, or in combination with CHX (50 μg/mL, 30 min before PCE incubation) or MG132 (25 μM, overnight), and ERα protein levels were analyzed in total extracts. Results are expressed as fold changes compared to the control, incubated only with DMSO, and represent the mean ± SE of 3 different experiments. ***$p < 0.001$ compared with the corresponding condition, control treated only with DMSO (0.32%) or PCE. **c** ERα half-life. Cells were incubated with 50 μg/mL cycloheximide for the indicated times either alone or in combination with PCE for 72 h, and total extracts were prepared. The levels of ERα protein were determined by western blot as described. The quantification of the bands was performed by image analysis and plotted using a logarithmic scale. In *black* ERα half-life in control cells (treated only with 0.32% of DMSO) and in *gray* ERα half-life in PCE-treated cells. Results are the mean ± S.E. of 3 independent experiments

SKBR3 decreases to a value comparable to MCF-7 (3–5 h) in the presence of PCE [45]. Altogether, the increase in protein synthesis concomitant to a decrease in protein stability could be due to an increase in ER-precursor levels and processing upon the addition of PCE.

In addition to CYP1A1, the levels of GADD45A and GDF15 were also increased in MCF-7 and SKBR3 cell lines after PCE treatment. Growth arrest and DNA-damage-inducible alpha, GADD45A, is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. GADD45A is also a candidate breast cancer susceptibility gene because its product participates in DNA repair, and it is a downstream gene of p53 and BRCA1, both of which are breast cancer susceptibility genes [46]. Its expression has been shown to be induced by polyphenols such as quercetin [47] and EGCG [48]. Growth differentiation factor 15, GDF15, also known as macrophage inhibitory cytokine and nonsteroidal anti-inflammatory drug-activated 1 is a member of the transforming growth factor beta superfamily and regulates tissue differentiation and maintenance. A pronounced induction of GDF15 expression by oxLDL, C6-ceramide, tumor necrosis factor, and hydrogen peroxide has been described in cultured human activated macrophages [49]. GDF15 has been shown to have antitumorigenic activity, and it is up-regulated in resveratrol-treated cancer cells [50, 51]. In MCF-7 cells, treatment with tocotrienol-rich fraction from palm oil extract (PTRF) induces a significant increase in the expression of GDF15 mRNA and protein levels. The authors suggest that the effects of PTRF on gene expression modulation in MCF-7 cells are due in part to its interaction with ER transcriptional pathway [52]. All these observations indicate that the increase in GADD45A and GDF15 mRNA levels upon incubation with PCE could be linked to the antioxidant properties of PCE [53].

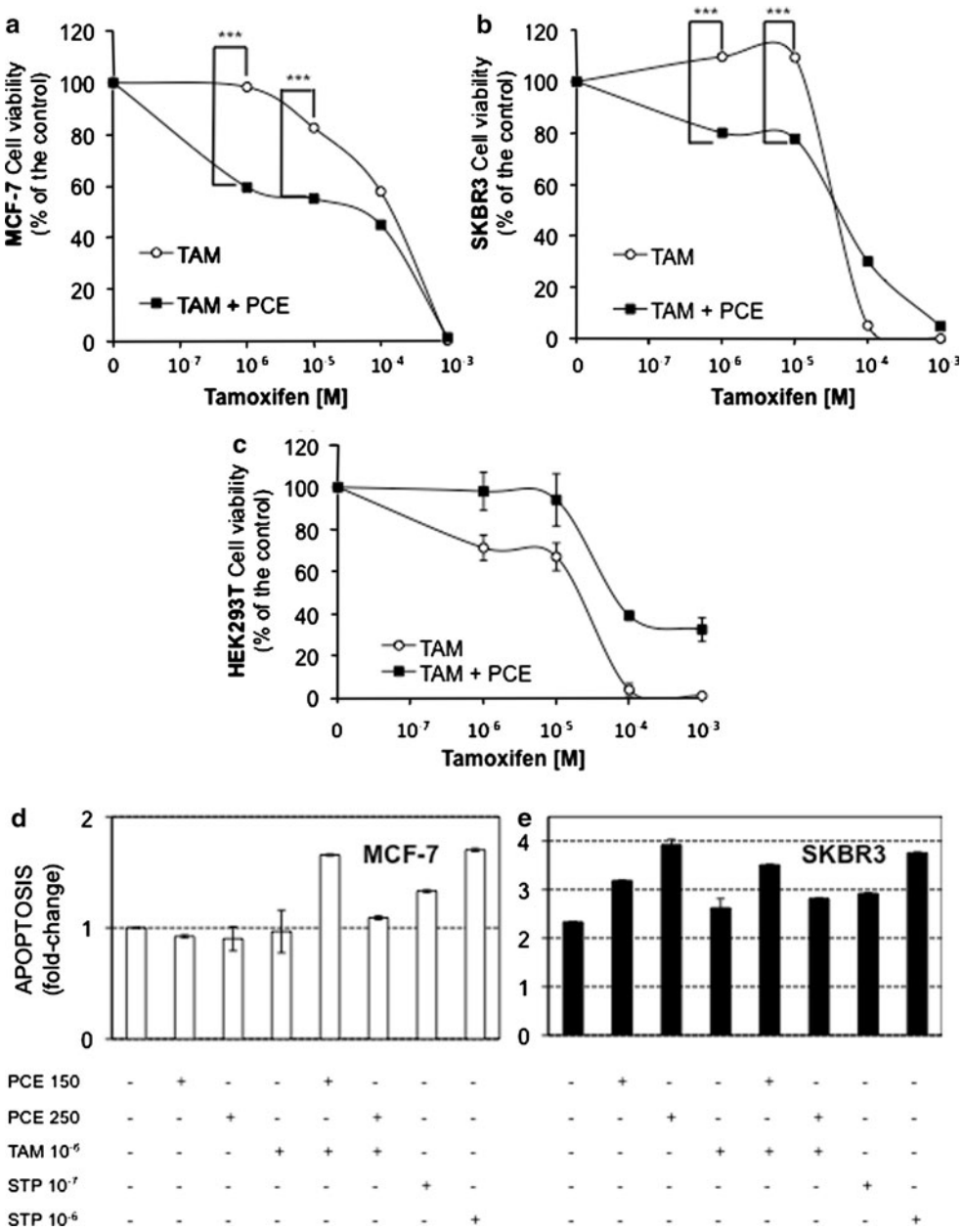

Fig. 5 Effects of tamoxifen plus PCE. Effect on cell viability. Cell viability was determined in MCF-7 (**a**) SKBR3 (**b**) and HEK293T (**c**) cells incubated with the indicated concentrations of Tamoxifen (TAM) either alone (*empty circles*) or in combination with PCE (250 ng/μL for 24 h, *filled squares*). Results are expressed as % of living cells compared to the control only with DMSO (0.22%) and represent the mean ± SE of 3 different experiments. ***$p < 0.001$. **d**, **e** TAM + PCE combination induces apoptotic levels. Levels of apoptosis are represented as fold changes compared to the level of apoptosis in control cells (MCF-7, *empty bars*; SKBR3, *filled bars*) for the different incubation conditions indicated in the figure (TAM; PCE; STP). The levels of apoptosis in the presence of staurosporine (STP) were determined as a positive control. Results represent the mean ± SE of 3 different experiments

Additionally, PCE could trigger an XRE-mediated transcriptional effect for the induction of GADD45A, taking into account that the GADD45A promoter contains an AhR element [54], and its expression is induced by TCDD [55].

The development of a combination therapy that increases the efficacy of tamoxifen has been evaluated. It has been described that the combination of tamoxifen and docetaxel synergistically inhibited the growth of MDA-MB-231, CEM-VBLr, and MCF-7ADr breast cancer cell lines [56]. Similarly, Shen et al. [57] demonstrated synergistic cytotoxicity when MDA-MB-435 cells were treated with tamoxifen and genistein. Synergism has also been reported in vivo, as complete inhibition of DMBA-induced mammary tumors in rats was achieved following treatment with both tamoxifen and 6-MCDF, an aryl hydrocarbon receptor antagonist [58]. EGCG in combination with 4-OH-TAM is synergistically cytotoxic to MDA-MB-231 cells at low concentrations of both tamoxifen and EGCG [34]. The combination of tamoxifen and EGCG elicits an earlier and enhanced apoptotic response in MDA-MB-231 cells [36] and synergistically inhibits the growth of MDA-MB-231 xenografts [35]. The combination of green tea extract and tamoxifen is better than either drug alone at suppressing the growth of MCF-7 xenografts and correlates with increased levels of apoptosis and a suppression of angiogenesis [59]. In our conditions, the cytotoxic effect of tamoxifen was enhanced by the combination with PCE in MCF-7 and SKBR3 cell lines. The presence of PCE caused

a synergistic effect, confirmed by the Chou-Talay method, which led to a decrease in cell viability of up to 40% in MCF-7 cells at tamoxifen concentrations that did not affect cell viability by themselves. Our study also showed the induction of apoptosis in this cell line, as part of the mechanism by which this combination was lethal for breast cancer cells. Additionally, in non-tumor cells, the presence of PCE in combination with tamoxifen was able to reduce the cytotoxic effect of tamoxifen.

In summary, the changes in CYP1A1 expression upon incubation with PCE could explain the antioxidant effect of flavonoids at the molecular level since this gene is involved in different oxidative pathways. Additionally, CYP1A1 overexpression might interfere with estrogen metabolism and the production of estrogen metabolites in breast cells. The increase in CYP1A1 activity may shift estrogen metabolism toward the production of 2-OHE2, a relatively non-genotoxic metabolite [60]. Moreover, the increase in estrogen metabolism could lead to the reduction in the levels of estrogens in mammary tumors, thus contributing to the cytotoxic effect of tamoxifen. Further in vivo studies are necessary to analyze the synergism between tamoxifen and cocoa and to establish the possible benefits of cocoa polyphenol consumption during breast cancer therapy.

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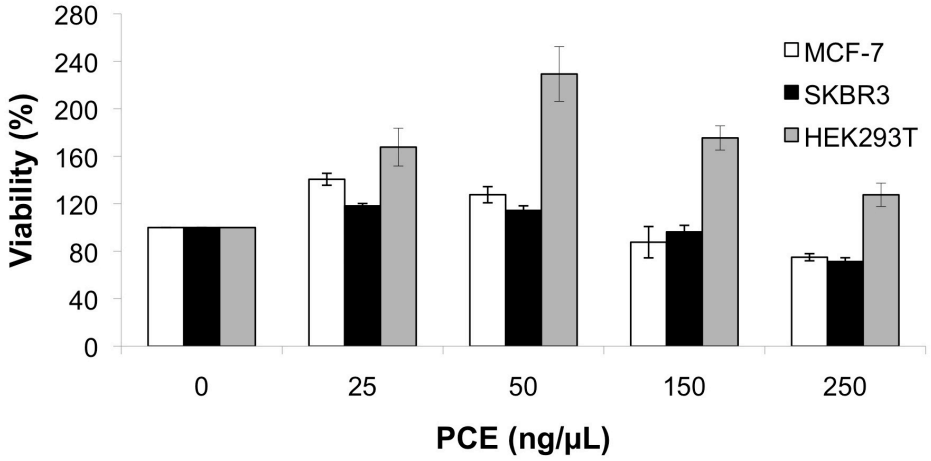
4.1.1 Anexo a ARTÍCULO I:

Al artículo I le acompaña un material suplementario (Online resources) en la página web de la revista científica ((Oleaga, C., et al., 2012)-Supplemetary material) que complementa la investigación. Estos resultados se adjuntan y detallan a continuación.

La citotoxicidad del extracto polifenólico de cacao se evaluó en las tres líneas celulares ensayadas (MCF-7, SKBR3 y HEK293T) y no se observó variación significativa en la viabilidad celular (Online Resource 1).

La incubación con PCE en los dos modelos de cáncer de mama provoca la alteración en la expresión de genes relacionados con toxicidad y estrés oxidativo. Los listados para los genes diferencialmente expresados de MCF-7 y SKBR3 se detallan en las Online Resources 2 y 3.

La combinación de Tamoxifeno y PCE ejerce un efecto citotóxico superior al de los compuestos individuales y al de la suma de los efectos individuales, por ello, es un efecto sinérgico. Con la ayuda del software informático CalcuSyn V2 de Biosoft basado en la ecuación del índice de combinación de Chou-Talalay se confirmó el sinergismo de la combinación TAM y PCE a las concentraciones de TAM 10^{-6} M en ambas líneas celulares y TAM 10^{-5} M en SKBR3. Se muestran las curvas de dosis respuesta (Online Resource 4 a&b) y los valores de CI (Online Resource 4 c&d) para ambas líneas celulares.



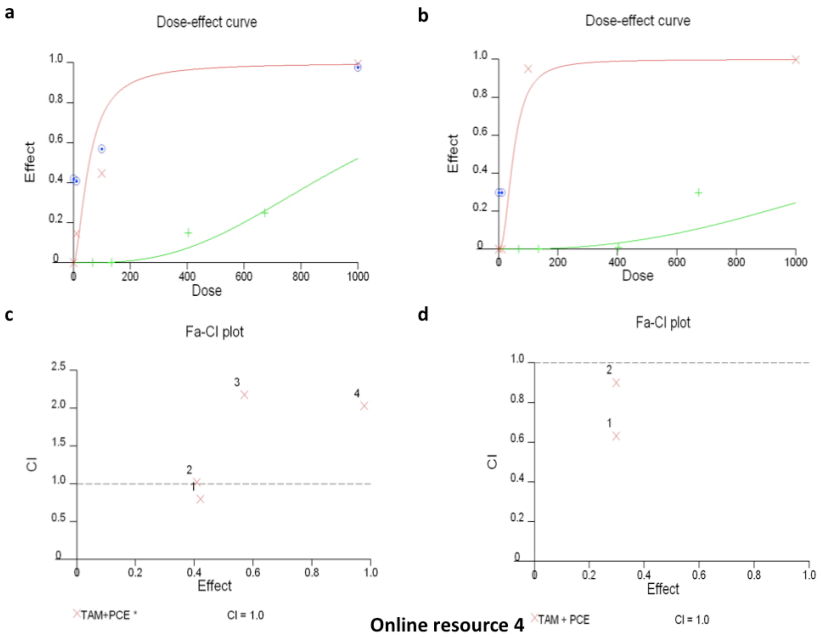
Online resource 1. Cytotoxicity of increasing concentrations of PCE in MCF-7 (white bars), SKBR3 (black bars) and HEK293T cells (grey bars) expressed as the percentage of living cells compared to the control (< 0.4% of DMSO).

Gene Symbol	Fold Difference	T-Test	Fold Up- or Down-Regulation
	Test Sample/ Control Sample	p value	Test Sample/ Control Sample
CYP1A1	17.50	0.0001	17.50
GADD45A	4.20	0.0264	4.20
GDF15	2,61	0.0001	2.61
GPX1	4.25	0.0163	4.25
RAD23A	13.92	0.0394	13.92
SERPINE1	0.02	0.0216	-49.90
TP53	2.26	0.0470	2.26
XRCC2	17.50	0.0356	17.50

Online resource 2. Genes differentially expressed in MCF-7 cells upon incubation with PCE. Genes upregulated (fold difference >2) or downregulated (fold difference <0.5) in MCF-7 treated with cocoa extract compared to control cells with a statistical significance of $p < 0.05$. The fold difference column corresponds to the average fold change in expression of each gene relative to the control. Three independent experiments were performed for each condition.

Gene Symbol	Fold Difference	T-Test	Fold Up- or Down-Regulation
	Test Sample/ Control Sample	p value	Test Sample/ Control Sample
CAT	22.68	0.0205	22.68
CYP1A1	155.29	0.0001	155.29
FMO5	6.55	0.0055	6.55
GADD45A	5.61	0.0027	5.61
GDF15	4.68	0.0046	4.68
HSPA5	78.15	0.0274	78.15
IL18	8.87	0.0241	8.87
LTA	2.51	0.0204	2.51
PTGS1	17.83	0.0058	17.83

Online resource 3. Genes differentially expressed in SKBR3 cells upon incubation with PCE. Genes upregulated (fold difference >2) in SKBR3 cells treated with cocoa extract compared to control cells with a statistical significance of $p < 0.05$. The fold difference column corresponds to the average fold change in expression of each gene relative to the control. Three independent experiments were performed for each condition.



Online resource 4. Synergistic analysis by Chou-Talalay method. The dose-effect curves, in which *Dose* corresponds to the ratio between a fixed PCE concentration and increasing TAM concentrations and *Effect* corresponds to the cytotoxicity, are represented for the different incubation conditions (PCE, TAM or PCE + TAM) in MCF-7 (a) and SKBR3 (b) cells. Fa-CI plots represent the fraction affected (fa; ratio between treated and control) against the Combination Index (CI). Synergism is demonstrated when CI < 1, TAM 10^{-6} M + PCE (x #1) in MCF-7 (c) and SKBR3 cells (d), and TAM 10^{-5} M + PCE (x #2) in SKBR3 cells.

Posteriormente, se llevaron a cabo experimentos de actividad transcripcional y silenciamiento para complementar el trabajo publicado, cuyos resultados se describen a continuación;

Para corroborar la activación de la transcripción del gen *CYP1A1* a través de las cajas XRE por la incubación con PCE se llevaron a cabo ensayos de actividad luciferasa.

La primera aproximación se realizó en un modelo celular diferente, las células HeLa derivadas de cáncer de cuello uterino, con el objetivo de estudiar la activación transcripcional de CYP1A1 mediada por PCE en un ambiente no influenciado por la sobreexpresión de receptores nucleares como ERα o HER2. Para ello, se transfectaron células HeLa (250.000) con un vector reportero que contenía cuatro cajas XRE del promotor del gen CYP1A1 (CYP1A1.LUC) o el vector vacío (LUC), acomplejados con el liposoma X-tremeGENE 9® (Roche Molecular Biochemicals) en una relación 1:3. Seis horas después de la transfección, las células se incubaron con PCE (250 ng/µL) para inducir la transcripción de CYP1A1. La actividad luciferasa se analizó 30 h después de la transfección y se normalizó cada condición con la cantidad de proteína total (Figura Art.I.6).

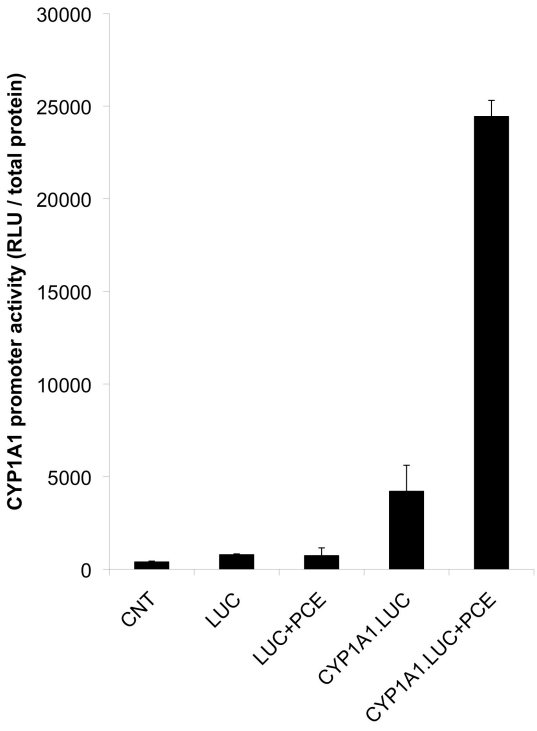


Figura Art.I.6. Actividad transcripcional del promotor de *CYP1A1* en células HeLa. Los vectores LUC o CYP1A1.LUC (1µg) se transfectaron en células HeLa. El tratamiento con PCE se realizó 6h después de la transfección. La actividad luciferasa (RLU) se normalizó con la cantidad total de proteína. Los valores absolutos representados corresponden a la media ±SE de 3 experimentos independientes.

Nuestros resultados demostraron que la activación de la transcripción de *CYP1A1* mediada por PCE ocurría a través de las cajas XRE presentes en el promotor del gen *CYP1A1* transfectado en células HeLa.

Los niveles basales de expresión de CYP1A1 en células SKBR3 son 18 veces superiores a los de MCF-7. En base a este resultado, se analizó el posible efecto que pueda plantear esta diferencia en la transcripción de *CYP1A1* a través de la inducción por PCE. Se llevó a cabo un nuevo ensayo de actividad luciferasa, utilizando las mismas condiciones que en las células HeLa, para detectar las diferencias en la actividad basal del promotor de *CYP1A1* y la inducción por PCE en las líneas MCF-7 y SKBR3 (Figura Art.I.7).

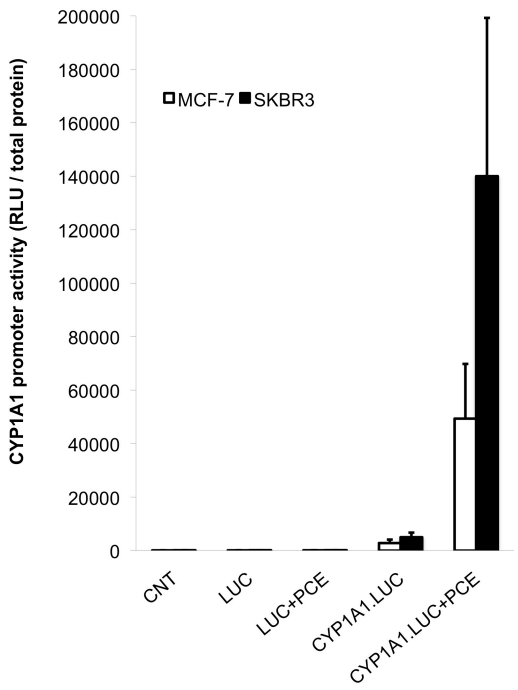


Figura Art.I.7. Actividad transcripcional del promotor de CYP1A1 en células MCF-7 y SKBR3. Los vectores LUC o CYP1A1.LUC (1µg) se transfectaron en células MCF-7 (barras blancas) o SKBR3 (barras negras). Posteriormente (6 h) se efectuó el tratamiento con PCE. La actividad luciferasa (RLU) se normalizó con la cantidad total de proteína. Los valores absolutos representados corresponden a la media ±SE de 3 experimentos independientes.

Los resultados en la Figura Art.I.7 demostraron una mayor actividad transcripcional basal del promotor de *CYP1A1* en las células SKBR3 en comparación con MCF-7 (1,8 veces mayor actividad transcripcional). Además, confirmaron que PCE inducía la transcripción de *CYP1A1* a través de las cajas XRE de su promotor en las líneas celulares MCF-7 y SKBR3. La mayor actividad promotora de las células SKBR3 se mantenía cuando se inducía la transcripción con PCE. Estos nuevos resultados completan los conocimientos sobre la regulación de la transcripción de *CYP1A1* modulada por PCE ya publicados.

Los tumores de cáncer de mama pueden clasificarse según la sobreexpresión de diferentes receptores nucleares y de membrana. Los tres receptores más comunes son el receptor de estrógeno (ER), el receptor de progesterona (PR) y el receptor del factor de crecimiento epidérmico (HER2) (Eroles, et al., 2012). Dependiendo del tipo de receptores que sobreexpresen los tumores mamarios, éstos se clasifican en varios subtipos moleculares (Tabla Art.I.1).

Subtipo Molecular	v (%)	ER/PR/HER2	Línea celular representativa	Dependencia de estrógenos
Basal-like	10-20	ER-, PR-, HER2+	SKBR3*	No
HER2-enriched	10-15	ER-, PR-, HER2+		
Normal breast-like	5-10	ER-/+, HER2-		
Claudin-low	12-14	ER-, PR-, HER2-		
Luminal A	50-60	ER+, PR-+ HER2-	MCF-7	Sí
Luminal B	10-20	ER+/-, PR+/-, HER2+/-	SKBR3*	Sí / No

Tabla Art.I.1. Clasificación de los subtipos de cáncer de mama en base a sus características moleculares (Eroles, et al., 2012). (v) Frecuencia. * SKBR3 no muestra una fuerte correlación con ninguno de los subtipos celulares, el subtipo celular que más se le asemeja es el *Luminal A* y posteriormente el *HER2-enriched* (Jonsson, et al., 2007).

Las dos líneas celulares de cáncer de mama empleadas en nuestro estudio representan dos modelos clásicos de cáncer de mama; el dependiente de estrógenos (ER+), MCF-7 (ER+, PR+, HER2-), y el no dependiente de estrógenos (ER-), SKBR3 (ER-, PR-, HER2+) (Finn, et al., 2009, Hevir, et al., 2011, Hu, et al., 2009).

Los receptores ERα y HER2 se encuentran en niveles distintos en las dos líneas de cáncer de mama estudiadas. Se determinaron los niveles de mRNA basales de los receptores ERα y HER2 en ambas líneas celulares para confirmar estas diferencias mediante RT-PCR a tiempo real con sondas “Assay on demand” (Life Technologies) específicas para cada gen. Las células MCF-7 expresan 120 veces más ERα que SKBR3, mientras que SKBR3 expresan 60 veces más HER2 que MCF-7 (Figuras Art.I.8 y Art.I.9).

Los resultados de los ensayos de co-inmunoprecipitación realizados previamente indicaban la participación de ERα en la transcripción de *CYP1A1* por medio de la vía de señalización de AhR en ambas líneas celulares. A partir de aquí, se planteó la cuestión de si la diferente expresión de los receptores ERα y HER2, en cada modelo celular, podía afectar la sobreexpresión de CYP1A1 inducida por PCE. Por esta razón, se diseñó un experimento donde se silenciaron los receptores (ERα o HER2) y, posteriormente, se analizaron los niveles de

mRNA de CYP1A1 en células control (siRNA) o células incubadas con PCE (siRNA + PCE).

Las células MCF-7 y SKBR3 (30.000) fueron transfectadas con los siRNAs específicos siERα (sc-29405), siHER2 (sc-29305) y los no relacionados siC (sc-44231) y siA (sc-37007) (Santa Cruz Biotechnology) acomplejados con el liposoma Metafecten Pro (Biontex). La concentración de 30nM fue escogida como efectiva y no citotóxica. Veinticuatro horas después se renovó el medio y se incubaron las células con PCE o con el vehículo. Después de 48 h de la transfección se analizaron los niveles de expresión de CYP1A1 de ambas líneas celulares (Figuras Art.I.8 y Art.I.9).

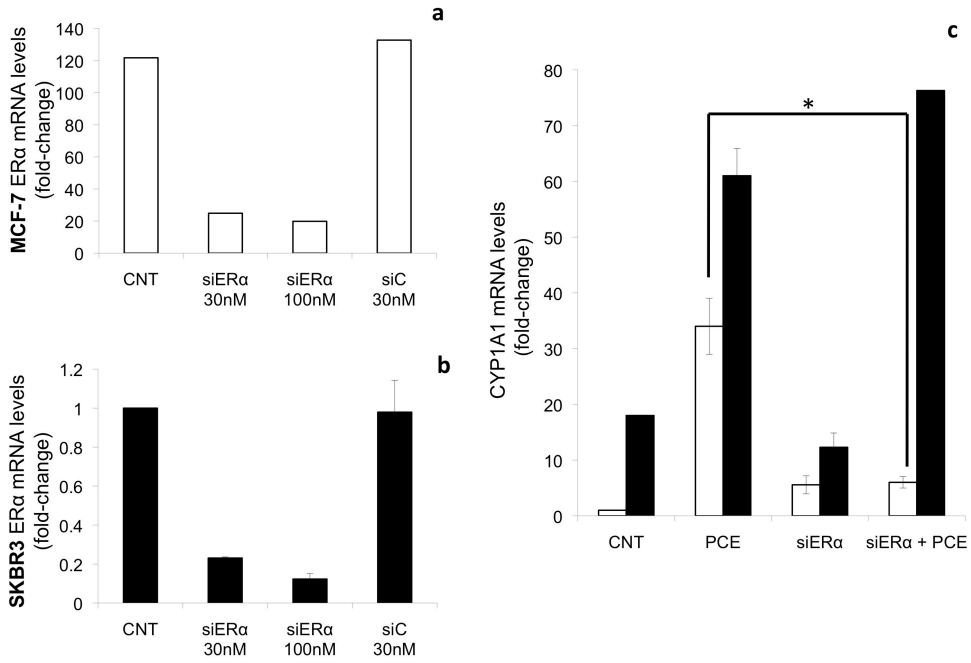


Figura Art.I.8. Efecto del silenciamiento de ERα en la expresión de CYP1A1. El efecto específico del siRNA contra ERα (siERα) a concentraciones de 30 y de 100 nM se demuestra en dos líneas de cáncer de mama, MCF-7 (a) y SKBR3 (b), mediante la determinación de los niveles de mRNA de ERα por RT-PCR a tiempo real. siRNA no relacionado (siC; 30 nM). (c) Los niveles de expresión de CYP1A1 se han analizado en MCF-7 (columnas blancas) y en SKBR3 (columnas negras) control, incubadas con PCE, transfectadas con siERα (30nM), o transfectadas con siERα (30nM) y tratadas con PCE (siERα +PCE). Los resultados representan la media ±SE de 3 experimentos independientes. *p<0.05 comparado con el control correspondiente.

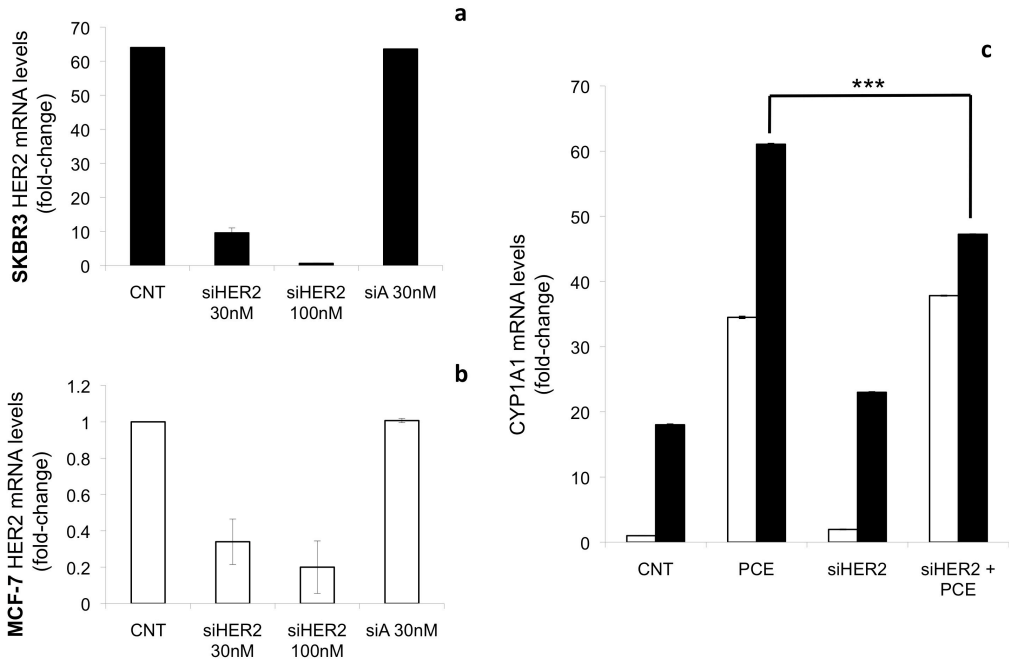


Figura Art.I.9. Efecto del silenciamiento de HER2 en la expresión de CYP1A1. El efecto específico del siRNA contra HER2 (siHER2) a concentraciones de 30 y de 100 nM se demuestra en dos líneas de cáncer de mama, MCF-7 (a) y SKBR3 (b), determinando los niveles de mRNA de HER2 por RT-PCR a tiempo real, siRNA no relacionado (siC; 30 nM). (c) Los niveles de expresión de CYP1A1 se han analizado en MCF-7 (columnas blancas) y en SKBR3 (columnas negras) control, incubadas con PCE, transfectadas con siHER2 (30nM), o transfectadas con siHER2 (30nM) y tratadas con PCE (siHER2 +PCE). Los resultados representan la media ±SE de 3 experimentos independientes. ***p<0.001 comparado con el control correspondiente.

Los resultados obtenidos de los ensayos de silenciamiento demostraron que las sobreexpresiones de los receptores ERα en MCF-7 y HER2 en SKBR3 son imprescindibles, cada uno específico para su línea celular, para la inducción de CYP1A1 mediada por PCE.

Finalmente, se analizó si la sobreexpresión ectópica de ERα era capaz de modificar la activación de *CYP1A1* mediada por PCE en MCF-7 y SKBR3. Para ello, se cotransfectó en ambas líneas celulares un vector de expresión para ERα junto con el vector reportero CYP1A1.LUC. Mediante un ensayo de actividad luciferasa, se analizó la actividad transcripcional del promotor de *CYP1A1* en ausencia y en presencia de PCE (Figura Art.I.10).

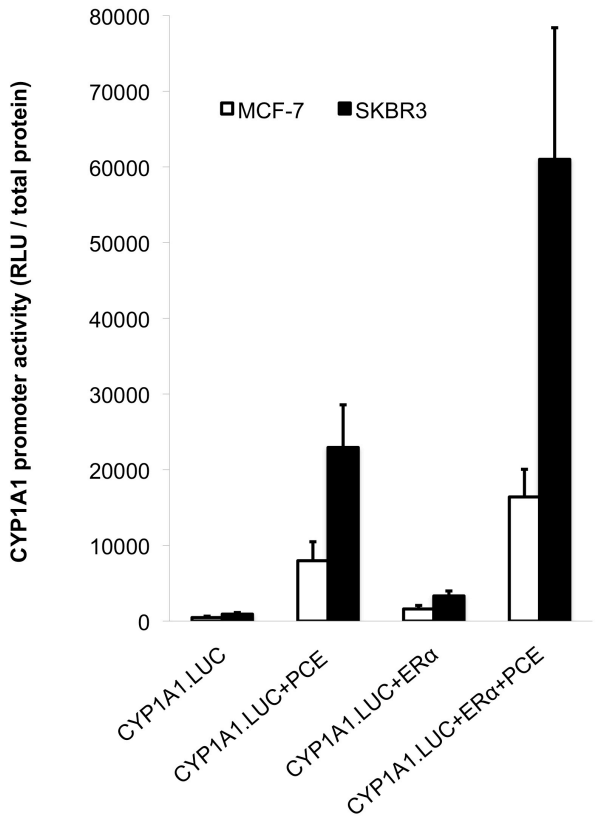


Figura Art.I.10. Efecto de la sobreexpresión de ERα sobre la actividad transcripcional del promotor de CYP1A1. Los vectores reportero LUC o CYP1A1.LUC (500ng) han sido transfectados en células MCF-7 (barras blancas) o SKBR3 (barras negras), solos o en combinación con el vector de expresión para ERα (500ng). Posteriormente (6 h) las células han sido tratadas, o no, con PCE. La actividad luciferasa (RLU) se ha normalizado con la cantidad total de proteína. Los valores absolutos representados son la media ±SE de 3 experimentos independientes.

El ensayo de cotransfección confirmó la implicación de ERα en la inducción de la actividad transcripcional de *CYP1A1* mediada por la incubación con PCE en ambas líneas celulares.

4.2 ARTÍCULO II:

COCOA FLAVANOL METABOLITES ACTIVATE HNF-3β, SP1 AND NFY MEDIATED TRANSCRIPTION OF APOLIPOPROTEIN AI IN HUMAN CELLS

Carlota Oleaga, Carlos J. Ciudad, Maria Izquierdo-Pulido & Véronique Noé

Manuscrito bajo segunda revisión (19 de Octubre 2012) al *Molecular Nutrition and Food Research Journal*. *(Índice de impacto: 4,301. Posición 2/128 en la categoría de Food Science and Technology según índice de impacto)*

Antecedentes: Las propiedades antiaterogénicas de las lipoproteínas de alta densidad (HDL) y su proteína mayoritaria, la Apolipoproteína AI (ApoAI), radican en transportar el colesterol desde tejidos extrahepáticos hacia el hígado para su procesado y excreción. Por ello, el control de los niveles de HDL es importante para la prevención de eventos cardiovasculares. La industria farmacéutica investiga sobre nuevos fármacos capaces de incrementar los niveles de ApoAI como estrategia para controlar los niveles de HDL. Se ha confirmado que algunos compuestos naturales de origen polifenólico como la epicatequina, presente en el cacao, como también algunos de sus metabolitos, son capaces de incrementar los niveles de HDL en plasma y orina.

Objetivos: Identificar el mecanismo de acción por el que la epicatequina y los metabolitos del cacao incrementan los niveles de HDL en humanos, mediante el análisis de los niveles de expresión de ApoAI, y la posible inducción que puedan ejercer los metabolitos del cacao sobre su regulación transcripcional.

Resultados: La incubación con Epicatequina (EPI), Atorvastatina (ATR), Estrona (E1) y los distintos metabolitos del cacao seleccionados (durante 48h) inducían la expresión del gen ApoAI en células de hepatoma HepG2. Ensayos de retardación de la movilidad electroforética mostraban que EPI y el metabolito 3-metil-epicatequina (3-M-EPI) generaban un patrón de 3 bandas proteína/DNA con una sonda correspondiente a la secuencia Site B del promotor del gen *ApoAI*. Además, la unión de los factores de transcripción HNF-3β, Sp1 y NFY a la secuencia Site B estaba inducida por EPI y 3-M-EPI, así como los niveles de proteína de estos factores de transcripción. Finalmente, ensayos de actividad luciferasa confirmaron que la activación de la transcripción de *ApoAI* por los

metabolitos del cacao ocurría a través de la secuencia Site B del promotor del gen.

Conclusiones: Los metabolitos del cacao activan la transcripción de ApoAI a través de la región Site B mediante la sobreexpresión de HNF-3β, NFY y Sp1. El metabolito 3-M-EPI induce la mayor activación transcripcional de *ApoAI*, a través del Site B en células HepG2.

De: Véronica Noé <vnoe@ub.edu>
Asunto: **Fwd: Molecular Nutrition and Food Research mnfr.201200507 - Decision on Manuscript # mnfr.201200507**
Fecha: 11 de octubre de 2012 11:04:17 a.m. GMT+02:00
Para: Carlota Oleaga <coleaga@ub.edu>, MARIA IZQUIERDO PULIDO <maria_izquierdo@ub.edu>, "Carles J. Ciudad" <cciudad@ub.edu>

Inicio del mensaje reenviado:

> **De:** <schreier@pzlc.uni-wuerzburg.de>
> **Fecha:** 11 de octubre de 2012 10:06:06 GMT+02:00
> **Para:** <vnoe@ub.edu>
> **Asunto:** **Molecular Nutrition and Food Research mnfr.201200507 - Decision on Manuscript # mnfr.201200507**
>
> 11-Oct-2012
>
> Dear Prof. Veronique Noe:
>
> Manuscript # mnfr.201200507 entitled "COCOA FLAVANOL METABOLITES ACTIVATE HNF-3β, Sp1 AND NFY MEDIATED TRANSCRIPTION OF APOLIPOPROTEIN AI IN HUMAN CELLS" which you submitted to Molecular Nutrition & Food Research has been reviewed. The comments of the referee(s) are included at the end of this email.
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COCOA FLAVANOL METABOLITES ACTIVATE HNF-3β, Sp1 AND NFY MEDIATED TRANSCRIPTION OF APOLIPOPROTEIN AI IN HUMAN CELLS

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Keywords:	Nutrigenomics, Cocoa metabolites, Polyphenols, ApoAI, SITE B

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COCOA FLAVANOL METABOLITES ACTIVATE HNF-3β, Sp1 AND NFY MEDIATED TRANSCRIPTION OF APOLIPOPROTEIN AI IN HUMAN CELLS

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ABBREVIATIONS: Apolipoprotein AI, **ApoAI**; Adenine Phosphoribosyltransferase, **APRT**; Atorvastatin, **ATR**; Site B luciferase construct, **B.LUC;** Diethyl pyrocarbonate, **DEPC**; Estrone, **E1;** Electrophoretic mobility shifted assay, **EMSA;** Epicatechin, **EPI**; Epicatechin-Sulphate, **EPI-S**; Epicatechin-3-Sulphate, **EPI-3-S**; Estrogen receptor, **ER;** Estrone, **E1;** Hepatic Nuclear Factor-3β, **HNF-3β;** pGL3 basic vector, **LUC;** Nuclear Extracts, **NE**; Relative Luminescence Units, **RLU;** Vanillic Acid, **VA**; 3-hydroxy-phenylacetic acid, **3-HFA**; 3,4-hydroxy-phenylacetic acid, **3,4-HFA**; 3-Methyl-Epicatechin, **3-M-EPI**; 4-Methyl-Epicatechin, **4-M-EPI**;

KEY WORDS Nutrigenomics, Cocoa metabolites, Polyphenols, ApoAI, SITE B.

For Peer Review

ABSTRACT

To identify the mechanisms by which cocoa induces HDL levels and since ApoAI is the major protein in HDL, we analyzed, upon incubation with cocoa metabolites, ApoAI mRNA levels, its transcriptional regulation and the levels of the transcription factors involved in this process.

Epicatechin and cocoa metabolites caused an increase in ApoAI expression in HepG2 cells. Electrophoretic mobility shift assays revealed the involvement of sites A and B of the *ApoAI* promoter in the induction of ApoAI mRNA. Using supershift assays we demonstrated the binding of HNF-3β, HNF-4, ERα and RXRα to Site A and the binding of HNF-3β, NFY and Sp1 to Site B. Luciferase assays performed with a construct containing site B confirmed its role in the up-regulation of ApoAI by cocoa metabolites. Overexpression of NFY, Sp1 or ERα induced *ApoAI* promoter transcription. Moreover, incubation with 3-Methyl-Epicatechin increased the activation of *ApoAI* transcription by NFY, Sp1 and ERα. Epicatechin and 3-Methyl-Epicatechin increased HNF-3β, ERα, Sp1 and NFY protein levels.

The activation of ApoAI transcription through Site B of the *ApoAI* promoter by cocoa flavanol metabolites is mediated by an increase in HNF-3β, Sp1 and NFY levels, as a mechanism for the protective role of these compounds in CVD.

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1. INTRODUCTION

Cardiovascular diseases (CVD) are the number one cause of death representing 30% of mortality in 2008 [1] and atherosclerosis is a mayor risk factor for CVD [2]. Dyslipidemia, an atherogenic precursor, occurs when an imbalance in lipid or lipid transporters (lipoproteins) content in blood is established. Lipoprotein molecules are particles able to carry hydrophobic substances in the hydrophilic environment of plasma, which have been classified, attending to their density into chylomicrons, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Control of lipoprotein metabolism through diet and lifestyle measures and medication has become an essential part of cardiovascular prevention [3].

Nowadays, besides healthy habits strategies and drug established therapies to prevent CVD [4], emerging pharmacological approaches have appeared in this field [5], based on the antiatherogenic properties of HDLs and specifically of their major protein, apolipoprotein AI (ApoAI) [6, 7]. These antiatherogenic particles transport cholesterol from extrahepatic tissues to the liver for further processing and excretion in bile acid. It has been described that retinoic acid, thyroid hormones, glucocorticoids, estrogens and androgens are *ApoAI* promoter activators leading to ApoAI upregulation [8-10]. Additionally, the discovery that naturally occurring compounds, ingested usually with the diet such as some flavonoids, are able to increase HDL and ApoAI levels [11-13], have enlarge the spectrum of molecules that may be effective on lipid homeostasis.

There is substantial interest in the potential role of cocoa and its derived products in prevention and management of CVD [14-18]. Several observational studies supported the association between high cocoa intake and reduced CVD risk and mortality [19, 20]. The potential protective effect of cocoa is ascribed to flavanols that occur as monomers

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(epicatechin and catechin), dimers, oligomers, and polymers, also called procyanidins [21]. Cocoa flavanols are supposed to reduce cardiovascular risk through effects on endotelial function, HDL colesterol, inflammation, platelet function, angiotensin-converting enzyme activity and glucose transport [21-23]. However, health effects of cocoa flavonoids depend on their bioavailability. Epicatechin, the main monomeric flavanol in cocoa, is relatively well absorbed as such from the human intestinal tract [24]. During transfer across the enterocyte and, subsequently, in the liver, epicatechin undergoes O-methylation and other conjugation reactions, namely, glucuronidation and sulfation. The biological activity of these metabolites is expected to be different of that of the parent flavonoids [25]. In addition, low molecular weight phenolics are also produced in the colon by microbial fermentation from procyanidins. These compounds are well absorbed and are excreted in the urine [26]. Scalbert et al [27] suggested that *in vitro* studies should be carried out using the conjugated metabolites, since polyphenols are essentially present in blood and tissues as conjugated metabolites, and there is still limited evidence that they can be deconjugated *in vivo*.

Using a nutrigenomic approach, the aim of our study was to get further insight of the mechanism by which epicatechin and some of its main conjugated metabolites, from phase II (epicatechin-sulphate, epicatechin-3-sulfate, 3-O-methyl-epicatechin, and 4-O-methyl-epicatechin) and microbial (3-hydroxy-phenylacetic acid, 3,4-dihydroxy-phenylacetic acid and vanillic acid) could induce HDL levels in humans. Since ApoAI is the major protein in HDL we analyzed ApoAI mRNA levels, its transcriptional regulation and the levels of transcription factors that could be involved in ApoAI induction upon incubation with cocoa metabolites. Based on our knowledge, this is the first time that cocoa flavanol metabolites have been used in an *in vitro* studied to elucidate the molecular mechanisms underlying the vascular health effects of cocoa.

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2. MATERIALS AND METHODS

Materials and Chemicals

Epicatechin (EPI) and Atorvastatin calcic (ATR) were resuspended in DMSO and Estrone (E1) was resuspended in methanol (all purchase from Sigma). Cocoa flavanol metabolites Epicatechin-Sulphate (EPI-S), Epicatechin-3-Sulphate (EPI-3-S), 3-Methyl-Epicatechin (3-M-EPI), 4-Methyl-Epicatechin (4-M-EPI), 3-hydroxy-phenylacetic acid (3-HFA), 3,4-hydroxy-phenylacetic acid (3,4-HFA) and Vanillic Acid (VA) were resuspended in DMSO as well.

Cell Culture

Human hepatocellular carcinoma HepG2 and Human Cervix Adenocarcinoma HeLa cell lines were routinely grown in Ham's F-12 medium supplemented with 7% fetal bovine serum (FBS, both from Gibco) at 37ºC in a 5% CO_2 humidified atmosphere. 250.000 to 2,500.000 cells were incubated with 10µM EPI [28] from 6 to 72 h. Incubations were also performed with 10µM of ATR [29], E1 (10µM) [30-32] or 10µM of cocoa metabolites (48h). The final concentrations of DMSO and methanol in the culture media were ≤ 0.5 or 0.005% respectively.

RT-Real Time PCR

Total RNA was extracted from HepG2 cells using Ultraspec (Biotex) in accordance with the manufacturer's instructions. Complementary DNA was synthesized as described in Mencia et. al. [33].

mRNA levels were determined in an StepOnePlus Real-Time PCR System (Applied Biosystems) using 3µL of the cDNA reaction. TaqMan technology was used for ApoAI analysis with the assay-on-demand Hs00163641_m1 for ApoAI and Hs00356991_m1 for APRT (all from Applied Biosystems). HNF-3β mRNA levels were determined with SYBR-Green (Biotools B&M Labs) and specific designed primers (Primer-Blast Tool):

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HNF-3β Transcript variant 1 (FW 3' GCCATGCACTCGGCTTCCAGT 5' and RV: 3' AGTAGCCCTCGGGCTCTGCATAGTA 5') and APRT (FW 3' GCAGCTGGTTGAGCAGCGGAT 5' and RV: 3' AGAGTGGGGCCTGGCAGCTTC 5'). The reactions were performed following the manufacturers recommendations. APRT mRNA was used as an endogenous control in both cases. Fold-changes in gene expression were calculated using the standard ΔΔCt method.

Nuclear Extracts

HepG2 or HeLa cells were plated ($5x10^6$) and treated with either EPI, 3-M-EPI or ATR (10μM) or DMSO (vehicle for the control). After 48h of incubation cells where harvested and nuclear extracts (NE) were prepared according to Noé et al. [34], but the amount of triton was reduced to 0.05% for HepG2 cells.

Electrophoretic Mobility Shift and Supershift Assays

The liver specific enhancer of *ApoAI* gene promoter is located between positions -220 and -110 relative to the transcription start site and contains multiple transcription binding sites [35]. The corresponding binding sequences to Site A (-214 to -192) and Site B (-169 to -146) were used as DNA probes for the gel retardation assay (Fig. 1B). Specific primer pairs

5'-TGAACCCTTGACCCCTGCC-3' & 5'-GGCAGGGGTCAAGGGTTCA-3' for site A and 5'-TTTGCCCACTCTATTTGCCCAGCCC-3'

& 5'-GGGCTGGGCAAATAGAGTGGGCAAA-3' for Site B were hybridized, gel purified and further end labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]-ATP (3,000Ci/mmol, Perkin Elmer) to generate the SITE A (19bp) and the SITE B probes (25bp), as in [36].

DNA/Protein binding assays were performed as described Noé et. al [37] using 2μg of nuclear extract, 20,000 cpm of the radioactive probes and 1 μg of Herring Sperm as

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unspecific competitor. After electrophoresis in 5% polyacrylamide and 5% glycerol native gels using 0.5X TBE buffer (89 mM Tris boric acid, 2.5 mM EDTA, pH 8.0), the bindings were visualized using a PhosphorImager with ImageQuant software v 5.2 (Molecular Dynamics).

In the supershift experiments 2µg of antibodies against ERα (rabbit polyclonal sc-544X), HNF-4 (rabbit polyclonal antibody sc-6554X), NFY (goat pAb sc-7711X), RXRα (rabbit pAb sc-553X), Sp1 (rabbit pAb PEP-2 sc-59X), Sp3 (rabbit pAb sc-644X) or 1µg of antibody against HNF-3β (goat pAb sc-6554, all from Santa Cruz Biotechnology Inc) were added to the reaction mixture and incubated on ice for 15 min after the addition of the probe.

Luciferase construct

Site B is a transcription binding sequence belonging to the *cis*-acting elements from the *ApoAI* promoter (-169 to -146) [38-41]. B.LUC luciferase construct was engineered by unidirectional cloning of a 31bp dsDNA sequence containing the site B response element from the *ApoAI* promoter (-169/-146) between the Mlu I and the Xho I sites of the reporter luciferase vector pGL3-basic (Promega) (Fig. 3A). Hybridization of specific primer pairs including the overhand terminus of the restriction enzymes Mlu I and Xho I (underlined) was performed to obtain the insert.

5' CGCGTTTTGCCCACTCTATTTGCCCAGCCCC 3' and

5' TCGAGGGGCTGGGCAAATAGAGTGGGCAAAA 3'

Transfection, Cotransfections and Luciferase Assay

2,5 x 10^5 HeLa cells were plated in 6-well dishes the day before transfection. The medium (2mL) was renewed before transfection that was performed with the X-tremeGENE 9 (Roche Molecular Biochemicals). For each well, the transfection reagent was incubated for 5 min with 100µL of serum & antibiotic-free medium, followed by

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the addtion of the plasmidic DNA and incubated for other 20 min (ratio of 3:1 (µL of transfection reagent: µg of plasmid DNA), all at room temperature. One µg of plasmidic DNA, either pGL3 basic vector (LUC) or B.LUC, were used. In cotransfections 500 ng of B.LUC and 500 ng of NFYA, Sp1, ERα or Sp3 expression vectors (pNFYA13 [42], pSp1CMV [43], pKCR2-ER [44], pSp3CMV [44]) where mixed before the addition of the transfection reagent.

Treatment with 10µM of EPI, ATR or cocoa selected metabolites was performed 6h after transfection, and luciferase activity was determined 30 h after transfection. Cell extracts were prepared by lysing the cells with 200µL of freshly diluted 1X Reporter Lysis Buffer (25mM Tris-Phosphate pH7.8, 2mM DTT, 2mM EDTA, 10% glycerol, 1% Triton X_100). The lysate was centrifugated at 12,000 g for 2 min (4ºC) to pellet the cell debries. The supernatants were transferred to a fresh tube. Fifteen µL of the extract was added to 15µL of the luciferase assay substrate (Promega) at room temperature. Samples luminescence were measured 2 sec after mixture in the Glomax™ 20/20 Luminometer, in which the light production (relative luminescence units; RLU) was measured with 5 sec integration during 10 sec. Each transfection was performed in three different experiments. Luciferase results were corrected by total protein concentration using the Bio-Rad protein assay reagent Bradford according to the manufacturer's protocol.

Database searching

Searching of the sequence of the *ApoAI* promoter was performed using human BLAST (UCSC Genome Bioinformatics Site). The presence of putative binding sites in the Site B sequence from the *ApoAI* promoter was analyzed using the Match™ 1.0 tool that uses a library of mononucleotide weight matrices from TRANSFAC® 6.0 in order to predict transcription factor binding sites (TFBS) in DNA sequences.

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Western blot

40 µg of NE were resolved on SDS-polyacrylamide gel (7/4 %) and transferred to PVDF membranes (Immobilon P, Millipore) using a semidry electroblotter. The membranes were probed with anti-HNF-3β (goat polyclonal antibody sc-6554), anti-Sp1 (rabbit pAb PEP-2 sc-59X), anti-ERα (rabbit pAb sc-544X) or anti-NFY (goat pAb sc-7711X). Signals were detected by secondary HRP-conjugated antibodies anti-goat (sc-2056) or anti-rabbit (P0399 Dako). Enhanced chemiluminiscence (ECL™ Prime Western Blotting Detection Reagent) and detection (ImageQuant LAS 4000 Mini) was performed as manufacturers recommendations (GE Healthcare, Amersham). Blots were reprobed with antibody Oct-1 (sc-232 (C-01) all purchase from Santa Cruz Biotechnology Inc) in order to normalize the results.

Statistical methods

Values are expressed as the mean ± SE. Data were evaluated by unpaired Student's t test when analyzing the difference between two conditions, control and treated. One-way ANOVA followed by Bonferroni post hoc multiple range test was used for different conditions that differ in one parameter (i.e. time). Both analyses were performed using the software PASW Statistics v18.0.0. Differences with p-values < 0.05 were taken as statistically significant.

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3. RESULTS

Epicatechin and Atorvastatin increase ApoAI mRNA levels in HepG2 cells

It had been previously described that cocoa polyphenols increased ApoAI protein levels in HepG2 cells [13]. To determine whether this increase in protein levels was a consequence of an increase in ApoAI mRNA levels, we treated HepG2 cells treated with EPI or ATR (10μM) for different time periods. Total RNA was extracted and ApoAI expression was analyzed by RT-Real Time PCR. A maximum in ApoAI overexpression (>1.5-fold) was observed upon incubation with EPI at 48h (Fig. 1A). ATR was used as a positive control since statins induce human ApoAI mRNA levels and promoter activity in HepG2 cells [45, 46].

Epicatechin increases the binding of nuclear proteins to Sites A and B from the *ApoAI* promoter in HepG2 cells

To evaluate if ApoAI overexpression at the mRNA level was a consequence of an increase in *ApoAI* promoter transcription, we analyzed the binding of transcription factors to sites A and B of the *ApoAI* promoter (Fig. 1B) by EMSA. As shown in Fig.1C, nuclear proteins from HepG2 cells bound to the DNA probes SITE A and SITE B, originating three shifted bands in both cases, that were increased when cells were incubated with 10μM EPI for 48h.

Binding of nuclear factors to Sites A and B from *ApoAI* promoter in HepG2 cells treated with EPI.

To identify the nuclear proteins responsible for the binding observed to sites A and B, we performed supershift assays with different antibodies against transcription factors known to bind to the *ApoAI* promoter [35] and nuclear extracts from either control HepG2 cells or treated with EPI. For the Site A probe, the band with the intermediate mobility corresponded to the binding of ERα, HNF-3β and HNF-4 and the band with

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higher mobility corresponded to the binding of HNF-4 and RXRα (Fig. 2A), taking into account the disappearance of the different shifted bands. For the Site B probe, the band with the intermediate mobility corresponded to the binding of HNF-3β (Fig. 2B).

Luciferase activity of construct-Site B (-169 to -146 *ApoAI* promoter region) upon incubation with EPI, E1, ATR and cocoa metabolites.

To analyze whether the increase of binding of transcription factors to site B led to a transcriptional activation of the *ApoAI* promoter, transient transfection assays with a luciferase reporter vector containing the Site B from the ApoAI promoter (B.LUC, Fig. 3A) were performed in HeLa cells. As shown in Fig. 3B, the presence of the sequence for site B induced luciferase activity 1.6 fold compared to the basic vector LUC. Treatment with EPI at 10μM increased by 41% the transcription activity of B.LUC (Fig. 3B). EMSA assays were performed to confirm that HeLa nuclear extracts were able to bind to Sites A and B probes with the same pattern observed in HepG2 cells (Fig. 3C). In addition, the effect of the seven selected metabolites from cocoa, ATR and estrone on the induction of ApoAI expression through the activation of Site B was also evaluated. Estrone (E1) is an estradiol precursor and was used as a positive control since it has been demonstrated to activate ApoAI expression through the Site B region [11].

As shown in Fig. 4, all compounds were able to increase luciferase activity from the B.LUC construct. Methylated and phenylacetic acid metabolites, as well as VA led to the highest luciferase activity, with a maximum for 3-M-EPI. An increase in luciferase activity was also observed with ATR in accordance with previous results in HepG2 cells with cerivastatin and pitavastatin [46]. Finally, the incubation of HeLa cells with estrone led to a 1.5-fold increase in luciferase activity.

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Cocoa metabolites increased ApoAI and HNF-3β mRNA levels

Total RNA was extracted from HepG2 cells treated with EPI, ATR or cocoa selected metabolites (10μM) for 48h. ApoAI and HNF-3β mRNA levels were analyzed by RT-Real Time PCR, to determine whether the stimulatory effect of cocoa metabolites, EPI and ATR on ApoAI transcription could involve the induction of HNF-3β since it binds to Site B. A maximum induction of ApoAI was observed with 4-M-EPI (2.67 fold), VA (1.8 fold), ATR (1.6 fold) and phenylacetic acid metabolites 3-HFA and 3,4-HFA (1.55 and 1.66 fold respectively). HNF-3β expression was induced by ATR (2.23 fold), EPI (1.85 fold), 3,4-HFA (1.92 fold), 3-HFA (1.85 fold) and 3-M-EPI (1.73 fold) (Fig. 5). Furthermore, incubation of HepG2 cells with 3-M-EPI originated the same binding pattern to the Site B probe as EPI in the gel-shift assays (data not shown).

NFY and Sp1 bind to Site B and induce ApoAI promoter activity through this binding sequence.

We have shown that the binding pattern to Site B probe generates three Protein-DNA complexes and the binding of HNF-3β corresponds to the intermediate shifted band (Fig. 2B). To identify the transcription factors responsible for the additional shifted bands, we searched out for other putative binding sequences within Site B using the Match bioinformatic tool. Putative transcription factor binding sites for NFY; 5'-tgcCCACTcta-3' (CORE > 0.89 and matrix > 0.83) and Sp1; 5'-gccCAGCCcc-3' (CORE > 0.96 and matrix > 0.94) were chosen for further validations (Fig. 6A). Transcriptional activation of the *ApoAI* Site B promoter was analyzed by cotransfecting B.LUC together with expression vectors encoding for the different transcription factors that might be binding to Site B. As shown in Fig. 6B, the overexpression of NFY or Sp1 in HeLa cells induce *ApoAI* promoter transcription through Site B, whereas Sp3 was not able to induce luciferase activity. Overexpression of ERα also increased luciferase

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activity to the same level as Sp1. Moreover, incubation with 3-M-EPI increased the activation of *ApoAI* transcription by NFY, Sp1 and ERα (Fig. 6B).

The binding of NFY and Sp1 to the Site B from the *ApoAI* promoter was demonstrated by supershift assays. Of the three shifted bands, the lower mobility bands were due to Sp1 and NFY binding, whereas the intermediated mobility band corresponded to Sp1, NFY and HNF-3β. An antibody against Sp3 was used as a negative control in the supershift experiments (Fig. 6C).

HNF-3β, ERα, Sp1 and NFY protein levels upon incubation with EPI, 3-M-EPI and ATR in HepG2 cells

The effect observed on HNF-3β mRNA levels was translated into protein levels. In HepG2 cells incubation with EPI, 3-M-EPI and ATR increased HNF-3β protein levels by at least 6-fold (Fig. 7). The levels of ERα, Sp1 and NFY proteins were also analyzed in the same conditions. In all cases an increase in the protein levels of at least 1.5-fold was observed for all the compounds used in cell incubations (Fig. 7).

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4. DISCUSSION

The main objective of our work was to get further insight into the mechanism by which cocoa polyphenols are able to increase the levels of the major protein of HDL, apolipoprotein AI.

The levels of ApoAI mRNA were analyzed after Epicatechin (EPI) and Atorvastatin (ATR) incubations in HepG2 cells. Both, ATR, used as a positive control, and EPI, the major monomeric flavanol in cocoa, induce ApoAI mRNA levels in agreement with Yasuda et al. [13]. The transcriptional regulation of ApoAI is controlled by a short sequence located in its promoter, the liver-specific enhancer. This region is a binding sequence for multiple transcription factors and it is divided in three principal sites (Site A, Site B and Site C) [35]. The binding of nuclear proteins to Sites A and B was analyzed after the induction of ApoAI expression by incubation with EPI. The binding of HepG2 nuclear extracts to both probes was determined as well as its induction upon incubation with EPI, indicating that a higher amount of transcription factors were able to bind to these sequences probably activating *ApoAI* transcription. Then we studied the interacting factors responsible for ApoAI overexpression mediated by cocoa. The presence of Site A interacting factors such as HNF-4 and RXRα were identified in accordance with previous results [35, 38, 41]. ERα was discarded as a possible binding factor due to the inexistence of the classical estrogen response element, even if two ERE half-palindromic sites are located within the Site A where ERα could be bound [11]. Additionally, we analyzed the binding of HNF-3β to the Site A; it has been reported that this factor cooperates with HNF-4 to facilitate the accession of the mediator and RNA polymerase II to initiate transcription [47], even though there are no binding sites for HNF-3β located in the Site A probe. The binding of HNF-3β to Site A was confirmed by the addition of a specific antibody against HNF-3β, that caused a reduction of the

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shifted band. Surprisingly, the combination of antibodies against HNF-3β and HNF-4 caused less reduction on the binding to Site A than the HNF-4 antibody alone, suggesting a possible competition between HNF-3β and HNF-4. In this direction it had been described that HNF-4 and HNF-3β activated synergistically the minimal enhancer region from the hepatocyte-specific enhancer in the 5'-flanking region of *ApoAI* [39]. In this case, synergy was dependent upon simultaneous binding of these factors to their cognate sites, but not due to cooperativity in DNA binding, and the authors suggested the existence of a cell type-restricted intermediary factor jointly recruited by HNF-4 and HNF-3β that participates in activation of the apoAI enhancer in liver cells. Using the Site B probe, the interaction of transcription factor HNF-3β was confirmed by a very clear supershift, which was not the case for ERα or HNF-4.

In addition to the characterization of the transcription factors bound to Sites A and B, promoter activity was also evaluated for the sequence corresponding to Site B. This site has been described to mediate the effects of the isoflavone genistein by Lamon-Fava et al. [11], who identified the Site B as the region required for ApoAI activation upon incubation with genistein as well as with estradiol. Taylor et al. [48] also determined the activation of ApoAI through Site B by estrogen. Our results from luciferase experiments demonstrate that EPI is able to induce ApoAI transcription through Site B, as well as cocoa metabolites EPI-S, EPI-3-S, 3-M-EPI, 4-M-EPI, 3-HFA, 3,4-HFA or VA, and ATR and E1. The cocoa metabolite 3-M-EPI led to the maximum activation of B.LUC and induced the binding of nuclear proteins such as HNF-3β, NFY and Sp1 to Site B. Even if the NFY consensus in Site B was not the typical CCAAT sequence, the binding of NFY to Site B had been previously reported [49], as well as its role as a positive activator of the *ApoAI* gene promoter [50]. The involvement of Sp1 in the transcription of the *ApoAI* promoter is well documented. The increase in Sp1 binding to the *ApoAI*

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promoter and the ability of exogenous Sp1 to induce *ApoAI* reporter gene expression has already been described upon insulin administration [51, 52]. Furthermore, activation of PKA or PKC increase *ApoAI* promoter transcription and the activity of both signaling pathways is mediated by the insulin-responsive core element, a motif that binds the transcription factor Sp1 [53]. Sp1 is also required for the actions of EGF mediated by the Ras-mitogen-activated protein, MAP kinase, to activate *ApoAI* transcription [32]. Protein levels for HNF-3β, NFY and Sp1 were analyzed upon EPI, ATR or 3-M-EPI incubations, showing that the three transcription factors were induced in all these conditions. Thus, the increase in Site B activity could be due to the overexpression of HNF-3β, NFY and Sp1 caused by these compounds. Changes in HNF-3β mRNA levels have been already described in diabetic rats, accompanied by significant increases in binding activity [54]. Furthermore, Vecchini et al. observed an increase in HNF-3β expression upon the addition of polyunsaturated fatty acids to the diet of ACT/I rats [55].

In summary our work extended the study of the mechanism by which cocoa is able to increase HDL-cholesterol in plasma. It is worth noting the use of the most representative cocoa flavanol metabolites since they are the responsible of the final effects observed in their targets. 3-M-EPI has been identified as the most potent *ApoAI* transcriptional activator through the Site B region of the liver-specific enhancer. In addition we show that the regulation of *ApoAI* through the Site B involves HNF-3β, NFY and Sp1 transcription factors through their direct binding to the promoter. ERα also participated in the regulation of *ApoAI* Site B promoter but through an indirect way.

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LEGENDS TO FIGURES

Figure 1. Epicatechin induces ApoAI mRNA levels as well as binding to Sites A and B from liver specific enhancer of *ApoAI* gene promoter

A) 250.000 HepG2 cells were incubated with either Epicatechin (EPI; empty bars) or Atorvastatin (ATR; filled bars) (10µM) from 6 to 72 h. ApoAI mRNA levels were determined by RT-Real Time PCR. Values are the mean ± SE of three independent experiments. Significant differences at all time points were evaluated by ANOVA analysis, plus post hoc Bonferroni comparisons. B) Schematic drawing of the liver-specific enhancer sequence from the *ApoAI* promoter showing the transcription factors binding sites A, B and C located at -214 to -192, -169 to -146 and -134 to -119 positions relative to the transcription start site, respectively. C) EMSA were performed using as probes the sequences corresponding to Site A (19bp) or Site B (25bp) within the *ApoAI* promoter and nuclear extracts (NE) from control HepG2 cells or cells treated with EPI (10µM) for 48h. Free Probes (Sites A or B) are indicated. GS stands for shifted bands.

Figure 2. Supershift assays

EMSA were performed using as probes the sequences corresponding to Site A (19bp) (A) or Site B (25bp) (B) within the *ApoAI* promoter and nuclear extracts (NE) from control HepG2 cells or cells treated with EPI (10µM) for 48h. The supershifts were performed with specific antibodies against ERα, HNF-3β, HNF-4 or RXRα. GS and SS stand for shifted and supershifted bands respectively.

Figure 3. Epicatechin induces *ApoAI* promoter transcription through Site B

A) Schematic representation of the Site B construct (B.LUC) in a pGL3 basic vector (LUC). The location of Site B in the liver-specific enhancer sequence from the *ApoAI* promoter is highlighted. B) 1 µg of LUC (empty bars) or B.LUC (filled bars) vectors were transfected in HeLa cells, and 6 hours later, Epicatechin (EPI, 10µM) was added.

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Luciferase activity (Relative light units, RLU) was determined 30 h after transfection and the values for each sample were normalized to total protein concentration (mg/mL). Absolute values (RLU/protein) of control or EPI treated cells represent the mean ± SE of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared with the corresponding control.

Figure 4. Site B promoter activity upon incubation with cocoa metabolites

1 µg of LUC (empty bars) or B.LUC (filled bars) vectors were transfected in HeLa cells, and 6 hours later 10µM EPI, E1, ATR or Cocoa metabolites EPI-S, EPI-3-S, 3-M-EPI, 4-M-EPI, 3-HFA, 3,4-HFA or VA were added to the culture medium. Luciferase activity (Relative light units, RLU) was determined 30 h after transfection and the values for each sample were normalized to total protein concentration (mg/mL). Values represent the mean ± SE of three independent experiments and results are expressed relative to the luciferase activity of the vector alone. *p<0.05, **p<0.01 and ***p<0.001 compared with the corresponding control.

Figure 5. ApoAI and HNF-3β mRNA levels are upregulated by cocoa metabolites.

250.000 HepG2 cells were incubated with 10µM EPI, E1, ATR or Cocoa metabolites EPI-S, EPI-3-S, 3-M-EPI, 4-M-EPI, 3-HFA, 3,4-HFA or VA for 48 h. ApoAI (empty bars) and HNF-3β (filled bars) mRNA levels were determined by RT-Real Time PCR. Values represent the mean ± SE of three independent experiments. *p<0.05 and **p<0.01 compared with the corresponding control.

Figure 6. 3-M-EPI induces the activation of *ApoAI* promoter transcription through the binding of HNF-3β, NFY and Sp1 transcription factors to Site B

A) Schematic drawing of the binding sequences for transcription factors HNF-3β, NFY and Sp1 in Site B of the *ApoAI* promoter. B) 500 ng of the B.LUC construct together with the expression vectors for NFY, Sp1, ERα, or Sp3 were cotransfected in HeLa

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cells, and 6 h later, 10μM of 3-M-EPI were added to the culture medium. Luciferase activity (Relative light units, RLU) was determined 30 h after transfection and the values for each sample were normalized by total protein concentration (mg/mL). Absolute values (RLU/protein) for control cells (empty bars) or 3-M-EPI treated cells (filled bars) represent the mean ± SE of three independent experiments. *p<0.05 and **p<0.01 compared with the corresponding control. C) EMSA was performed using as a probe the sequence for Site B (25bp) and nuclear extracts (NE) from control HepG2 cells or cells treated with 3-M-EPI (10μM) for 48h. Supershift mobility assays were performed with specific antibodies against HNF-3β, NFY, Sp1 or Sp3. GS and SS stand for shifted and supershifted bands respectively.

Figure 7. HNF-3β, ERα, NFY, and Sp1 protein levels in HepG2 cells upon incubation with EPI, 3-M-EPI and ATR.

The protein levels of HNF-3β (black bars), ERα (dark grey bars), Sp1 (light grey bars) and NFY (white bars) were determined by Western blot in control or treated cells. Blots were normalized to total protein loading. Values represent the mean ± SE of 3 different experiments and are expressed relative to control (100%).

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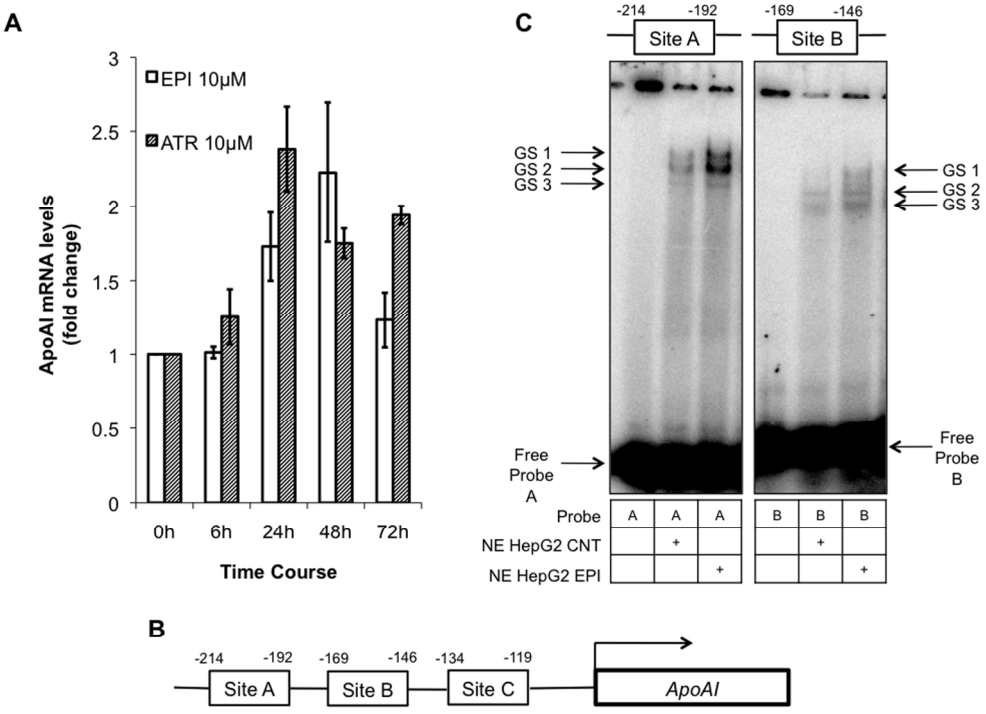

Figure. 1

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Review

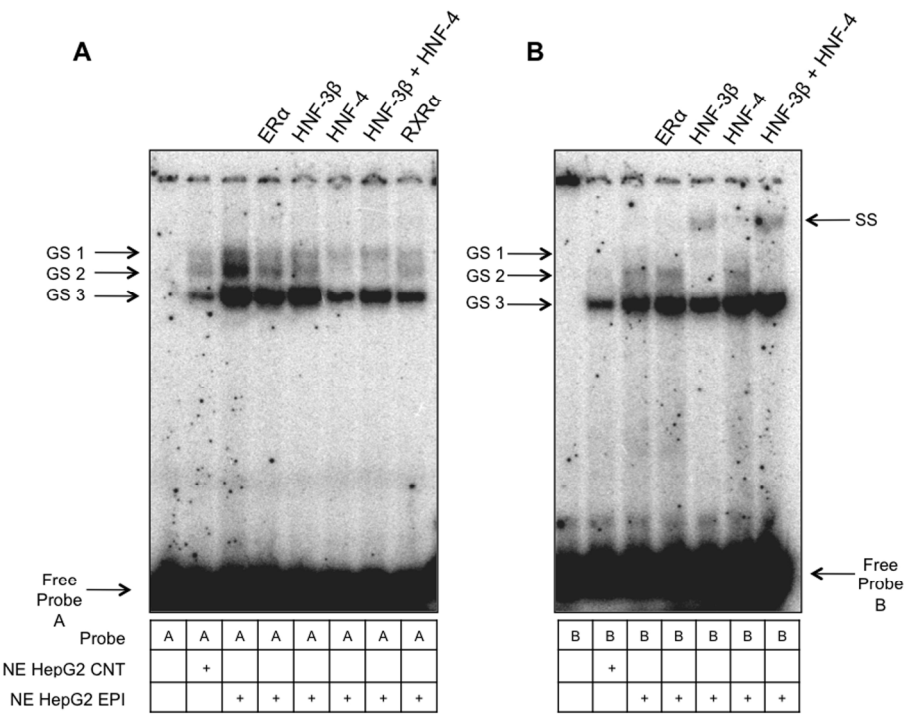

Figure. 2

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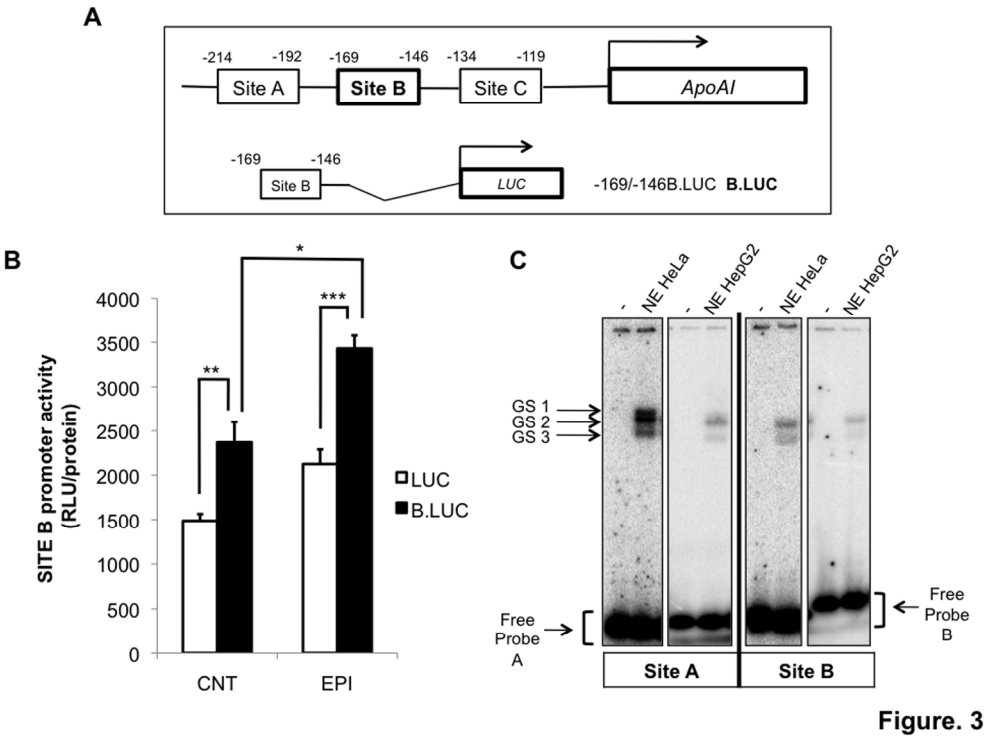

Figure. 3

126x93mm (600 x 600 DPI)

Review

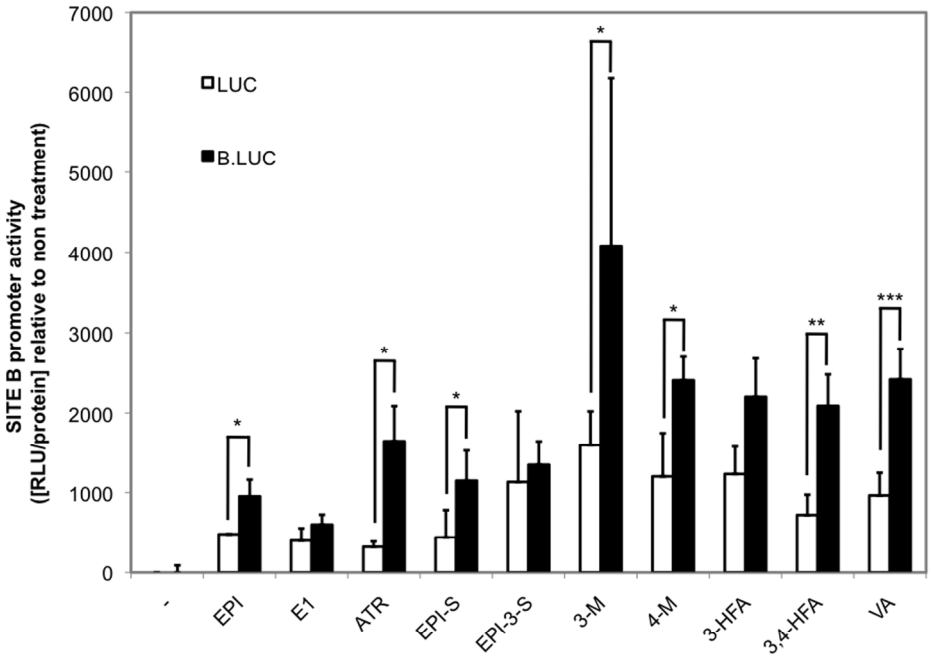

Figure. 4

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Review

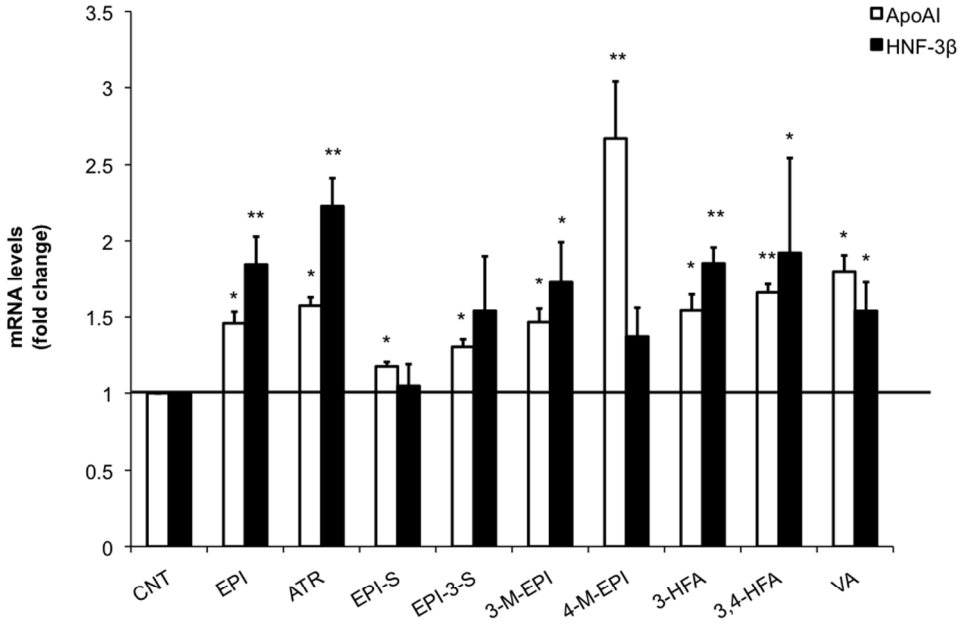

Figure. 5

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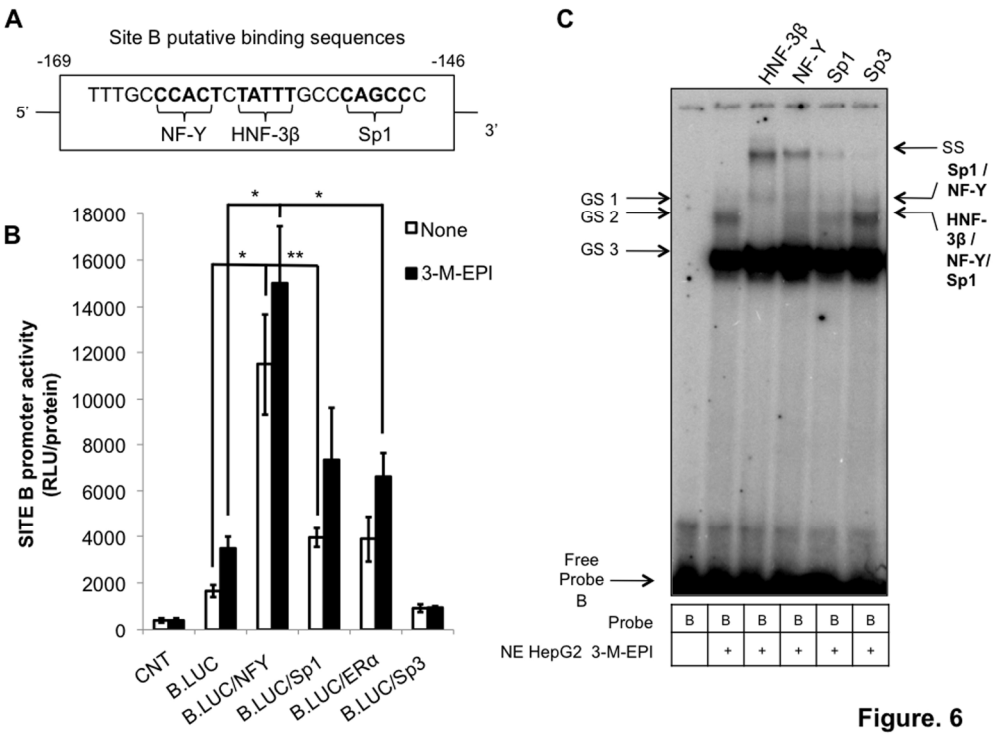

Figure. 6

124x91mm (600 x 600 DPI)

Review

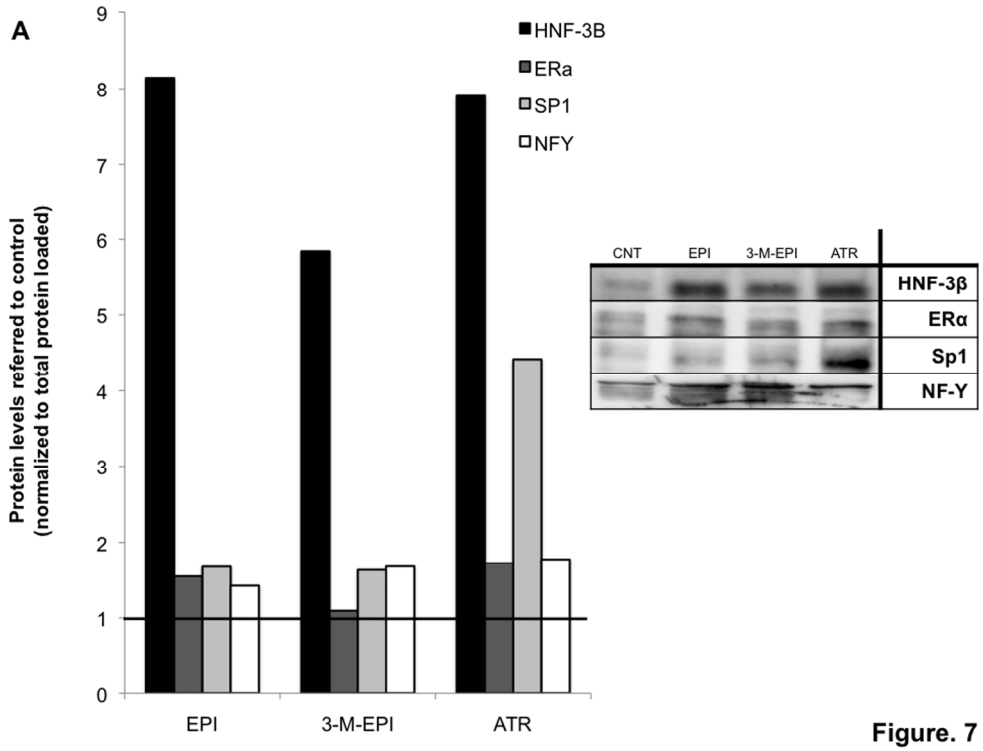

Figure. 7

128x96mm (600 x 600 DPI)

Review

4.3 ARTÍCULO III:

COFFEE POLYPHENOLS CHANGE THE EXPRESSION OF STAT5B AND ATF-2 MODIFYING CYCLIN D1 LEVELS IN CANCER CELLS.

Carlota Oleaga, Carlos J. Ciudad, Véronique Noé & Maria Izquierdo-Pulido

Oxidative Medicine and Cellular Longevity 2012, *2012*, 1-17. (Article ID 390385).
(Índice de impacto: 2,841. Posición 104/180 en la categoría de Cell Biology según índice de impacto)

Antecedentes: Diversos estudios epidemiológicos han demostrado que el consumo de café provoca un efecto inversamente proporcional al riesgo de desarrollar ciertos tipos de cáncer, pero el mecanismo biológico preciso todavía se desconoce. Existen datos experimentales que otorgan características antitumorales a los compuestos fenólicos que contiene el café.

Objetivo: Realizar un análisis genómico funcional para determinar cómo el ácido cafeico (CA) del café afecta la expresión del genoma humano. El modelo escogido para realizar este análisis ha sido el de cáncer de colon por la asociación descrita entre el consumo de café y menor riesgo de desarrollar este tipo de cáncer.

Se han llevado a cabo experimentos con microarrays después de incubar células humanas de adenocarcinoma de colon HT29 con CA o con una solución de café soluble cafeinado (ICC), para comparar qué genes aparecen diferencialmente expresados y son comunes a ambos tratamientos.

Resultados: El tratamiento con CA inducía la sobreexpresión de 12 genes y la infraexpresión de 32, mientras que la incubación con ICC generaba más variaciones con 57 genes sobreexpresados y 161 infraexpresados. A partir de los genes modulados en común a ambos tratamientos y mediante el uso de una base de datos bibliográfica, se construyó una red de asociación biológica (BAN). Esta red o BAN conecta los diferentes genes por conocimientos bibliográficos previos y persigue resaltar los nodos más interconectados, que probablemente serán los de mayor relevancia. Los genes *Signal transducer and activator of transcription 5B (STAT5B)* (sobreexpresado en un 23,8% en ICC y en un 33,4% en CA) y *Activating transcription factor 2 (ATF-2)* (infraexpresado en un 32,5% en ICC y en un 26% en CA) aparecían como los nodos más interconectados del BAN. La diana

STAT5B fue validada a nivel de mRNA por RT-PCR a tiempo real y a nivel de proteína por Western blot en ambos tratamientos. De la misma manera se validó la diana ATF-2, exceptuando a nivel de proteína en el caso del tratamiento con ICC.

Ambos factores de transcripción STAT5B y ATF-2 regulan la ciclina D1. La ciclina D1 es una proteína perteneciente a la familia de las ciclinas y su actividad es necesaria para la transición de G1/S en el ciclo celular. Esta proteína se encuentra sobreexpresada en el 50% de los casos de cáncer de mama.

Se evaluaron los niveles de proteína ciclina D1 en una línea tumoral de cáncer de mama, MCF-7, así como también en HT29, junto con los de STAT5B y ATF-2 modulados por los tratamientos con ICC y CA. En el modelo de cáncer de mama observamos una reducción drástica en los niveles de ciclina D1 mediada por el aumento de STAT5B. Por otro lado, el modelo de cáncer de colon se comportó de manera diferente frente a los tratamientos con ICC o CA, produciendo un aumento en la ciclina D1 a pesar del aumento de STAT5B, o la disminución de ATF-2 a cargo del CA.

Conclusiones: La cantidad de CA presente en una taza de café es capaz de modular los niveles de STAT5B y ATF-2 en dos líneas celulares, HT29 y MCF-7. Además esta regulación afecta a los niveles de ciclina D1 en ambos modelos celulares produciendo un drástico descenso en los niveles de ciclina D1 en MCF-7 a cargo de un aumento de STAT5B, y un aumento de esta ciclina en HT29.

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Research Article

Coffee Polyphenols Change the Expression of STAT5B and ATF-2 Modifying Cyclin D1 Levels in Cancer Cells

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Background. Epidemiological studies suggest that coffee consumption reduces the risk of cancer, but the molecular mechanisms of its chemopreventive effects remain unknown. *Objective*. To identify differentially expressed genes upon incubation of HT29 colon cancer cells with instant caffeinated coffee (ICC) or caffeic acid (CA) using whole-genome microarrays. *Results*. ICC incubation of HT29 cells caused the overexpression of 57 genes and the underexpression of 161, while CA incubation induced the overexpression of 12 genes and the underexpression of 32. Using Venn-Diagrams, we built a list of five overexpressed genes and twelve underexpressed genes in common between the two experimental conditions. This list was used to generate a biological association network in which STAT5B and ATF-2 appeared as highly interconnected nodes. STAT5B overexpression was confirmed at the mRNA and protein levels. For ATF-2, the changes in mRNA levels were confirmed for both ICC and CA, whereas the decrease in protein levels was only observed in CA-treated cells. The levels of cyclin D1, a target gene for both STAT5B and ATF-2, were downregulated by CA in colon cancer cells and by ICC and CA in breast cancer cells. *Conclusions*. Coffee polyphenols are able to affect cyclin D1 expression in cancer cells through the modulation of STAT5B and ATF-2.

1. Introduction

Polyphenols are the most abundant antioxidants in the diet. Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers, and osteoporosis suggesting a role of these antioxidants in the prevention of neurodegenerative diseases and diabetes mellitus [1].

It is well established that polyphenol ingestion results in an increase of the plasma-antioxidant capacity. However, there is still some uncertainties about their efficiency to enhance the protection of cellular components, such as lipids or DNA, against oxidative stress in humans [2]. Polyphenols and other antioxidants were thought to protect cell constituents against oxidative damage by scavenging free radicals. However, this concept now appears to be an oversimplified view of their mode of action [3]. More likely, cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions [4]. This could also apply to the anticarcinogenic effects of polyphenols, which properties may be explained by many different mechanisms.

Hydroxycinnamic acids are a major class of polyphenols found in almost every plant [2]. The major representative of hydroxycinnamic acids is caffeic acid, which occurs in food mainly as an ester with quinic acid named chlorogenic acid (5-caffeoylquinic acid). Coffee is a major source of chlorogenic acid in the human diet; the daily intake in coffee drinkers is 0.5–1 g whereas coffee abstainers will usually ingest <100 mg/day. Studies have shown that approximately the 33% of ingested chlorogenic acid and the 95% of caffeic acid are absorbed intestinally [5]. Thus, about two-thirds of ingested chlorogenic acid reach the colon where it is probably metabolized to caffeic acid [6].

Bioavailability data suggest that the biological effects of chlorogenic acid would become apparent after its metabolism to caffeic acid, and hence the need of studying the effects of this acid. Chlorogenic acid and caffeic acid are

antioxidants *in vitro* [7], and they might inhibit the formation of mutagenic and carcinogenic N-nitroso compounds since they are inhibitors of the N-nitrosation reaction *in vivo* [8]. Furthermore, chlorogenic acid can inhibit DNA damage *in vitro* [9] as it inhibits lipid peroxidation-induced DNA adduct formation [10] and suppresses reactive oxygen species-mediated nuclear factor (NF-κB), activator protein-1 (AP-1), and mitogen-activated protein kinase activation by upregulating antioxidant enzymes [11]. These studies suggested that coffee polyphenols are potent chemopreventive agents.

Recent meta-analyses demonstrate inverse associations between coffee intake and the risk of colon, liver, breast, and endometrial cancer [12–15]. Moreover, in prospective population-based cohort studies, the inverse association between coffee consumption and risk of cancer has been shown. The group of Naganuma [16] found that the consumption of at least one cup of coffee per day was associated with a 49% lower risk of upper gastrointestinal cancer in a Japanese population, while Wilson and collaborators [17] found that men who regularly drink coffee appeared to have a lower risk of developing a lethal form of prostate cancer. The lower risk was evident when consuming either regular or decaffeinated coffee. It has been proposed that the inverse association between coffee intake and colon cancer could be explained, at least in part, by the presence of chlorogenic acid in coffee [18]. Ganmaa et al. [19] observed a general protective effect of caffeine intake on breast cancer risk for both ER subtypes, but the effect was only found to be significant for ER-positive breast cancers. In this study, the association between caffeine and breast cancer was stronger among postmenopausal women with estrogen-receptor and progesterone-receptor-positive breast cancer than those with estrogen-receptor and progesterone-receptor negative breast cancer [19]. In another study, coffee drinking specifically reduced the risk of developing ER-negative breast cancer but not ER-positive breast cancer [20].

Although there is enough evidence from epidemiological data supporting that coffee seems to reduce the risk of certain cancers, the molecular mechanisms underlying the chemopreventive effects of coffee remain unknown. For this reason, the aim of our study was to determine the effect at the molecular level of coffee polyphenols at low concentrations equivalent to one cup of coffee, using as a model a human colon cancer cell line HT29 in a nutrigenomic approach. Furthermore, the effect of coffee polyphenols was also evaluated in breast cancer cells.

2. Materials and Methods

2.1. Materials and Chemicals. Cells were incubated with Instant Caffeinated Coffee (ICC) (regular lyophilized instant coffee) and Caffeic acid (CA, Sigma). Compounds were dissolved either in DMSO (CA), or sterile water (ICC), and stored at -20°C.

2.2. Cell Culture. Colon adenocarcinoma HT29 and breast cancer MCF-7 cell lines were routinely grown in Ham's F12 medium supplemented with 7% fetal bovine serum (FBS, both from Gibco) at 37°C in a 5% CO_2 humidified atmosphere in 10 cm dish, or in 33 mm plate.

Cells were incubated with ICC or CA at concentrations equivalent to one cup of coffee. The concentrations used in cell incubations, 7 μg/mL in H_2O mQ for ICC and 1.68 μg/mL in DMSO for CA, respectively, took into account the amount of these compounds in one cup of coffee and their distribution in a regular human body with 75% water content. These concentrations did not cause any cytotoxic effect in the cell incubations as determined by the MTT assay [21].

2.3. Microarrays. Gene expression was analyzed by hybridization to The GeneChip Human Genome U133A plus 2.0 microarrays from Affymetrix, containing 47,000 transcripts and variants. HT29 cells were incubated with ICC and CA for 24 h. Total RNA was prepared from triplicate samples using Speedtools Total RNA Extraction Kit (Biotools) following the recommendations of the manufacturer. RNA quality was tested by 2100 Bioanalyzer Eukaryote Total RNA Nano Series II (Agilent Technologies). Labeling, hybridization, and detection were carried out following the manufacturer's specifications at the IDIBAPS Genomic Service (Hospital Clínic, Barcelona).

2.4. Microarray Data Analyses. Quantification was carried out with GeneSpring GX v.11.5.1 software (Agilent Technologies), which allows multifilter comparisons using data from different experiments to perform the normalization, generation of lists, and the functional classification of the differentially expressed genes. The input data was subjected to preprocess baseline transformation using the Robust Multiarray Average summarization algorithm using the median of control samples. After grouping the triplicate of each experimental condition, list of differentially expressed genes could be generated by using volcano plot analysis. The expression of each gene is reported as the ratio of the value obtained after each condition relative to control condition after normalization and statistical analysis of the data. The corrected P value cutoff applied was of <0.05; then the output of this statistical analysis was filtered by fold expression, selecting specifically those genes that had a differential expression of at least 1.3-fold. Gene classification was established by the Gene Ontology database.

2.5. Common Genes between ICC and CA Treatments. Common genes were selected from the lists of differentially expressed genes for each treatment using Venn-Diagrams. The newly generated list contained both over and underexpressed genes.

2.6. Generation of Biological Association Networks. BANs were constructed with the aid of the Pathway Analysis within the GeneSpring v.11.5.1 (Agilent) as described in Selga et al. [22] with the list of common genes differentially expressed in both treatments. A filtered screening was processed by the program between our data and bibliographic interaction

databases up to a total of 100 related genes. Network associations were confirmed in the literature.

2.7. RT Real-Time PCR. Total RNA was extracted from HT29 cells using Ultraspec (Biotex) in accordance with the manufacturer's instructions.

Complementary DNA was synthesized as described in Selga et al. [23] and the cDNA product was used for amplification by real time PCR. STAT5B and ATF-2 mRNA levels were determined in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 3 μL of the cDNA reaction and the assays-on-demand Hs00560035_m1 for STAT5B, Hs00153179_ml for ATF-2, and Hs00356991_m1 for APRT (all from Applied Biosystems). APRT mRNA was used as an endogenous control. The reaction was performed following the manufacturers recommendations. Fold changes in gene expression were calculated using the standard $\Delta\Delta$Ct method.

2.8. Western Blot. Whole extracts were obtained from 2.5×10^6 control or treated cells according to Selga et al. [23]. Five μL of the extract was used to determine protein concentration by the Bradford assay (Bio-Rad). The extracts were frozen in liquid N_2 and stored at -80°C. Total extracts (50 μg) were resolved on SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon P, Millipore) using a semidry electroblotter.

The SNAP i.d. protein detection system technology (Millipore) was used to probe the membranes. This system applies vacuum through the membrane to actively drive reagents to protein locations, unlike the traditional technique of diffusion over the membrane as a reagent transport. Table 1 compiles the antibodies used in the different determinations.

Signals were detected by secondary horseradish peroxidase-conjugated antibody, either anti-rabbit (1 : 5000 or 1 : 10000 dilution; Dako) or anti-mouse (1 : 2500 dilution, Amersham NIF 824) and enhanced chemiluminescence using the ECL method, as recommended by the manufacturer (Amersham). Chemiluminescence was detected with ImageQuant LAS 4000 Mini technology (GE Healthcare).

2.9. Statistical Methods. For the RT-PCR and Western blot analyses, values are expressed as the mean ± SE of three different experiments. Data were evaluated by unpaired Student's *t* test, and analyses were performed using the PASW Statistics v. 18.0.0. software.

3. Results

3.1. Effect of ICC and CA Incubations in HT29 Gene Expression. The expression profile of over 47,000 transcripts and variants included in the microarray HG U133 plus 2.0 from Affymetrix was compared between HT29 control cells and cells incubated with either CA or ICC, at nontoxic concentrations for 24 h. GeneSpring GX software v.11.5.1 was used to analyze the results. A list of differentially expressed genes by 1.3-fold with a *P* value cutoff of <0.05 was generated as described in Methods. When HT29 cells were incubated with ICC, 57 genes were overexpressed whereas 161 genes were underexpressed. Among the overexpressed genes, 24% belonged to the Transcription factors category and 19% to Cell cycle or to Biosynthetic processes. Within the underexpressed genes, the category corresponding to cell cycle was the most affected (53% of the genes) followed by Transcription factors (19%) and Biosynthetic processes (12%). Upon incubation with CA, 12 genes were overexpressed whereas 32 genes were underexpressed. Among the overexpressed genes, 33% belonged to the Transcription factors category, 25% to Cell cycle, and 16,7% to Biosynthetic processes or immune response. Within the underexpressed genes, again the category corresponding to Cell cycle was the most affected (30% of the genes) followed by Biosynthetic processes (15%) and Transcription factors (12%). The lists of differentially expressed genes are presented as Tables 2, 3, 4, and 5. The data presented in this work have been deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number [GSM867162].

3.2. Generation of Biological Association Networks. A Biological Association Network (BAN) was constructed using the Pathway Analysis within GeneSpring v.11.5.1 as described in Methods using as the starting list the common genes differentially expressed upon incubation with CA and ICC. This list included five overexpressed genes and twelve underexpressed genes (Table 6). In the generated network, signal transducer and activator of transcription 5B (STAT5B) and activating transcription factor 2 (ATF-2) appeared as highly interconnected nodes (Figure 1). These two main nodes were selected for further validations. STAT5B was overexpressed with respect to the control by 23.8% in cells treated with ICC and by 33.4% in cells treated with CA, whereas ATF-2 was found underexpressed in HT29 incubated with ICC (32.5% decrease compared to the control) and with CA (26% decrease).

3.3. Validation of STAT5B and ATF-2 Changes at the mRNA and Protein Levels. STAT5B overexpression in HT29 cells upon incubation with CA and ICC was confirmed at the mRNA (1.16- and 1.3-fold compared to the control, respectively) and protein levels (1.5- and 1.2-fold compared to the control, respectively) (Figures 2(a) and 2(c)). In the case of ATF-2, the changes in mRNA levels were confirmed for both CA and ICC (0.88- and 0.86-fold compared to the control, respectively), whereas the decrease in protein levels was only observed in CA-treated cells (0.62-fold compared to the control) (Figures 2(b) and 2(d)).

3.4. Expression of Cyclin D1 upon Incubation with ICC and CA. Cyclin D1 is overexpressed at the mRNA and protein level in over 50% of the breast cancers either in the presence or absence of gene amplification, and it is one of the most commonly overexpressed proteins in breast cancer [24, 25]. Cyclin D1 transcription is regulated by STAT5 [26–29] and ATF-2 [30–32].

Figure 1: Biological association network (BAN) of differentially expressed genes in common between CA and ICC. The list of common genes between both treatments was used to construct a BAN with the Pathway Analysis software within GeneSpring v.11.5.1. An expanded network was constructed by setting an advanced filter that included the categories of binding, expression, metabolism, promoter binding, protein modification, and regulation. Only proteins are represented. The BAN shows the node genes STAT5B and ATF-2 that were further studied.

Table 1

Antibody	Molecular weight (KDa)	Dilution used	Supplier
STAT5B	95	1: 200	sc-835, Santa Cruz Biotechnology Inc.
ATF-2	72	1: 200	sc-6233, Santa Cruz Biotechnology Inc.
Cyclin D1	38	1: 200	sc-8396, Santa Cruz Biotechnology Inc.
β-actin	42	1: 200	A2066, Sigma
Tubulin	60	1: 100	CP06, Calbiochem

We analyzed the levels of cyclin D1 by western blot in MCF-7 and HT29 cells upon incubation with ICC and CA. As shown in Figure 3(a), incubation of MCF-7 cells with either CA and ICC led to a drastic decrease in the levels of cyclin D1 protein, together with an increase in the levels of STAT5B, but not to a decrease in the levels of ATF-2. In HT29 cells, incubation with CA did not affect cyclin D1 levels, whereas the presence of ICC led to an increase in cyclin D1 levels 3 (b).

4. Discussion

In this work we analyzed the gene expression profile of human cancer cells treated with either ICC or CA. Caffeic acid was chosen since it is the main representative of hydroxycinnamic acids. Using microarrays we identified the differential expression of specific genes involved in several biological pathways. The changes in mRNA expression of two outlier genes, STAT5B and ATF-2, observed in the microarrays were confirmed by RT real-time PCR, and the changes in protein levels were also analyzed by Western blot. The selection of STAT5B and ATF-2 was made according to the results obtained in the construction of a biological association network. Finally, the modulation of cyclin D1, a target of STAT5B and ATF-2 transcription factors, upon incubation with coffee polyphenols was also established.

We show that ICC and the amount of CA of one cup of coffee are able to induce STAT5B mRNA and protein

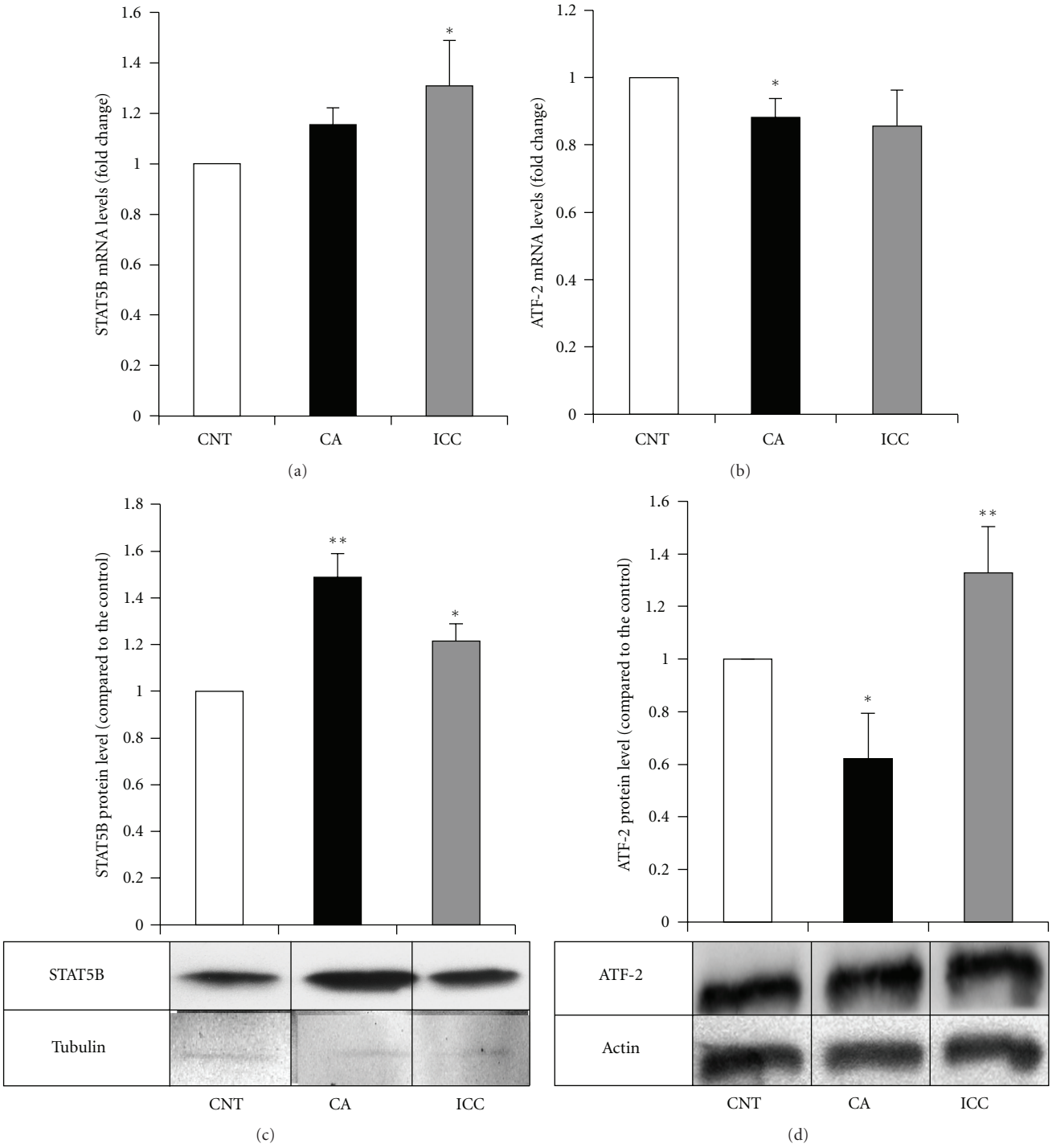

Figure 2: Quantitation of mRNA and protein levels for STAT5B and ATF-2 in HT29 cells. The mRNA levels of STAT5B (a) and ATF-2 (b) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) and instant caffeinated coffee (ICC, grey bars) by RT real-time PCR as described in Methods. Results are expressed in fold changes compared to the control and are the mean ± SE of 3 different experiments. $^*P < 0.05$ compared with the corresponding control. The protein levels of STAT5B (c) and ATF-2 (d) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) and instant caffeinated coffee (ICC, grey bars) by Western blot. Blots were reprobed with an antibody against β-actin or tubulin to normalize the results. Results represent the mean ± SE of 3 different experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the corresponding control.

Table 2: List of overexpressed genes in HT29 cells upon incubation with instant caffeinated coffee.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
CALM3	Calmodulin 3 (phosphorylase kinase, delta)	0.016	1.3	Up
CDC42EP1	CDC42 effector protein (Rho GTPase binding) 1	0.027	1.3	Up
FOXN3	Forkhead box N3	0.022	1.3	Up
KIR2DL1	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 1	0.023	1.3	Up
ORAI2	ORAI calcium release-activated calcium modulator 2	0.011	1.3	Up
RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1	0.022	1.3	Up
STH	Saitohin	0.031	1.3	Up
SLC39A3	Solute carrier family 39 (zinc transporter), member 3	0.028	1.3	Up
ZNF397OS	Zinc finger protein 397 opposite strand	0.024	1.3	Up
ZP4	Zona pellucida glycoprotein 4	0.046	1.3	Up
FGFRL1	Fibroblast growth factor receptor-like 1	0.035	1.31	Up
ITGA9	Integrin, alpha 9	0.002	1.31	Up
IRAK1	Interleukin-1 receptor-associated kinase 1	0.038	1.31	Up
OBSL1	Obscurin-like 1	0.008	1.31	Up
RPS17L4	Ribosomal protein S17-like 4	0.026	1.31	Up
STAT5B	Signal transducer and activator of transcription 5B	0.007	1.31	Up
TRABD	TraB domain containing	0.043	1.31	Up
MYO9B	Myosin IXB	0.041	1.32	Up
NME7	Nonmetastatic cells 7, protein expressed in (nucleoside-diphosphate kinase)	0.037	1.32	Up
RPS6KA4	Ribosomal protein S6 kinase, 90 kDa, polypeptide 4	0.014	1.32	Up
SIRPA	Signal-regulatory protein alpha	0.019	1.32	Up
TBX20	T-box 20	0.035	1.32	Up
TCF20	Transcription factor 20 (AR1)	0.022	1.32	Up
ALDH3B1	Aldehyde dehydrogenase 3 family, member B1	0.005	1.33	Up
BGN	Biglycan	0.029	1.33	Up
GNB4	Guanine nucleotide binding-protein (G protein), b-polypeptide 4	0.044	1.33	Up
IFNA17	Interferon, alpha 17	0.026	1.33	Up
KY	Kyphoscoliosis peptidase	0.013	1.33	Up
SCARF1	Scavenger receptor class F, member 1	0.025	1.33	Up
SERPINB8	Serpin peptidase inhibitor, clade B (ovalbumin), member 8	0.01	1.33	Up
FST	Follistatin	0.025	1.34	Up
MOGAT1	Monoacylglycerol O-acyltransferase 1	0.009	1.34	Up
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	0.015	1.34	Up
SUCLG2	Succinate-CoA ligase, GDP-forming, beta subunit	0.011	1.34	Up
SULT1B1	Sulfotransferase family, cytosolic, 1B, member 1	0.018	1.34	Up
TBX10	T-box 10	0.011	1.34	Up
ZNF503	Zinc finger protein 503	0.022	1.34	Up
HBA1	Hemoglobin, alpha 1	0.04	1.35	Up
MEPE	Matrix, extracellular phosphoglycoprotein with ASARM motif	0.001	1.35	Up
PPP1CB	Protein phosphatase 1, catalytic subunit, beta isoform	0.03	1.35	Up
ARV1	ARV1 homolog (*S. cerevisiae*)	0.011	1.36	Up
BCL3	B-cell CLL/lymphoma 3	0.034	1.36	Up
CTRC	Chymotrypsin C (caldecrin)	0.045	1.36	Up
EPOR	Erythropoietin receptor	0.008	1.37	Up
HMGA1	High-mobility group AT-hook 1	0.039	1.37	Up
IL19	Interleukin 19	0.018	1.38	Up
ABCC12	ATP-binding cassette, subfamily C (CFTR/MRP), member 12	6.00E-04	1.39	Up
RAI1	Retinoic acid induced 1	0.017	1.39	Up

Table 2: Continued.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
KLF5	Kruppel-like factor 5 (intestinal)	0.028	1.4	Up
CBWD1	COBW domain containing 1	0.044	1.41	Up
ASAH3	N-acylsphingosine amidohydrolase (alkaline ceramidase) 3	0.039	1.43	Up
ABHD14B	Abhydrolase domain containing 14B	0.03	1.45	Up
TLN1	Talin 1	0.049	1.45	Up
ARHGAP23	Rho GTPase-activating protein 23	0.024	1.65	Up
HINT3	Histidine triad nucleotide binding protein 3	0.002	1.77	Up
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	0.034	1.83	Up
CALR	Calreticulin	0.007	1.93	Up

The table shows the list of overexpressed genes by 1.3-fold with a P value < 0.05 obtained in cells treated with instant caffeinated coffee and includes the gene symbol for all genes, and their associated description. The ratio columns correspond to the absolute fold change in expression relative to the control group and the type of regulation (up: upregulation).

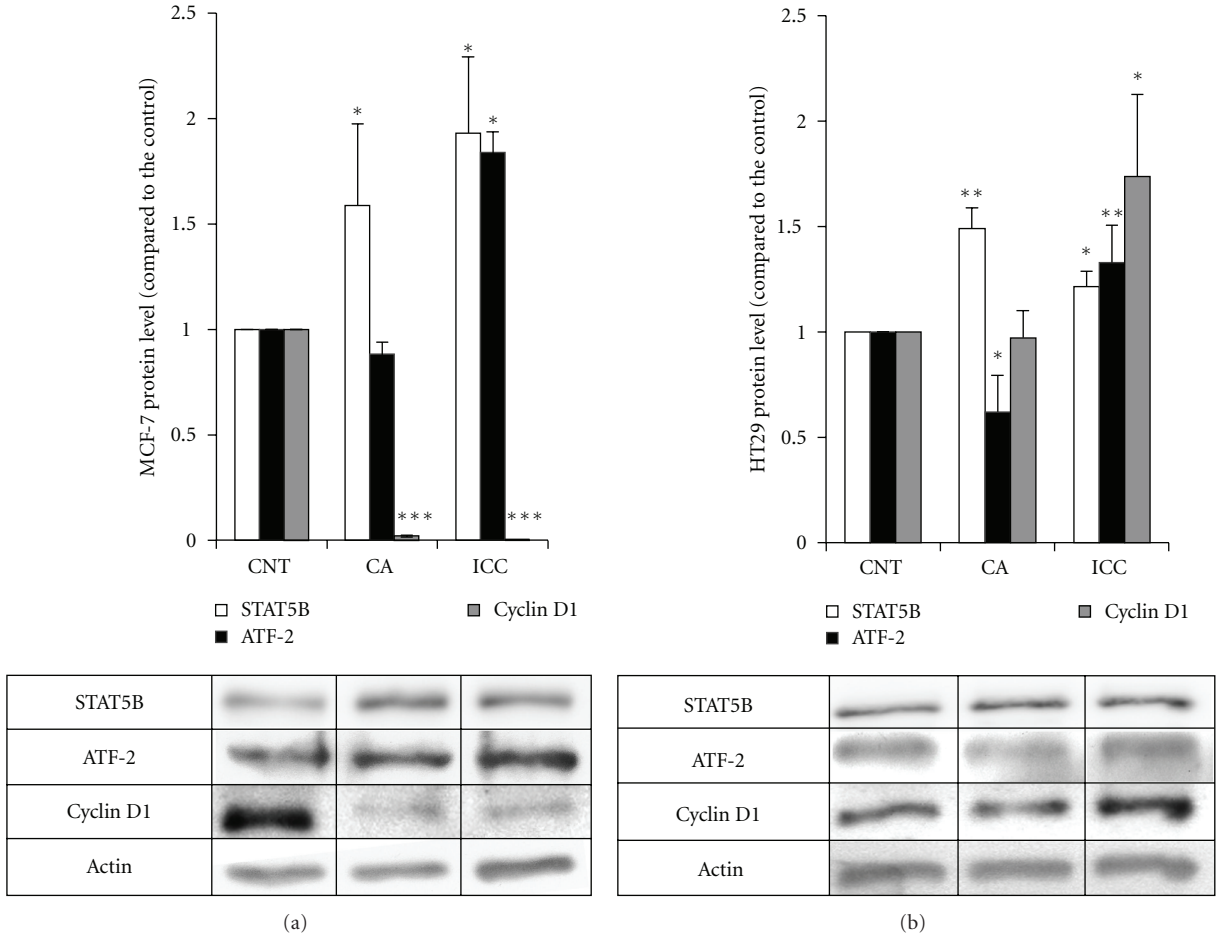

Figure 3: Expression of cyclin D1 upon incubation with ICC and CA in HT29 and MCF-7 cells. (a) Quantitation of STAT5b (empty bars), ATF-2 (filled bars), and cyclin D1 (grey bars) protein levels in MCF-7 cells. The protein levels were determined in control MCF-7 cells (CNT) and cells treated with caffeic acid (CA) and instant coffee (ICC) by Western blot. Blots were reprobed with an antibody against β-actin to normalize the results. Results represent the mean ± SE of 3 different experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the corresponding control. (b) Quantitation of STAT5b (empty bars), ATF-2 (filled bars), and cyclin D1 (grey bars) protein levels in HT29 cells. The protein levels were determined in control HT29 cells (CNT) and cells treated with caffeic acid (CA) and instant coffee (ICC) by Western blot. Blots were reprobed with an antibody against β-actin to normalize the results. Results represent the mean ± SE of 3 different experiments.$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the corresponding control.

Table 3: List of underexpressed genes in HT29 cells upon incubation with instant coffee.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
ACBD5	Acyl-coenzyme A binding domain containing 5	0.017	1.3	Down
CXADR	Coxsackie virus and adenovirus receptor	0.015	1.3	Down
FANCD2	Fanconi anemia, complementation group D2	0.047	1.3	Down
FRYL	FRY-like	0.039	1.3	Down
NUB1	Negative regulator of ubiquitin-like proteins 1	0.029	1.3	Down
PBRM1	Polybromo 1	0.004	1.3	Down
PRKACB	Protein kinase, cAMP-dependent, catalytic, beta	0.033	1.3	Down
RIF1	RAP1 interacting factor homolog (yeast)	0.012	1.3	Down
SLC39A6	Solute carrier family 39 (zinc transporter), member 6	0.022	1.3	Down
TMEM170	Transmembrane protein 170	0.032	1.3	Down
WDR26	WD repeat domain 26	0.028	1.3	Down
RNGTT	RNA guanylyltransferase and 5′-phosphatase	0.04	1.3	Down
CTDSPL2	CTD small phosphatase like 2	0.03	1.3	Down
ZC3H11A	Zinc finger CCCH-type containing 11A	0.014	1.3	Down
TMOD3	Tropomodulin 3 (ubiquitous)	0.0171	1.3	Down
CPD	Carboxypeptidase D	0.002	1.31	Down
CBL	Cas-Br-M ecotropic retroviral transforming sequence	0.008	1.31	Down
CDC42SE2	CDC42 small effector 2	0.022	1.31	Down
CLN5	Ceroid-lipofuscinosis, neuronal 5	0.001	1.31	Down
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	0.027	1.31	Down
FGFR1OP2	FGFR1 oncogene partner 2	0.049	1.31	Down
LRRFIP1	Leucine-rich repeat (in FLII) interacting protein 1	0.026	1.31	Down
PDCD4	Programmed cell death 4	0.005	1.31	Down
REPS2	RALBP1-associated Eps domain containing 2	0.046	1.31	Down
SLC7A6	Solute carrier family 7, member 6	0.002	1.31	Down
TFRC	Transferrin receptor (p90, CD71)	0.038	1.31	Down
TMEM19	Transmembrane protein 19	0.024	1.31	Down
AGPS	Alkylglycerone phosphate synthase	0.001	1.31	Down
SLC4A7	Solute carrier family 4, member 7	0.028	1.31	Down
SPTAN1	Spectrin, alpha, nonerythrocytic 1 (alpha-fodrin)	0.02	1.31	Down
GPD2	Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	0.033	1.31	Down
BICD1	Bicaudal D homolog 1 (*Drosophila*)	0.008	1.31	Down
FBXW11	F-box and WD repeat domain containing 11	0.025	1.31	Down
BCLAF1	BCL2-associated transcription factor 1	0.025	1.32	Down
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	0.011	1.32	Down
CLK4	CDC-like kinase 4	0.049	1.32	Down
PTAR1	Protein prenyltransferase alpha subunit repeat containing 1	0.027	1.32	Down
SMEK2	SMEK homolog 2, suppressor of mek1 (*Dictyostelium*)	0.012	1.32	Down
CEPT1	Choline/ethanolamine phosphotransferase 1	0.038	1.32	Down
SAR1A	SAR1 gene homolog A (*S. cerevisiae*)	0.033	1.32	Down
PDGFC	Platelet-derived growth factor C	0.02	1.32	Down
NFAT5	Nuclear factor of activated T-cells 5, tonicity responsive	0.045	1.32	Down
FRS2	Fibroblast growth factor receptor substrate 2	0.03	1.32	Down
BMS1P5	BMS1 pseudogene 5	0.036	1.33	Down
GLS	Glutaminase	5.00E-04	1.33	Down
LMAN1	Lectin, mannose binding, 1	7.00E-04	1.33	Down
ARHGAP18	Rho GTPase-activating protein 18	8.00E-04	1.33	Down

Table 3: Continued.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
ARHGAP5	Rho GTPase-activating protein 5	0.006	1.33	Down
CCNE2	Cyclin E2	0.036	1.33	Down
SPCS3	Signal peptidase complex subunit 3 homolog (*S. cerevisiae*)	0.008	1.33	Down
NCOA2	Nuclear receptor coactivator 2	0.005	1.33	Down
SRPRB	Signal recognition particle receptor, B subunit	0.018	1.33	Down
TLK1	Tousled-like kinase 1	0.04	1.33	Down
NCOA3	Nuclear receptor coactivator 3	0.048	1.33	Down
STRN3	Striatin, calmodulin-binding protein 3	2.00E-04	1.33	Down
AP1G1	Adaptor-related protein complex 1, gamma 1 subunit	0.004	1.34	Down
B3GALNT2	Beta-1,3-N-acetylgalactosaminyltransferase 2	0.034	1.34	Down
PPHLN1	Periphilin 1	2.00E-04	1.34	Down
SNX13	Sorting nexin 13	0.001	1.34	Down
TMED2	Transmembrane emp24 domain-trafficking protein 2	0.041	1.34	Down
BRWD1	Bromodomain and WD repeat domain containing 1	0.011	1.34	Down
HLA-B	Major histocompatibility complex, class I, B	0.028	1.34	Down
CHP	Calcium-binding protein P22	0.002	1.34	Down
MTMR9	Myotubularin-related protein 9	0.026	1.34	Down
DCUN1D4	DCN1, defective in cullin neddylation 1, domain containing 4	0.031	1.34	Down
ARL6IP2	ADP-ribosylation factor-like 6 interacting protein 2	0.02	1.35	Down
GLIS3	GLIS family zinc finger 3	0.01	1.35	Down
LARP4	La ribonucleoprotein domain family, member 4	0.019	1.35	Down
PTPLB	Protein tyrosine phosphatase-like member b	0.036	1.35	Down
TRAM1	Translocation-associated membrane protein 1	0.002	1.35	Down
TMEM64	Transmembrane protein 64	0.001	1.35	Down
CBFB	Core-binding factor, beta subunit	0.005	1.35	Down
SELT	Selenoprotein T	0.002	1.35	Down
PEX13	Peroxisome biogenesis factor 13	0.011	1.35	Down
TNKS2	TRF1-interacting ankyrin-related ADP-ribose polymerase 2	0.034	1.35	Down
TMPO	Thymopoietin	0.001	1.35	Down
LIN7C	Lin-7 homolog C (*C. elegans*)	0.007	1.35	Down
MTA2	Metastasis-associated 1 family, member 2	0.013	1.36	Down
TMEM168	Transmembrane protein 168	0.035	1.36	Down
CREBZF	CREB/ATF bZIP transcription factor	0.016	1.36	Down
OSTF1	Osteoclast-stimulating factor 1	0.002	1.36	Down
WDR57	WD repeat domain 57 (U5 snRNP specific)	0.001	1.36	Down
GLT25D1	Glycosyltransferase 25 domain containing 1	0.008	1.36	Down
NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma	0.015	1.36	Down
CCDC126	Coiled-coil domain containing 126	0.039	1.37	Down
LASS6	LAG1 homolog, ceramide synthase 6	0.005	1.37	Down
MYSM1	Myb-like, SWIRM and MPN domains 1	0.021	1.37	Down
CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1	0.007	1.37	Down
PDE4DIP	Phosphodiesterase 4D interacting protein (myomegalin)	0.024	1.37	Down
SAP30L	SAP30-like	0.012	1.37	Down
PTPRJ	Protein tyrosine phosphatase, receptor type, J	0.011	1.37	Down
PGGT1B	Protein geranylgeranyltransferase type I, beta subunit	9.00E-04	1.37	Down
ASPH	Aspartate beta-hydroxylase	0.011	1.37	Down
SEMA3C	Sema domain, (semaphorin) 3C	0.036	1.38	Down
WDR76	WD repeat domain 76	0.016	1.38	Down

Table 3: Continued.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
ATP13A3	ATPase-type 13A3	0.002	1.38	Down
LMBR1	Limb region 1 homolog (mouse)	0.014	1.38	Down
GLUD1	Glutamate dehydrogenase 1	0.001	1.39	Down
GSTCD	Glutathione S-transferase, C-terminal domain containing	0.029	1.39	Down
SPTLC1	Serine palmitoyltransferase, subunit 1	0.02	1.39	Down
U2AF1	U2 small nuclear RNA auxiliary factor 1	9.00E-04	1.39	Down
UHMK1	U2AF homology motif (UHM) kinase 1	0.007	1.39	Down
ARGLU1	Arginine and glutamate-rich 1	6.00E-04	1.39	Down
ANKRD12	Ankyrin repeat domain 12	0.03	1.39	Down
PPP3R1	Protein phosphatase 3, regulatory subunit B, alpha isoform	0.023	1.39	Down
XRN1	5′-3′ exoribonuclease 1	0.019	1.4	Down
CLSPN	Claspin homolog (*Xenopus laevis*)	0.013	1.4	Down
CXADRP1	Coxsackie virus and adenovirus receptor pseudogene 1	0.034	1.4	Down
G3BP1	GTPase-activating protein- (SH3 domain) binding protein 1	0.002	1.4	Down
TMEM30A	Transmembrane protein 30A	0.01	1.4	Down
CLCN3	Chloride channel 3	0.035	1.41	Down
STK4	Serine/threonine kinase 4	0.039	1.41	Down
ZNF644	Zinc finger protein 644	0.02	1.41	Down
TCP11L1	T-complex 11 (mouse)-like 1	0.014	1.41	Down
SFRS6	Splicing factor, arginine/serine-rich 6	0.031	1.41	Down
NPL	N-acetylneuraminate pyruvate lyase	0.006	1.41	Down
G3BP2	GTPase-activating protein- (SH3 domain) binding protein 2	0.001	1.42	Down
HNRNPU	Heterogeneous nuclear ribonucleoprotein U	0.01	1.42	Down
TBL1XR1	Transducin (beta)-like 1 X-linked receptor 1	0.001	1.42	Down
PHTF2	Putative homeodomain transcription factor 2	0.002	1.42	Down
ADAM10	ADAM metallopeptidase domain 10	0.011	1.43	Down
ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)	0.01	1.43	Down
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1	0.04	1.43	Down
SCARB2	Scavenger receptor class B, member 2	0.001	1.43	Down
CANX	Calnexin	0.043	1.43	Down
CASP2	Caspase 2, apoptosis-related cysteine peptidase	0.033	1.43	Down
TRPS1	Trichorhinophalangeal syndrome I	0.005	1.44	Down
ZFX	Zinc finger protein, X-linked	0.033	1.44	Down
SGPL1	Sphingosine-1-phosphate lyase 1	0.04	1.44	Down
PTPN11	Protein tyrosine phosphatase, nonreceptor type 11	0.045	1.44	Down
SFRS11	Splicing factor, arginine/serine-rich 11	0.045	1.45	Down
B3GNT5	Beta-1,3-N-acetylglucosaminyltransferase 5	0.021	1.45	Down
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	0.019	1.45	Down
SNHG4	Small nucleolar RNA host gene (nonprotein coding) 4	0.004	1.46	Down
PARD6B	Par-6 partitioning defective 6 homolog beta (*C. elegans*)	0.04	1.46	Down
ROD1	ROD1 regulator of differentiation 1 (*S. pombe*)	0.001	1.46	Down
SPTBN1	Spectrin, beta, nonerythrocytic 1	0.02	1.48	Down
TXNDC1	Thioredoxin domain containing 1	0.013	1.48	Down
ATF2	Activating transcription factor 2	0.005	1.48	Down
RDX	Radixin	0.043	1.48	Down
SCAMP1	Secretory carrier membrane protein 1	0.009	1.48	Down
PTAR1	Protein prenyltransferase alpha subunit repeat containing 1	0.018	1.49	Down
RC3H2	Ring finger and CCCH-type zinc finger domains 2	0.0037	1.49	Down

Table 3: Continued.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
ADAM17	ADAM metallopeptidase domain 17	0.007	1.49	Down
FAM76B	Family with sequence similarity 76, member B	0.014	1.5	Down
ITGB8	Integrin, beta 8	1.00E-04	1.5	Down
TRIM23	Tripartite motif-containing 23	0.005	1.5	Down
CASC5	Cancer susceptibility candidate 5	0.019	1.52	Down
SLC16A1	Solute carrier family 16, member 1	0.002	1.52	Down
FNBP1	Formin-binding protein 1	0.037	1.53	Down
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha	9.00E-04	1.53	Down
B4GALT1	Beta 1,4-galactosyltransferase, polypeptide 1	0.035	1.55	Down
MDM4	Mdm4 p53-binding protein homolog (mouse)	0.011	1.58	Down
FGD4	FYVE, RhoGEF, and PH domain containing 4	0.001	1.59	Down
UBA6	Ubiquitin-like modifier activating enzyme 6	8.00E-04	1.62	Down
ZDHHC21	Zinc finger, DHHC-type containing 21	0.036	1.64	Down
REEP3	Receptor accessory protein 3	7.00E-04	1.65	Down
SSR3	Signal sequence receptor, gamma	0.014	1.65	Down
ZDHHC20	Zinc finger, DHHC-type containing 20	0.003	1.66	Down
EIF2S3	Eukaryotic translation initiation factor 2, subunit 3 gamma	0.001	1.7	Down
HNRNPH1	Heterogeneous nuclear ribonucleoprotein H1	0.011	1.79	Down
ATL3	Atlastin 3	0.001	2.02	Down

The table shows the list of underexpressed genes by 1.3-fold with a P value < 0.05 obtained in cells treated with instant caffeinated coffee and includes the gene symbol for all genes, and their associated description. The ratio columns correspond to the absolute fold change in expression relative to the control group and the type of regulation (down: downregulation).

Table 4: List of overexpressed genes in HT29 cells upon incubation with caffeic acid.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
SULT1B1	Sulfotransferase family, cytosolic, 1B, member 1	0.02	1.3	Up
BCL6B	B-cell CLL/lymphoma 6, member B (zinc finger protein)	3.00E-04	1.3	Up
KCNJ5	Potassium inwardly-rectifying channel, subfamily J, member 5	0.01	1.31	Up
EPOR	Erythropoietin receptor	0.02	1.32	Up
DNAJC21	DnaJ (Hsp40) homolog, subfamily C, member 21	0.049	1.33	Up
STAT5B	Signal transducer and activator of transcription 5B	0.012	1.33	Up
FST	Follistatin	0.021	1.37	Up
CD84	CD84 molecule	0.033	1.37	Up
THRA	Thyroid hormone receptor, alpha	0.017	1.37	Up
MAPK8IP3	Mitogen-activated protein kinase 8 interacting protein 3	0.028	1.4	Up
SIAE	Sialic acid acetylesterase	0.01	2.42	Up
HINT3	Histidine triad nucleotide-binding protein 3	0.033	2.6	Up

The table shows the list of overexpressed genes by 1.3-fold with a P value < 0.05 obtained in cells treated with caffeic acid and includes the gene symbol for all genes, their associated description. The ratio columns correspond to the absolute fold change in expression relative to the control group and the type of regulation (up: upregulation).

levels in HT29 cells. STAT5 was originally described as a prolactin-induced mammary gland factor [33]. The cloning of two closely related STAT5 cDNAs, from both mouse and human cDNA libraries, showed two distinct genes, STAT5A and STAT5B that encoded two STAT5 proteins [34–37].

In addition to prolactin, STAT5 proteins are activated by a wide variety of cytokines and growth factors, including IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, granulocyte-macrophage colony-stimulating factor, erythropoietin, growth hormone, thrombopoietin, epidermal growth factor, and platelet-derived growth factor. The key function of STAT5B is to mediate the effects of growth hormone [38, 39]. Modulation of STAT5 levels or transcriptional activity has already been described in cells treated with natural compounds such as nobiletin, a citrus flavonoid [40], thea flavins [41], and silibinin, a natural polyphenolic flavonoid which is a major bioactive component of silymarin isolated from *Silybum marianum* [42]. Furthermore, it has been reported that

Table 5: List of underexpressed genes in HT29 cells upon incubation with caffeic acid.

Gene symbol	Gene title	*P* value	FC absolute	Regulation
MFSD7	Major facilitator superfamily domain containing 7	1.00E-04	1.3	Down
MSI2	Musashi homolog 2 (*Drosophila*)	0.027	1.3	Down
CDA	Cytidine deaminase	2.00E-04	1.31	Down
DEFB1	Defensin, beta 1	0.026	1.31	Down
PIP5K1A	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	0.027	1.31	Down
ZDHHC20	Zinc finger, DHHC-type containing 20	0.005	1.31	Down
ZDHHC21	Zinc finger, DHHC-type containing 21	0.016	1.31	Down
SLC4A7	Solute carrier family 4, member 7	0.0249	1.32	Down
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	0.0459	1.32	Down
PDZRN3	PDZ domain containing RING finger 3	0.002	1.32	Down
WDR62	WD repeat domain 62	0.005	1.32	Down
FAM76B	Family with sequence similarity 76, member B	0.036	1.32	Down
TCF21	Transcription factor 21	0.029	1.33	Down
TBL1XR1	Transducin (beta)-like 1 X-linked receptor 1	6.00E-04	1.33	Down
CLK4	CDC-like kinase 4	0.021	1.33	Down
CYP2A13	Cytochrome P450, family 2, subfamily A, polypeptide 13	0.009	1.34	Down
CXCR4	Chemokine (C-X-C motif) receptor 4	0.0488	1.34	Down
ATF2	Activating transcription factor 2	0.0158	1.35	Down
PDE10A	Phosphodiesterase 10A	0.03	1.35	Down
METT10D	Methyltransferase 10 domain containing	0.003	1.35	Down
PRMT2	Protein arginine methyltransferase 2	7.00E-04	1.36	Down
GLS	Glutaminase	5.70E-04	1.37	Down
SLC38A5	Solute carrier family 38, member 5	0.043	1.37	Down
TINAG	Tubulointerstitial nephritis antigen	0.043	1.38	Down
AQP1	Aquaporin 1 (Colton blood group)	0.0221	1.4	Down
JMJD6	Jumonji domain containing 6	0.004	1.4	Down
SAP30L	SAP30-like	0.021	1.4	Down
FGD4	FYVE, RhoGEF, and PH domain containing 4	0.026	1.52	Down
S100A2	S100 calcium-binding protein A2	0.005	1.53	Down
CTSZ	Cathepsin Z	0.045	1.53	Down
SLC4A4	Solute carrier family 4, member 4	9.00E-04	1.54	Down
AGR3	Anterior gradient homolog 3 (*Xenopus laevis*)	0.011	1.69	Down

The table shows the list of underexpressed genes by 1.3-fold with a P value < 0.05 obtained in cells treated with caffeic acid and includes the gene symbol for all genes, their associated description. The ratio columns correspond to the absolute fold change in expression relative to the control group and the type of regulation (down: downregulation).

butein, the major biologically active polyphenolic component of the stems of *Rhus verniciflua*, downregulated the expression of STAT3-regulated gene products such as Bcl-xL, Bcl-2, cyclin D1, and Mcl-1 [43].

STAT5B participates in diverse biological processes, such as growth development, immunoregulation, apoptosis, reproduction, prolactin pathway, and lipid metabolism. STAT5B deficiency is a recently identified disease entity that involves both severe growth hormone-resistant growth failure and severe immunodeficiency [44–46]. The induction of STAT5B expression upon incubation with CA and ICC could represent a nutritional tool to upregulate this transcription factor and suggests novel research strategies for natural therapies in Crohn's disease and inflammatory bowel disease in which STAT5B appears to maintain the mucosal barrier integrity and tolerance [47, 48]. In colorectal cancer both STAT5a and STAT5b play important roles in progression and downregulation of both STAT5A and STAT5B results in a gradual decrease in cell viability, predominantly attributed to G1 cell cycle arrest, and apoptotic cell death [49]. In this context the increase in STAT5B caused by ICC and CA would have a negative effect on colorectal cancer patients, as it would trigger cell proliferation and survival.

In human breast cancer, STAT5A/B has been shown a dual role in the mammary gland as an initiator of tumor formation as well as a promoter of differentiation of established tumors. STAT3, STAT5A, and STAT5B are overexpressed or constitutively activated in breast cancer [50–52] and active STAT5A/B in human breast cancer predicted favorable clinical outcome [53]. Prolactin receptor signal

Table 6: Common differentially expressed genes in HT29 treated-cells.

Gene symbol	FC absolute ICC	*P* value	Regulation	FC absolute CA	*P* value	Regulation
FST	1.343	0.025	Up	1.375	0.022	Up
SULT1B1	1.349	0.018	Up	1.304	0.020	Up
EPOR	1.372	0.008	Up	1.321	0.021	Up
HINT3	2.410	0.040	Up	2.607	0.033	Up
STAT5B	**1.312**	**0.007**	**Up**	**1.334**	**0.012**	**Up**
GLS	1.335	0.001	Down	1.370	0.001	Down
PPP3R1	1.397	0.023	Down	1.423	0.026	Down
ATF2	**1.481**	**0.005**	**Down**	**1.354**	**0.016**	**Down**
SLC4A7	1.314	0.029	Down	1.322	0.025	Down
MARCH3	1.330	0.016	Down	1.319	0.005	Down
TBL1XR1	1.426	0.001	Down	1.332	0.001	Down
SAP30L	1.375	0.013	Down	1.405	0.021	Down
FGD4	1.593	0.001	Down	1.523	0.027	Down
ZDHHC20	1.665	0.004	Down	1.314	0.005	Down
ZDHHC21	1.642	0.037	Down	1.318	0.016	Down
FAM76B	1.506	0.014	Down	1.325	0.037	Down
CLK4	1.326	0.049	Down	1.339	0.021	Down

Common differentially expressed genes in HT29 treated-cells with a P value < 0.05 and a minimum fold of 1.3. Column ICC correspond to cells treated with instant caffeinated coffee and column CA corresponds to cells treated with caffeic acid. Overexpressed genes are indicated on the upper part of the table, whereas underexpressed genes are depicted in the lower part. The genes in bold, STAT5B and ATF-2, were chosen for further analysis.

transduction through the Jak2-STAT5 pathway has been considered to be essential for proliferation and differentiation of normal mammary epithelial cells [54–56]. It has been shown that the levels of NUC-pYSTAT5 decreased as breast cancer progressed from normal to *in situ*, to invasive, and then to nodal metastases [57]. Additionally Peck et al. [57] found that the absence of detectable NUC-pYStat5 in tumors of patients how where under antiestrogen therapy was associated with poor breast cancer-specific survival. We analyzed STAT5B modulation through the PRL pathway in response to coffee polyphenols in a breast cancer cell line. The MCF-7 cell line was chosen because expression of the prolactin receptor is more often found in estrogen receptor-positive breast tumors [58]. In our conditions, incubation with CA and ICC led to an increase in STAT5B protein levels in MCF-7 cells, and this result could be the basis for a possible inclusion of coffee polyphenols in the diet of breast cancer patients.

ATF-2 is a member of the ATF-cAMP response element-binding protein (CREB) family of transcription factors that can bind to the cAMP response element (CRE) found in many mammalian gene promoters [59, 60]. ATF-2 exhibits both oncogenic and tumor suppressor functions [61]. CREs are found in several genes involved in the control of the cell cycle, for example, the cyclin D1 gene, and ATF-2 binding to this sequence stimulates the transcription of cyclin D1 [30, 31]. ATF-2 mediated cyclin D1 promoter induction can be stimulated by a number of growth-promoting agents, such as estrogen [31], hepatocyte growth factor [62], and regenerating gene product [63]. ATF-2 has been correlated with proliferation, invasion, migration, and resistance to DNA-damaging agents in breast cancer cell lines.

The downregulation of ATF-2 expression after CA and ICC incubation in HT29 cells reported here is in accordance with the observed decrease in activity of ATF-2 in gastric cells when incubating with chlorogenic acid, the precursor of caffeic acid [64]. Surprisingly, the validation of the protein levels showed the upregulation of ATF-2 protein with ICC, but not with CA, both in HT29 and MCF-7 cells. This differential behavior could be due to other ICC components besides CA. In this direction Rubach et al. [64] reported a different response in ATF-2 activity after incubation of a gastric cell line with different coffee compounds. The presence of pyrogallol, catechol, βN-alkanoylhydroxytryptamides, and N-methylpyridinium increased ATF-2 activity, whereas chlorogenic acid and caffeine decrease it [64]. In our conditions incubation of HT29 cells with ICC caused a modest decrease in ATF-2 mRNA levels. However this effect was not translated at the protein level. We hypothesize that ICC contains other polyphenols in addition to caffeic acid that are able to increase ATF-2 protein levels through an increase of the translation of its mRNA, the increase of stability of the protein or an inhibition of its degradation. In this direction several plant polyphenols such as (-)-epigallocatechins-3-gallate (EGCG), genistein, luteolin, apigenin, chrysin, quercetin, curcumin, and tannic acid have been described to possess proteasome-inhibitory activity [65, 66].

The regulation of ATF-2 transcriptional activity, mostly at the level of its phosphorylation status, has been described upon treatment of cancer cells with several natural compounds. In MCF-7 cells, the anticancer agent 3,30-Diindolylmethane, derived from *Brassica* vegetables, activates both JNK and p38 pathways, resulting in c-Jun and ATF-2 phosphorylation, and the increase of binding of the

c-Jun–ATF-2 homodimers and heterodimers to the proximal regulatory element of IFN-γ promoter [67]. Biochanin-A, an isoflavone, existing in red clover, cabbage and alfalfa, has an inhibitory and apoptogenic effect on certain cancer cells by blocking the phosphorylation of p38 MAPK and ATF-2 in a dose-dependent fashion [68]. The JNK stress-activated pathway is one of the major intracellular signal transduction cascades involved in intestinal inflammation [69, 70], and upregulation of ATF-2 has been shown in Crohn's disease [71, 72]. Thus CA could represent potential therapeutical properties in different states of intestinal inflammation due to its combined effects on STAT5B and ATF-2 in HT29 cells.

Finally, the modulation of cyclin D1, a target of STAT5B and ATF-2 transcription factors, upon incubation with coffee polyphenols was established in colon and breast cancer cells. Cyclin D1 overexpression is common in colorectal cancer, but the findings regarding its prognostic value are conflicting. In a recent study, positive expression of cyclin D1 protein was detected in 95 of 169 colonic adenocarcinoma specimens, and increased cyclin D1 levels were associated with poorer prognosis [73]. Furthermore, there was a significant correlation between the positive expression of p-Stat5 and cyclin D1 in patients with colonic adenocarcinoma. However, in a second study, cyclin D1 overexpression was associated with improved outcome in a total of 386 patients who underwent surgical resection for colon cancer, classified as TNM stage II or III. Belt et al. [74] showed that low p21, high p53, low cyclin D1, and high AURKA were associated with disease recurrence in stage II and III colon cancer patients. In this context the effect of ICC on cyclin D1 levels could represent either a positive or a negative effect in colon cancer cells, depending on tumor progression. The increase in cyclin D1 levels could represent a marker of better outcome since it has been recently established that cyclin D1 expression is strongly associated with prolonged survival in male colorectal cancer and that lack of cyclin D1 is associated with a more aggressive phenotype in male patients [75]. However, several natural compounds such as anthocyanins, anthocyanidins, apigenin, luteolin, and fisetin have all been described to induce experimentally cell-cycle arrest and apoptosis through the decrease of cyclin D1 levels in HT29 cells [76–80]. In accordance to these data, the increase observed in cyclin D1 levels in HT29 cells upon incubation with ICC could probably be the consequence of the presence of different compounds other than polyphenols in ICC.

In MCF-7 breast cancer cells, cyclin D1 was down-regulated upon incubation with coffee polyphenols. The rationale for the choice of MCF-7 cell line was based on the observation that although cyclin D1 overexpression is present across multiple histologic subtypes of breast cancer, it has been shown that the large majority of cyclin D1–overexpressing breast cancers are ER positive [24, 25, 81]. Cyclin D1 overexpression has been reported between 40 and 90% of cases of invasive breast cancer, while gene amplification is seen in about 5–20% of tumors [24, 81–83]. In cyclin D1-driven cancers, blocking cyclin D1 expression by targeting the cyclin D1 gene, RNA, or protein should increase the chances for therapeutic success. Cell culture studies have raised the possibility that certain compounds might act in this way [84, 85] and approaches to blocking cyclin D1 expression using antisense, siRNA, or related molecules specifically target the driving molecular lesion itself [86–88]. It is believed that compounds that modulate cyclin D1 expression could have a role in the prevention and treatment of human neoplasias. For instance, flavopiridol, a synthetic flavonoid based on an extract from an Indian plant for the potential treatment of cancer, induces a rapid decline in cyclin D1 steady-state protein levels [89]. Taking all these results together, inhibition of cyclin D1 expression appears to be a good approach for cancer treatment. In this direction our observation that coffee and caffeic acid are able to drastically reduce the expression of cyclin D1 in breast cancer cells could suggest that some coffee components could be used as a coadjuvant therapeutic tool in the treatment of breast cancer.

Abbreviations

APRT: Adenine phosphoribosyltransferase
ATF-2: Activating transcription factor
BAN: Biological association network
CA: Caffeic acid
DMSO: Dimethyl sulfoxide
DEPC: Diethyl pyrocarbonate
ICC: Instant caffeinated coffee
RT-PCR: Reverse transcription-polymerase chain reaction
STAT5B: Signal transducer and activator of transcription 5B.

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4.4 ARTÍCULO IV:

NUTRITIONAL GENOMICS. A NEW APPROACH IN NUTRITION RESEARCH

Carlota Oleaga, Carlos J. Ciudad, Véronique Noé & Maria Izquierdo-Pulido

Recent Advances in Pharmaceutical Sciences II, 2012: ISBN: 978-81-7895-569-8.

Los resultados obtenidos en los Artículos I y III han sido utilizados como ejemplo de estudios nutrigenómicos en este capítulo, en el que además se realiza una revisión de las bases de la genómica nutricional y de los avances más recientes.

Los estudios en genómica nutricional empiezan a generar evidencias que pueden ayudar a mejorar la salud pública a través de un ajuste más personalizado de las recomendación dietéticas a la población. Esta meta se hará palpable cuando se desvelen los mecanismos por los cuales la alimentación puede reducir el riesgo de enfermedades de origen poligénico. La genómica nutricional emplea tecnologías de alto rendimiento de la genómica funcional, así como otras herramientas de biología molecular para la investigación nutricional, favoreciendo un conocimiento más profundo sobre la interacción nutriente-genoma en situaciones de equilibrio o desequilibrio de la homeostasis. La comprensión de la interrelación entre genes, productos de genes y hábitos dietéticos es esencial para discernir entre beneficio o riesgo en una intervención nutricional. El objetivo final de este campo es mejorar la salud pública mediante una estrategia nutricional más efectiva basada en las investigaciones realizadas.

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4. Nutritional genomics. A new approach in nutrition research

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Abstract. There is an increasing evidence that nutritional genomics represents a promise to improve public health. This goal will be reached by highlighting the mechanisms through which diet can reduce the risk of common polygenic diseases. Nutritional genomics applies high throughput functional genomic technologies and molecular tools in nutrition research, allowing a more precise and accurate knowledge of nutrient-genome interactions in both health and disease. Understanding the inter-relationships among genes, genes products, and dietary habits is fundamental to identify those who will benefit the most or be placed at risk by nutritional interventions. This chapter provides an overview of this novel nutritional approach, including the most relevant results of our recent research on the nutrigenomic effects of food polyphenols on cancer cells. Those studies would highlight the molecular mechanisms underlying the chemopreventive effects of those bioactive food compounds.

Introduction

Until recently, nutrition research concentrated on nutrient deficiencies and impairment of health. The importance of diet to sustain health,

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prevention and treatment of diseases has been known for a long time. The advent of genomics –high-throughput technologies for the generation, processing, and application of scientific information about the composition and functions of genomes – has created unprecedented opportunities for increasing our understanding of how nutrients modulate gene and protein expression influencing cellular and organismal metabolism and thus, ultimately impacting human health and well-being. Notably, the knowledge of the human genome has dramatically broadened the scope of studies in nutrition science [1-4].

Nutritional genomics is a relatively new and very fast-moving field of research and combines molecular biology, genetics, and nutrition [3, 5]. It provides a genetic understanding for how diet, nutrients or other food components affect the balance between health and disease by altering the expression and/or structure of an individual's genetic makeup. The conceptual basis for this new branch of genomic research is built on the following premises [1,6]:

- Diet and dietary components can alter the risk of disease development by modulating multiple processes involved with the onset, incidence, progression, and/or severity;
- Diet and dietary components can act on the human genome, either directly or indirectly, to alter the expression of genes and gene products.
- Diet and dietary components could potentially compensate for or accentuate effects of genetic polymorphisms.

The term nutritional genomics frequently is used as an umbrella term for two research specialties: **nutrigenomics** and **nutrigenetics**. However, it is important to note the difference between the terms nutrigenomics and nutrigenetics because although these terms are closely related they are not interchangeable. Nutrigenomics focuses on with the effects of nutrients on genes, proteins, and metabolic processes, whereas nutrigenetics involves determining the effect of individual genetic variation on the interaction between diet and disease [2,7]. Thus, those working in nutrigenomics investigate the role of nutrients in gene expression, and those working in nutrigenetics determine how genetic polymorphisms (mutations) affect responses to nutrients [7,8]. Moreover, when reviewing scientific literature, other terms appear, such as epigenetics, transcriptomics, proteomics or metabolomics. All of them describe processes, new tools or situations of this emerging field of nutrition (Table 1). The key challenge is to determine

whether it is possible to utilize this information meaningfully to provide reliable and predictable personalized dietary recommendations for specific health outcomes.

Nutrigenetics and nutrigenomics hold much promise for providing better nutritional advice to the public generally, genetic subgroups and individuals [11]. In the future, the integration of nutrition and genomics may lead to the enhanced use of personalized diets to prevent or delay the onset of disease and to optimize and maintain human health. The objectives of this chapter are to provide an overview of this novel nutritional approach. Moreover, we will also include the most relevant results of our research on the nutrigenomic effects of food polyphenols on cancer cells. In addition to the essential nutrients, such as calcium, zinc, selenium or vitamins, there are a variety of classes of nonessential nutrients and bioactive components, such as polyphenols, that seem to significantly influence health. Those bioactive components are known to modify a number of cellular processes associated with health and disease prevention, including carcinogen metabolism, hormonal balance, cell signaling, cell cycle control, apoptosis, and angiogenesis. Our studies are focused to highlight the molecular mechanisms underlying the chemopreventive effects of those bioactive food compounds.

Table 1. Definitions of terms used in nutritional genomics [9,10].

Term	Definition
Nutrigenomics	Investigates the effects of nutrients and other food components on genes, proteins, and metabolic processes. Transcriptomics, proteomics and metabolomics are used in nutrigenomics research
Nutrigenetics	Investigates the effect of individual genetic variation on the interaction between diet and disease. Genomics are often used in nutrigenetics studies
Epigenetics	Investigates the genome modifications that are copied from a generation to another but not implying changes on DNA sequence
Transcriptomics	Investigates gene expression changes at the mRNA level in response to different stimuli. Utilizes variety of technologies, most commonly microarrays and next-generation sequencing
Proteomics	Analyses all the proteins in a biological system, their interactions and their functional states although effectively, usually only the most abundant subset of 300 or so proteins is relatively easily analyzed
Metabolomics	Investigates the metabolome that consists of all of non-proteinaceous, small molecules present in a biological system. Changes in the metabolome content reflect the biological responses to external stimuli (nutrients among others), which involves altered gene expression and protein production/ activity associated with metabolic pathways

1. Nutrigenetics

Nutrigenetics focuses on the effects of genetic variations have on the binomial diet/disease or on the nutritional requirements and recommended intakes for individuals and populations. To achieve its objectives, the methodology used in nutrigenetics includes the identification and characterization of genetic variants that are associated with, or are the responsible for a different response to certain nutrients or food components [6,11]. These variations generically designated as polymorphisms, including the polymorphisms of a single nucleotide (SNP, single-nucleotide polypmorphisms), differences in the number of copies, inserts, deletions, duplications and rearrangements or reorganizations. Undoubtedly, SNPs are the most frequent as they appear every 1,000 base pairs [12].

These differences may determine the susceptibility of an individual to have a disease related to diet or to one or some diet components, as well as to influence in the individual's response to diet changes. There is certain parallelism between nutrigenetics and phamacogenetics, although in the field of nutrition is more difficult to draw conclusions, since there are important differences between drugs and food components, such as chemical purity, number of therapeutic targets and duration of the exposure, among others [3, 9, 11].

One of the best-described examples of the effect of SNPs is the relationship between folate and the gene encoding for MTHFR (5,10-methylenetetrahydrofolate reductase) [13]. MTHFR has a role in supplying 5-methylenetetrahydrofolate, which is necessary for the re-methylation of homocysteine to form methionine. Methionine is essential to many metabolic pathways including production of neurotransmitters and regulation of gene expression. Folate is essential to the efficient functioning of this MTHFR. There is a common polymorphism in the gene for MTHFR that leads to two forms of protein: the wild type (C), which functions normally, and the thermal-labile version (T), which has a significantly reduced activity. People with two copies of the wild-type gene (CC) or one copy of each (CT) appear to have normal folate metabolism. Those with two copies of the unstable version (TT) and low folate accumulate homocysteine and have less methionine, which increases their risk of vascular disease and premature cognitive decline [14].

Thus, in people with low folic acid intake, higher serum homocysteine levels would be detected in TT homozygotes compared with other genotypes, which would lead them to an increased risk of cardiovascular disease (Figure 1). However, when the intake of folic acid in diet is higher, this increased

amount of folic acid would compensate the defect in DNA and homocysteine serum concentrations in the TT people would not reach such high values and not show hyperhomocysteinemia. According to this example of gene-diet interaction, a practical application for cardiovascular disease prevention would be to recommend a higher daily consumption of folic acid-rich food to those people with the TT genotype, since due to genetic susceptibility, these individuals have higher folic acid requirements than those for the general population [14,15].

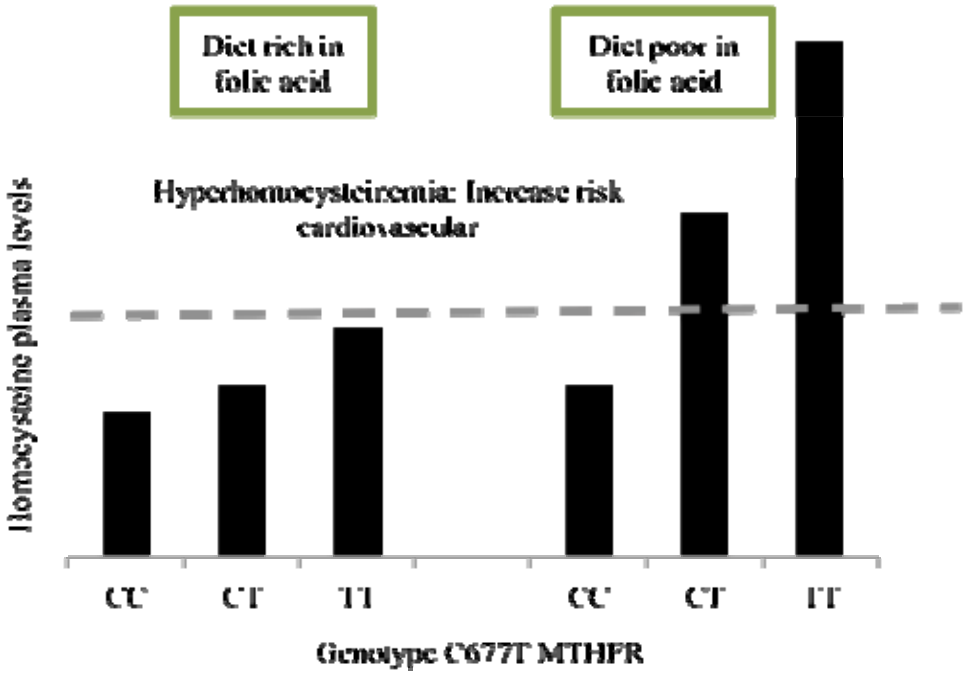

Figure 1. Gene-diet interaction. Folic acid intake may modulate the genetic risk of hyperhomocysteinemia conferred by the C677T polymorphism in the MTHFR gene. Hyperhomocysteinemia only would happen when the mutation occurs with a low folate intake [Adapted from 15].

Another of the genes on which a very active research has been developed is the one that encodes for the synthesis of the lipoprotein APOA1 [16]. APOA1 is the main component of plasma HDL and seems to play an important role in the transport of cholesterol. It has been reported that a polymorphism in the promoter of the gene promoter the -75 A/G (substitution of guanine by adenine), has an influence on the response individual intake of polyunsaturated fatty acids (PUFA). Thus, women with the A/A genotype showed higher HDL-cholesterol levels in plasma after ingestion of PUFA, whereas those with genotypes A/G and G/G (wild type) did not show HDL-cholesterol changes or even though a certain decrease in response to the

PUFA from diet (Figure 2). Therefore, for the individuals with the genotype A/A the ingestion of PUFA could be a good diet recommendation since it increases HDL. Those results illustrate the complexity of polymorphism-phenotype associations and underscore the importance of accounting for interactions between genes and environmental factors in population genetic studies.

The examples cited here and many others that can be found in the literature published until now [10,11,17-20] illustrate perfectly why nutrigenetics is also termed personalized nutrition, since its major goal is to identify and characterize of genes, and nucleotide variants within these, that are associated (or account for) the differential responses to nutrients. In addition to providing a more rational basis for giving personalized dietary advice, the knowledge gained by applying genomic information to nutrition research will also improve the quality of evidence used for making population-based dietary recommendations. The sequencing of an individual's genome has fueled interest in the field of personalized medicine [21,22], but replicating and validating nutrigenetic studies need to remain a priority before personalized nutrition can be considered a worthwhile approach to improve human health [23].

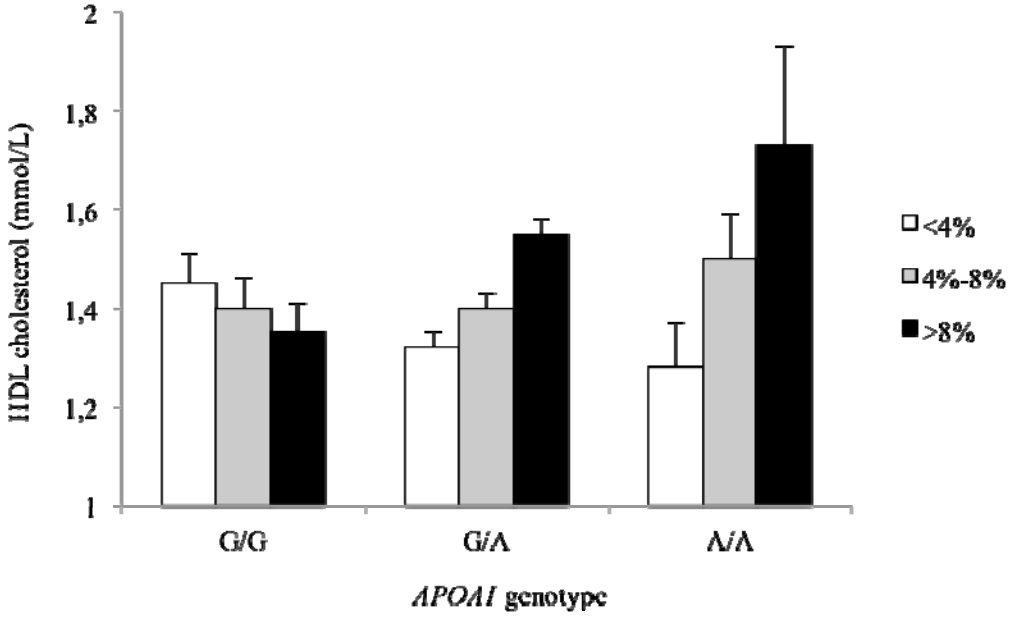

Figure 2. Effect of polyunsaturated fatty acid intake (>4%, 4-8% and >8% of energy) on high-density lipoprotein (HDL) cholesterol blood levels in women. Means were adjusted for age, body mass index, alcohol consumption, tobacco smoking, and intakes of energy, saturated fatty acids, monounsaturated fatty acids, and PUFAs [Adapted from 16].

2. Nutrigenomics

The term nutrigenomics was coined ten years ago to describe a branch of nutrition and food research that applies new profiling techniques for transcripts, proteins and metabolites to better understand the interplay of the genome with its nutritional environment. In this respect, nutrigenomics is still in its infancy and it will need time until it really delivers what was originally hoped [3,6,9].

The field of nutrigenomics harnesses multiple disciplines and includes dietary effects on genome stability (DNA damage at the molecular and chromosome level), epigenome alterations (DNA methylation), RNA and micro-RNA expression (transcriptomics), protein expression (proteomics) and metabolite changes (metabolomics), all of which can be studied independently or in an integrated manner [11, 24]. In this approach, nutrients, other food components, and even whole diets, are considered as "dietary signals" that are detected by "cellular sensors". These sensors, that are part of cellular signaling cascades, can affect, in turn, all the processes involved in the cell function. Therefore, they influence the transcription, translation and protein expression and on different metabolic pathways, which ultimately form the phenotype [25, 26].

Using the current genomic tools that include transcriptomics, proteomics and metabolomics, there are two approaches in nutrigenomic research. The first would identify genes, proteins or metabolites that are affected by the diet (nutrients or bioactive compounds) and know which are the mechanisms involved in this interaction and, consequently, figure out the regulation pathways through which the diet induces changes. In the second approach, early biomarkers are sought (genes, proteins or metabolites) that are linked with certain dietary compounds or to the whole diet [1,24]. Those biomarkers could act as a "warning signals" about changes in the homeostasis with could have implications for the health [10,11,24].

There are numerous examples [9,11,27,28] that illustrate the interaction between food components and the genome, from mammalian cells in culture to human studies. However, most applications are still of descriptive nature. As example of a typical nutrigenomic approach research, we will explain our research that has as the main goal to study mechanisms underlying the potential chemopreventive effects of a certain type of well-known food compounds called polyphenols.

Polyphenols are the most abundant antioxidants in the diet. Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Vegetables, cereals, cocoa, chocolate, and dry legumes also contribute to the total polyphenol intake. Their total dietary intake could

be as high as 1g/d, which is much higher than that of all other classes of phytochemicals and known dietary antioxidants [29]. Despite their wide distribution in plants, the health effects of dietary polyphenols have come to the attention of nutritionists only rather recently. Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers, and osteoporosis and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus [30]. However, our knowledge still appears too limited for formulation of recommendations for the general population or for particular populations at risk of specific diseases.

For many years, polyphenols and other antioxidants were thought to protect cell constituents against oxidative damage through scavenging of free radicals. However, this concept now appears to be an oversimplified view of their mechanism of action [31,32]. More likely, cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions [33]. Both antioxidant and prooxidant effects of polyphenols have been described, with contrasting effects on cell physiologic processes. As antioxidants, polyphenols may improve cell survival; as prooxidants, they may induce apoptosis and prevent tumor growth [30, 32]. However, the biological effects of polyphenols may extend well beyond the modulation of oxidative stress. One of the best-known examples involves the interaction of soy isoflavones with estrogen receptors and the effects of these compounds on endocrine function. These effects could explain the prevention by isoflavones of bone resorption among postmenopausal women [30]. A detailed understanding of the molecular events underlying these various biological effects is essential for evaluation of the overall impact on disease risk and progression.

2.1. Coffee polyphenols and breast cancer: A transcriptomics approach

Coffee is one of the most popular and widely consumed beverages throughout the world. Recent meta-analyses demonstrate inverse associations between coffee intake and the risk of colon, liver, breast and endometrial cancer [34-37]. In prospective population-based cohort studies, the inverse association between coffee consumption and risk of cancer has also been showed. The group of Naganuma et al. [38] found that the consumption of at least one cup of coffee per day was associated with a 49% lower risk of upper gastrointestinal cancer in a Japanese population, while Wilson et al. [39] found that men who regularly drink coffee appeared to have a lower risk of developing a lethal form of prostate cancer. The lower risk was evident when consuming either regular or decaffeinated coffee.

It has been proposed that the inverse association between coffee intake and colon cancer could be explained, at least in part, by the presence of phenolics compounds in coffee [40]. Among the different phenolic compounds in coffee, the most abundant are hydroxycinnamic acids, which exist mainly in the esterified form. The best example is chlorogenic acid (5-caffeoylquinic acid). In fact, coffee is the major source of chlorogenic acid in the human diet; the daily intake in coffee drinkers ranges from 0.5 to 1 g of chlorogenic acid whereas coffee abstainers will usually ingest <100 mg/day. Studies have showed that approximately the 33% of ingested chlorogenic acid and 95% of caffeic acid are absorbed intestinally [41]. Thus, about two-thirds of ingested chlorogenic acid reaches the colon where it is probably metabolized to caffeic acid [42]. Bioavailability data suggest that the biological effects of chlorogenic acid would become apparent after its metabolism to caffeic acid, and hence studying the effects of this acid is necessary.

As mentioned before, there is enough evidence from epidemiological data supporting that coffee seems to reduce the risk of certain type of cancers; however, the molecular mechanisms underlying the chemopreventive effects of coffee remain unknown. Using a transcriptomics approach, the effect at the molecular level of the main coffee phenolic compound, caffeic acid, at concentrations equivalent to one cup of coffee on human colon cancer cells (HT29) was studied. Furthermore, the effect of coffee polyphenols was also evaluated in breast cancer cells.

Colon adenocarcinoma HT29 cells were incubated with caffeic acid at a concentration equivalent to one cup of coffee for 24 hours. Previously, it was checked that this concentration did not cause any cytotoxic effect in the cell incubations. Then, the gene expression was analysed by hybridization to the GeneChip Human Genome U133A plus 2.0 microarrays from Affymetrix, containing 47,000 transcripts and variants. Quantification was carried out with GeneSpring GX v.11.5.1 software (Agilent Technologies), which allows multi-filter comparisons using data from different experiments to perform the normalization, generation of lists and the functional classification of the differentially expressed genes.

A list of differentially expressed genes by 1.3-fold with a p-value cut-off of <0.05 was generated. Upon incubation with caffeic acid, 12 genes were overexpressed whereas 32 genes were underexpressed. Among the overexpressed genes, 33% belonged to the Transcription factors category, 25% to Cell cycle, and 17% to Biosynthetic processes or Immune response. Within the underexpressed genes, again the category corresponding to Cell cycle was the most affected (30% of the genes) followed by Biosynthetic processes (15%) and Transcription factors (12%). Using these data, a

Biological Association Network (BAN) was constructed using the Pathway Analysis within the GeneSpring v.11.5.1, as described in Selga et al. [43]. Signal transducer and activator of transcription 5B (STAT5B) and Activating transcription factor 2 (ATF-2) appeared as highly interconnected nodes (Figure 3). In fact, STAT5B was overexpressed with respect to the control by 33,4% in cells treated with caffeic acid, whereas ATF-2 was found underexpressed in HT29 incubated with caffeic acid (26% decrease compared to the control).

The changes in mRNA expression of these two main (STAT5B and ATF-2) nodes were confirmed by RT-PCR and at the level of protein by Western blot analysis (Figure 4). The key function of STAT5B is to mediate

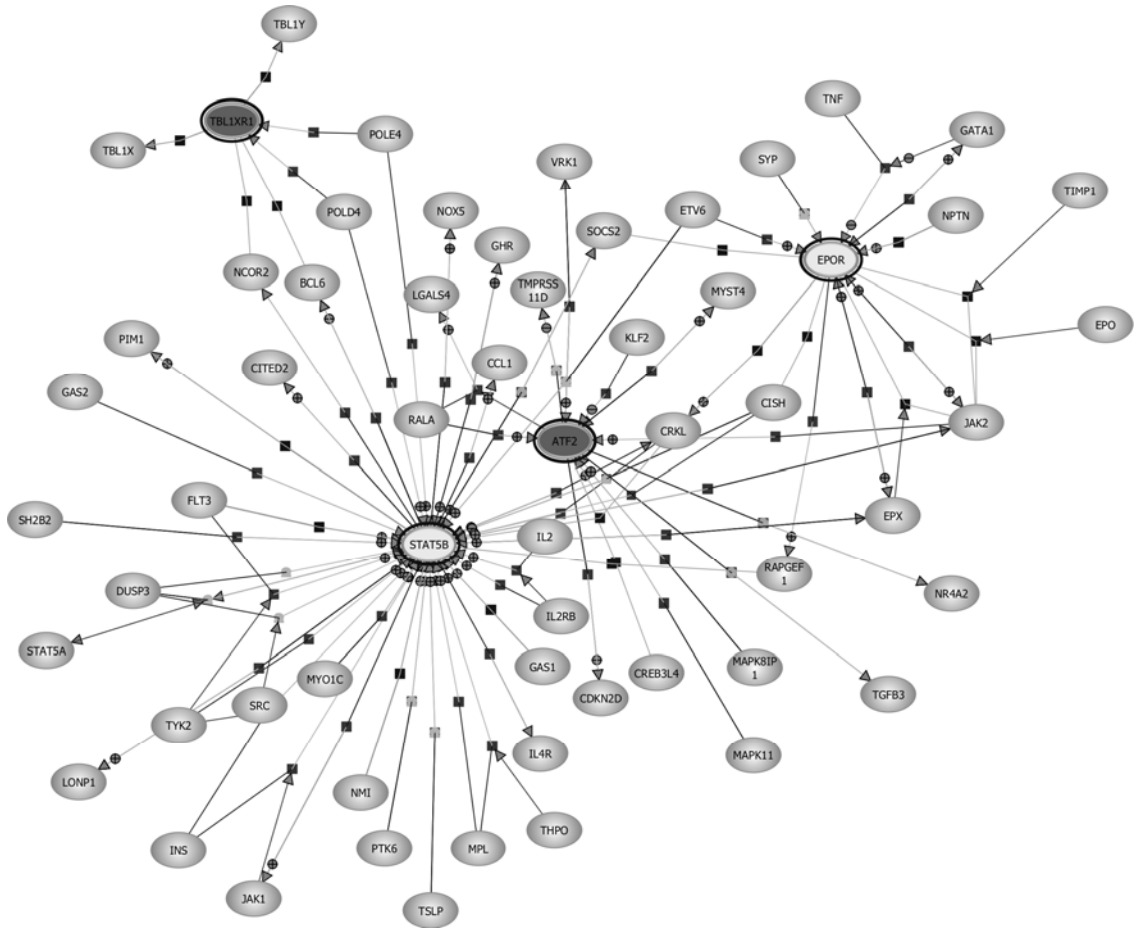

Figure 3. Biological Association Network (BAN) of differentially expressed genes under caffeic treatment. The BAN was constructed with the Pathway Analysis software within GeneSpring v11.5.1. An expanded network was constructed by setting an advanced filter that included the categories of binding, expression, metabolism, promoter binding, protein modification and regulation. Only proteins are represented. The BAN shows the node genes STAT5B and ATF-2 that were further studied.

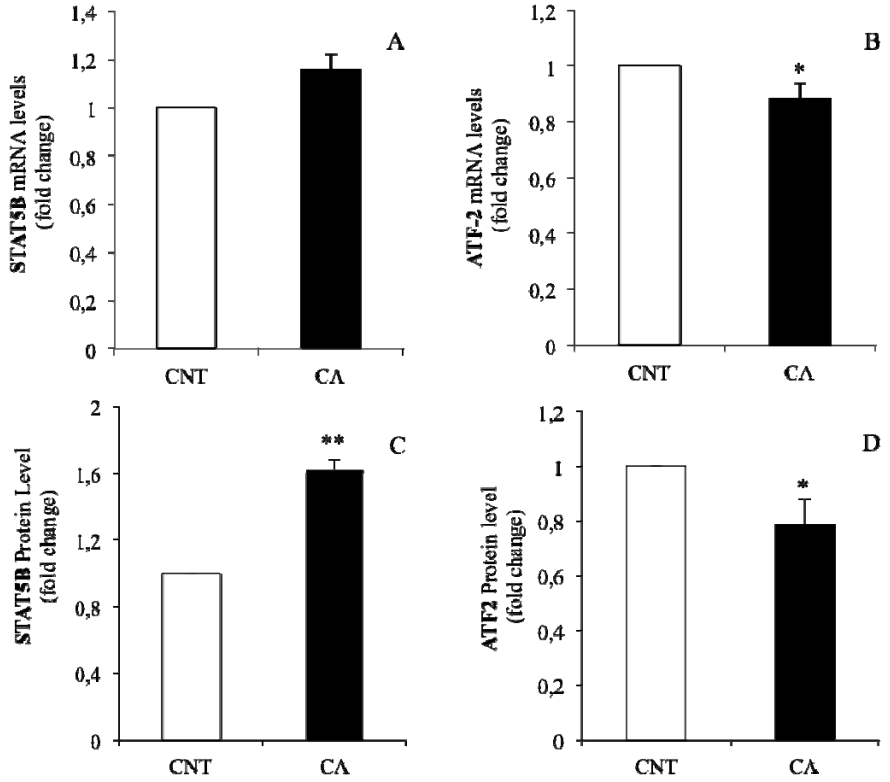

Figure 4. Quantification of mRNA and protein levels for STAT5B and ATF-2 in HT29 cells. The mRNA levels of STATB5 (A) and ATF-2 (B) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) by RT-Real Time. Results are expressed in fold-changes compared to the control, and are the mean + SE of 3 different experiments. *$p<0.05$ compared with the corresponding control. The protein levels of STAT5B (C) and ATF-2 (D) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) by Western blot. Blots were reprobed with an antibody against β-actin or tubulin to normalize the results. Results represent the mean ± SE of 3 different experiments. *$p<0.05$ and **$p<0.01$ compared with the corresponding control.

the effects of growth hormone, as STAT5B-null mice failed to respond effectively to this hormone [44]. Modulation of STAT5 levels or transcriptional activity has already been described in cells treated with natural compounds such as nobiletin, acitrus flavonoid [45] thea flavins [46] and silibinin, a natural polyphenolic flavonoid which is a major bioactive component of silymarin isolated from *Silybum marianum* [47]. Activation of STAT5A/B in human breast cancer has been shown to positively correlate with the differentiation status of the tumour. STAT5 have been also shown to transcriptionally regulate E2-sensitive proliferative genes such as cyclin D1 and c-Myc [48] suggesting that STAT5 may play a role in E2-stimulated breast cancer growth. STAT5 activation has also been linked to regulating the expression of the cell cycle control protein cyclin D1 both directly and indirectly [48-50].

On the other hand, ATF-2 is a member of the ATF-cAMP response element-binding protein (CREB) family of transcription factors that can bind to the cAMP response element (CRE) found in many mammalian gene promoters [51]. ATF-2 exhibits both oncogenic and tumor suppressor functions [52)] CREs are found in several genes involved in the control of the cell cycle, e.g., the cyclin D1 gene and ATF-2 binding to this sequence stimulates the transcription of cyclin D1 [53]. ATF-2 has been correlated with proliferation, invasion, migration, and resistance to DNA-damaging agents in breast cancer cell lines.

Therefore, the two main nodes identified in our work regulate cyclin D1 transcription. Cyclin D1 is an important regulator of G1-S phase transition, and its expression in breast cancer cells is sensitive to estrogens and antiestrogens [54]. Cyclin D1 is overexpressed at the mRNA and protein level in over 50% of the breast cancers either in the presence or absence of gene amplification and it is one of the most commonly overexpressed proteins in breast cancer [55]. In order to know the influence that the caffeic acid could have in the levels of cyclin D1, since the expression of STATB5 and ATF-2 is modified by this phenolic compound, the levels of cyclin D1 by Western blot in MCF-7 cells upon incubation with caffeic acid were analyzed. As shown in Figure 5, incubation of MCF-7 cells with caffeic acid led to a drastic decrease in the levels of cyclin D1 protein, together with an increase in the levels of STAT5B, but not a decrease in the levels of ATF-2.

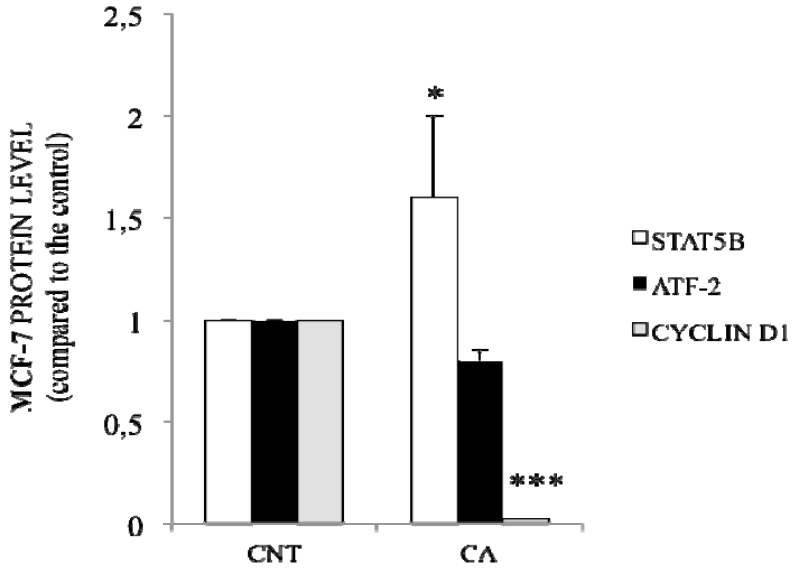

Figure 5. Expression of cyclin D1 upon incubation with caffeic acid in MCF-7 cells. The protein levels were determined in control MCF-7 cells (CNT) and in cells treated with caffeic acid (CA) by Western blot. Blots were reprobed with an antibody against β-actin to normalize the results. Results represent the mean ± SE of 3 different experiments. *$p<0.05$ and ***$p<0.001$ compared with the corresponding control.

It is believed that compounds that modulate cyclin D1 expression could have a role in the prevention and treatment of human neoplasia. For instance, flavopiridol, a synthetic flavonoid based on an extract from an Indian plant for the potential treatment of cancer, induces a rapid decline in cyclin D1 steady-state protein levels [54]. Taking all these results together, inhibition of cyclin D1 expression appears to be a good approach for cancer treatment. In this direction our observation that coffee and caffeic acid are able to drastically reduce the expression of cyclin D1 in breast cancer cells could suggest that some coffee components could be use as a coadjuvant therapeutic tool in the treatment of breast cancer.

2.2. Cocoa polyphenols and changes in the CYP1A1 gene expression

Cocoa is rich in polyphenols. In fact, cocoa has the highest flavanol contents of all foods on a per-weight basis and is a significant contributor to the total dietary intake of flavonoids [56]. The main subclasses of flavonoids found in cocoa are flavanols, particularly the flavanol monomers catechin and epicatechin, and their oligomers, also known as procyanidins [57]. Many examples of the health benefits of cocoa consumption can be found in the literature [58].

Epidemiologic studies of cocoa intake and cancer risk are few, and those assessing overall mortality provide only weak support for a benefit of cocoa. However, human intervention trials indicate that cocoa favours intermediary factors in cancer progression—specifically, markers of antioxidant status [59]. Moreover, there is growing evidence that polyphenols may play a role in regulating apoptosis [60]. Apoptosis may be triggered intrinsically, through the mitochondrial pathway or extrinsically by death ligands and receptors. It is the external pathway that may potentially be modulated by bioactive food components. Flavanols found in cocoa have exhibited pro-apoptotic effects. Proanthocyanadins inhibited growth of human lung cancer cells in vitro and in vivo [61], and epicatechin synergistically enhanced apoptosis in lung cancer cells treated with epigallocatechin-3-gallate (EGCG) [62]. Cocoa polyphenols have also been found to inhibit the mutagenic activity of heterocyclic amines *in vitro* and *ex vivo* [63].

It has been reported that catechins from green tea could be effective in modulating estrogen-induced breast carcinogenesis, either interfering with receptor mediated pathways or reducing the production of genotoxic estrogen metabolites [64,65]. In our functional genomic study, we sought to evaluate the effect of cocoa flavonoids in a type of breast cancer cells (MCF-7), which are estrogen-receptor (ER)- dependent [66]. Estrogens are implicated in the initiation and promotion stages of breast cancer, and lifetime estrogen

exposure is a major risk factor for breast cancer [67]. Estrogens exert their carcinogenic effects by both estrogen receptor (ER)-dependent and independent mechanisms [68]. Most human breast cancers are initially positive for ER, and their growth can be stimulated by estrogens and inhibited by antiestrogens such as tamoxifen.

For that purpose, MCF-7 cells were incubated for 24h with a purified polyphenol cocoa extract (PCE). PCE was used as representative of the wide flavonoid spectrum (monomers and oligomers) present in cocoa and the concentrations used were not toxic. The differential gene expression analysis was done using PCR arrays. In particular, the expression profile of the 84 genes included in the Stress & Toxicity PathwayFinder™ PCR Array was analyzed in MCF-7 cells both control and treated with a PCE. It was observed that the exposition to PCE decreased the expression of serpine 1 and up-regulated the expression of the CYP1A1, GADD45A, GDF15, GPX1, RAD23A, TP53, and XRCC2 genes (Table 2).

Among those genes, CYP1A1 was chosen for further validation since: (a) it was one of the most overexpressed gene upon incubation with PCE, (b) its overexpression in response to polyphenols had already been described, and (c) it plays an important role in the oxidative metabolism of estrogens. CYP1A1 is a candidate gene for low-penetrance breast cancer susceptibility because it plays an important role in the metabolism of xenobiotics or carcinogens as well as in the oxidative metabolism of estrogens [2004]. CYP1A1 encodes aryl hydrocarbon hydroxylase (AHH) that catalyzes a

Table 2. List of under- and overexpressed genes in MCF-7 cells upon incubation with PCE for 24hours[1].

MCF-7 Gene symbol	Fold-up or down-regulation Test sample / control sample	p-value
CYP1A1	17.60	0.0001
GADD45A	4.20	0.0264
GDF15	2.60	0.0001
GPX1	4.25	0.0183
RAD23A	13.90	0.0394
SERPINE1	- 49.90	0.0216
TP53	2.26	0.0470
XRCC2	17.50	0.0356

[1]The expression of each gene was reported as the fold change obtained after each treatment relative to control after normalization of the data. A cut-off of 2-fold was chosen since small changes in gene expression may represent important changes downstream those differentially expressed genes. Lists of differentially expressed genes, with a p-value<0.05, were generated from three independent experiments.

hydroxylation reaction in Phase I metabolism as a first step to increase the polarity of different molecules. Some of these metabolites can be more active than the initial molecules and behave as electrophilic compounds, thus initiating or promoting tumorigenic processes. Additionally, other metabolites may behave as chemoprotectors, such as the result of 2-hydroxylation in E1 and E2 metabolism [70].

Therefore, the differential expression of CYP1A1 mRNA in control versus treated cells was validated by RT- Real Time PCR (Figure 6A). Next, we investigated whether the changes at the RNA level were translated into protein. PCE treatment for 24 h led to a very modest increase in CYP1A1 protein levels (1.2-fold). A time course incubation during 24, 48, 72 and 96 h led to an increase in CYP1A1 protein in MCF-7 cells of 3.9-fold after 48 h (Figure 6B). The difference between mRNA levels and the corresponding protein levels may indicate that many of the mRNA molecules does not reach

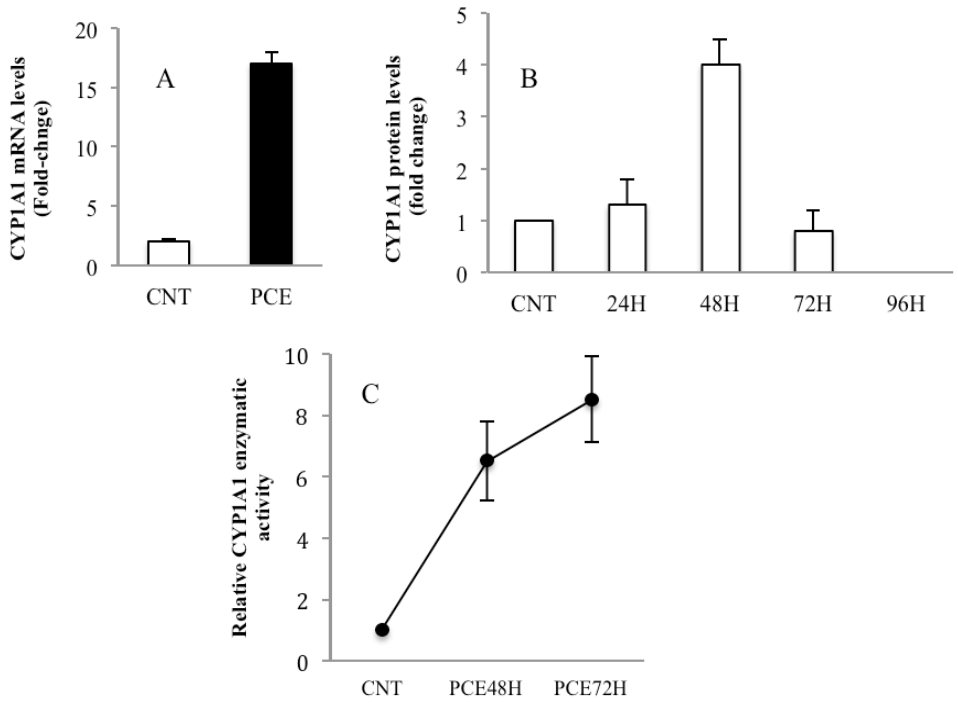

Figure 6. CYP1A1 overexpression in MCF-7 cells treated with PCE. (A) Determination of CYP1A1 mRNA levels. Results are expressed in fold changes compared to MCF-7 control and are the mean ± SE of 3 different experiments. (B) Determination of CYP1A1 protein levels. Results represent the mean ± SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA plus post hoc Bonferroni comparison. (C) Determination of CYP1A1 activity in MCF-7 treated cells. Results are expressed relative to the activity of the control and represent the mean ± SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA, plus post hoc Bonferroni comparison.

the translational machinery, probably because the translation mechanism is saturated in these conditions. Finally, CYP1A1 activity was determined upon incubation with PCE. An increase in CYP1A1 activity in good correlation with the observed increased in CYP1A1 protein levels was determined for both cell lines (Figure 6C).

The changes in CYP1A1 expression upon incubation with PCE could explain the antioxidant effect of flavonoids at the molecular level since this gene is involved in different oxidative pathways. Additionally, CYP1A1 overexpression might interfere with estrogen metabolism and the production of estrogen metabolites in breast cells. The increase in CYP1A1 activity may shift estrogen metabolism toward the production of 2-OHE2 (2-hydroxyoestradiol), a relatively non-genotoxic metabolite [71].

Finally, we wanted to test whether cocoa polyphenols would exert a synergistic effect in combination with TAM, since it has been described a synergistic effect for the combination of tamoxifen (TAM) with EGCG in breast cancer cells [72,73]. Thus, MCF-7 cells were incubated with increasing concentrations of TAM (10^{-6}–10^{-3}M) either alone or in combination with PCE (250 ng/µL). Then, cell viability was determined after 48 h. The presence of PCE, which did not cause significant cell death by itself, increased the cytotoxic effect of TAM in MCF-7 cells (Figure 7).

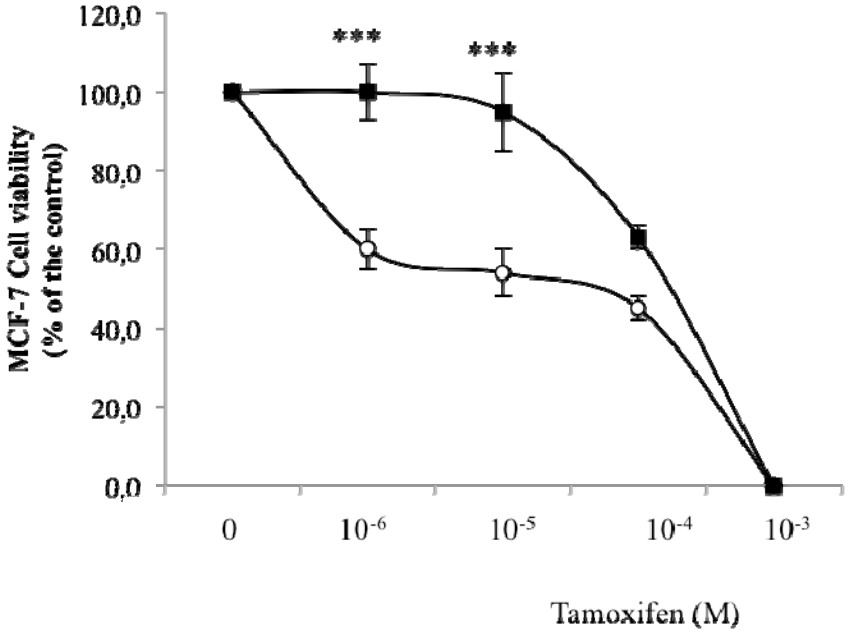

Figure 7. Effect of tamoxifen plus PCE on MCF-7 viability. Tamoxifen (TAM) either alone (filled squares) or in combination with PCE (250 ng/µL for 24H, empty circles). Results are expressed as % of living cells compared to the control only with DMSO (0.22%) and represent the mean ± SE of 3 different experiments. ***$p<0.001$.

The reduction in cell viability reached an increase of 44% when combined with 10^{-6}M TAM. Thus, in our conditions, the cytotoxic effect of TAM was enhanced by the combination with PCE in MCF-7 cells. The presence of PCE caused a synergistic effect, confirmed by the Chou-Talay method, which led to a decrease in cell viability of up to 40% in MCF-7 cells at tamoxifen concentrations that did not affect cell viability by themselves. A plausible explanation of the synergistic effect observed could be that the increase in estrogen metabolism, induced by the PCE on CYP1A1, could lead to the reduction in the levels of estrogens in mammary tumours, thus contributing to the cytotoxic effect of tamoxifen. Nevertheless, further *in vivo* studies are necessary to analyse the synergism between tamoxifen and cocoa and to establish the possible benefits of cocoa polyphenol consumption during breast cancer therapy.

3. Conclusions

Current global trends in food consumption may have an impact on disease progressions observed worldwide. The impact may occur by means of nutrient regulation of genes, or by other unclear that are yet to be discovered. The "omics" and associated technology will surely provide a greater understanding of the environmental and behavioral factors that influence phenotype and its relationship to health and wellness. It is highly likely that during the next decade the nutritional supplement and functional food industries will experience robust growth in response to advances in nutritional genomics research and its applications.

Parallel to this growth will be impressive progress in understanding the specific influence of certain food components on metabolic pathways and their role in health and disease. It will become increasingly less expensive to generate genetic information about individual persons, and such data are likely to redefine the current concept of preventive medicine. Moreover, through nutrigenomic research, new nutritional regulation of gene expression will hopefully come to light. If specific nutrient regulation of genes closely related to disease onset or progression is identified new arenas for disease prevention and potential for treatment will come to the foreground of nutritional medicine and preventive medicine. Discoveries made in the field of nutrigenomics and nutrigenetics should translate into more effective dietary strategies to improve overall health by identifying unique targets for prevention.

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5.DISCUSIÓN

Los diferentes estudios realizados en esta Tesis se enmarcan dentro del campo de la investigación en Nutrigenómica que, como se ha comentado anteriormente, tiene como objetivo estudiar cómo influyen la dieta y/o determinados compuestos de la misma sobre la expresión del genoma. Los resultados generarán nuevos conocimientos que permitirán conocer la repercusión que un determinado tipo de dieta o de componentes (nutrientes y otros compuestos presentes en los alimentos) pueda tener sobre la salud, por lo que las expectativas son muy elevadas.

La búsqueda de compuestos bioactivos y el estudio de sus efectos beneficiosos en el organismo, así como el mecanismo de acción por el que actúan, es uno de los objetivos principales de la nutrigenómica (Kris-Etherton, et al., 2004). Los avances en la tecnología y el amplio conocimiento del genoma en la actualidad (Afman and Muller, 2006) harán posible alcanzar estos objetivos y, seguramente en un futuro no muy lejano, se podrán incorporar recomendaciones dietéticas, en base a los resultados nutrigenómicos, con un cierto grado terapéutico (Bouchard and Ordovas, 2012).

La discusión de los resultados obtenidos en esta tesis se han organizado teniendo en cuenta los genes y las vías de señalización afectados en cada bloque de resultados.

5.1 CYP1A1, AhR, Arnt y ER

La incubación con un extracto de polifenoles de cacao (PCE), a concentraciones no citotóxicas, en dos líneas celulares de cáncer de mama, MCF-7 dependiente de estrógenos y SKBR3 no dependiente de estrógenos, modula la expresión de varios genes relacionados con estrés y toxicidad. De los genes diferencialmente expresados, el gen *CYP1A1* (*Citocromo p450 1A1*) resulta ser el más sobreexpresado en ambas líneas celulares después del tratamiento con PCE. Para confirmar el incremento en la expresión de CYP1A1, observado a través de la plataforma de PCR Array, determinamos la expresión de CYP1A1 por la tecnología de las sondas TaqMan de Applied Biosystems en una reacción de RT-PCR a Tiempo Real. También observamos que el incremento del mRNA de CYP1A1 inducido por PCE se traducía en una mayor cantidad y actividad enzimática de la proteína correspondiente.

La proteína CYP1A1 pertenece a la superfamilia de las isoenzimas citocromo p450 o hemoproteínas, caracterizadas por contener un grupo hemo en su estructura. Estas isoenzimas se localizan principalmente en la bicapa lipídica del retículo endoplasmático de los hepatocitos, encargándose de reacciones de metabolización de fase I (Figura D.1). Su función es la hidroxilación de moléculas susceptibles de ser eliminadas haciéndolas más hidrófilas.

$$NADPH + H^+ + O_2 + RH \rightarrow NAPD^+ + H_2O + ROH$$

Figura D.1. Estequiometría de la reacción de oxidación catalizada por las isoenzimas CYP p450. "RH" representa el sustrato oxidable por las isoenzimas y "ROH" el metabolito generado con su grupo hidroxilo (Bibi, 2008).

El gen *CYP1A1* (perteneciente a la familia CYP1 y subfamilia A) se localiza en el cromosoma 15 y comparte 25 kb de promotor con *CYP1A2*, otro gen de la misma subfamilia (Figura D.2). Se expresa principalmente en pulmones, glándulas mamarias, placenta y linfocitos, y también en hígado, aunque en menor cantidad (Bibi, 2008). Este enzima juega un papel importante en la metabolización de xenobióticos y carcinógenos, así como, en el metabolismo oxidativo de los estrógenos (Delescluse, et al., 2000), aunque se desconoce si existe un sustrato endógeno a día de hoy.

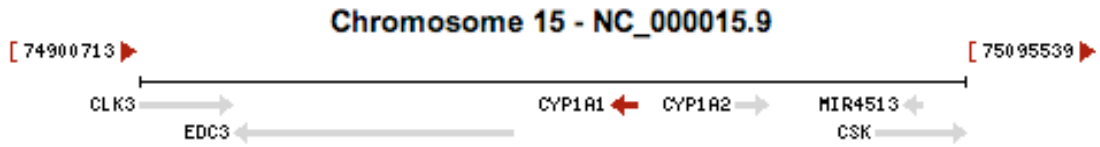

Figura D.2. Localización del gen *CYP1A1* en el cromosoma 15 (NCBI, nucleotide NC_000015.9)

Los metabolitos generados por la metabolización catalizada por CYP1A1 pueden tener mayor o menor actividad que el sustrato original. Así, pueden no tener ningún efecto, simplemente ser eliminados, o bien pueden ejercer una función en el organismo igual o diferente a la del compuesto original. Por ejemplo, los compuestos hidrocarburos son sustrato de CYP1A1 y, algunos de los metabolitos generados son capaces de promover la iniciación y promoción de procesos tumorogénicos. También los estrógenos E1 (estrona) y E2 (estradiol) son sustrato de CYP1A1, pero en este caso sus metabolitos tienen un papel protector en el cáncer de mama (Okey, et al., 1994). La industria farmacéutica aprovecha el proceso de metabolización para la activación de moléculas en los casos en que el pro-fármaco tenga una mayor biodisponibilidad o una mayor estabilidad durante el proceso de fabricación, así como, en el estudio de nuevas indicaciones para los diferentes metabolitos activos (Gad, 2003).

La inducción de CYP1A1, tanto los niveles de mRNA como de proteína y actividad, por la quercetina en la línea celular MCF-7 ya había sido demostrada (Ciolino, et al., 1999). Estudios posteriores demostraron que la especificidad de la activación de CYP1A1 con este polifenol dependía de la actividad de la unión entre este compuesto

y AhR (Ciolino, et al., 1999, Ramadass, et al., 2003). Teniendo en cuenta que el extracto de polifenoles de cacao utilizado en este trabajo contiene, entre otros, quercetina, los resultados observados de la inducción de CYP1A1 en MCF-7 y SKBR3 después de la incubación con PCE estarían en concordancia con Ciolino y col. 1999 (Ciolino, et al., 1999) y Ramadass y col. 2003 (Ramadass, et al., 2003).

La metabolización de estrógenos la llevaban a cabo algunas isoenzimas del grupo p450. Los metabolitos resultantes de su hidroxilación (fase I de su metabolismo) son 2-OHE1, 2-OHE2, 4-OHE1, 4-OHE2, 16α-OHE1, 16α-OHE2. De estos metabolitos, 4-OHE1 y 16α-OHE2, continúan teniendo capacidad estrogénica y actúan como carcinógenos por su capacidad de formar aductos con el DNA. Por otro lado, los metabolitos hidroxilados en posición 2 poseen propiedades anticarcinogénicas después de la acción de la *O*-metiltransferasa (Clemons and Goss, 2001). El *ratio* entre las enzimas que hidroxilan en las posiciones 2 y 16α controlará las proporciones de metabolitos carcinogénicos o anticarcinogénicos generados en la degradación de estrógenos. Actualmente, este *ratio* se utiliza como prueba diagnóstica para calcular el riesgo a desarrollar un proceso tumoral dependiente de estrógenos, mediante la detección de metabolitos de estrógenos en orina por espectroscopía de masas-HPLC o bien ELISA (Dallal and Taioli, 2010). El enzima CYP1A1 cataliza la hidroxilación en la posición 2, generando los catecoles 2-OHE2 y 2-OHE1, por lo que un incremento en la actividad de este enzima, como el inducido por PCE o el ya descrito por compuestos indólicos de crucíferas (Michnovicz and Bradlow, 1990) favorecerá el ratio 2/16 otorgando un papel protector frente a procesos tumorogénicos (Clemons and Goss, 2001, Longcope, 1990). La Figura D.3 resume el proceso de metabolización de estrógenos descrito en este párrafo.

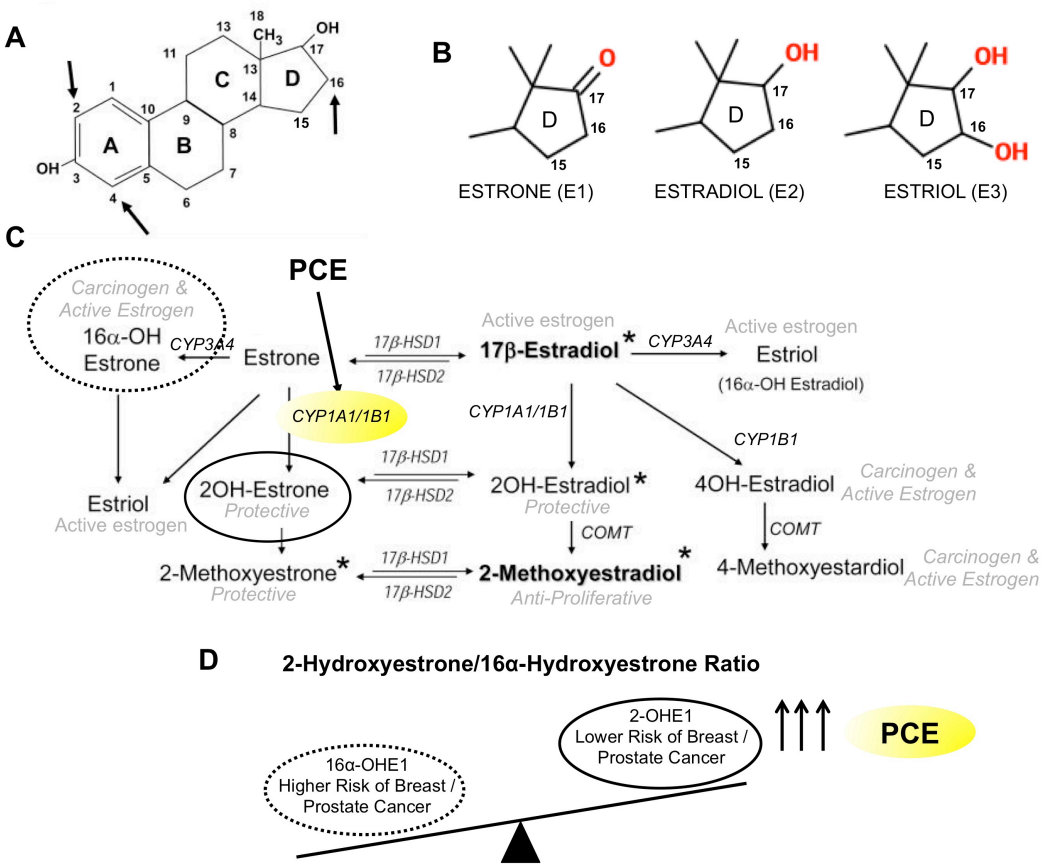

Figura D.3. Esquema de la metabolización de estrógenos. El metabolismo oxidativo de estrógenos ocurre en las posiciones C2, C4 y C16 de su estructura (A y B) llevando a la formación de metabolitos que pueden tener diferentes propiedades biológicas (C). Un aumento en la actividad 2-hidroxilasa, como el observado con PCE, podría favorecer el ratio 2/16 de los catecoles estrogénicos reduciendo el riesgo de un desarrollo tumoral (Clemons and Goss, 2001, Michnovicz and Bradlow, 1990, Schneider, et al., 1984, Tofovic, 2010)).

En la determinación de los niveles de mRNA de CYP1A1 por RT-PCR a tiempo real, se observó que la línea celular SKBR3 expresaba 16 veces más mRNA que la línea MCF-7 (Figura 1.A. del Artículo I). Los niveles de expresión de CYP1A1 varían en diferentes líneas tumorales y no tumorales de mama (Spink, et al., 1998). Esta diferencia en los niveles de expresión basales de CYP1A1 podría ser debida a que la línea celular MCF-7 es dependiente de estrógenos y puesto que el enzima CYP1A1 es metabolizador de éstos, estas células podrían disponer de un sistema para reprimir, o mantener bajos los niveles de CYP1A1.

Para estudiar el mecanismo de acción por el que el PCE es capaz de inducir a CYP1A1, nuestro trabajo se centró en la regulación transcripcional de este gen por la vía de señalización del receptor Aril (AhR). AhR se encuentra en el citoplasma formando un complejo heterogéneo donde participa la chaperona Hsp90. En presencia de su ligando, AhR se separa de su complejo citoplasmático y se une a su ligando por

el que presenta una mayor afinidad. Una vez unidos podrán translocarse al núcleo donde AhR forma un heterodímero con Arnt, el translocador nuclear del receptor Aril. El dímero AhR/Arnt tiene afinidad por una secuencia específica de DNA, 5'-GCGTG-3', la secuencia "core" de los elementos de respuesta a xenobióticos (XRE). De esta manera el dímero AhR/Arnt puede activar la transcripción de los genes que contengan cajas de unión o elementos de respuesta XRE en sus promotores, como *CYP1A1* (Balan, et al., 2006, Trapani, et al., 2003, Wijayaratne and McDonnell, 2001) (Figura D.4).

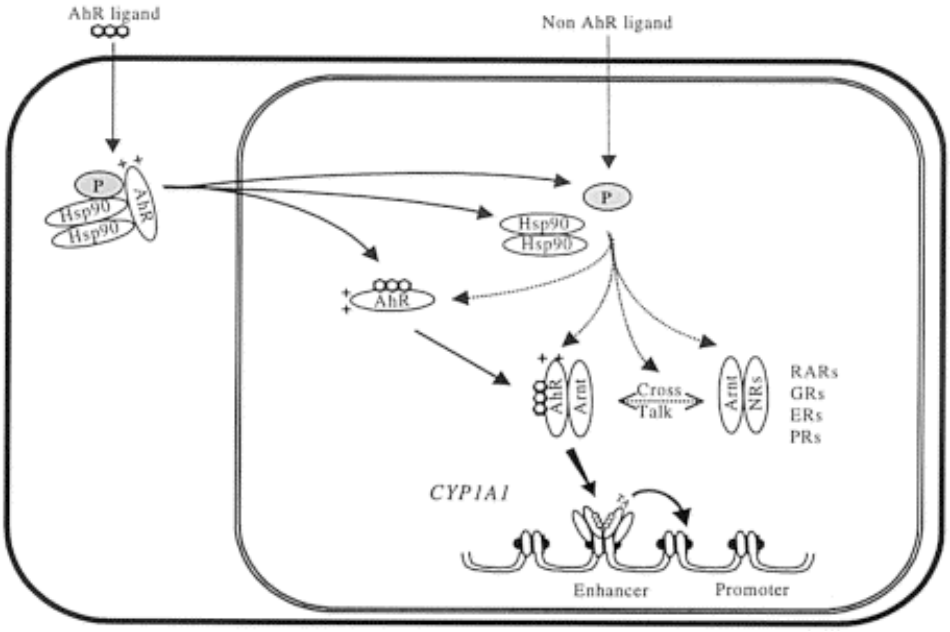

Figura D.4. Esquema de la vía de señalización de AhR (Delescluse, et al., 2000)**.**

Los resultados de los ensayos de gel-shift y supershift demostraron la participación de AhR en ambas líneas celulares. En la línea celular MCF-7, la incubación con PCE es capaz de inducir la expresión de CYP1A1 a través del incremento en la unión del AhR a las cuatro cajas XRE presentes en el fragmento de 327 pb específico del promotor de *CYP1A1*, sugiriendo que hay una activación en la vía de señalización de AhR (Figura 2. del Artículo I). Estos resultados concuerdan con la inducción de la actividad transcripcional por la incubación con PCE a través de las cajas XRE del promotor de *CYP1A1,* determinada posteriormente en ensayos de actividad luciferasa (Figura Art.I.9). Por otro lado, los niveles basales superiores de CYP1A1 en SKBR3 se traducen en una mayor unión AhR/DNA inicial con respecto a MCF-7, aunque la unión proteína/DNA responde de manera diferente ante la incubación con PCE, ya que se debilita. Aún y así, la actividad transcripcional de CYP1A1, en SKBR3, incrementa en presencia de PCE (Figura Art.I.9), correspondiendo al aumento en los niveles de expresión del gen y de su actividad enzimática (Figura 1. del Artículo I). Este cambio

sugiere la participación de otra vía de señalización paralela a la de AhR pero con el mismo efecto final, incrementar la actividad transcripcional de *CYP1A1* por PCE.

La disminución de la unión AhR/DNA en SKBR3 por PCE no se debe a una disminución de los niveles de proteína AhR. Nuestras observaciones demostraron un incremento de los niveles de AhR junto con los de CYP1A1 en SKBR3, que se hace máximo a las 72h de incubación con PCE. En las células MCF-7 estas dos proteínas también están inducidas con PCE, pero con un máximo más temprano, a las 48h de la incubación (Figura 3. del Artículo I).

Puesto que está descrito que AhR es capaz de comunicarse con otros receptores en el núcleo, como RARs, GRs, ERs o PRs (Delescluse, et al., 2000), y las dos líneas celulares con las que trabajamos se diferencian en sus niveles de receptor de estrógeno, se determinaron los niveles de esta proteína ERα en respuesta a la incubación con PCE. El tratamiento con PCE altera el perfil de ambas líneas celulares, incrementando la cantidad de ERα en SKBR3 (ER-negativas) junto con AhR y CYP1A1, y disminuyendo el nivel de ERα en MCF-7 (ER-positivas) coincidiendo con la máxima inducción de CYP1A1 (Figura 3. del Artículo I).

La posibilidad de que el PCE esté afectando de manera diferente la comunicación entre AhR y ERα en las líneas celulares estudiadas nos llevó a determinar si el PCE podía modular la interacción entre estos dos receptores. Mediante un ensayo de co-inmunoprecipitación, se comprobó esta unión y no se observaron modificaciones en respuesta a la incubación con PCE (Figura 3. del Artículo I).

La comunicación o “crosstalk” entre AhR y ERα ya había sido demostrada anteriormente, e incluso se habían descrito cambios en el mecanismo de interacción dependiendo del tipo celular estudiado (Matthews and Gustafsson, 2006, Safe, et al., 2000). Este “crosstalk” conlleva a una competición por afinidades entre ERα y Arnt por la unión a AhR en varios promotores (Safe, et al., 2000). Nuestra hipótesis plantea que un elevado nivel de ERα, como en las células MCF-7, puede bloquear, reprimir o interponerse en la unión del complejo AhR/Arnt a sus elementos de respuesta XRE, resultando en una unión menor al DNA, tal como se observó en los ensayos de retardación. No obstante, el nivel elevado de ERα no afectaría a la activación de la transcripción, puesto que la inducción de CYP1A1 por PCE se da en ambas líneas celulares, independientemente de los niveles de proteína ERα basales. Así, en la incubación con PCE, es posible una mayor translocación de AhR al núcleo en MCF-7 que consigue desbloquear la unión al DNA reprimida por el exceso de ERα, consiguiendo un incremento en la unión proteína/DNA. La incubación con PCE en las células SKBR3 conlleva a un aumento en el nivel de proteína ERα, y este aumento bloquea o reprime la unión del dímero AhR/Arnt a las cajas XRE. Sin embargo, la

sobreexpresión de ERα mediante la transfección de un vector de expresión si induce, aunque de manera leve, la transcripción de CYP1A1, en ambas líneas celulares. Esta leve inducción se ve incrementada en combinación con el tratamiento de PCE (Figura Art.I.10).

Los estudios de Krishnan y col. 1995 (Krishnan, et al., 1995) y Bjornstrom y Sjoberg 2005 (Bjornstrom and Sjoberg, 2005) sobre el mecanismo inhibitorio del "crosstalk" demuestran la participación de ERα que, dimerizado con Sp1, puede unirse a secuencias ricas en GCs. El promotor del gen *catepsina D* contiene elementos de respuesta para el dímero ERα/Sp1. La secuencia de estos elementos coincide en 5 nucleótidos (5'-GCGTG-3') con parte de la secuencia de los elementos de respuesta XRE. En el caso del promotor de *CYP1A1*, la unión del dímero ERα/Sp1 a la secuencia "GC-rich Sp1-ERE", dentro del elemento de respuesta XRE quedaría interrumpida en presencia de AhR. La sonda que se utilizó de 327pb con cuatro cajas XRE también presenta las secuencias correspondientes a la unión de ERα/Sp1 (Figura D.5), pudiendo darse el mismo caso. En presencia de elevados niveles de ERα, éste se unirá al DNA junto con Sp1, pudiendo reprimir la transcripción de *CYP1A1*, pero la translocación al núcleo de AhR, inducida por PCE, desplaza la unión del dímero ERα/Sp1 al DNA y facilita la unión de AhR a XRE induciendo la transcripción de *CYP1A1*.

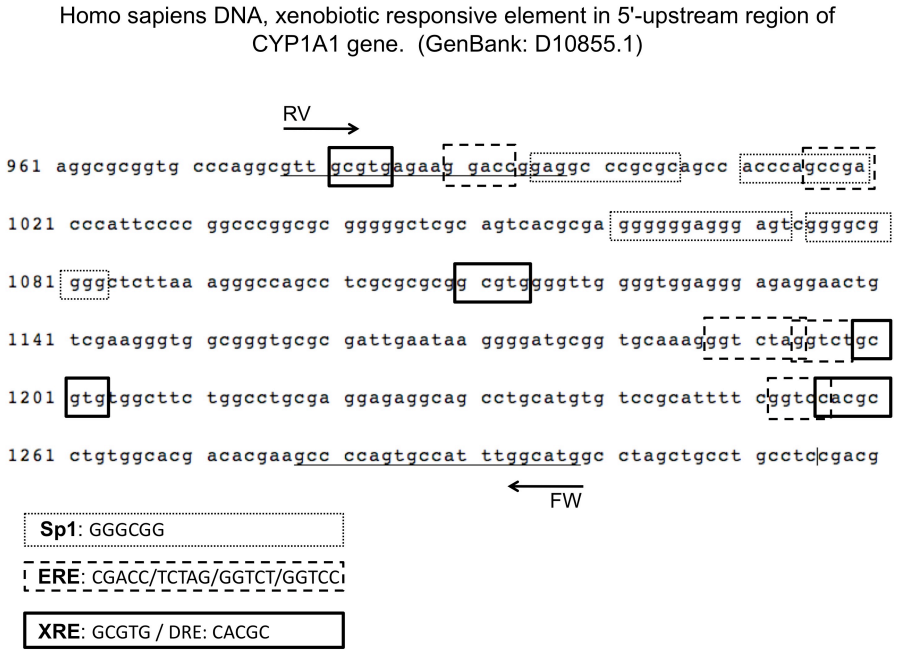

Figura D.5. Secuencia de la sonda XRE. La secuencia de 327pb correspondientes a una sección del promotor *CYP1A1* contiene cuatro cajas XRE (o DRE). Utilizando el programa Match ™ de Gene-Regulation se localizaron secuencias de unión para Sp1 y ERα que podrían reproducir la situación del promotor de *catepsina D*.

La incubación con PCE en ambos modelos conlleva al aumento en la expresión de CYP1A1, pero el mecanismo por el que se lleva a cabo se diferencia según el tipo celular. Por ello, se analizaron los niveles de ERα en ambas líneas celulares y su variación ante la incubación con PCE. En las células MCF-7, el PCE provoca una disminución en los niveles de ERα. ERα es una proteína precursora y su activación resulta de una actividad similar a la de la quimiotripsina en el proteosoma, así pues, es necesaria su degradación para que sea activa (Balan, et al., 2006). Por otro lado, el PCE en las células SKBR3 induce un incremento en los niveles de la forma precursora ERα y, al mismo tiempo, disminuye su semivida hasta valores similares a los determinados en MCF-7 (3-5h) (Alarid, et al., 1999), debido a la aceleración del proceso de degradación o activación por el proteosoma (Figura 4.b del Artículo I). El PCE no afecta a los niveles de expresión de ERα, pero sí induce su traducción y su degradación en las células SKBR3 (Figura 4.a&c del Artículo I).

Los polifenoles del cacao podrían estar comportándose como un SERM (*selective estrogen receptor modulator* o modulador selectivo del recetor de estrógeno) actuando como agonistas o antagonistas del receptor de estrógeno en cada modelo celular. La estabilidad y actividad transcripcional de ERα dependerá de la conformación que obtenga al unirse a su ligando (Wijayaratne and McDonnell, 2001).

Independientemente de cómo afecte el PCE a los niveles de proteína ERα, nuestros resultados han demostrado que un silenciamiento de ERα no afecta a la capacidad de PCE para inducir la expresión de CYP1A1 en las células SKBR3, pero sí incapacita la inducción de CYP1A1 por PCE en las células MCF-7 (Figura Art.I.8). El PCE afecta los niveles de ERα en SKBR3 pero no parece que este receptor nuclear tenga un gran peso en la activación de CYP1A1 en esta línea celular. Las células SKBR3 se consideran no dependientes de estrógenos puesto que no sobreexpresan ERα, pero si sobreexpresan el receptor HER2/c-erb-2, una proteína perteneciente a la familia de los receptores de factores de crecimiento epidérmicos (*EGFRs; Epidermal Growth Factor Receptors*) (Slamon, et al., 1989, Yu and Hung, 2000). El silenciamiento de este receptor en las células SKBR3 bloquea la inducción de CYP1A1 mediada por PCE, pero no afecta a su inducción en la línea MCF-7 (Figura Art.I.9). Esta observación abre las puertas a la participación de otra molécula en el mecanismo de sobreexpresión de CYP1A1 en las células SKBR3.

Son varios los estudios realizados para incrementar la eficacia de tratamientos con Tamoxifeno mediante la combinación con otros compuestos, tanto fármacos como compuestos naturales. La Tabla D.1 recopila algunos de estos estudios.

Combinación de TAM y:	Modelo	Efecto	Referencia
Docetaxel	MDA-MB-231, CEM-VBLr y MCF-7ADr	Inhibición del crecimiento celular sinérgico	(Ferlini, et al., 1997)
Genisteína	MDA-MB-435	Actúa de manera sinérgica aumentando la citotoxicidad	(Shen, et al., 1999)
6-MCDF (un antagonista selectivo del AhR)	Tumor mamario inducido por DMBA en hembras de female Sprague Dawley (*in vivo*)	Inhibición del crecimiento del tumor por la combinación de ambos compuestos.	(McDougal, et al., 2001)
EGCG (Epigalocatequina galato)	MDA-MB-231	Efecto sinérgico induciendo la citotoxicidad celular a bajas concentraciones de ambos compuestos.	(Chisholm, et al., 2004)
		La combinación induce la apoptosis celular.	(Stuart, et al., 2007)
	Xenógrafos de MDA-MB-231	La combinación induce inhibición del crecimiento tumoral mayor que con los compuestos por separado.	(Scandlyn, et al., 2008)
	PC-9	Aumento en la apoptosis en la combinación de los compuestos	(Suganuma, et al., 1999)
Extracto de te verde	Xenógrafos de MCF-7	La combinación induce una inhibición del crecimiento tumoral mayor que con los compuestos por separado. Aumenta la apoptosis y se suprime la angiogénesis.	(Sartippour, et al., 2006)

Tabla D.1. Estudios publicados que investigan en la mejora del tratamiento con Tamoxifeno combinando este fármaco con otros compuestos.

En nuestras condiciones experimentales, el efecto del Tamoxifeno aumenta cuando se combina con PCE en las líneas tumorales MCF-7 y SKBR3, pero no en una línea no tumoral HEK293T. La combinación de ambos compuestos, a concentraciones no tóxicas por separado, reduce la viabilidad celular en un 20% en SKBR3 y un 40% en MCF-7. Además se ha evaluado el sinergismo de la combinación por el método de Chou-Talalay (Online resource 4). La actividad sinérgica observada de la combinación podría ser debida a varios factores, como por ejemplo; **i**) el Tamoxifeno es un compuesto precursor y para activarse necesita un proceso de metabolización que los cataliza precisamente, CYP1A1 (Crewe, et al., 2002) para generar 4-OHT (metabolito

activo). De esta manera, sería posible que la inducción de CYP1A1 estimulada por PCE ayude a la activación de la molécula precursora. Además el compuesto 4-OHT induce la expresión y actividad de CYP1A1 (DuSell, et al., 2010). **ii)** Un aumento de la actividad enzimática de CYP1A1 acelerará el metabolismo de estrógenos, traduciéndose en una doble privación de estrógenos en las células ERα-positivas. Para evaluar si la apoptosis es un posible mecanismo de citotoxicidad para la combinación de TAM y PCE, se analizaron los niveles de apoptosis en las dos líneas tumorales. Nuestros resultados muestran que la combinación de PCE y TAM induce citotoxicidad por un aumento de la apoptosis en la línea celular MCF-7.

En resumen, la tecnología PCR Array nos ha permitido detectar la sobreexpresión de CYP1A1 inducida por el tratamiento con un extracto de polifenoles de cacao. La inducción de este gen que está relacionado con procesos de oxidación podría explicar el papel que juegan los polifenoles como antioxidantes. PCE actúa a través de la vía de señalización de AhR que se comunica con ERα para la inducción de CYP1A1 en las líneas celulares MCF-7 y SKBR3. Los efectos citotóxicos de la combinación sinérgica entre PCE y TAM deben estudiarse mediante ensayos *in vivo* para poder establecer beneficios del consumo de cacao durante una terapia de cáncer de mama.

5.2 ApoAI, HNF-3β, NFY & Sp1

El aumento de los niveles de HDL en plasma conseguido por metabolitos del cacao (Corti, et al., 2009, Duenas, et al., 2012, Schewe, et al., 2008) podría ser la base para una posible recomendación dietética como prevención de procesos aterogénicos y enfermedades o eventos cardiovasculares (CVD) (Duffy and Rader, 2006, Tall, 1990). Comprender el mecanismo de acción por el que estos metabolitos son capaces de inducir los niveles de HDL fue el objetivo planteado de este estudio.

El principal favanol del cacao, la epicatequina (EPI), induce la expresión de ApoAI, la proteína mayoritaria de las HDL, en células HepG2, como ya había demostrado Yasuda y col. 2011 (Yasuda, et al., 2011) al igual que el control positivo utilizado, la Atorvastatina (ATR) (Maejima, et al., 2004, Martin, et al., 2001). La regulación transcripcional del gen *ApoAI* esta controlada principalmente por una secuencia en la parte proximal del promotor llamada "*liver-specifc enhancer*" donde existen cajas de unión para numerosos factores de transcripción. Esta secuencia se divide en tres regiones Site A, Site B y Site C (Malik, 2003). La Figura D.6, muestra las posiciones de estas tres regiones en la parte proximal del promotor. Las regiones Site A y Site B de esta secuencia generan un patrón de tres bandas con la unión de proteínas nucleares de células HepG2. Ante un tratamiento con EPI, la unión proteína/DNA se ve inducida

en HepG2 y HeLa. Los factores de transcripción HNF-4 y RXRα, ya descritos anteriormente (Hargrove, et al., 1999, Malik, 2003, Rottman, et al., 1991), participan en la unión a la región Site A tanto en ausencia como en presencia de EPI. ERα, a pesar de tener dos cajas de unión "half-palindromic sites" en el Site A (Lamon-Fava and Micherone, 2004), no parece estar participando directamente en la unión al DNA, según nuestros resultados. El factor de transcripción HNF-3β, a pesar de no tener caja de unión en la región Site A, se une indirectamente puesto que en presencia de un anticuerpo específico contra HNF-3β genera una retardación en la segunda banda del patrón proteína/DNA. Esta unión indirecta podría justificarse por su cooperación con HNF-4 para facilitar el acceso a la maquinaria de transcripción (Cirillo, et al., 2002). La comunicación entre los factores hepáticos nucleares HNF-4 y HNF-3β puede actuar de manera sinérgica para la activación del promotor, en nuestro caso la presencia de los dos anticuerpos específicos (anti-HNF-4 y anti-HNF-3β) desvela una competición por el Site A, no siendo el caso en la región Site B. En base a nuestras observaciones, sugerimos que la posible sinergia sea inducida por la unión de cada factor a su correspondiente caja o por la participación, dependiente del ambiente celular, de un factor intermediario que comunique a los factores HNF-4 y HNF-3β (Harnish, et al., 1996). También se comprobó la unión del factor de transcripción HNF-3β a la región Site B, y su inducción por EPI, no siendo el caso para HNF-4 ni ERα.

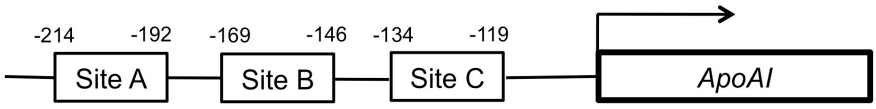

Figura D.6. Esquema de las regiones que componen la secuencia "*liver-specific enhancer*" del promotor proximal del gen *ApoAI*. La región Site A (22nt) se encuentra en la posición más 5', a 214 nucleótidos del inicio de transcripción. En dirección 3' continúan las regiones Site B (23nt) y Site C (15nt).

Para caracterizar la unión del factor de transcripción HNF-3β a la región Site B estudiamos la actividad promotora de esta región. La selección del Site B para generar una construcción reportera de actividad luciferasa se basó en los trabajos de Lamon-Fava y Micherone 2004 (Lamon-Fava and Micherone, 2004) y Taylor y col. 2000 (Taylor, et al., 2000), que habían descrito previamente la capacidad activadora de la isoflavona genisteína y el estradiol sobre la transcripción de *ApoAI* a través de la región Site B del promotor. Los ensayos de actividad luciferasa demostraron que EPI activa la transcripción de *ApoAI* induciendo la unión de factores de transcripción como

HNF-3β a la región Site B. También E1 y ATR, así como los metabolitos flavanoles del cacao estudiados, inducen la transcripción de ApoAI a través de la región Site B.

El metabolito con mayor actividad promotora sobre la región Site B es 3-M-EPI y su efecto implica la participación de los factores de transcripción HNF-3β, NFY y Sp1. La región Site B presenta tres cajas de unión, una para cada uno de estos factores. Pese a que la caja de unión para NFY no es la clásica secuencia CCAAT, su unión a la región Site B ya había estado descrita con anterioridad por Papazafiri y col. 1991 (Papazafiri, et al., 1991), así como su participación en la activación de la transcripción de *ApoAI* (Novak and Bydlowski, 1997). La unión de Sp1 a la región Site B y su capacidad para inducir la transcripción también había estado descrita anteriormente en respuesta a insulina (Haas, et al., 2004, Lam, et al., 2003). Sp1 puede además actuar de manera indirecta, ya que la unión de Sp1 a los elementos de respuesta a insulina activará a PKC y PKA, ambos inductores de la transcripción de ApoAI (Zheng, et al., 2000). La participación de Sp1 también es necesaria para el complejo inductor de ApoAI formado por EGF y la MAP quinasa activada EGF "*Ras-mitogen*" (Zhang, et al., 2001).

Los niveles de expresión de ApoAI y HNF-3β aumentan con EPI, ATR y los metabolitos estudiados. El metabolito 3-M-EPI, así como EPI y ATR, incrementan los niveles de proteína para HNF-3β, NFY y Sp1. La sobreexpresión de HNF-3β ya había sido detectada anteriormente en respuesta a ácidos grasos poliinsaturados en la dieta de ratas ACT/I (Vecchini, et al., 2005) y en ratas diabéticas, donde la sobreexpresión iba acompañada de una inducción significativa en la unión del factor de transcripción a su región en el DNA (Freitas, et al., 2009).

El incremento en la actividad transcripcional del Site B en las cotransfecciones con los vectores de expresión para NFY, Sp1 y ERα y su posterior inducción en presencial de 3-M-EPI, así como los resultados comentados anteriormente, sugieren que el incremento de la actividad de la región Site B por los metabolitos del cacao se debe a la sobreexpresión de HNF-3β, NFY y Sp1.

En resumen, el mecanismo por el cual el cacao y sus metabolitos incrementan los niveles de HDL resulta de la sobreexpresión de su proteína mayoritaria, ApoAI, a través de la activación de los factores de transcripción HNF-3β, NFY y Sp1. La implicación de ERα en la activación promotora de *ApoAI* podría tener lugar a través de una vía indirecta que no implica la unión a la región Site B. Finalmente, 3-M-EPI es el metabolito con mayor capacidad activadora de la transcripción de *ApoAI* a través de la región Site B localizada en la secuencia "*liver-specifc enhancer*" de su promotor.

5.3 STAT5B, ATF-2 & ciclina D1

El ácido cafeico (CA) es el compuesto fenólico más representativo y con mayor biodisponibilidad entre los ácidos hidroxicinámicos del café. En este proyecto, se han analizado los cambios en el perfil de expresión de células tumorales humanas mediados por el tratamiento con ICC (café soluble cafeinado) o con CA. Mediante la tecnología de Microarrays, se ha identificado la variación en la expresión de genes específicos relacionados con diferentes procesos biológicos. Los genes STAT5B y ATF-2 aparecían como genes más interconectados en la red de asociación biológica (BAN) generada a partir de los listados de genes diferencialmente expresados comunes a ambos tratamientos. La validación de la variación de la expresión de estos dos genes fue confirmada posteriormente por RT-PCR a tiempo real. También analizamos los niveles de proteína por *Western blot* de STAT5B y ATF-2 y la variación de una proteína regulada por ambas, la Ciclina D1.

Nuestros resultados muestran que ICC y CA, en cantidades equivalentes a las de una taza de café, provocan la sobreexpresión de STAT5B a nivel de mRNA y de proteína en las células HT29.

El gen *STAT5B* está localizado en el cromosoma 17 del genoma humano, flanqueado por los genes *GHDC* y *STAT5A*, con quien comparte el promotor. La proteína STAT5B pertenece a la familia de los factores de transcripción STAT, capaces de ser fosforilados por citoquinas y factores de crecimiento a través de receptores asociados a quinasas. Una vez fosforilados forman homo- o heterodímeros y se translocan al núcleo donde actuarán como factores de transcripción.

Esta familia la componen siete miembros; Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b y Stat6. STAT5A fue descubierta en tejido mamario ovino y se nombro "*mammary gland factor (MGF)*" (Schmitt-Ney, et al., 1991). Posteriormente se descubrió la isoforma B, en glándula mamaria de ratón, que a pesar de ser codificada por otro gen conserva un 96% de identidad a nivel de proteína (Wakao, et al., 1994). Ambas proteínas comparten estructura y funcionalidades semejantes con el resto de miembros de la familia. STAT5A y STAT5B se diferencian en la secuencia de aminoácidos de la cadena C-terminal, siendo únicos 20 aminoácidos para 5A y ocho para 5B (Darnell, 1997, Liao, et al., 2010, Tan and Nevalainen, 2008).

La principal función de STAT5B es la regulación de los efectos de la hormona del crecimiento (Teglund, et al., 1998, Udy, et al., 1997). Las moléculas conocidas como activadoras de stat5a/b son la eritropoyetina, la trombopoyetina, el factor estimulante de colonias de granulocitos y macrófagos (GM-CSF), la hormona del crecimiento, la insulina, la prolactina y las interleucinas (IL-2, IL-3, IL-5 y IL-7) (Darnell, 1997, Liao, et

al., 2010, Tan and Nevalainen, 2008). Cambios en la regulación del factor de transcripción STAT5B se habían descrito para otros compuestos de origen natural como el flavonoide cítrico nobiletina (Kanda, et al., 2012), flavonoides del té (Chattopadhyay, et al., 2009) y el mayor compuesto bioactivo del *Silybum marianum*, silibinina (Singh, et al., 2009). También un estudio con buteína, un compuesto polifenólico del *Rhus verniciflua*, demostró la capacidad para infraexpresar Bcl-xL, Bcl-2, ciclina D1 y Mcl-1, todos regulados por STAT3 (Pandey, et al., 2009).

STAT5B participa en diferentes procesos biológicos a parte de la regulación del crecimiento, en inmunoregulación, apoptosis, reproducción, regulación de la prolactina y en el metabolismo lipídico. Recientemente se ha relacionado su deficiencia como una enfermedad que comporta anomalías en el crecimiento, debidas a una resistencia a la hormona del crecimiento, e inmunodeficiencia severa (Nadeau, et al., 2011, Rotwein, 2012, Scaglia, et al., 2012). La inducción de STAT5B mediada por CA o ICC podría representar una herramienta nutricional para regular los niveles de este factor de transcripción, o bien sugerir nuevas estrategias para las alteraciones que afectan a la mucosa intestinal donde STAT5B participa en el mantenimiento y la tolerancia como la enfermedad de Crohn o el síndrome del colon irritable (Han, et al., 2006, Han, et al., 2009). STAT5A y STAT5B juegan un papel importante en la progresión del cáncer de colon, la infraexpresión de estos factores comporta un descenso en la viabilidad celular en respuesta al paro del ciclo celular en la fase G1 y la muerte apoptótica (Du, et al., 2012). La sobreexpresión de STAT5B inducida por CA e ICC podría tener un efecto negativo en el contexto del cáncer de colon puesto que estaría promoviendo la proliferación y la supervivencia de las células malignas.

En cáncer de mama, STAT5A/B participan tanto en la iniciación tumoral, así como en la promoción de la diferenciación a un tumor establecido en la glándula mamaria. Además STAT3, STAT5A y STAT5B están sobreexpresados en cáncer de mama (Garcia, et al., 2001, Garcia, et al., 1997, Yamashita, et al., 2003) y la activación de STAT5A/B es un factor favorable en el desarrollo de la enfermedad (Tan and Nevalainen, 2008). La vía de señalización de la prolactina se considera esencial para la proliferación y diferenciación de las células epiteliales mamarias (Frasor, et al., 2001, Frasor and Gibori, 2003, Yamashita, et al., 2003). Los niveles de NUC-pYSTAT5 descienden a medida que el cáncer de mama evoluciona del estado normal al invasivo y finalmente al metastático. Adicionalmente, la ausencia de NUC-pYSTAT5 en los tumores de pacientes en terapia antiestrogénica esta asociado con un peor pronóstico de supervivencia (Peck, et al., 2011). Nosotros hemos evaluado la modulación de STAT5B regulada por la vía de señalización de la prolactina a través de los polifenoles del café en una línea celular de cáncer de mama. Los tumores dependientes de

estrógenos suelen expresar de manera más clara el receptor de prolactina, por esta razón, se escogió la línea celular MCF-7 que sobreexpresa el receptor de estrógeno (Perotti, et al., 2008). El incremento observado en los niveles de STAT5B en las células MCF-7 producido por las incubaciones con CA y ICC podrían ser el inicio para una futura recomendación dietética en pacientes con cáncer de mama.

ATF-2 es un factor de transcripción perteneciente a la familia ATF-cAMP, proteínas que se unen a los elementos de respuesta cAMP (CRE) localizados en muchos promotores de genes en mamíferos (Benbrook and Jones, 1990, Hai, et al., 1989). Presenta dos funciones opuestas ya que puede actuar como oncogen o supresor tumoral (Bhoumik and Ronai, 2008). Los elementos CRE se encuentran en varios genes relacionados con el ciclo celular, como la ciclina D1, para la que ATF-2 estimula la transcripción (Beier, et al., 1999, Sabbah, et al., 1999). La unión de ATF-2 al promotor de ciclina D1 puede estar inducida por diferentes agentes promotores del crecimientos como estrógeno (Sabbah, et al., 1999), factores de crecimiento hepático (Recio and Merlino, 2002) o el factor Reg (*Regenerating gene product*) (Takasawa, et al., 2006). En líneas celulares de cáncer de mama se ha asociado ATF-2 con proliferación, invasión, migración y resistencia a agentes nocivos del DNA.

CA e ICC inducen la infraexpresión de ATF-2 en células HT29. Rubach y col. ya habían observado, en células gástricas, una disminución en la actividad ATF-2 después de incubación con ácido clorogénico, precursor de CA (Rubach, et al., 2012). Curiosamente, la infraexpresión de ATF-2 no llega a traducirse en una reducción de los niveles de proteína, sino en un aumento en el tratamiento con ICC pero no con CA, tanto en HT29 como en MCF-7. Este efecto podría atribuirse a otros compuestos que pueda contener el ICC, a parte del CA. Los estudios de Rubach y col. también analizaron el efecto de otros compuestos del café, a parte del ácido clorogénico, y su efecto sobre ATF-2. Pirogalol, catecol, βN -alcanoilhidroxitriptamina y N-metilpiridinio inducen la actividad de ATF-2, mientas que el CA y la cafeína la reducen (Rubach, et al., 2012). En nuestras condiciones experimentales, ICC reduce los niveles de ATF-2 de manera ligera en células HT29, pero se altera el nivel de proteína. Otros polifenoles contenidos en el ICC podrían inducir los niveles de proteína de ATF-2 mediante un aumento de la traducción, un aumento en la estabilidad de la proteína o una inhibición de su degradación. La inhibición del proteosoma ya se ha descrito anteriormente para diferentes polifenoles como la epigalocatequina galato, la genisteína, la luteolina, la apigenina, la crisina, la quercetina, la curcumina y el ácido tánico (Mujtaba and Dou, 2012, Shen, et al., 1999), indicando un posible mecanismo mediante el cual otros compuestos presentes en el ICC inhibiesen la degradación de ATF-2.

Diversos estudios con compuestos de origen natural han descrito alteraciones en la regulación transcripcional sobre el estado de fosforilación de ATF-2. En células MCF-7, el agente anticanceroso 3,30-Diindolimetano (DIM), derivado de plantas del género *Brassica*, activa las vías JNK y p38 resultando en la fosforilación de c-Jun y ATF-2 y un incremento en la unión del homodímero y heterodímero c-Jun-ATF-2 al elemento de respuesta del promotor de IFN-Y (Xue, et al., 2005). La isoflavona bichanina-A presente en el trébol rojo, el repollo y la alfalfa comporta un efecto inhibitorio y apoptótico en algunas líneas celulares tumorales mediante el bloqueo de la fosforilación de p38, MAPK y ATF-2 de manera dosis dependiente (Kole, et al., 2011). Por otro lado, la activación por estrés de la vía de JNK es una de las cascadas de señalización intracelular más afectadas en la inflamación intestinal (Ip and Davis, 1998, Romier, et al., 2009) y también está descrita la sobreexpresión de ATF-2 en la enfermedad de Crohn (Derer, et al., 2009, Hollenbach, et al., 2005). La acción combinada de CA sobre STAT5B y ATF-2 en células HT29 y los datos bibliográficos, sugieren que este ácido fenólico presenta propiedades como potencial agente terapéutico en diferentes estados de inflamación intestinal.

Por último, en nuestro estudio, se ha analizado como los polifenoles del café modulan la ciclina D1, regulada transcripcionalmente por STAT5B y ATF-2, en células de cáncer de colon y mama. La sobreexpresión de ciclina D1 es habitual en tumores colorrectales, pero su repercusión en el pronóstico es dudosa. Un estudio reciente describe la detección de la sobreexpresión de ciclina D1 en 96 individuos de 169 que padecían adenocarcinoma de colon, y su asociación con un mal pronóstico (Mao, et al., 2011). También se ha demostrado una correlación significativa entre pSTAT5 y ciclina D1 en adenocarcinoma de colon. En un segundo estudio se asoció la sobreexpresión de ciclina D1 con la mejoría de 386 pacientes de cáncer de colon en estados II y III, según el sistema TNM (tumor, nodo y metástasis), después de la resección quirúrgica del colon. También en pacientes en estados II y III (TNM) de cáncer de colon se relacionó un riesgo de recurrencia con niveles bajos de p21 y ciclina D1 y altos de p53 y AURKA (Belt, et al., 2012). Teniendo en cuenta estos efectos, la inducción de ciclina D1 provocada por ICC podría representar efectos positivos o negativos, dependiendo del contexto de la progresión tumoral. Por ejemplo, el efecto de ICC será positivo en el caso de un cáncer colorrectal en pacientes de sexo masculino, puesto que la sobreexpresión de ciclina D1 está asociada a una supervivencia prolongada y su ausencia con un fenotipo más agresivo (Wangefjord, et al., 2011). Son numerosos los compuestos naturales capaces de inducir el paro del ciclo celular e inducir apoptosis, dando mayor peso a la posibilidad de que sean otros compuestos no polifenólicos de ICC, los responsables de la inducción de ciclina D1 en

células HT29 (Hsu, et al., 2012, Lim, et al., 2007, Seelinger, et al., 2008, Suh, et al., 2009, Turktekin, et al., 2011).

En la línea celular de cáncer de mama MCF-7 los polifenoles del café consiguen reducir los niveles de proteína ciclina D1. La elección de la línea celular MCF-7 se debe, como comentamos anteriormente, a que la sobreexpresión de ciclina D1 está presente en numerosos subtipos de cáncer de mama mayoritariamente ER-positivos (Buckley, et al., 1993, Gillett, et al., 1998, Zukerberg, et al., 1995). La sobreexpresión de ciclina D1 se observa entre el 40 y el 90% de los casos de cáncer de mama invasivo, mientras que la amplificación génica se observa entre el 5 y el 20% de los tumores (Gillett, et al., 1998, Simpson, et al., 1997, Weinstat-Saslow, et al., 1995, Zukerberg, et al., 1995).

En los tumores asociados a la sobreexpresión de ciclina D1, el bloqueo de su expresión utilizando como diana el propio gen, RNA o proteína, debería incrementar las posibilidades de éxito de la terapia. Varios estudios en cultivos celulares han analizado compuestos que actúan en esta vía (Hsiang and Straus, 2002, Sawatsri, et al., 2001), y también diferentes aproximaciones para bloquear la expresión de ciclina D1 con moléculas antisentido, siRNA, u otras moléculas semejantes dirigidas específicamente contra la molécula diana (Arber, et al., 1997, Kornmann, et al., 1998, Sauter, et al., 1999). Los compuestos que modulan la ciclina D1 juegan un papel importante en la prevención y el tratamiento de neoplasias humanas. Un ejemplo es el flavonoide sintético flavopiridol, sintetizado basándose en un extracto de una planta india, por el potencial que tiene en el tratamiento del cáncer ya que reduce de manera drástica los niveles de ciclina D1 (Carlson, et al., 1999). Todos estos resultados indican que la inhibición de la expresión de la ciclina D1 es una buena aproximación para el tratamiento del cáncer. Nuestra observación de la reducción drástica de los niveles de proteína ciclina D1 con ICC o CA en células de cáncer de mama sugiere que algunos componentes del café puedan ser utilizados como terapia coadyuvante en el tratamiento del cáncer de mama.

La complejidad de la dieta hace que recomendaciones más básicas sean todo un desafío. Los alimentos no son elementos puros que actúen sobre dianas únicas, sino que son mezclas de moléculas muy complejas que pueden modular muchos procesos biológicos en el hospedador (Milner, 2003). A lo largo de esta tesis hemos utilizado tres tipos de aproximaciones para el estudio de compuestos bioactivos. (i) **Extractos de un alimento**, concentran todos los compuestos bioactivos con propiedades químicas similares. Este tipo de muestra abarca todos los compuestos de un determinado alimento de la dieta y suele emplearse cuando no hay estudios previos

que adjudiquen las propiedades beneficiosas de dicho alimento a una molécula en particular pero sí al conjunto. Esta aproximación es utilizada en estudios más iniciales o donde se quiera analizar el conjunto de compuestos bioactivos de ese alimento. No se podrá atribuir el efecto estudiado a una determinada molécula y la mezcla de compuestos bioactivos puede favorecer el efecto cuando todos lo compuestos actúen de la misma manera, o pueden desmerecer el efecto de unos compuestos por la presencia de otros, enmascarando una acción interesante. (ii) **Compuestos puros o moléculas aisladas**, el uso de un compuesto con características de compuesto bioactivo da un valor añadido a las investigaciones realizadas porque se puede otorgar un determinado efecto a una determinada molécula. Esta aproximación es posible cuando previamente se ha denominado compuesto bioactivo a una molécula en concreto. (iii) **Metabolitos de compuestos bioactivos**, generados en el organismo después de pasar por fases de metabolización, o en el aparato digestivo al ser metabolizados por la microbiota intestinal o por enterocitos. Son las moléculas que interaccionarán con las dianas y, por tanto, pueden dar una información más cercana a la situación real. Las tres aproximaciones tienen pros y contras, pero ninguna desmerece las investigaciones realizadas ya que ayudan a obtener información desde diferentes puntos de vista.

Para poder hacer recomendaciones de una ingesta seleccionada o concentrada en un compuesto en concreto, por encima de las necesidades alimentarias estándar, es imprescindible tener un conocimiento científico profundo para asegurar la eficacia y la salud pública. Diseñar una dieta para mejorar la salud fisiológica es un objetivo loable pero conlleva retos importantes (Milner, 2003).

6.CONCLUSIONES

6.1 El tratamiento con un extracto de polifenoles de cacao induce la expresión de CYP1A1, así como sus niveles de proteína y la actividad enzimática en dos líneas celulares de cáncer de mama, MCF-7 y SKBR3.

6.2 La inducción de CYP1A1 mediada por PCE tiene lugar a través de la vía de señalización de AhR. PCE induce la transcripción de *CYP1A1* a través de la unión de AhR a las cajas XRE de su promotor en ambas líneas celulares.

6.3 PCE induce los niveles de ERα en la línea celular SKBR3 aumentando la síntesis de la proteína, pero su vida media se reduce aproximadamente 10 horas en comparación con la proteína basal.

6.4 La incubación con PCE y TAM a concentraciones no citotóxicas inducen un efecto sinérgico provocando un descenso en la viabilidad de dos líneas de cáncer de mama, MCF-7 y SKBR3, pero no en la línea no tumoral HEK293T.

6.5 EPI y los metabolitos del cacao inducen la expresión de ApoAI en la línea celular HepG2.

6.6 El tratamiento con EPI en células HepG2 induce la unión de factores de transcripción a dos regiones del "*liver specific enhancer*", Site A y Site B. En la unión a Site A participan los factores de transcripción HNF-4, RXRα y ERα, además de HNF-3β de manera indirecta. En la unión a Site B participa el factor de HNF-3β. EPI y los metabolitos del cacao estudiados inducen los niveles de mRNA de HNF-3β.

6.7 La transcripción de ApoAI se induce por el tratamiento con EPI y los metabolitos del cacao estudiados a través del Site B, siendo 3-M-EPI el metabolito que consigue una mayor activación transcripcional.

6.8 Los factores de transcripción NFY y Sp1, además de HNF-3β, participan en la unión al Site B inducida por la incubación con 3-M-EPI. La sobreexpresión de los factores de transcripción NFY y Sp1 induce la transcripción de ApoAI a través del Site B.

6.9 El tratamiento con CA y con ICC genera un cambio en el perfil de expresión de la línea tumoral HT29.

6.10 Se han identificado dos genes nodo, STAT5B y ATF-2, tras la generación de una red de asociación biológica a partir de los genes diferencialmente expresados comunes a ambos tratamientos, CA e ICC.

6.11 La validación de los cambios en la expresión de ambos genes confirma la sobreexpresión de STAT5B e infraexpresión de ATF-2 inducida por los tratamientos con CA e ICC. También se ha confirmado el aumento en la proteína STAT5B inducida por el tratamiento con CA y con ICC y la disminución de los niveles de ATF-2 debidos al tratamiento con CA.

6.12 El tratamiento con CA e ICC modula los niveles de ciclina D1, proteína regulada por STAT5B y ATF-2 en dos líneas celulares de cáncer. En HT29, el tratamiento con CA reduce los niveles de ciclina D1, mientras que ICC induce los niveles de ciclina D1 al igual que los de ATF-2. En MCF-7 ambos tratamientos, CA e ICC, consiguen una disminución drástica de los niveles de proteína ciclina D1, a pesar de no acompañarles la disminución de ATF-2.

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ANEXOS

a) ARTÍCULO V:

Identification of novel Sp1 targets involved in proliferation and cancer by functional genomics

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Identification of novel Sp1 targets involved in proliferation and cancer by functional genomics

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ABSTRACT

Sp1 is a transcription factor regulating many genes through its DNA binding domain, containing three zinc fingers. We were interested in identifying target genes regulated by Sp1, particularly those involved in proliferation and cancer. Our approach was to treat HeLa cells with a siRNA directed against Sp1 mRNA to decrease the expression of Sp1 and, in turn, the genes activated by this transcription factor. Sp1-siRNA treatment led to a great number of differentially expressed genes as determined by whole genome cDNA microarray analysis. Underexpressed genes were selected since they represent putative genes activated by Sp1 and classified in six Gene Onthology categories, namely proliferation and cancer, mRNA processing, lipid metabolism, glucidic metabolism, transcription and translation. Putative Sp1 binding sites were found in the promoters of the selected genes using the Match™ software. After literature mining, 11 genes were selected for further validation. Underexpression by qRT-PCR was confirmed for the 11 genes plus Sp1 in HeLa cells after Sp1-siRNA treatment. EMSA and ChIP assays were performed to test for binding of Sp1 to the promoters of these genes. We observed binding of Sp1 to the promoters of RAB20, FGF21, IHPK2, ARHGAP18, NPM3, SRSF7, CALM3, PGD and Sp1 itself. Furthermore, the mRNA levels of RAB20, FGF21 and IHPK2 and luciferase activity for these three genes related to proliferation and cancer, were determined after overexpression of Sp1 in HeLa cells, to confirm their regulation by Sp1. Involvement of these three genes in proliferation was validated by gene silencing using polypurine reverse hoogsteen hairpins.

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1. Introduction

The Sp1 transcription factor which belongs to the Sp family of transcription factors (TF) is characterized by three Cys2His2 zinc fingers required for the sequence-specific DNA binding to GC-rich promoter elements. Sp1 is ubiquitously expressed in mammalian cells and regulates the transcriptional activity of multiple target genes involved in many cellular processes such as cell differentiation, cell cycle progression and oncogenesis [1–3]. Sp1 encodes a 105 kDa protein containing two glutamine-rich transactivation subdomains that stimulate transcription. Next to the glutamine-rich domains there are serine/threonine subregions involved in posttranslational modifications, such as glycosylation, acetylation, phosphorylation and sumoylation, which regulate Sp1 activity [4]. Single or multiple Sp1-binding sites have been mapped in promoters of genes involved in almost all cellular processes. In these promoters, Sp1 is usually a transcriptional activator, whereas Sp3, another TF that belongs to the Sp family, can act as an activator as well as a repressor. Since both transcription factors bind to the same binding site, the Sp1/Sp3 ratio in a cell will determine the activation state of the promoter [3].

Previously, we investigated the regulation of Sp1 by cloning the 5′ region of this gene, determining its transcriptional start site, and analyzing its promoter for putative TF binding sites [5]. The Sp1 promoter is regulated by Sp1, Sp3, NF-Y and E2F [5,6]. Sp1-dependent transcription can also be influenced by changes in Sp1 abundance, as it increases during the G1-phase of the cell cycle, by DNA binding activity and by interaction with other nuclear factors [1], such as the interaction of Sp1 with retinoblastoma protein in a complex that enhances the transcriptional activation of Sp1 [7].

Abbreviations: FDR, false discovery rate; GEO, gene expression omnibus; GO, gene ontology; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NE, nuclear extract; NP-40, nonyl phenoxypolyethoxylethanol; PPRHs, polypurine reverse hoogsteen hairpins; RMA, robust multichip average; siNR, siRNA non related; siSp1, siRNA directed against Sp1; TF, transcription factor.

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Sp1 also interacts with CDK4, SKP2, Rad51, BRCA2 and p21, and these proteins are also able to activate the Sp1 promoter. Furthermore, Sp1 mRNA expression is increased upon transient overexpression of CDK4, Rad51, E2F, p21 or Stat3, whereas its mRNA levels are decreased upon overexpression of NF-κB and p53 [8].

Sp1 knockout embryos are retarded in development, show a broad range of abnormalities and die around day 11 of gestation whereas mice lacking Sp3 are postnatal lethal and show cardiac malformations. Knocking out Sp1 and Sp3 is lethal, and heterozygous Sp1/Sp3 mice show embryonic lethality accompanied by a range of developmental abnormalities such as morphological alterations of the lung, impaired ossification, anemia, and placental defects [9]. It can be concluded that the Sp family plays an important role in the normal development of tissues and organs.

Deregulation of Sp1 and Sp3 can be seen in many cancers and diseases. Genes associated to Sp1 such as Rb, p53 and E2F have been found to play important roles in cancer hallmarks [10]. Angiogenesis is an important aspect in the growth and metastasis of cancers and tumor cells are able to produce their own angiogenic factors. Of these angiogenic factors, VEGF is considered to be one of the most potent factors. The expression of the VEGF gene is stimulated by TNF-α, through the action of Sp1 [11]. Transfection of cells with Sp1 decoy oligonucleotides suppressed the expression of VEGF and reduced the invasiveness and proliferation of A459 lung adenocarcinoma and U251 glioblastoma cells [12]. Sp1 protein was found to be highly expressed in the nuclei of gastric tumor cells, whereas minimal levels of Sp1 protein were detected in stromal or normal glandular cells within or surrounding the tumor [13,14]. Furthermore, the survival of patients with high Sp1 protein levels was significantly decreased when compared to patients with low to non-detectable Sp1 protein levels [13].

siRNAs against Sp1, Sp3 and Sp4 have been used to investigate the role of these TFs in angiogenesis and cell growth in Panc-1 cells. siRNAs for Sp3, but not for Sp1 or Sp4, inhibited the phosphorylation of retinoblastoma protein, blocked transition to the G1/S phase and upregulated p27 promoter activity in pancreatic cells [15].

The aim of this work was to identify Sp1 targets, with special emphasis in those involved in proliferation and cancer. It is known that Sp1 is able to regulate a large number of genes through its DNA binding domain, but not much is known about which genes this TF actually regulates *in vivo*. We determined changes in gene expression in HeLa cells upon treatment with a siRNA against Sp1 by using whole human genome microarrays and show evidence for 8 genes newly described to be regulated by Sp1.

2. Materials and methods

2.1. Cell culture

HeLa human cervical carcinoma cells were grown in Ham's F-12 medium containing 7% fetal bovine serum (FBS) (Gibco-Invitrogen-Life Technologies SA, Madrid, Spain). Cell cultures were maintained at 37 °C in a humidified 5% CO_2 environment.

2.2. Sp1 knockdown and overexpression

Sp1 siRNA (siSp1) was designed using the iRNAi 2.1 software (Alexander Griekspoor & Tom Groothuis) and synthesized by Thermo (Thermo Fisher Scientific SL, Madrid, Spain), against the following sense sequence in the Sp1 mRNA: 5′-AACAGCGTTTCTG-CAGCTACC-3′ (GC content: 47.8% and ΔG3′: 7.5; ΔG5′: 7.8; Dif: −0.3). Non-related siRNAs (siNR) were purchased from Ambion (Life Technologies SA, Madrid, Spain).

Transfection with siSp1 was performed using Metafectene (Biontex, Martinsried/Planegg, Germany). For each 35 mm well, 2 μl of metafectene in 100 μl of serum-free F-12 medium were added to 100 nM of the siRNAs in 100 μl of serum-free F-12. Complexes were incubated 20 min at room temperature and were added to the cells for 48 h.

Overexpression of Sp1 was accomplished by transient transfection (48 h) of 1 μg of an expression vector for Sp1 (pCMV-Sp1) using Fugene 6® (Roche, Barcelona, Spain) following the manufacturer's instructions. Briefly, 3 μl of Fugene in 100 μl of serum-free medium was incubated at room temperature for 5 min. The vector was added to the mixture and incubated at room temperature for 20 min before its addition to the cells.

2.3. Real time RT-PCR (qRT-PCR)

Total RNA was extracted from HeLa cells (30,000), either control or treated with siSp1 or pCMV-Sp1, using the Ultraspec™ RNA reagent (Biotecx, Ecogen, Barcelona, Spain) in accordance with the manufacturer's instructions. Complementary DNA was synthesized from 1 μg of total RNA as described in [16]. Sp1 mRNA levels were determined in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Life Technologies SA, Madrid, Spain) using 3 μl of the cDNA mixture and the assays-on-demand Hs00412720_m1 for Sp1 and Hs99999901_s1 for 18S RNA (Applied Biosystems, Life Technologies SA, Madrid, Spain). 18S RNA was used as endogenous control. The reaction was performed following the manufacturer's recommendations.

RAB20, FGF21, IHPK2, ARHGAP18, NPM3, SFRS7, CALM3, PGD, SLC2A3, CEBPδ and CBFβ mRNA levels were determined by SYBR-Green qRT-PCR (Applied Biosystems, Life Technologies SA, Madrid, Spain) and the pairs of primers listed below (Table 1) using 18S RNA as endogenous control. Fold-changes in gene expression were calculated using the standard ΔΔCt method.

2.4. Western blot analysis

Whole extracts were obtained from control or siSp1 treated cells (30,000) for 48 h according to [17]. Total extracts (40 μg) were resolved on a SDS-7%-polyacrylamide gels (AppliChem, Ecogen, Barcelona, Spain) and transferred to PVDF membranes (Immobilon P, Millipore, Madrid, Spain) using a semidry electroblotter. The membranes were probed with anti-Sp1 antibody (sc-59) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) 1:100 dilution, OVN at 4 °C. Signals were detected with anti-rabbit secondary horseradish peroxidase–conjugated antibody (P0399) (Dako, Barcelona, Spain) 1:5000 dilution, for 1 h at room temperature, and enhanced chemiluminescence, as recommended by the manufacturer (Amersham, GE Healthcare Life Sciences, Barcelona, Spain). To normalize the results blots were reprobed with an antibody against tubulin (Cp06) (Calbiochem, Millipore, Merck, Madrid, Spain) 1:500 dilution, OVN at 4 °C and detected with anti-mouse (NIF 824) (Amersham, GE Healthcare Life Sciences, Barcelona, Spain) 1:2500 dilution, for 1 h at room temperature.

2.5. Cell survival studies

Cell survival was measured by the MTT test as described in [18] (Sigma–Aldrich Quimica S.A., Madrid, Spain). Results were expressed as the percentage of survival with respect to the control.

2.6. Microarrays

Gene expression was analyzed by hybridization to GeneChip® Human Genome U133 PLUS 2.0 Affymetrix microarrays, containing

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Table 1
Primers used for mRNA determination by qRT-PCR. The sequences and product sizes for each of the selected genes are indicated.

Gene	Forward primer	Reverse primer	Size (bp)
18S	5′-gcgaaagcatttgccaagaa-3′	5′-catcacagacctgttattgc-3′	508
RAB20	5′-cgccttctacctgaagcagtg-3′	5′-gccggtgattcacatcatag-3′	140
FGF21	5′-cttgaagccgggagttattc-3′	5′-gcttcggactggtaaacattg-3′	162
IHPK2	5′-ccaagggaacatcagttctac-3′	5′-gagtgaatttgcgcatctcag-3′	55
ARHGAP18	5′-cactgaatcgcagtcacttag-3′	5′-gggatcagcttctcttcacc-3′	93
NPM3	5′-ggcaccagattgttacgatg-3′	5′-gaaacttgtcagggaacagg-3′	191
SRSF7	5′-gattgtcatcgttacagccg-3′	5′-cttgatcgtcgaggagatgc-3′	140
CALM3	5′-gatcaatgaggtggatgcag-3′	5′-catctccatcgatgtcagcc-3′	248
PGD	5′-gacatcatcattgacggagg-3′	5′-cacagcagggttctccagttc-3′	229
SLC2A3	5′-gtcaacctgttggctgtcac-3′	5′-ggaaggatggtaaaacccag-3′	293
CEBPδ	5′-ctgcgagagaagctaaacgtg-3′	5′-cttagctgcatcaacaggag-3′	91
CBFβ	5′-gaacagcgacaaacacctag-3′	5′-cccataccatccagtctttg-3′	143

54,675 transcripts and variants. Total RNA for cDNA arrays was prepared from triplicate samples from both control and siSp1-treated cells using RNAeasy Mini kit (Qiagen, Barcelona, Spain) following the recommendations of the manufacturer. The integrity of the RNA species was checked using the Bioanalyzer 2100 system (Agilent Technologies, Madrid, Spain). Labeling, hybridization and detection were carried out following the manufacturer's specifications. Microarray data are available from GEO as series accession number GSE37935.

2.7. Microarray data analysis

Quantification was carried out with GeneSpring GX 11.5.1 software (Agilent Technologies, Madrid, Spain), which allows multi-filter comparisons using data from different experiments to perform the normalization, generation of lists and the functional classification of the differentially expressed genes. The input data was subjected to preprocess baseline transformation using the RMA summarization algorithm using the median of control samples. After grouping the triplicates of each experimental condition, lists of differentially expressed genes could be generated by using volcano plot analysis. Unpaired *t*-Test was applied using asymptotic *p*-value computation and multiple testing correction of Benjamini–Hochberg FDR. The expression of each gene was reported as the ratio of the value obtained for each condition relative to the control condition after normalization and statistical analysis of the data. The corrected *p*-value cut-off applied was of <0.01; then the output of this statistical analysis was filtered by fold expression, selecting specifically those genes that had a differential expression of at least 2-fold. Finally, the obtained gene list was classified according to gene ontology biological processes. After data mining, the selected genes could be grouped into the categories of (i) proliferation and cancer; (ii) mRNA processing; (iii) lipid metabolism; (iv) glucidic metabolism; (v) transcription; and (vi) translation.

2.8. Database searching

Searching for the different promoter sequences analyzed was performed using human BLAT (UCSC Genome Bioinformatics Site). The presence of putative Sp1 binding sites in the promoters was analyzed using the Match™ 1.0 software [19] that uses a library of mononucleotide weight matrices from TRANSFAC® 6.0. The values of the settings for core and matrix were changed depending on the stringency required for the search.

2.9. Electrophoretic mobility shift, supershift and competition assays

Nuclear extracts were prepared as described in [5] from exponentially growing HeLa cells or HeLa cells (5×10^5) transiently transfected (48 h) with 1 μg of an expression vector for Sp1 (pCMV-Sp1) using Fugene 6® (Roche, Barcelona, Spain). The probes were constructed by annealing commercially synthesized complementary single-stranded oligodeoxynucleotides corresponding to putative Sp1 DNA binding sites (underlined) present in the RAB20 (5′-CCCCGCCCCCGCCCCCGGGCC-3′), FGF21 (5′-GGGTGATTGGGCGGGCCTGTC-3′) or IHPK2 (5′-GCGGGACTCCGCCCATGCCAC-3′) gene promoters. Each ds oligonucleotide was gel-purified, end-labeled with T4 polynucleotide kinase (New England Biolabs, Barcelona, Spain) and [γ-^{32}P]-ATP (3000 Ci/mmol, Perkin Elmer, Madrid, Spain), and used as the probe in gel shift experiments. DNA/Protein binding assays were performed as described [20] using 2 μg of nuclear extract, 20,000 cpm of the radioactive probes and 1 μg of poly[d(I-C)] (Sigma–Aldrich Quimica S.A., Madrid, Spain) as unspecific competitor. After electrophoresis in 5% polyacrylamide and 5% glycerol native gels, the bindings were visualized using a PhosphorImager with ImageQuant software v 5.2 (Molecular Dynamics, GE Healthcare Life Sciences, Barcelona, Spain).

In the supershift experiments, 2 μg of rabbit polyclonal antibody PEP-2 (sc-59) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) against Sp1 or 2 μg of rabbit polyclonal antibody (D-20) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) against Sp3 were added to the reaction mixture and incubated on ice for 15 min after addition of the probe.

Competition assays where performed incubating the FGF21 radioactive probe with increasing fold excess (5, 20, 50 and 100×) of competitor ds probes corresponding to RAB20, IHPK2 and FGF21 itself. The addition of the cold probes was performed 15 min before the labeled probe.

2.10. Chromatin immunoprecipitation (ChIP analysis)

HeLa cells were washed twice with phospate-buffered saline (PBS) and subsequently incubated with 1% formaldehyde for 10 min, at room temperature. The crosslinking reaction was terminated by addition of 0.125 M glycine dissolved in PBS. Cells were washed three times with ice-cold PBS, scraped off the culture dish in 2 ml PBS containing 1 mM PMSF and centrifuged at 4 °C for 10 min. The pellet was resuspended in lysis buffer (5 mM Pipes pH 8.0, 85 mM KCl, 0.5% NP40 and protease inhibitors) and after 30 min incubation on ice, cells were dounced on ice to aid nuclei release. After centrifugation at 4 °C for 10 min, the pellet was resuspended in sonication buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 0.1% SDS, 0.5% deoxycholic acid and protease inhibitors). All previous chemical reagents where purchase from AppliChem (Ecogen, Barcelona, Spain). Cells were sonicated in an ice-water bath to obtain chromatin fragments of an average length of 500–1500 bp. This was usually achieved by sonicating the chromatin 8 rounds for 20 s, with 30 s between each round of sonication (cycle was set at 1, amplitude at 50) in a Ultrasonic processor UP 200 S (GmbH, Valladolid, Spain). Sonicated chromatin was then either processed immediately or stored at −80 °C.

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For immunoprecipitation, 100 μl of sonicated chromatin was diluted in 900 μl ChIP dilution buffer (16.7 mM Tris–HCl pH 8.1, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl and protease inhibitors leupeptin and PMSF) and pre-cleared with 20 μl pre-washed protein A/agarose (sc-2001) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) by incubating for 2 h at 4 °C on a rotating wheel. Samples were centrifuged 13,000 rpm for 2 min at 4 °C and the supernatant was then incubated overnight (4 °C, on a rotating wheel) with 5 μg of rabbit polyclonal anti-Sp1 antibody (sc-59 X) (Santa Cruz Biotechnology Inc., Heidelberg, Germany), mouse IgG1 negative control (x0931) (Dako, Barcelona, Spain), or no antibody (beads-only control). After immunoprecipitation, 40 μl of pre-washed protein A/agarose (pre-blocked overnight with 1 μg/μl herring sperm DNA) was added per sample, and incubation continued for 1 h at 4 °C with rotation. The chromatin-antibody-protein A/agarose complexes were collected by centrifugation (13,000 rpm, 2 min, 4 °C). Supernatant from the beads-only control was collected and saved as input. Samples were sequentially washed with 1 ml of low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 150 mM NaCl, twice for 10 min), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 500 mM NaCl, once for 10 min), LiCl buffer (0.25 M LiCl, 0.5% NP40, 0.5% deoxycholic acid, 10 mM Tris–HCl pH 8.1, 1 mM EDTA, once for 5 min) and TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0, three times for 5 min). The protein/DNA complexes were then eluted from the protein A/agarose by incubation with 250 μl elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at room temperature. Elution was repeated and eluates were combined. Crosslinking was reversed by addition of 20 μl 5 M NaCl to the eluates and 500 μl of the saved input DNA and overnight heating of the samples to 65 °C. The next day, all samples were digested with 5 μl proteinase K (10 mg/ml) for 1 h at 55 °C. DNA was recovered by phenol/chloroform extraction. DNA precipitation was carried out by addition of 1 ml of absolute ethanol to each sample plus 5 μl of glycogen blue. The resulting pellets were resuspended in TE (in 60 μl TE for the immunoprecipitations and 200 μl TE for the input) and stored at −20 °C until needed for PCR.

PCR was performed to amplify the immunoprecipitated and input DNAs. Primers were designed in the promoter regions for each of the selected genes with PrimerBLAST tool; in such a way that the region amplified contained at least one Sp1 transcription factor binding-site. Table 2 contains primer sequences and information for each of the selected genes.

1–2 μl of immunoprecipitated DNA was used for amplification of the selected genes. PCR reaction mix contained 5% DMSO to facilitate DNA strand separation and to improve PCR efficiency. The reactions were performed in a MJ Research Thermocycler (Ecogen, Barcelona, Spain) under standard conditions in a final volume of 50 μl for at least 32 cycles.

For each sample, 10 μl of the PCR products was electrophoresed in a 5% acrylamide gel. The gel was run at 160 V in TBE 1x, and the amplified fragments were visualized after ethidium bromide staining.

2.11. Luciferase constructs, cotransfections and luciferase assay

Luciferase constructs RAB20.LUC, FGF21.LUC or IHPK2.LUC were engineered by unidirectional cloning of the dsDNA sequence containing the same Sp1 DNA binding sites (underlined) present in the RAB20, FGF21 or IHPK2 gene promoters as those used in the EMSA experiments, between the Mlu I and the Xho I sites of the reporter luciferase vector pGL3-basic (Promega, Madrid, Spain). Hybridization of the following specific primer pairs including the overhang terminus of the restriction enzymes Mlu I and Xho I (highlighted): RAB20-MX-FW (5′-**CGCGT**CCCCGCCCCCGCCCCCGGGCC**C**-3′) & RAB20-MX-RV (5′-**TCGAG**GGCCCGGGGGCGGGGGCGGGG**A**-3′), FGF21-MX-FW (5′-**CGCGT**GGGTGATTGGGCGGGCCTGTC**C**-3′) & FGF21-MX-RV (5′-**TCGAG** GACAGGCCCGCCCAATCACCC**A**-3′) or IHPK2-MX-FW (5′-**CGCGT** GCGGGACTCCGCCCATGCCAC**C**-3′) & IHPK2-MX-RV (5′-**TCGAG** GTGGCATGGGCGGAGTCCCGC**A**-3′) was performed to obtain the inserts.

For luciferase assays, 2.5×10^5 HeLa cells were plated in 6-well dishes the day before transfection. The medium (2 mL) was renewed before transfection that was performed with Fugene 6® (Promega, Madrid, Spain). For each well, the transfection reagent was incubated for 5 min with 100 μL of serum & antibiotic-free medium, followed by the addition of plasmid DNA and incubated for 20 min (ratio of 3:1 (μL of transfection reagent:μg of plasmid DNA), all at room temperature. 500 ng of either RAB20.LUC, FGF21.LUC or IHPK2.LUC and 500 ng of the Sp1 expression vector pCMV-Sp1 [21] were mixed before the addition of the transfection reagent. Luciferase activity was determined 30 h after transfection. Cell extracts were prepared by lysing the cells with 200 μL of freshly diluted 1× Reporter Lysis Buffer (25 mM Tris–Phosphate pH7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% Triton X_100). The lysate was centrifugated at 12,000 × *g* for 2 min (4 °C) to pellet the cell debries. The supernatants were transferred to a fresh tube. 15 μL of the extract was added to 15 μL of the luciferase assay substrate (Promega, Madrid, Spain) at room temperature. Luminescence of the samples was measured 2 s after mixture in the Glomax™ (Promega, Madrid, Spain) 20/20 Luminometer, in which the light production (relative luminescence units; RLU) was measured with 5 s integration during 10 s. Three different experiments were performed for each transfection.

Luciferase results were corrected by total protein concentration, which was determined with the Bio-Rad protein assay reagent Bradford (Bio-Rad, Barcelona, Spain) according to the manufacturer's protocol.

Table 2
ChIP analysis primer sequences. The sequences of the primers to detect the immunoprecipitated promoters bound to Sp1 are indicated as well as the PCR product sizes.

Gene	Forward primer	Reverse primer	Size (bp)
Sp1	5′-cggacaccaggcacgcaact-3′	5′-gaggcaagcgaacccggacc-3′	216
GAPDH	5′-atggttgccactggggatct-3′	5′-tgccaaagcctaggggaaga-3′	174
RAB20	5′-ggagctcaagagaggaagcgc-3′	5′-ctcggctggagaactcggatg-3′	228
FGF21	5′-ctgtagctcctgccaaatgg-3′	5′-gtggtttagaattggtgccag-3′	217
IHPK2	5′-gttcgaagtagcgtgggaag-3′	5′-cctgcctgtctgcatctaac-3′	181
ARHGAP18	5′-gcgatcctgacacagagaag-3′	5′-cttcgcctcctttaaccaaag-3′	222
NPM3	5′-gctgtaagagccttcttcaaac-3′	5′-gagacactcttccgacacag-3′	297
SRSF7	5′-gcggaaggaactgaagagac-3′	5′-gattgttaaggctgagggtcc-3′	217
CALM3	5′-ccaattcctgtgcagggtg-3′	5′-gagcacggggatcaaggttc-3′	283
PGD	5′-gtcctgcgtgagttgctatg-3′	5′-cgacgcctgttagaccatc-3′	206
SLC2A3	5′-gcaatcttgtgatctctcgg-3′	5′-cctccaggcttttctggtag-3′	233
CEBPδ	5′-ctctgcgtccaagcgaggc-3′	5′-ggcaccctcctgcaacgtg-3′	253
CBFβ	5′-ggagttgtgaatggtgcgtc-3′	5′-cgttccggggagtctgag-3′	245

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Table 3
PPRHs against Sp1 targets. The sequences of PPRHs hairpins to knock down Sp1 selected target genes are indicated.

PPRH name	Sequence
Hp-RAB20	5′-aagggagagaggaagagagaaggggttttggggggaagagagaaggaga gagggaa-3′
Hp-FGF21	5′-agggaggaagggaaggaggggggttttgggggggaggaagggaaggaggga-3′
Hp-IHPK2	5′-gagggaaagaggagaggaaaagaggaagttttgaaggagaaaaggagagga gaaagggag-3′
Hp-sc	5′-aaaagaagaagaagaagaagaagaagaaggtttttggaagaagaagaagaaga agaagaagaaaa-3′
Hp-nr	5′-cctccttccttcctccctcttccttttccttttttaggaaaaggaagagggaggaagg aaggagg-3′

2.12. PPRH design to knock down RAB20, FGF21 and IHPK2 expression

PPRHs hairpins (Hp) against RAB20, FGF21 and IHPK2 genes were designed as described in de Almagro et al. [22] in order to functionally validate these targets. Non-related (Hp-nr) and scrambled (Hp-sc) PPRHs were assessed as negative controls. Table 3 describes PPRHs sequences used. HeLa cells (10,000) were plated in 35-mm plates and PPRHs transfection (100 nM) was performed using 10 μM DOTAP reagent (Roche, Barcelona, Spain) as in de Almagro et al. [22]. Five days after transfection cell survival was analyzed as previously described in this section. Results were expressed as the percentage of survival with respect to control cells.

3. Results

3.1. Effects of siRNA against Sp1 on its mRNA and protein levels, and on cell survival

Exponentially growing HeLa cells were treated with siSp1 or with a siNR, both at a concentration of 100 nM. After 48 h, Sp1 mRNA levels were measured by qRT-PCR and cell survival was determined by the MTT assay. Sp1 mRNA levels were decreased by 90% in cells treated with siSp1 when compared to cells treated with siNR (Fig. 1A). Knockdown of Sp1 after siSp1 treatment was confirmed by Western blot analysis. Sp1 protein was decreased in cells treated with siRNA against Sp1 when compared to cells treated with siNR and cells treated with no siRNA as a control (Fig. 1B). Cell survival decreased by 50% in cells treated with siSp1, whereas survival of cells treated with siNR was unaffected (Fig. 1C). On the other hand, transient overexpression of Sp1 using a eukaryotic expression vector (3 μg) for this protein caused an increase in cell proliferation of 40%. These results are in accordance with previous studies showing decreased cell growth upon inhibition of Sp-family members [12,15,23].

3.2. Functional genomics upon cell incubation with siSp1

Affymetrix whole genome arrays were used to determine genes that were differentially expressed upon knocking down Sp1 by siSp1 treatment. Genomic analysis was processed using the GeneSpring GX software. Fig. 2 shows a volcano plot of the genes differentially expressed upon Sp1 knockdown. Genes that were underexpressed by at least 2-fold, at a cut-off of $p < 0.01$, were selected for further analysis, since they represent those genes that are putatively activated by Sp1. The underexpressed genes were classified in six GO categories (Table 4).

3.3. Search of Sp1 sites within the promoters of the underexpressed genes

All 36 genes in Table 4 with the exception of one, the lamin B receptor, showed putative Sp1 binding sites when using values for Core > 0.9 and Matrix > 0.8 in the Match™ analyses. Then, a more stringent search for Sp1 binding sites was performed using values of Core = 1 and Matrix > 0.9, shortening the list down to 21 genes. This list was subjected to literature mining to finally select a total of 11 genes according to their bibliographic interest. The 11 selected genes were grouped in 4 GO categories (proliferation, mRNA processing, glucidic metabolism and transcription), which included 3 genes (SLC2A3, C/EBPδ, and CBFβ) already reported as regulated by Sp1 [24–26]. These 3 genes and Sp1 itself [5,6] were taken as positive controls. Additionally, the accuracy of Match™ was positively checked by running this software using the

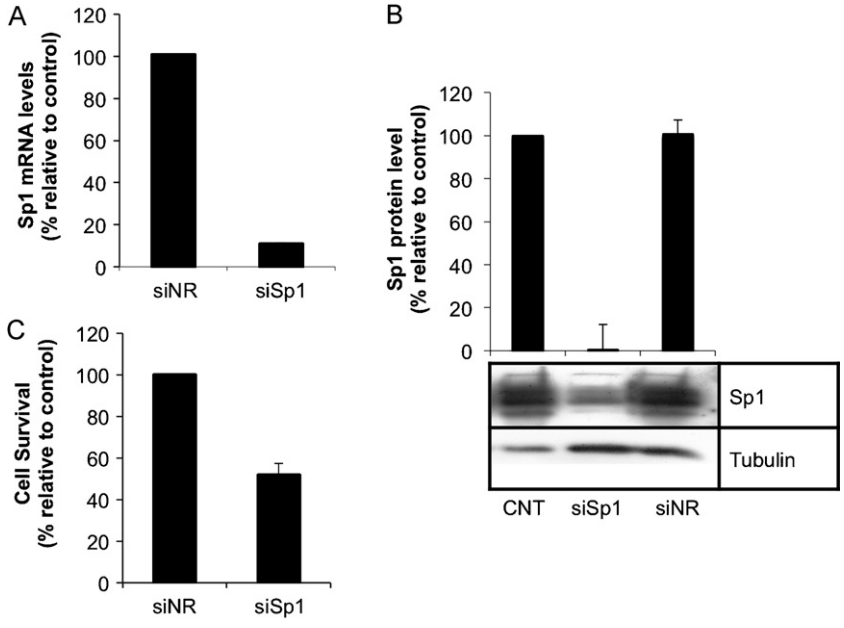

Fig. 1. Cell survival and Sp1 mRNA and protein levels upon siSp1 incubation. Exponentially growing HeLa cells were treated with 100 nM of either siRNA against Sp1 RNA (siSp1) or control (siNR). (A) Sp1 mRNA levels were determined by qRT-PCR. (B) Sp1 protein was determined by Western blot using PEP-2 antibody. (C) Cell survival was assessed by the MTT assay 48 h after the siRNA treatment. Values are the mean ± SE of three independent experiments.

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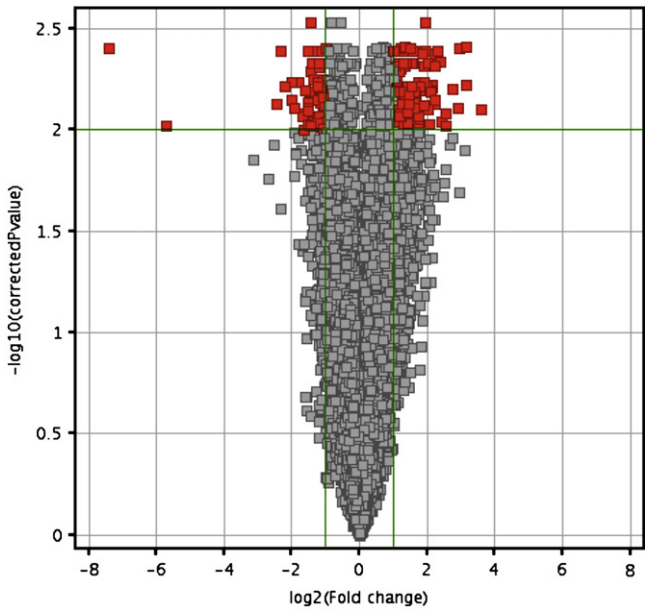

Fig. 2. Identification of genes differentially expressed upon knocking down Sp1. Affymetrix complete genome microarrays (HG U133 PLUS 2.0) were used to identify the changes in gene expression in HeLa cells after treatment with siSp1. Lists of genes differentially expressed in siSp1-treated cells were generated using GeneSpring GX v11.5.1. The image shows a volcano plot representation and the genes differentially expressed by 2-fold with a *t*-test *p*-value of <0.01 are shown in red. Overexpressed genes are located in the upper-right side and underexpressed genes in the upper-left side. (grey scale for printed version).

sequences corresponding to the binding motifs extracted from ChIP-seq technology for the transcription factors STAT1 [27], NF-κβ [28], NRSF [29] and HSF [30]. The sequences of the Sp1 sites of the 11 selected genes, along with the Sp1-box core position, are shown in Table 5.

3.4. Validation at the RNA level of the effects caused by siSp1

Validation of the underexpression caused by siSp1 was determined by qRT-PCR for 11 genes (RAB20, FGF21, IHPK2, ARHGAP18, NPM3, SRSF7, CALM3, PGD, SLC2A3, C/EBPδ and CBFβ). Underexpression was confirmed for all the 11 genes as well as Sp1 (Fig. 3).

3.5. Sp1 regulates RAB20, FGF21 and IHPK2 mRNA levels

To confirm the role of Sp1 in the regulation of the selected genes, the effect of overexpression of Sp1 on the putative targets involved in proliferation and cancer was determined by transfecting HeLa cells with an expression vector for Sp1. After 48 h, RNA was extracted and mRNA levels were determined by qRT-PCR. Sp1 overexpression caused an increase in the mRNA levels of RAB20, FGF21 and IHPK2 genes (Fig. 4).

3.6. Sp1 binds to RAB20, FGF21 and IHPK2 promoters

To test that the putative Sp1-boxes were indeed susceptible to be bound by transcription factor Sp1, we performed EMSA using double stranded 21-nucleotide length probes corresponding to the natural sequence of the Sp1 binding sites within the RAB20, FGF21 and IHPK2 gene promoters. Nuclear extracts were prepared from control HeLa cells or cells transfected with an expression vector for Sp1 (pCMV-Sp1). Fig. 5 shows the binding pattern for the different probes analyzed. The major band corresponds to Sp1 binding and two minor bands correspond to Sp3 binding, as determined in the supershift experiments using Sp1 and Sp3 antibodies. The binding corresponding to Sp1 was increased for the RAB20 (42%), IHPK2 (12%) and FGF21 (9%) gene promoters when using nuclear extracts from cells transfected with pCMV-Sp1.

3.7. Similarity of RAB20, FGF21 and IHPK2 Sp1 binding sequences vs the consensus

Sp1 consensus sequence (G/T)(G/A)GG(C/A)G(G/T)(G/A)(G/A) contains 3 binding subsites for the union of the three zinc fingers of Sp1 [31]. Sequence comparison among RAB20, FGF21 and IHPK2 Sp1 binding sequences and the consensus for this TF was carried out to explain the different Sp1 binding to each promoter when overexpressing Sp1. RAB20 matches with higher similarity with the Sp1 consensus sequence than FGF21 and IHPK2 (Fig. 6A). Competition binding assays between RAB20, FGF21 and IHPK2 Sp1 binding sequences demonstrates that RAB20 sequence shows the highest affinity for Sp1 (Fig. 6 B and C). This observation correlates with its higher similarity with the consensus.

3.8. Sp1 binds to RAB20, FGF21, IHPK2, ARHGAP18, NPM3, SFSR7, CALM3 and PGD in vivo

To investigate whether Sp1 binds *in vivo* to the promoters of the selected genes, ChIP experiments were performed with chromatin from HeLa cells and a specific antibody against Sp1. The promoter regions of the selected genes were amplified from the immunoprecipitated DNA to check for Sp1 binding to these regions. Fig. 7 shows the PCR products from the immunoprecipitations with Sp1 and mouse IgG1 antibodies, in relation to the input. Sp1 protein was found to bind to the promoters of RAB20, FGF21, IHPK2, ARHGAP18, NPM3, SRSF7, CALM3, and PGD. Sp1 promoter was used as positive control.

3.9. Luciferase activity of RAB20, FGF21 and IHPK2 Sp1 binding sites upon Sp1 overexpression

To analyze whether RAB20, FGF21 and IHPK2 promoters could be regulated by transcription factor Sp1, transient transfection assays with the luciferase reporter vectors containing the selected Sp1 binding sequence for each gene (RAB20.LUC, FGF21.LUC and IHPK2.LUC) were performed in HeLa cells. As shown in Fig. 8, overexpression of Sp1 induced luciferase activity of the three genes by more than 2-fold compared to the controls.

3.10. Functional validation of Sp1 proliferation and cancer targets using PPRHs silencing methodology

In order to functionally validate RAB20, FGF21 and IHPK2 as proliferation and cancer target genes we knocked down these genes using specific PPRHs. The evaluation of cell survival was performed afterwards by the MTT assay and a cytoxicity of 40% for IHPK2 and >95% for RAB20 and FGF21 in HeLa cells was observed (Fig. 9). These effects were specific since cytotoxicity was not induced by the non-related and scrambled PPRHs. Thus, our results confirm the implication of these three genes regulated by Sp1, in proliferation and cancer.

4. Discussion

The main objective of this work was to identify gene targets activated *in vivo* by Sp1. HeLa cells were treated with a siRNA against Sp1 and expression microarrays were carried out to identify changes in gene expression. Since Sp1 is a general transcription factor, there were a substantial number of differentially expressed genes that can be observed by accessing the

Table 4
Gene ontology classification of genes underexpressed by 2-fold. GeneSpring GX was used to classify the genes underexpressed by 2-fold with a *t*-test *p*-value of <0.01 by gene ontology (biological process). The ratio column corresponds to the expression of each gene after Sp1-siRNA treatment relative to the control. The Match™ software and the TRANSFAC® database were used to search for putative Sp1 binding sites in the promoters of the classified genes. R indicates that a reference exists of Sp1 binding to the gene, and the genes selected for further studies are marked with a √.

GOntology biological process	Ratio	Gene symbol	Gene title	Sp1 box Core > 0.9 Matrix > 0.8	Stringent Sp1 box Core = 1 Matrix > 0.9	Ref.	Choice
Proliferation and cancer	0.20	GLULD1	Glutamate-ammonia ligase (glutamine synthetase) domain containing 1	+			
	0.35	RAB20	RAB20, member RAS oncogene family	+	+		√
	0.36	FGF21	Fibroblast growth factor 21	+	+		√
	0.37	INHBE	Inhibin, beta E	+			
	0.44	LBR	Lamin B receptor				
	0.45	ZNF364	Zinc finger protein 364	+	+		
	0.45	IHPK2	Inositol hexaphosphate kinase 2	+	+		√
	0.49	GADD45B	Growth arrest and DNA-damage-inducible, beta	+			
	0.49	ZNRF1	Zinc and ring finger 1	+	+		
	0.42	OSMR	Oncostatin M receptor	+			
	0.44	IPO5	Importin 5	+			
	0.42	ARHGAP18	Rho GTPase activating protein 18	+	+		√
mRNA processing	0.26	SFRS1	Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	+			
	0.49	NPM3	Nucleophosmin/nucleoplasmin, 3	+	+		√
	0.49	SFRS7	Splicing factor, arginine/serine-rich 7, 35kDa	+	+		√
	0.49	SFRS12	Splicing factor, arginine/serine-rich 12	+			
Lipid metabolism	0.36	ACSL3	Acyl-CoA synthetase long-chain family member 3	+			
	0.46	LEPROTL1	Leptin receptor overlapping transcript-like 1	+	+		
	0.50	ELOVL5	ELOVL family member 5,elongation of long chain fatty acids (FEN1/Elo2,SUR4/Elo3-like,yeast)	+			
Glucidic metabolism	0.32	AGXT2L1	Alanine-glyoxylate aminotransferase 2-like 1	+	+		
	0.37	PDE7B	Phosphodiesterase 7B	+			
	0.39	RPE	Ribulose-5-phosphate-3-epimerase	+	+		
	0.47	GFPT2	Glutamine-fructose-6-phosphate transaminase 2	+	+		
	0.47	CALM3	Calmodulin 3 (phosphorylase kinase, delta)	+	+		√
	0.47	ENO3	Enolase 3 (beta, muscle)	+			
	0.50	PGD	Phosphogluconate dehydrogenase	+	+		√
	0.25	SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	+	+	R	C
Transcription	0.31	SNAPC3	Small nuclear RNA activating complex, polypeptide 3, 50kDa	+	+		
	0.35	LOC57228	Small trans-membrane and glycosylated protein	+			
	0.38	ATF7	Activating transcription factor7	+			
	0.49	PNN	Pinin, desmosome associated protein	+	+		
	0.35	PSIP1	PC4 and SFRS1 interacting protein 1	+	+		
	0.36	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	+	+	R	C
	0.38	CBFB	Core-binding factor, beta subunit	+	+	R	C
Translation	0.42	FNBP1	Formin binding protein 1	+	+		
	0.50	GFM1	G elongation factor, mitochondrial 1	+			

microarray data deposited in GEO. In our approach, we focused on those underexpressed genes that displayed a high statistical significance ($p < 0.01$) upon knockdown of Sp1, since they represent genes activated *bona fide* by Sp1. From the original 36 genes that met these criteria, a further selection was made of those containing stringent putative binding sites (Sp1 boxes) in their promoters, gathering 21 genes that could be bound by Sp1 with high probability. This selection of genes susceptible to be activated by Sp1 was subjected to literature data mining, leading to a limited list of 11 interesting genes that fell within the categories of proliferation, mRNA processing, glucidic metabolism and transcription. We found in our list 3 genes (SLC2A3, C/EBPδ, and CBFβ) already reported as regulated by Sp1 [24–26], leaving a list of 8 genes newly described to be under the regulation of Sp1.

Confirmation of mRNA underexpression for the 11 selected genes was carried out by qRT-PCR. Furthermore, ChIP assays were performed to verify binding of Sp1 to the promoters of these genes. The results of the ChIP assays confirmed that Sp1 binds to the promoter regions of RAB20, FGF21, IHPK2, ARHGAP18, NPM3, SRSF7, CALM3 and PGD, in addition of the Sp1 gene itself. Functional validation was achieved by determining that the endogenous mRNA levels of the three Sp1 gene-targets within the category of proliferation, RAB20, FGF21 and IHPK2, were increased upon Sp1 overexpression. Binding by Sp1 to the promoters of these three genes was verified by gel shift analyses, which was increased by Sp1 overexpression. Among these three genes, the Sp1 binding sequence in the RAB20 promoter showed the highest affinity for Sp1 according to the competition experiments. This site shows the highest similarity for the consensus Sp1 binding site reported in Marco et al. [31]. This affinity for RAB20 may explain the higher response in terms of Sp1 binding and mRNA levels when Sp1 is overexpressed. Luciferase assays using specific constructs containing the same endogenous Sp1 boxes for each of these 3 genes as those used in the EMSA experiments, confirmed that Sp1 was responsible for the activation of these genes, which complement the binding analysis studies.

RAB20 belongs to the family of RAS oncogenes and its overexpression has previously been characterized in exocrine pancreatic carcinoma [32], in colorectal adenomas by a process of gene amplification [33] and in triple negative breast cancers [34]. The binding of Sp1 to the promoter of RAB20 indicates that it might play a role in the activation of this gene. An interesting observation

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Table 5
Sp1-box sequences in the promoters of selected genes. It is presented 33 nt of the sequences of the promoters of the selected genes with the Sp1-boxes (in bold and underlined) and the position of the core with respect to the translational start site. (*) Sequences reported as the complementary strand of the Sp1 binding site.

Gene	Sp1 box core position	5′-3′ Sequence of Sp1 boxes
RAB20	−264*	CCCCGCCCCC**GCCCCCGCCCCCG**GGCCCTTTCA
FGF21	−210	AGGGAGGGTG**ATTGGGCGGGCCTG**TCTGGGTAT
IHPK2	−252	GTTCTTGCGG**GACTCCGCCCATG**CCACCCACCT
	−231	CTATGAGCTC**ATGGGGCGGGGTT**AGATGCAGAC
ARHGAP18	−133	TTCACACGGG**GAGGGGCGGGGTC**TGTCCAGGGA
NPM3	−26	GCCTTCTTCA**AACTCCGCCCCCG**ACACGCACAA
	−73	GGCCAGTTCC**GGCCCCGCCCATT**AAAGGAGACG
SFRS7	−256	ACACGCGGGT**CATGGGCGGAGGG**ATACGTTTCT
	−326	TTCCCCATGC**TGTCCCGCCCCCT**CCTCTCCCCG
CALM3	−141	TGAGGGACCG**TTGGGGCGGGAGG**CGGCGGCGGC
	−193 and −178	GG**GTGGGGCGGGGCC**GA**GCGAGGCGGGGCG**CGC
	−369	AAGTGGGCCC**GGGAGGCGGGGCG**CGCGGCGAGG
PGD	−203	GGCCCCCGGG**GGTGGGCGGGCAC**GTTTGCCTCG
	−279	AAGCTCTGCG**CGTGGGCGGGGAG**CCCGGAGCCT
SLC2A3	−302* and −308*	CAGAACT**ACCCCCGCCCCCGCCCCAC**AGACAAT
CEBPδ	−97*	CTGACGTGCA**CGCCCCGCCCCGA**CTCCGGCACC
	−146	GCCGCGGGGG**AAGGGGCGGGGGC**GCCCTGGGAG
	−175	GGAGCCCCCC**GGAGCCGCCCCGA**GCCTTCCCGG
	−472	TCCTGTCGCC**GGAGGGCGGGGGG**TGGAGACCCG
CBFβ	−7	GGCCGGCCGGCGCGGCC**TCAGGGCGGGAAG**ATG
	−134	CGGGAGCCCA**CGCGGGCGGGCGC**CTGAAACAAA
	−175	GTTGGGCTCG**AGCGGGCGGCGGC**GCCTCAGACT
	−260	CGGCGGCGGC**CGGGGGCGGTGAG**CGCTGGGGCT
	−304 and −313	GGCCGG**CGGGGGCGGGGTGGGCGGTGAG**AGGAA
	−349	CGGAGTTGGA**GGCGGGCGGGCGC**GCGAGGAGGA
	−421	CCGCTCCGCC**AGCGGGCGGGTGG**CGCATGCGCG

from our results is that Sp1 overexpression leads to overexpression of RAB20. Thus, Sp1 may play a role in the development of the abovementioned types of cancer.

The protein encoded by FGF21 is a member of the fibroblast growth factor (FGF) family, involved in a wide range of biological

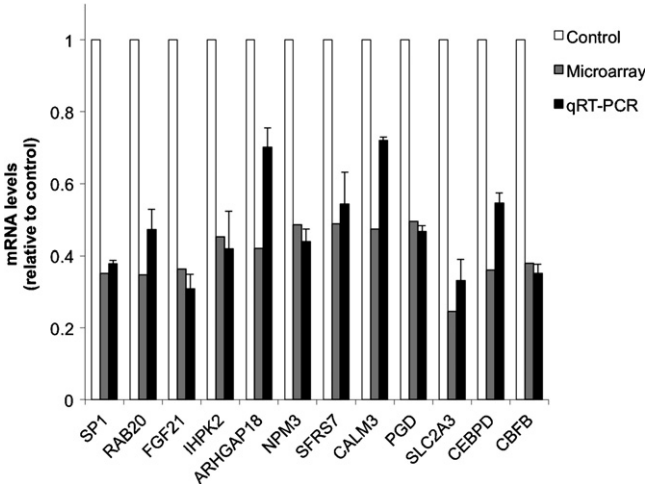

Fig. 3. Validation of downregulated genes. Underexpression of selected genes was confirmed by qRT-PCR (black bars). Values are the mean ± SE of three independent experiments of the changes in the mRNA levels relative to control (white bars). For comparison purposes the values found in the microarray (grey bars) are also represented.

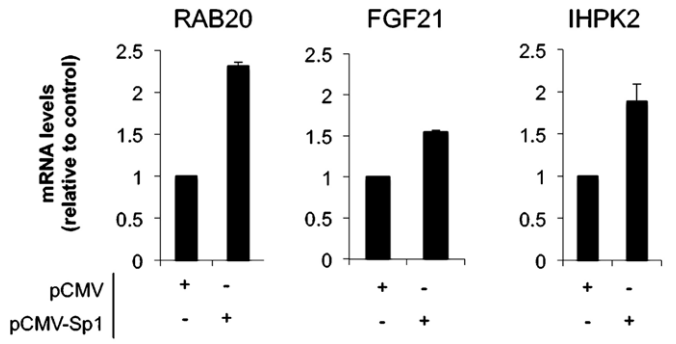

Fig. 4. Sp1 regulates gene expression. Exponentially growing HeLa cells were treated either with the expression vector for Sp1 (pCMV-Sp1) or with an empty vector. Forty-eight hours later, RNA was extracted and mRNA levels for RAB20, FGF21 and IHPK2 were determined. Values are the mean ± SE of three independent experiments.

processes including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. Recently, it has been reported to play a role as a metabolic regulator [35], and it causes an increase in ERK1/2 phosphorylation in mouse liver *in vivo* [36].

Inositol hexaphosphate kinase 2 (IHPK2) encodes a protein considered to be involved in the conversion of inositol hexakisphosphate (InsP6) to diphosphoinositol pentakisphosphate (InsP7/PP-InsP5) and 1,3,4,5,6-pentakisphosphate (InsP5) to PP-InsP4. Overexpression of IHPK2 sensitized ovarian carcinoma cell lines to the growth-suppressive and apoptotic effects of interferon beta (IFN-β), IFN-α2, and γ-irradiation [37,38]. According to our results, Sp1, which binds to the promoter of IHPK2, could play a role in IHPK2 gene expression and its effects on IFN-β in ovarian cancers.

For both, FGF21 and IHPK2, we also studied the effect of Sp1 overexpression, resulting in increased mRNA levels.

ARHGAP18 is a Rho-GTPase activating protein, which modulates cell signaling [39]. Recently, ARHGAP18 has been identified as a putative oncogene which expression in murine mammary gland cells altered their growth kinetics and caused their morphological transformation [40]. The results of ChIP analysis confirmed that Sp1 binds to its promoter. Further research is needed to determine the effects of Sp1 on this gene.

NPM3 and SRSF7 are both classified as mRNA processing genes. NPM3 likely functions as a molecular chaperone in the cell nucleus and it has been associated with mitogenesis in tumors and enhanced activator-dependent transcription, suggesting that its overexpression might lead to uncontrolled cell proliferation [41]. For instance, diffuse large B-cell lymphoma (DLBCL) is a more aggressive tumor with a higher cell growth fraction than follicular lymphoma (FL) and it has been shown that NPM3 levels were higher in DLBCL than in FL [42]. SRSF7 is a member of the serine/arginine (SR)-rich protein family involved mainly in pre-mRNA splicing [43]. SRSF7 is believed to be involved in both constitutive splicing and alternative splicing of many pre-mRNAs. In addition, it helps mRNA export to the cytoplasm and enhances the expression of unspliced mRNA [44]. A recent study has demonstrated that splicing misregulation of adult-specific exon 10 of microtubule-associated protein tau results in expression of abnormal ratios of tau isoforms, leading to frontotemporal dementia with Parkinsonism. SRSF7 strongly inhibits inclusion of tau exon 10 indicating that it plays a key role in regulation of exon 10 splicing and implying a pathogenic role for this factor in neurodegenerative diseases [45,46].

CALM3 is a calmodulin involved in differentiation and maturation of specific brain or muscle regions [47]. It has been

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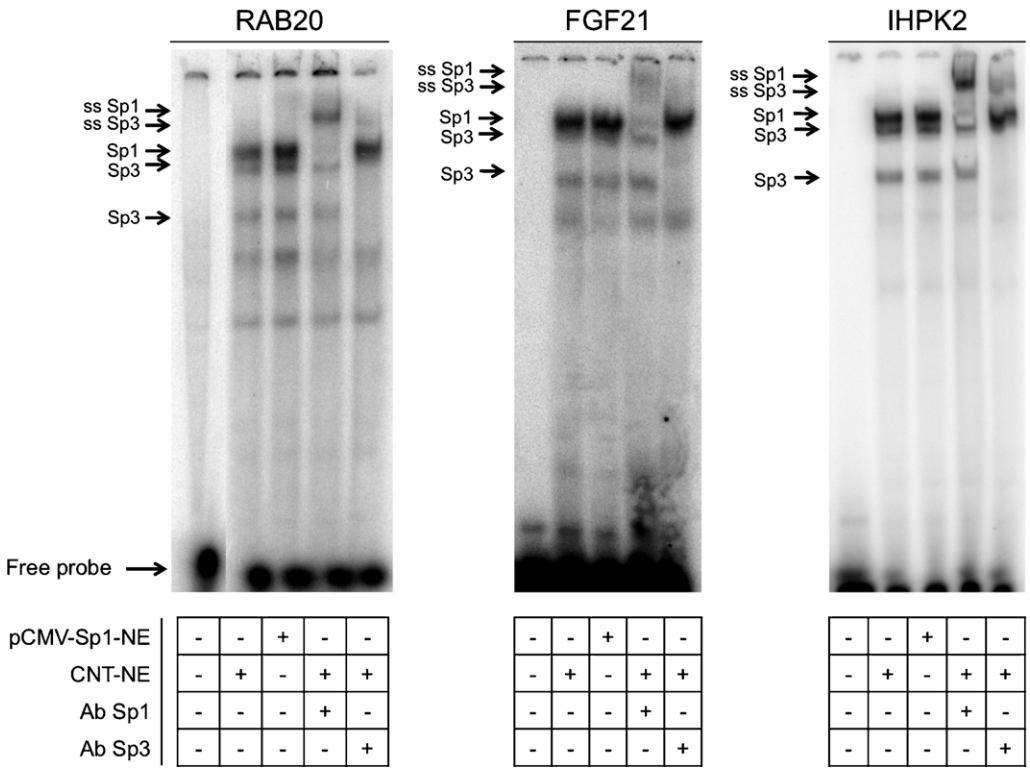

Fig. 5. Characterization of Sp1 binding to RAB20, FGF21 and IHPK2 promoters. EMSA were performed using sequences corresponding to Sp1 sites within the RAB20, FGF21 and IHPK2 promoter and nuclear extracts (NE) from either exponentially growing parental HeLa cells or transfected with an expression vector for Sp1 (pCMV-Sp1). Binding reactions were performed with 20,000 cpm of [γ-^{32}P]ATP-labeled ds probes, 2 μg of NE and 1 μg of poly[d(I-C)] used as nonspecific competitor. Supershift mobility assays were performed in the presence of specific antibodies against Sp1 or Sp3. Shifted and supershifted (ss) bands are indicated by arrows.

shown that the CALM3 transcript increases in proliferating human teratoma cells as well as in differentiating of neuroblastoma cells [48,49]. PGD, which is also classified as playing a role in the glucidic metabolism, is a phosphogluconate dehydrogenase and is the second enzyme in the pentose phosphate pathway. A reduction in the activity of PGD has been reported to induce cell death in the hepatoma cell line HA22T/VGH by interfering with redox state regulation [50]. The malignant transformation of oval

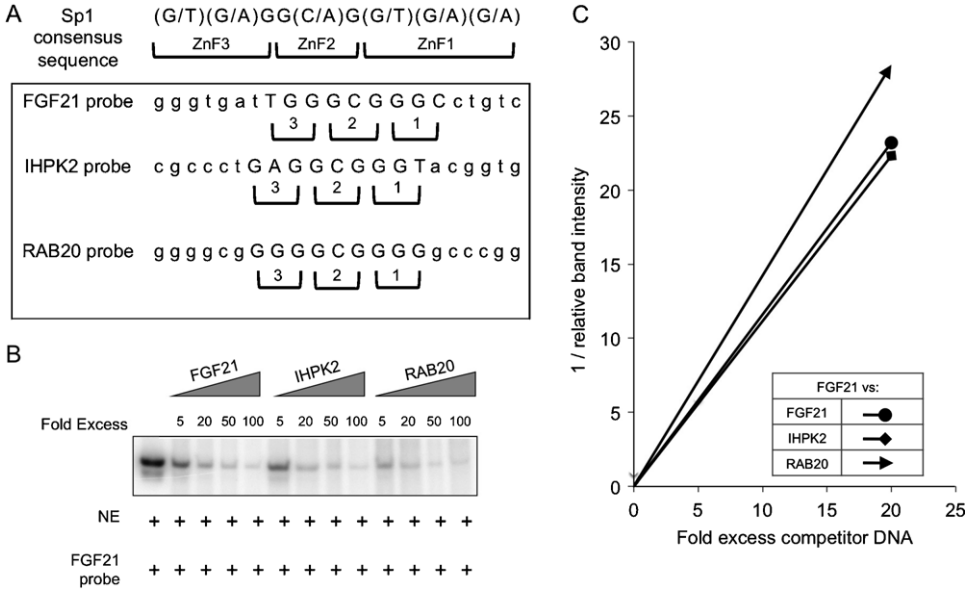

Fig. 6. Sp1 binding sites affinity for the RAB20, FGF21 and IHPK2 promoters. (A) Sequence comparison among RAB20, FGF21 and IHPK2 Sp1 binding sequences with the consensus sequence for Sp1. The position of the 3 zinc finger (ZnF) binding sites are also shown. (B) Competition analysis between FGF21 labeled probe and increasing fold excess (5, 20, 50 and 100×) of FGF21, IHPK2 and RAB20 cold probes. Binding reactions were performed with 20,000 cpm of each [γ-^{32}P]ATP-labeled ds probe, 2 μg nuclear extracts (NE) from exponentially growing HeLa cells and 1 μg of poly[d(I-C)] used as nonspecific competitor. (C) The reciprocal of the relative amount of bound probe (the value in the absence of competitor taken as one) is plotted *versus* the fold excess of the competitor DNA, with a best fit straight line fitted to the points by regression analysis. The slope of this line is proportional to the relative binding affinity of the competitor DNA. The slopes for each promoter are: RAB20: 1.42, FGF21: 1.16 and IHPK2: 1.12.

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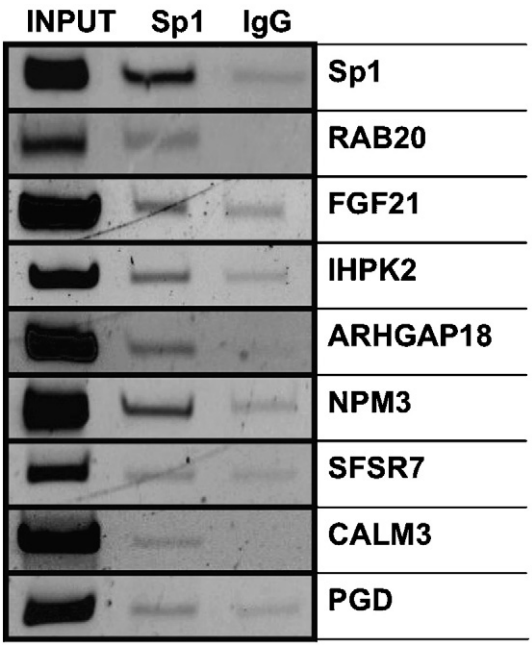

Fig. 7. ChIP analysis of the selected promoters. ChIP analysis of Sp1 binding to the selected promoters was performed using HeLa cells subjected to Sp1 and IgG immunoprecipitation. DNA bound to the immunoprecipitated Sp1 using specific antibody, was amplified by PCR. Mouse IgG was used as negative control. Representative images of the PCR products corresponding to the amplification of RAB20, FGF21, IHPK2, ARHGAP18, NPM3, SRSF7, CALM3, PGD and Sp1 (specific primers are described in Table 2) promoter fragments are shown. The input lane corresponds to the whole (non immunoprecipitated) DNA and the Sp1 and IgG lanes correspond to the immunoprecipitations with Sp1 and IgG, respectively.

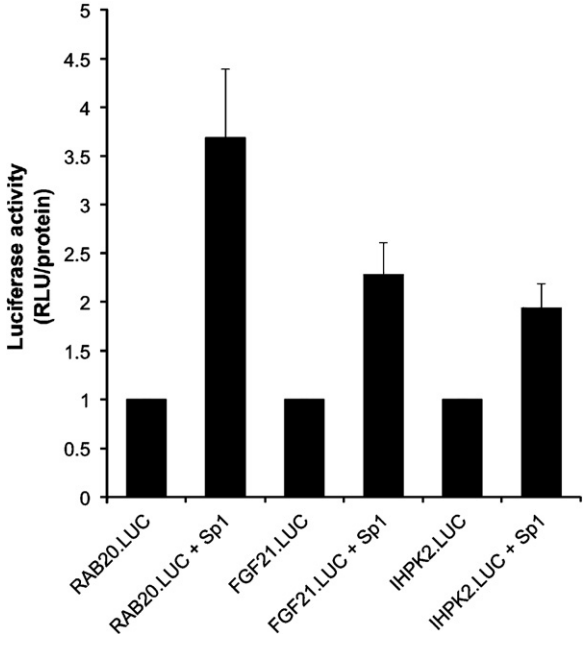

Fig. 8. Luciferase activity of RAB20, FGF21 and IHPK2 promoters upon Sp1 overexpression. 500 ng of either RAB20.LUC, FGF21.LUC or IHPK2.LUC constructs in the presence or in the absence of the Sp1 expression vector (500 ng) were cotransfected in HeLa cells. Luciferase activity (relative light units, RLU) was determined 30 h after transfection and the values for each sample were normalized by total protein concentration (mg/mL). Luciferase activity is expressed as fold change between the values obtained in the presence *vs.* the absence of Sp1. Values correspond to the mean ± SE of three independent experiments.

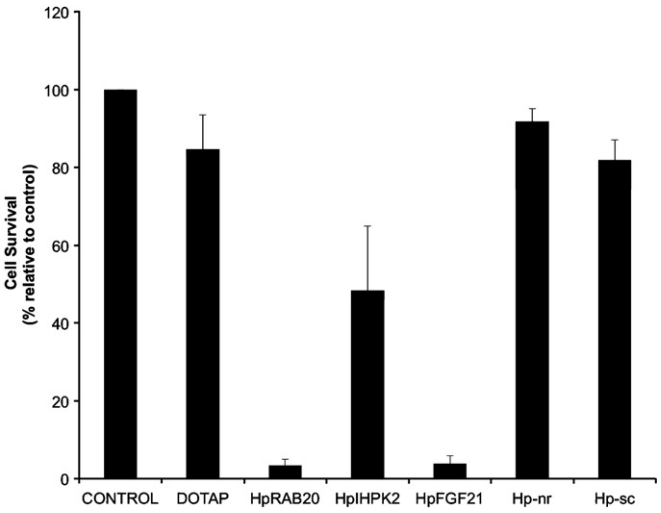

Fig. 9. Effect of knocking down RAB20, FGF21 and IHPK2 using PPRHs. 100 nM of Hp-RAB20, Hp-FGF21 and Hp-IHPK2 and the negative controls Hp-wc and Hp-sc were transfected using 10 μM DOTAP in HeLa cells. Five days after transfection, the MTT assay was carried out. Values are referred as % to control and correspond to the mean ± SE of three independent experiments.

cells, a type of liver epithelial cells that proliferate during the early stages of hepatocarcinogenesis, was accompanied by an increase in the activity of 6-phosphogluconate dehydrogenase [51]. In a genome wide association study to identify susceptibility variants for hepatitis B virus related to hepatocellular carcinoma, the 1p36.22 locus was found to be highly associated with this type of carcinoma. The 1p36.22 locus contains the PGD gene, in addition to two other genes [52]. Altogether, these findings suggest a role for PGD-related pathways in the pathogenesis of hepatocarcinomas.

In conclusion, we identified 8 new gene targets whose expression can be activated by the binding of Sp1 protein to their promoter regions. Four of them, RAB20, FGF21, IHPK2 and ARHGAP18 are involved in proliferation and cancer, NPM3, SRSF7 are related to mRNA processing, and CALM3 and PGD play a role in glucidic metabolism. Further extensive studies are needed to generalize the results since the data obtained in HeLa cells may be cell type specific. However, the findings of this work open now the possibility to explore the role of these Sp1-activated genes in their specific pathways. The effects observed by silencing RAB20, FGF21 or IHPK2 genes using PPRHs hairpins confirm the role of these three genes in proliferation.

Conflict of interest

The authors declare no potential conflicts of interest.

Acknowledgements

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b) ARTÍCULO VI:

Networking of differentially expressed genes in human cancer cells resistant to methotrexate

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Research

Networking of differentially expressed genes in human cancer cells resistant to methotrexate

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Abstract

Background: The need for an integrated view of data obtained from high-throughput technologies gave rise to network analyses. These are especially useful to rationalize how external perturbations propagate through the expression of genes. To address this issue in the case of drug resistance, we constructed biological association networks of genes differentially expressed in cell lines resistant to methotrexate (MTX).

Methods: Seven cell lines representative of different types of cancer, including colon cancer (HT29 and Caco2), breast cancer (MCF-7 and MDA-MB-468), pancreatic cancer (MIA PaCa-2), erythroblastic leukemia (K562) and osteosarcoma (Saos-2), were used. The differential expression pattern between sensitive and MTX-resistant cells was determined by whole human genome microarrays and analyzed with the GeneSpring GX software package. Genes deregulated in common between the different cancer cell lines served to generate biological association networks using the Pathway Architect software.

Results: Dikkopf homolog-1 (*DKK1*) is a highly interconnected node in the network generated with genes in common between the two colon cancer cell lines, and functional validations of this target using small interfering RNAs (siRNAs) showed a chemosensitization toward MTX. Members of the UDP-glucuronosyltransferase 1A (*UGT1A*) family formed a network of genes differentially expressed in the two breast cancer cell lines. siRNA treatment against *UGT1A* also showed an increase in MTX sensitivity. Eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*) was overexpressed among the pancreatic cancer, leukemia and osteosarcoma cell lines, and siRNA treatment against *EEF1A1* produced a chemosensitization toward MTX.

Conclusions: Biological association networks identified *DKK1*, *UGT1A*s and *EEF1A1* as important gene nodes in MTX-resistance. Treatments using siRNA technology against these three genes showed chemosensitization toward MTX.

Background

The large amount of information obtained with high-throughput technologies like expression microarrays needs to be processed in order to be comprehensible to molecular biologists. In this regard, many computational methods have been developed to facilitate expression data analysis. Gene clustering, gene ontology and pathway analyses are commonly used [1,2]. Pathways are manually generated diagrams that represent knowledge on molecular interactions and reactions [3] and they can be used to visualize the involvement of the differentially expressed genes in specific molecular, cellular or biological processes. However, the complexity of higher organisms cannot be explained solely as a collection of separate parts [4]; in organisms, pathways never exist in isolation, they are part of larger networks, which are more informative and real [5]. Gene networks are capable of describing a large number of interactions in a concise way, and provide a view of the physiological state of an organism at the mRNA level. Biochemical networks can be constructed at several levels and can represent different types of interactions. Literature mining allows the extraction of meaningful biological information from publications to generate networks [6]. Taking into account the progress in gene expression profiling, elucidating gene networks is an appropriate and timely step on the way to uncovering the complete biochemical networks of cells [5].

In this work, we use biological association networks (BANs) as a tool to define possible targets for gene therapy in combination with methotrexate (MTX). This approach could serve to minimize the development of MTX resistance acquired by cancer cells, which remains a primary cause of therapy failure in cancer treatment [7]. A role in MTX resistance was established for the three node genes selected, namely those encoding Dikkopf homolg 1 (*DKK1*), UDP-glucuronosyltransferases (UGTs; *UGT1A*s) and Eukaryotic translation elongation factor 1A1 (*EEF1A1*).

Methods

Cell lines

Cell lines representative of five types of human cancer were used: HT29 and Caco-2 for colon cancer, MCF-7 and MDA-MB-468 for breast cancer, MIA PaCa-2 for pancreatic cancer, K562 for erythroblastic leukemia, and Saos-2 for osteosarcoma. These cell lines are sensitive to MTX, with IC50s of 1.67 x 10^{-8} M MTX for HT29, 4.87 x 10^{-8} M MTX for MDA-MB-468 and 1.16 x 10^{-8} M MTX for MIA PaCa-2 cells. IC50 values were calculated using GraphPad Prism 5 version 5.0a for Macintosh (GraphPad Software, San Diego, CA, USA). Resistant cells were obtained in the laboratory upon incubation with stepwise concentrations of MTX (Lederle) as previously described [8]. HT29, Caco-2 and K562 resistant cells were able to grow in 10^{-5} M MTX; MIA PaCa-2, Saos-2, MCF-7 and MDA-MB-246 cells were resistant to 10^{-6} M MTX.

Cell culture

Human cell lines were routinely grown in Ham's F12 medium supplemented with 7% fetal bovine serum (both from Gibco/Invitrogen, Grand Island, NY, USA) at 37°C in a 5% CO_2 humidified atmosphere. Resistant cells were routinely grown in selective DHFR medium lacking glycine, hypoxanthine and thymidine (-GHT medium; Gibco), the final products of dihydrofolate reductase (DHFR) activity. This medium was supplemented with 7% dialyzed fetal bovine serum (Gibco).

Microarrays

Gene expression was analyzed by hybridization to the GeneChip® Human Genome U133 PLUS 2.0 from Affymetrix, containing over 54,000 transcripts and variants. Total RNA for oligo arrays was prepared from triplicate samples of every sensitive and resistant cell line using the RNAeasy Mini kit (Qiagen, Germantown, Maryland, USA) following the recommendations of the manufacturer. Labeling, hybridization and detection were carried out following the manufacturer's specifications.

Microarray data analyses

Gene expression analyses were performed using three samples of both sensitive and resistant cells for each of the seven cell lines studied. These analyses were carried out with the GeneSpring GX software v 7.3.1 (Agilent Technologies, Santa Clara, CA, USA), using the latest gene annotations available (March 2009). This software package allows multi-filter comparisons using data from different experiments to perform the normalization, generation of restriction (filtered) lists and functional classifications of the differentially expressed genes. Normalization was applied in two steps: 'per chip normalization', by which each measurement was divided by the 50th percentile of all measurements in its array; and 'per gene normalization', by which all the samples were normalized against the median of the control samples (sensitive cells). The expression of each gene was reported as the ratio of the value obtained for each condition relative to the control condition after normalization of the data. Then, data were filtered using the control strength, a control value calculated using the Cross-Gene Error model on replicates [9] and based on average base/proportional value. Measurements with higher control strength are relatively more precise than measurements with lower control strength. Genes that did not reach this value were discarded. Additional filtering was performed to determine differentially expressed genes. A first filter was performed by selecting the genes that displayed a *P*-value corrected by false discovery rate (Benjamini and Hochberg false discovery rate) of less than 0.05. The output of this analysis was then filtered by fold expression. Thus, lists of genes differentially expressed by at least twofold were generated for each of the seven resistant cell lines.

Table 1

Sense strand sequences of the siRNAs used

Target gene	siRNA name	siRNA sequence (5'- 3')
DKK1	siDKK1	AGGTGCTGCACTGCCTATT
UGT1A family	siUGT1A	GTGCTGGGCAAGTTTACTT
EEF1A1	siEEF1A1	CGGTCTCAGAACTGTTTGT
DHFR	siDHFR	CCTCCACAAGGAGCTCATT
Luciferase	NR-siRNA	TAAGGCTATGAAGAGATAC

The sequences for the sense strand of the siRNAs used against the target genes are provided.

Common genes between cell lines

The lists of genes differentially expressed by at least twofold with a *P*-value <0.05 including multiple testing correction for each cell line were divided into two groups: overexpressed and underexpressed genes. Comparisons of lists of overexpressed genes were performed using Venn diagrams in GeneSpring GX. Lists of underexpressed genes were also compared using the same approach. All lists were compared in pairs and lists of genes in common between each pair were generated.

Generation of biological association networks

BANs were constructed with the aid of Pathway Architect software v3.0 (Stratagene-Agilent). Briefly, this software package generates interaction networks starting with the genes in a given list (entities) taking into account the information present in a database of known molecular interactions. The lists correspond to the collection of differentially expressed genes under specific conditions. The database of molecular interactions is composed of more than 1.6 million interactions divided into different classes (binding, regulation, promoter binding, transport, metabolism, protein metabolism and expression). The interactions are extracted from the literature using a Natural Language Processing (NLP) tool run on Medline abstracts (NLP references), plus those obtained from external curated databases like BIND (Biomolecular Interaction Network Database) [10] and MINT (Molecular INTeraction) [11]. Interactions in the interaction database are scored according to five different categories: maximum, high, medium, low and minimal. Curated interactions (BIND and MINT sources) are given the maximum quality score, as are any interactions that have at least three NLP references. Pathway Architect gathers all that information to construct novel views as to how the entities in a list could be interacting with each other, even including entities not present in the original list (neighbors resulting from the expanded interaction). Customized analyses were performed to select relevant interaction networks with an associated high confidence index since such networks are likely to mirror biological significance. One-step expansion (using the expand network command) of the original set of entities with maximum score interaction were then analyzed by setting an advanced filter that included the categories of binding, expression, metabolism, promoter binding, protein modification and regulation. This procedure gives a final representation formed of a collection of nodes with different degrees of interrelationship. Some gene products from the original lists were not significantly connected with other members or neighbors and, therefore, were removed from the final view. Finally, members of the network were matched with expression levels.

Transfection of small interfering RNAs against selected genes

HT29 cells (30,000) were plated in 1 ml of -GHT medium and transfection was performed 18 hours later. For each well, Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) in 100 l of serum free -GHT medium was mixed in Eppendorf tubes with 100 nM of small interfering RNA (siRNA) in 100 l of serum free -GHT medium. The mixture was incubated at room temperature for 20 minutes before addition to the cells. MTX (2×10^{-8} M) was added 48 hours after siRNA treatment and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays [12] were performed 3 days after MTX addition. Treatment of MDA-MB-468 and MIA PaCa-2 cells was performed following the same protocol but using Metafectene™ (Biontex, Martinsried, Germany). A non-related siRNA was used as negative control; it was transfected in parallel with the other siRNAs and used to normalize the results.

The siRNAs were designed using the software iRNAi v2.1. (The Netherlands Cancer Institute, Amsterdam, The Netherlands) Among the possible alternatives, sequences rich in A/T at the 3' end of the target were chosen. Then, BLAST resources in NCBI were used to assess the degree of specificity of the sequence recognition for these siRNAs. Only the siRNAs that reported the target gene as the only mRNA hit, or some family members in the case of siUGT1A, were selected. The sequences for the sense strand of all siRNAs used are available in Table 1.

Heat map generation

A global comparison of all cell lines was performed using GeneSpring GX v 7.3.1. The triplicate samples for each condition in each of the seven cell lines (42 samples) were imported into one single experiment. Normalization was performed in two steps: 'per chip normalization' (as described above) and 'per gene normalization', by which the samples were normalized against the median of all samples. Lists of genes displaying a false discovery rate-corrected *P*-value <0.05 were generated for each cell line. As a filter, these values had to appear in at least two out of the seven cell lines. A hierarchical clustering method in GeneSpring was used to group genes on the basis of similar expression patterns over all samples. The distance matrix used was Pearson correlation, and average linkage was used as clustering

algorithm. The same clustering method was used to group the cell lines on the basis of similar patterns of gene expression.

Real-time RT-PCR

Gene mRNA levels were determined by real-time RT-PCR. Total RNA was extracted from cells using Ultraspec™ RNA reagent (Biotecx, Houston, TX, USA) following the recommendations of the manufacturer. For determining gene-node mRNA levels upon siRNA treatment, cells were treated as described above and total RNA was prepared 48 hours after transfection using the same reagent. In either case, complementary DNA was synthesized in a total volume of 20 l by mixing 500 ng of total RNA and 125 ng of random hexamers (Roche, Mannheim, Germany) in the presence of 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 units of RNasin (Promega, Madison, WI, USA), 0.5 mM dNTPs (AppliChem, Darmstadt, Germany), 200 units of M-MLV reverse transcriptase (Invitrogen) and 50 mM Tris-HCl buffer, pH 8.3. The reaction mixture was incubated at 37°C for 60 minutes. The cDNA product was used for subsequent real-time PCR amplification using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with 25 ng of the cDNA mixture, the assays-on-demand from Applied Biosystems Hs00758822_s1 for DHFR and Hs00356991_m1 for Adenine Phosphoribosyltransferase (APRT) or the following primers: *DKK1*, 5'-AGTACTGCGCTAGTCCCACC-3' and 5'-CTGGAATACCCATCCAAGGTGC-3'; *UGT1A*, 5'-TAAGTGGCTACCCCAAAACG-3' and 5'-CTCCAGCTCCCTTAGTCTCC-3'; *EEF1A1*, 5'-CGTCATTGGACACGTAGATTCGGG-3' and 5'-GGAGCCCTTTCCCATCTCAGC-3'.

Gene copy number determination

Genomic DNA from either sensitive or resistant cells was obtained with the Wizard™ Genomic DNA Purification Kit (Promega) following the manufacturer's recommendations. One hundred nanograms of DNA and the assays-on-demand Hs00758822_s1 for DHFR and Hs99999901_s1 for 18S were used for real-time PCR amplification.

Preparation of total extracts for western blotting

Total extracts from cells, either sensitive or MTX-resistant, were used to assay DHFR protein levels. Cells were washed twice with ice-cold phosphate-buffered saline and scraped in 200 ml lysis buffer (50 mM Hepes, 500 mM NaCl, 1.5 M $MgCl_2$, 1 mM EGTA, 10% glycerol (v/v), 1% Triton X-100 and protease inhibitor cocktail). Cells were incubated in ice for 1 hour with vortexing every 15 minutes and then centrifuged at 14,000 rpm at 4°C for 10 minutes. Five microliters of the extract were used to determine protein concentration by the Bradford assay (Bio-Rad, Munich, Germany). The extracts were frozen in liquid N_2 and stored at -80°C. Fifty micrograms of both sensitive and resistant cell total extracts were resolved by 15% SDS-PAGE. Transference to PVDF membranes (Immobilon P, Millipore, Bedford, MA, USA) using a semidry electroblotter was followed by incubation with an antibody against DHFR (Davids Biotechnologie, Regensburg, Germany), and detection was accomplished using secondary horseradish peroxidase-conjugated antibody and enhanced chemiluminescence, as recommended by the manufacturer (Amersham/GE Healthcare, Buckinghamshire, UK). To normalize the results, blots were re-probed with an antibody against actin (Sigma, St. Louis, MO, USA).

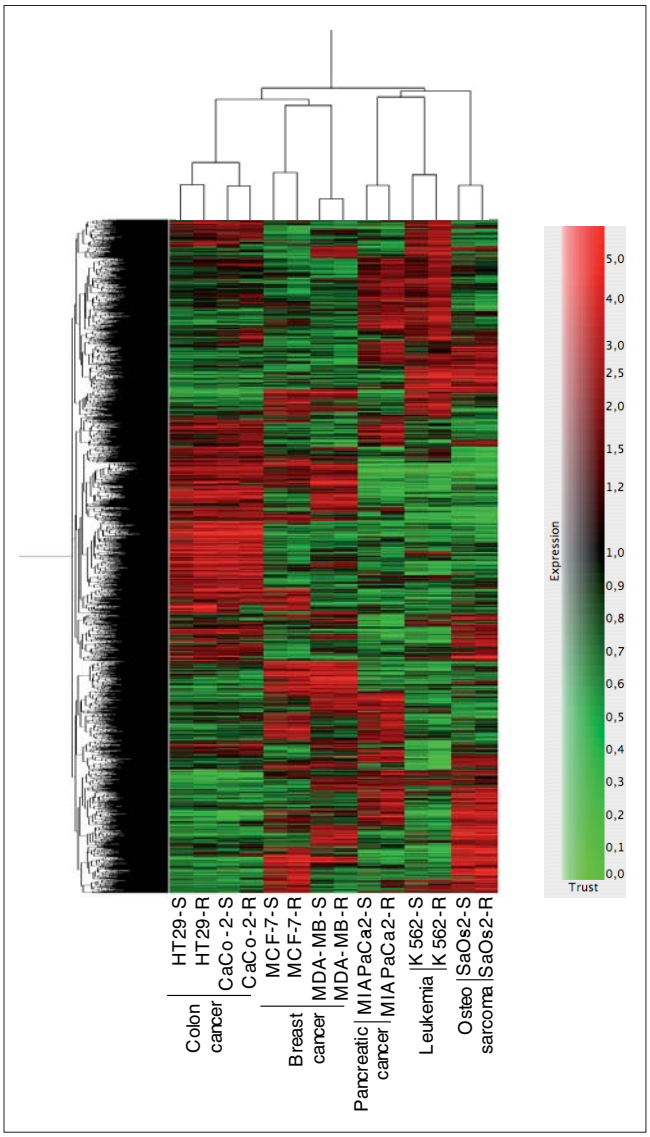

Figure 1

Heat map of differentially expressed genes. Lists of differentially expressed genes with a *t*-test *P*-value <0.05 including multiple testing correction were generated for each cell line. A hierarchical clustering method in GeneSpring GX v 7.3.1 was used to construct both the gene tree and the sample tree, as described in Methods. Data are shown in a matrix format: each row represents a single gene, and each column represents a cell line. Red indicates overexpressed genes (expression levels over the median) and green indicates underexpressed genes (expression levels under the median; see legend). The pattern and length of the branches in the dendrograms reflect the relatedness of the samples or the genes.

Table 2

Validation of *dhfr* overexpression and copy number determination in the different cell lines

	Expression			
Cell line	Microarray	RT-PCR validation	Copy number	Protein
HT29	7.1	10.8 ± 0.7	16.1 ± 1.4	++
Caco-2	46.7	49.7 ± 1.1	83.4 ± 8.1	ND
MCF-7	31.1	33.2 ± 0.7	58.1 ± 0.8	+++++
MDA-MB-468	1.8	3.4 ± 0.1	0.9 ± 0.1	ND
MIA PaCa-2	9.5	8.2 ± 1.1	32.2 ± 2.2	+++
K562	9.4	9.8 ± 0.2	1.9 ± 0.1	++++
Saos-2	4.1	4.1 ± 1.1	0.6 ± 0.1	+

The overexpression of DHFR was validated at the mRNA and protein levels by real-time RT-PCR and western blotting using a specific antibody, respectively. DHFR expression levels are presented both as the values found in the microarrays and as validated by RT-PCR. *dhfr* copy number was determined by real-time PCR. Values are the mean (in fold change relative to the sensitive cells) of three independent experiments ± SE. Protein levels were qualitatively assessed (+ to +++++) from the western blot images. ND, non-determined.

Transfections, co-transfections and luciferase assays

HT29 cells, either sensitive or MTX-resistant, were seeded into six-well plates the day before transfection at a density of 2 x 10^5 cells/well in Ham's F12 medium containing 7% fetal bovine serum. Transfection was performed using FUGENE™ HD (Roche). For each well, 6 l of FUGENE™ HD in 100 l of serum-free medium was incubated at room temperature for 5 minutes. The mixture was added to 1 g of TOPFLASH (Millipore) and incubated at room temperature for 20 minutes before addition to the cells. In co-transfections, 1 g of TOPFLASH was mixed together with 2 g of pBATEM2-CDH before the addition of FUGENE™ HD in serum-free medium. The total amount of DNA was kept constant at 3 g, adding empty vector when necessary. Luciferase activity was assayed 30 hours after transfection.

In all cases, cell extracts were prepared by lysing the cells with 200 l of freshly diluted 1x Reporter Lysis Buffer (Promega). The lysate was centrifugated at 13,000 g for 2 minutes to pellet the cell debris and the supernatants were transferred to a fresh tube. A 15- l aliquot of the extract was added to 15 l of the luciferase assay substrate (Promega) and the luminiscence of the samples was read immediately on a Gloomax 20/20 luminometer (Promega); light production (relative light units) was measured for 10 s. Each transfection was performed in triplicate. Protein concentration was determined by the Bradford assay and used to normalize the results.

Statistical analyses

Data are presented as mean ± standard error (SE). Statistical analyses were performed using the unpaired *t*-test option in GraphPad InStat version 3.1a for Macintosh (GraphPad Software). *P*-values <0.05 were considered to be statistically significant.

Results

Genes deregulated in methotrexate-resistant cancer cell lines

In a previous study, we analyzed the differential gene expression between sensitive and MTX-resistant cells derived from the human colon cancer cell line HT29 [13]. In the present work we extend the study of gene expression profiles associated with MTX resistance by including another six MTX-resistant cell lines. Together, the studied cell lines represent colon cancer (CaCo2 and HT29), breast cancer (MCF-7 and MDA-MB-468), pancreatic cancer (MIA PaCa-2), erythroblastic leukemia (K562) and osteosarcoma (SaOs-2). Total RNA was extracted for the seven pairs of sensitive and MTX-resistant cell lines, and the expression profile of the 54,700 transcripts and variants included in the HG U133 PLUS 2.0 microarray from Affymetrix was compared between each pair using GeneSpring GX software v7.3.1. Upon normalization and statistical filtering of the data, lists of genes differentially expressed by at least twofold were built as described in Methods. These lists are presented as Additional data files 1 to 7. The data discussed in this report have been deposited in the Gene Expression Omnibus (GEO) [14] and are accessible through GEO series accession number [GSE16648].

Hierarchical clustering of genes and cell lines

We compared the gene expression patterns of all the studied cell lines together. Lists of genes displaying a false discovery rate-corrected *P*-value <0.05 were generated for each cell line. Then, hierarchical clustering in GeneSpring GX was used to construct a heat map displaying both a gene tree and a sample tree (Figure 1), as described in Methods. Two facts could be extracted from this representation. First, there is a high correlation between cell lines sharing the same tissue origin. The two colon cancer cell lines studied (HT29 and Caco-2) are more highly correlated in gene expression with each other than with all the other cell lines. The breast cancer cell lines studied (MCF-7 and MDA-MB-468) also showed similar gene expression, although the degree of correlation is slightly lower than that for the colon cancer cell lines. The other three cell lines studied (MIA PaCa-2, K562 and Saos-2) displayed different gene expression from the colon or the breast cancer cell lines, and thus cluster apart from them. Second, gene expression of the resistant cells is more closely correlated with that of their sensitive cell counterparts than with any other sample or cell line.

Table 3

Genes differentially expressed in common among colon cancer cell lines resistant to MTX

GenBank ID	Gene name	Description	Ratio HT29	Ratio Caco-2
AI144299	*DHFR*	Dihydrofolate reductase	7.25	46.35
BC005238	*FXYD3*	FXYD domain containing ion transport regulator 3	7.24	2.20
BC003584	*DHFR*	Dihydrofolate reductase	6.96	50.23
BC000192	*DHFR*	Dihydrofolate reductase	6.89	38.31
NM_002380	*MATN2*	Matrilin 2	6.62	4.70
BU078629	*ZFYVE16*	Zinc finger, FYVE domain containing 16	6.06	22.73
NM_001975	*ENO2*	Enolase 2 (gamma, neuronal)	5.98	2.04
NM_017954	*CADPS2*	Ca^{2+}-dependent activator protein for secretion 2	5.60	2.27
AI991103	*AXIIR*	Similar to annexin II receptor	5.14	2.03
NM_000791	*DHFR*	Dihydrofolate reductase	4.71	21.17
U05598	*AKR1C2*	Aldo-keto reductase family 1, member C2	4.63	10.15
M33376	*AKR1C2*	Aldo-keto reductase family 1, member C2	4.41	8.89
NM_012242	*DKK1*	Dickkopf homolog 1	4.25	2.56
NM_014867	*KBTBD11*	Kelch repeat and BTB (POZ) domain containing 11	4.22	2.14
AB037848	*KIAA1427*	Synaptotagmin XIII	4.13	9.56
NM_014733	*ZFYVE16*	Zinc finger, FYVE domain containing 16	4.11	15.6
NM_001353	*AKR1C1*	Aldo-keto reductase family 1, member C1	3.94	8.87
NM_002439	*MSH3*	MutS homolog 3	3.87	4.00
S68290	*AKR1C1*	Aldo-keto reductase family 1, member C1	3.66	9.45
J04810	*MSH3*	MutS homolog 3	3.27	8.23
NM_000691	*ALDH3A1*	Aldehyde dehydrogenase 3 family, member A1	2.86	3.81
AI718385	*SLC26A2*	Solute carrier family 26 member 2	2.76	2.02
NM_003069	*SMARCA1*	SWI/SNF related, regulator of chromatin a1	2.60	2.49
AB029026	*TACC1*	Transforming, acidic coiled-coil containing protein 1	2.35	3.89
NM_006283	*TACC1*	Transforming, acidic coiled-coil containing protein 1	2.31	3.26
AF188298	*DAB2*	Disabled homolog 2	2.29	2.11
NM_020299	*AKR1B10*	Aldo-keto reductase family 1, member B10	2.26	22.95
BC006471	*MLLT11*	Myeloid/lymphoid or mixed-lineage leukemia	2.18	2.60
W93554	*SH3PXD2A*	SH3 and PX domains 2A	2.14	3.45
NM_014778	*NUPL1*	Nucleoporin like 1	0.46	0.43
NM_006033	*LIPG*	Lipase, endothelial	0.40	0.42
NM_012338	*TM4SF12*	Transmembrane 4 superfamily member 12	0.39	0.38
NM_007150	*ZNF185*	Zinc finger protein 185 (LIM domain)	0.38	0.42
AB014605	*MAGI2*	Membrane associated guanylate kinase	0.38	0.30
AB033831	*SCDGF*	Platelet derived growth factor C	0.35	0.35
NM_021021	*SNTB1*	Syntrophin, beta 1	0.35	0.23
NM_021822	*APOBEC3G*	Apolipoprotein B, catalytic polypeptide-like 3G	0.30	0.08
AB039791	*ARP11*	Actin-related protein Arp11	0.29	0.46
NM_013352	*SART2*	Squamous cell carcinoma antigen	0.22	0.31

Continued overleaf

Table 3

Continued

GenBank ID	Gene name	Description	Ratio HT29	Ratio Caco-2
NM_004362	*CLGN*	Calmegin	0.21	0.19
AI912583	*GLIPR1*	GLI pathogenesis-related 1 (glioma)	0.21	0.45
Z19574	*KRT17*	Keratin 17	0.19	0.46
NM_003186	*TAGLN1*	Transgelin	0.19	0.14
NM_014059	*RGC32*	Response gene to complement 32	0.17	0.44
BE872674	*CLEC3A*	C-type lectin domain family 3, member A	0.15	0.24
NM_003212	*TDGF1/3*	Teratocarcinoma-derived growth factor 1/3	0.13	0.47
BC000069	*RARRES2*	Retinoic acid receptor responder 2	0.07	0.33
AF110400	*FGF19*	Fibroblast growth factor 19	0.06	0.26
NM_006851	*GLIPR1*	GLI pathogenesis-related 1	0.05	0.28
NM_006169	*NNMT*	Nicotinamide N-methyltransferase	0.04	0.24
AF208043	*IFI16*	Interferon, gamma-inducible protein 16	0.03	0.38
BG256677	*IFI16*	Interferon, gamma-inducible protein 16	0.02	0.40
NM_006169	*NNMT*	Nicotinamide N-methyltransferase	0.02	0.24

Genes differentially expressed by at least twofold with a *P*-value <0.05 including multiple testing correction were compared between HT29 and Caco-2 cells using Venn diagrams in GeneSpring GX software v 7.3.1. The table includes the GenBank IDs for all genes, their respective common names and the associated description. The ratio column corresponds to the fold change in expression of each gene relative to its sensitive counterpart.

Dihydrofolate reductase status in all cell lines studied

As DHFR is the target for MTX, and was upregulated in MTX-resistant cells, we validated its overexpression in all cell lines studied. Real-time RT-PCR was used to quantify the mRNA levels, and DHFR protein levels were determined by western blotting in both sensitive and MTX-resistant cell lines (Table 2). Copy number determination revealed *dhfr* amplification only in HT29, Caco-2, MCF-7 and MIA PACA-2 resistant cells (Table 2).

Identification of genes differentially expressed in common among different cell lines resistant to MTX

Lists of genes differentially expressed by at least twofold between sensitive and resistant cell lines were generated for each cell line. Each list was split in two, one group including the overexpressed genes and the other including the underexpressed genes. Then, Venn diagrams were used to compare the lists of overexpressed and underexpressed genes between HT29 and Caco-2 cell lines, between MCF-7 and MDA-MB-468 cell lines, and among MIA PaCa-2, K562 and Saos-2 cell lines (Tables 3, 4 and 5, respectively). This approach allowed us to identify differentially expressed genes with a common trend in expression among the cell lines compared.

Detection of nodes upon generation of biological association networks

BANs were constructed using the Pathway Architect software as described in Methods starting with the lists of genes differentially expressed in common between both colon cancer cell lines, both breast cancer cell lines and among the pancreas cancer, leukemia and osteosarcoma cell lines (Figure 2a,b,c, respectively). In the BANs generated, *DKK1* is a highly interconnected node in the colon cancer cell lines, *UGT1A* family members formed a network of genes differentially expressed in breast cancer, and *EEF1A1* was commonly overexpressed in pancreatic cancer, leukemia and osteosarcoma. A BAN including all the genes of the three lists of differentially expressed genes was constructed (Figure 3). *DKK1*, *UGT1A* and *EEF1A1* all seemed to be important nodes of this newly constructed network, and thus were selected for further study.

Effect on MTX sensitivity of siRNAs designed against the mRNAs of node genes

Given that the node genes *DKK1*, *UGT1A*s and *EEF1A1* were overexpressed in cells resistant to MTX (Table 6), we investigated the effect of decreasing their mRNA levels by means of siRNAs on the sensitivity to this chemotherapeutic agent. We also performed treatments with siDHFR in order to assess the role of DHFR in MTX resistance. HT29 and MDA-MB-468 cell lines were used as models of colon and breast cancer, respectively, and MIA PaCa-2 cells were selected as the model for the other three cell lines. Previously, it was confirmed that the mRNA levels of the three genes were decreased 48 hours after siRNA treatment (Figure 4a-c). Cells were pre-incubated with individual siRNAs for 48 hours before the addition of methotrexate. The presence of 100 nM of either siDKK1 or siDHFR caused increases in MTX cytotoxicity in HT29 cells of 50% and 65%, respectively, com-

Table 4

Genes differentially expressed in common among breast cancer cell lines resistant to MTX

GenBank 468	Gene name	Description	Ratio MCF7	Ratio MDA-MB-
NM_019093	*UGT1A1/3/4/5/6/7/8/9/10*	UDP glucuronosyltransferase 1, polypeptides A1/3/4/5/6/7/8/9/10	24.36	27.93
NM_000463	*UGT1A1/4/6/8/9/10*	UDP glucuronosyltransferase 1, polypeptides A1/4/6/8/9/10	15.31	17.66
NM_021027	*UGT1A1/4/6/8/9/10*	UDP glucuronosyltransferase 1, polypeptides A1/4/6/8/9/10	13.55	17.05
NM_001072	*UGT1A1/3/4/5/6/7/8/9/10*	UDP glucuronosyltransferase 1, polypeptides A1/3/4/5/6/7/8/9/10	13.21	16.88
AV691323	*UGT1A1/3/4/5/6/7/8/9/10*	UDP glucuronosyltransferase 1, polypeptides A1/3/4/5/6/7/8/9/10	13.05	16.82

Genes differentially expressed by at least twofold with a *P*-value <0.05 including multiple testing correction were compared between MCF7 and MDA-MB-468 cells as described in Table 3.

pared to MTX alone (Figure 4d). Incubation with 100 nM of either siUGT1A or siDHFR in MDA-MB-468 cells caused increases in cytotoxicity of 36% and 50%, respectively, compared to MTX alone (Figure 4e). Treatment of MIA PaCa-2 cells with 30 nM of either siEEF1A1 or siDHFR resulted in increased cytotoxicity of 35% and 65%, respectively, compared to MTX alone (Figure 4f). The same approach was conducted in the resistant counterparts of the HT29, MDA-MB-468 and MIA PaCa-2 cell lines, but no significant changes in cytotoxicity were observed ($P > 0.05$; data not shown).

Given that the UGT1A family is involved in the metabolism of other drugs, we also performed combination treatments with siUGT1A and SN38, the active metabolite of the anticancer drug irinotecan. Transfection of the siRNA was performed as described above, and 1 nM SN38 was added 48 hours after siRNA treatment. These incubations led to a significant ($P < 0.01$) increase of 46% in SN38 sensitivity.

A non-related siRNA against the luciferase gene was used as a negative control in all experiments. Transfection of this siRNA was performed in parallel with the other siRNAs, and was used to normalize the results.

DKK1 is overexpressed in HT29 MTX-resistant cells due to higher activation of the Wnt pathway

As *DKK1* is known to be transcriptionally regulated by the Wnt pathway, we investigated the degree of activation of this signaling pathway in MTX-resistant HT29 cells compared with their sensitive counterpart. Cells were transiently transfected with the reporter plasmid TOPFLASH, bearing three T-cell factor (TCF) binding sites. A transcriptional activation of 26-fold resulted from the transfection of TOPFLASH in the resistant cells, while no significant activation was observed upon transfection in the sensitive cells (Figure 5). Additionally, co-transfections of TOPFLASH with an expression plasmid for E-cadherin (pBATEM2-CDH) were performed in both cell lines. As shown in Figure 5, overexpression of E-cadherin in the resistant cells led to a marked decrease in TOPFLASH activity, down to basal activity. No significant changes in transcriptional activity were observed when these co-transfections were performed in the sensitive cells.

Table 5

Genes differentially expressed in common among colon cancer cell lines resistant to MTX

GenBank	Gene name	Description	Ratio MIA PaCa-2	Ratio K562	Ratio Saos-2
BC003584	*DHFR*	Dihydrofolate reductase	16.97	17.78	4.06
BC000192	*DHFR*	Dihydrofolate reductase	12.56	13.57	5.28
AI144299	*DHFR*	Dihydrofolate reductase	12.1	9.45	8.94
NM_000791	*DHFR*	Dihydrofolate reductase	9.66	6.76	5.57
BE622627	*PIK3R3*	Phosphoinositide-3-kinase, regulatory subunit 3	5.23	2.09	4.44
AW469790	*EEF1A1*	Eukaryotic translation elongation factor 1 alpha 1	2.29	2.75	2.05
NM_012446	*SSBP2*	Single-stranded DNA binding protein 2	2.2	5.04	2.03
AF318326	*APG10L*	ATG10 autophagy related 10 homolog	2.08	7.39	2.19

Genes differentially expressed by at least twofold with a *P*-value <0.05 including multiple testing correction were compared between MIA PaCa-2, K562 and Saos-2 cells as described in Table 3.

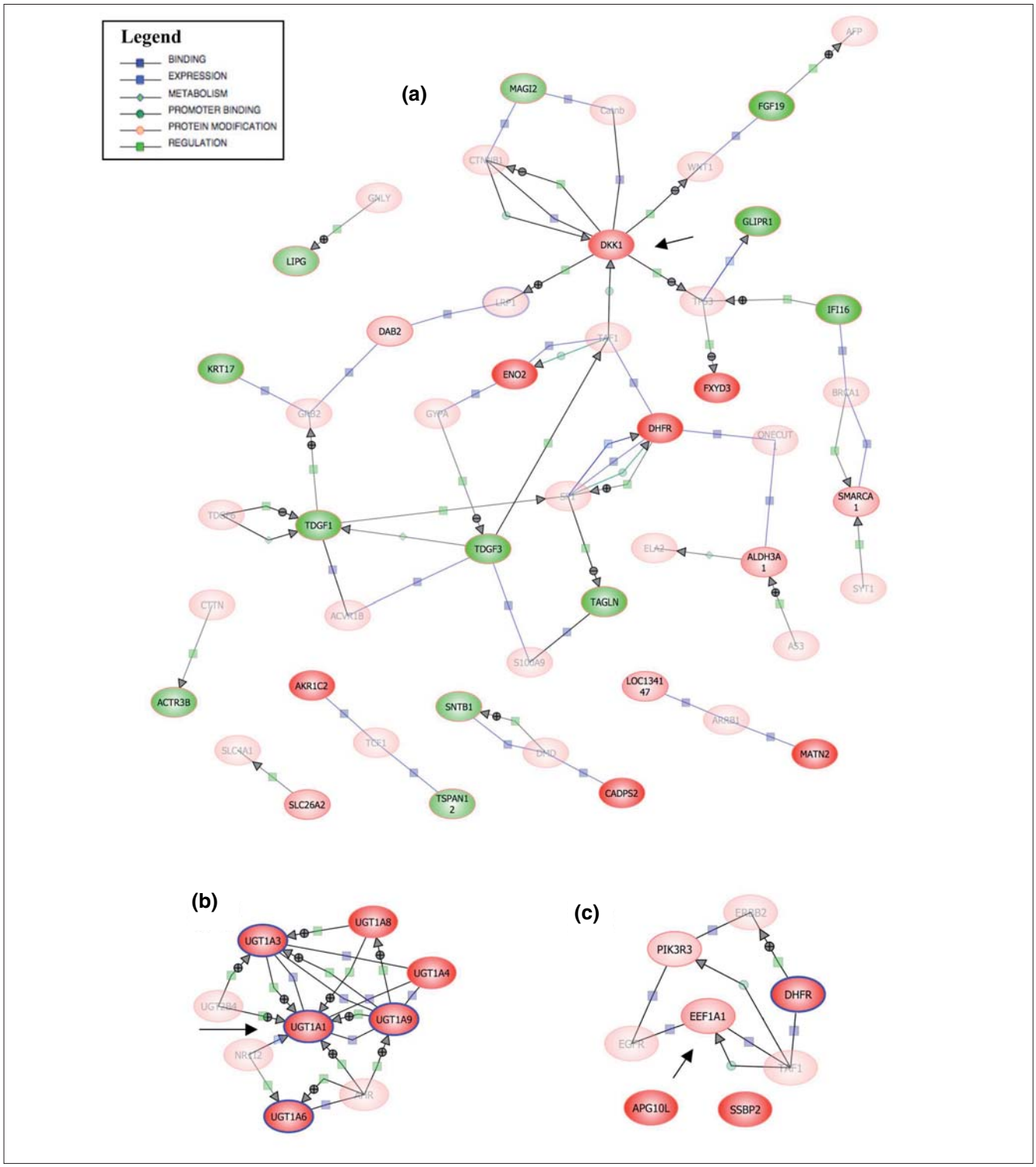

Figure 2
BANs of differentially expressed genes in common between cell lines. The lists of common genes between both colon cancer cell lines, between both breast cancer cell lines, and among the other three cell lines studied (representative of pancreatic cancer, leukemia and osteosarcoma) were used to construct BANs with the Pathway Architect software. Expanded networks were constructed for each list - **(a)** colon cancer, **(b)** breast cancer and **(c)** the other three cell lines - by setting an advanced filter that included the categories of binding, expression, metabolism, promoter binding, protein modification and regulation (see legend). Only proteins are represented. Overlapping of the expression levels was also performed (red for overexpressed genes and green for underexpressed genes; translucent shading represents genes that were not in the list and were added by the program from the interactions database). The BANs show some node genes that were studied further (those with arrows pointing to them).

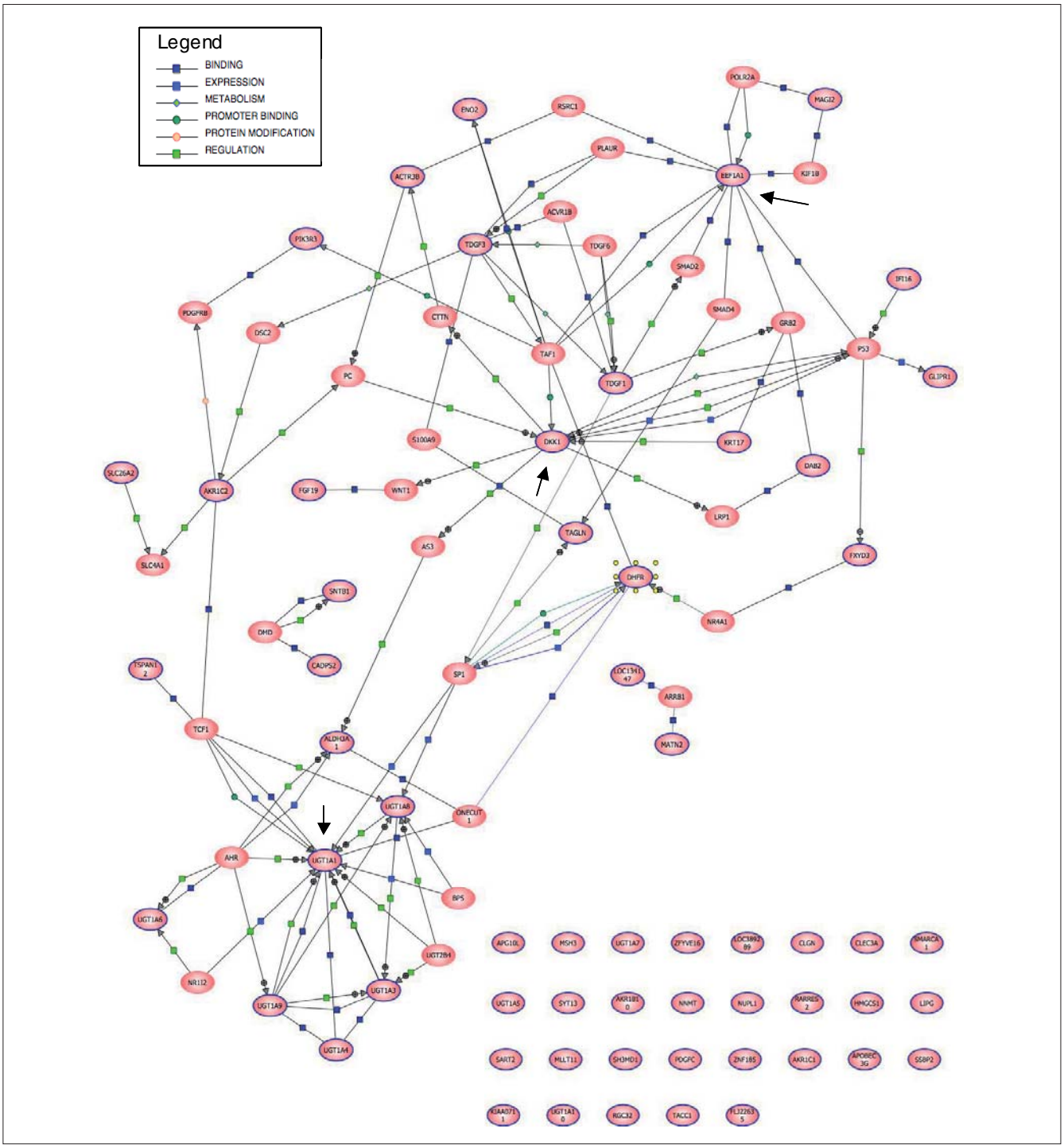

Figure 3
BAN of all common genes. A BAN was constructed as previously described with all the genes included in any of the three lists of common genes (encircled in blue). Genes added by the program from the interaction database are not outlined. Node genes are those with arrows pointing to them.

Discussion

The main objective of this work was to explore whether node genes could be identified from BANs constructed starting from genes differentially expressed in MTX-resistant cells from different human cancer cell lines, representative of five tissues. Those putative node genes may then be used as targets to increase the sensitivity toward MTX.

We started by determining and comparing the patterns of differential gene expression associated with MTX resistance

Table 6

Validation of *DKK1*, *UGT1A* and *EEF1A1* overexpression in the resistant cells

		Expression	
Gene	Cell line	Microarray	RT-PCR validation
DKK1	HT29	4.25	5.66 ± 0.23
	Caco-2	2.56	1.96 ± 0.03
UGT1A	MCF-7	15.90	23.41 ± 0.94
	MDA-MB-468	19.27	16.28 ± 0.19
EEF1A1	MIA PaCa-2	2.29	3.89 ± 0.16
	K562	2.75	2.38 ± 0.47
	Saos-2	2.05	1.85 ± 0.15

The expression levels for the three node genes (*DKK1*, *UGT1A* and *EEF1A1*) derived from both the microarray data (average of the overexpressed *UGT1A* family members) and real-time RT-PCR are given. All the results are expressed as fold changes relative to the sensitive cells and values are the mean of triplicate experiments ± SE.

in seven cell lines. The only differentially expressed gene in common among all the cell lines studied was *dhfr*. Its overexpression, at both the mRNA and protein levels, was confirmed in the MTX-resistant cells studied. The mRNA upregulation can be explained either by gene amplification of the *dhfr* locus, a well recognized mechanism for MTX resistance [15-17], or by an increase of *dhfr* transcription rate. In accordance with this, HT29, CaCo-2, MCF-7 and MIA PACA-2 resistant cells display an increased *dhfr* copy number. Indeed, amplification of the 5q14 locus, including *dhfr* and flanking genes, has been described in HT29 MTX-resistant cells [13]. On the other hand, the other cell lines studied bear no changes in *dhfr* copy number. Thus, drug resistance in MDA-MB-468, K562 and Saos-2 cells may be caused by any of the other known mechanisms for MTX resistance [18-22]. Additionally, one has to take into account that MTX causes the differential expression of many genes that may be direct or indirect regulators of cell proliferation, survival or apoptosis, and that this expression pattern can contribute to modulation of the resistance phenotype. As described in this work, the overexpression of *DKK1*, *UGT1A*s or *EEF1A1* could represent a mechanism, parallel to *DHFR* overexpression, that plays a role in MTX resistance; the possible contribution of the overexpression of each of these genes is addressed below. Other work has determined genes that correlate with the capability of parental cells to resist treatment using concentrations assessed to be clinically achievable in tumor tissue [23]. Although this approach is different to the goal of our work, to identify genes differentially expressed in cells with acquired resistance to high concentrations of MTX, we compared the results obtained by Gyorffy *et al.* [23] with ours and found only six genes in common, namely *CD99*, *CKMT1*, *DHRS2*, *IGFBP7*, *MAP7* and *MYO1E*.

Hierarchical clustering of all samples indicated that the MTX-resistant cells and their sensitive counterparts were highly correlated with regards to gene expression with each other. This is in accordance with reports showing that two breast tumor samples from the same patient before and after treatment with doxorubicin pair together in a hierarchical clustering [24]. Moreover, the same authors proposed that the molecular program of a primary tumor could generally be retained in its metastases. Similar results were obtained with leukemic cells from patients [25].

Interestingly, as shown in Figure 1, the gene expression patterns for the cell lines from the same tissue origin were very similar. Sets of coordinately expressed genes provide gene expression signatures that can indicate where to find targets suitable for gene therapy. Thus, we generated a list of genes differentially expressed in common between the two colon cancer cell lines. This list included, among others, genes encoding DHFR, the target for MTX, three members of the AKR family and ENO2, which we have previously studied as modulators of MTX resistance [8,13], and DKK1.

BAN construction using the genes differentially expressed in common between both colon cancer cell lines identified *DKK1* as a highly interconnected node of the network, which could as such be a candidate druggable gene. DKK1 is a secreted protein involved in embryonic development [26] and is classically considered to function as an inhibitor of the canonical Wnt signaling pathway [27] (see [28] for a review). However, it does not take an active part in the Wnt/β-catenin pathway in colon cancer cells, as mutation of adenomatous polyposis coli (one of the components of Wnt pathway) occurs in most human colon cancers [29,30], thus disconnecting the effector part of the signaling cascade from the Wnt receptors, where DKK1 exerts its inhibitory effect [31,32]. This situation led us to hypothesize that DKK1 could have other cellular functions aside from its role in the Wnt pathway. Indeed, a role for DKK1 overexpression in cancer [26], including hepatobastomas [33] and breast cancer bone metastasis [34], aggressive tumors, epithelial-mesenchymal transition [35] and proliferation [36], has been previously suggested, although its precise mechanism of action has not yet been elucidated. In the case of HT29 MTX-resistant cells, the role of DKK1 is unclear, although it seems to be related to the resistant phenotype, since treatment with the siRNA against *DKK1* mRNA showed a chemosensitization toward MTX. In keeping with this, Katula and collaborators [37] showed that folate deficiency led to the downregulation of DKK1, and that MTX inhibited *DKK1* transcription. Thus, *DKK1* overexpression in HT29 MTX-resistant cells could constitute a mechanism to overcome the transcriptional repression exerted by MTX.

It is worth noting that we had previously proposed the activation of the Wnt/ß-catenin pathway to be an important step in MTX resistance in HT29 colon cancer cells [13]. In

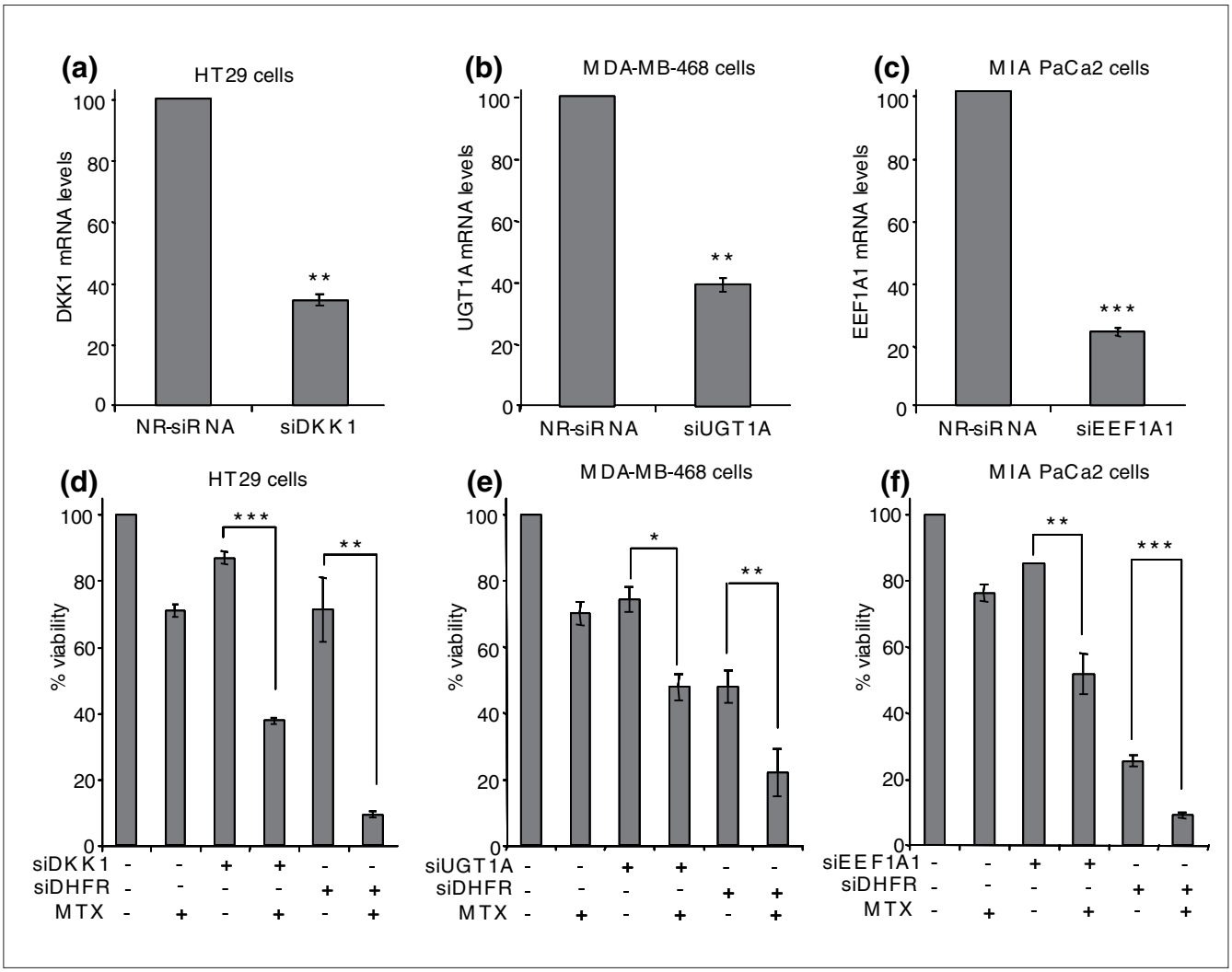

Figure 4

Effects on MTX sensitivity of treatment with siRNAs against *DKK1*, *UGT1A*s or *EEF1A1*. **(a-f)** Treatment with siDKK1 was performed in HT29 cells (a,d), siUGT1A was transfected in MDA-MB-468 cells (b,e) and the effects of siEEF1A1 were determined in MIA PaCa-2 cells (c,f). Treatments were performed as described in Methods, and MTX was added after 48 h. Cell viability was determined 3 days after MTX treatment (d-f). (d-f) A siRNA against *dhfr* was transfected in each of the three cell lines, and its effects on cell viability are presented. For determination of mRNA levels, real-time RT-PCR was performed with 500 ng of total RNA extracted 48 h after siRNA transfection (a-c). All results are expressed as percentages relative to the non-related negative control siRNA (NR-siRNA). Values are the mean of three independent experiments ± SE. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

this cell line, E-cadherin is chromosomically lost and underexpressed, thus allowing β-catenin to play its function in gene transcription. In order to shed some light on the possible role of the Wnt pathway in the overexpression of *DKK1* in HT29 resistant cells, we performed transient transfection experiments with a luciferase reporter of β-catenin-mediated transcriptional activation. These experiments showed that the Wnt pathway was more active in the HT29 resistant cells than in HT29 sensitive cells, and that re-expression of E-cadherin in the resistant cells resulted in lower β-catenin-mediated transcriptional activation, probably due to recruitment of β-catenin to the adherent junctions. Interestingly, *DKK1* is transcriptionally regulated by Wnt/β-catenin signaling. Thus, constitutive activation of this signaling pathway through β-catenin, downstream of adenomatous polyposis coli, could represent a mechanism for *DKK1* overexpression in HT29 MTX-resistant cells.

*UGT1A*s were the only genes differentially expressed in common between both breast cancer cell lines analyzed. UGTs comprise a family of membrane glycoproteins that come from one single gene located on chromosome 2q37, rendering nine functional UGT1A proteins by alternative splicing of 13 different first exons with the common exons 2 to 5 [38]. UGTs are involved in phase II metabolism of a wide range of metabolites, both endogenous and exogenous [39]. Glucuronidation is an important metabolic process, as it carries out the biotransformation of lipophilic substrates into

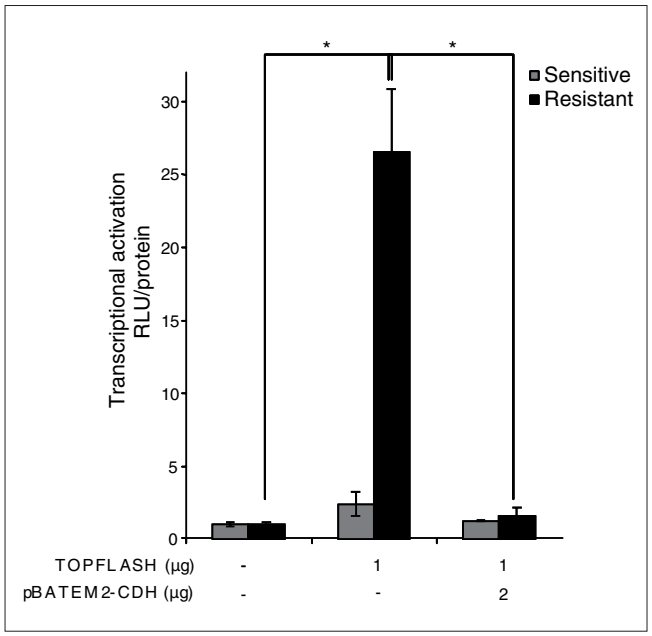

Figure 5
Transcriptional activation of the Wnt pathway in MTX-resistant cells leads to *DKK1* overexpression. HT29 cells, either sensitive or resistant, were transiently transfected with 1mg of a luciferase reporter of b-catenin-mediated transcriptional activation (TOPFLASH) using Fugene™ HD in the presence or absence of 2 mg of E-cadherin expression vector (pBATEM2-CDH). Thirty hours after transfection, luciferase activity (relative light units (RLU)) was assayed. The protein content was used to normalize the luciferase activity for each sample and is expressed relative to that of pGL3 basic vector (mean ± standard error of the mean for triplicate wells). $*P < 0.05$.

hydrophilic glucuronides, which can be more easily removed from the body. Among the different products that can undergo glucuronidation, we find analgesics, sex hormones, flavonoids, rifampicin, bilirubin and tobacco-specific carcinogens [40,41]. Some anticancer drugs, such as topotecan, irinotecan, SN-38 (the active metabolite of irinotecan), doxorubicin and 4-hydroxytamoxifen, have also been described to be substrates of UGT1A activity [42-47]. Metabolism by UGT1A family members has been described to induce resistance toward daunorubicin (in both cell lines and rat hyperplastic liver nodules) [48,49], mycophenolic acid [50,51], mitoxantrone [52], SN-38 [53,54], camptothecin [55] and other drugs [56,57]. Indeed, drug inactivation by metabolism within tumor cells is recognized as an important mechanism of drug resistance, and, specifically, glucuronidation by UGT enzymes has been proposed to contribute to multidrug resistance of several chemotherapeutic drugs [56]. From our results using a siRNA against *UGT1A* mRNA we can point out a role for this family of genes in MTX resistance in MDA-MB-468 breast cancer cells. In fact, Hanioka *et al.* [58] reported that β-naphthoflavone induced *UGT1A* mRNA levels. This could be a feasible explanation for the *UGT1A* overexpression we observed in the breast cancer MTX-resistant cells used in our study.

EEF1A1 was overexpressed in common among MIA PaCa-2, K562 and Saos-2 resistant cell lines. EEF1A1 is a ubiquitously expressed protein elongation factor that recruits amino-acetylated tRNAs to the A site of the ribosome (see [59] for a review). Although it has been traditionally described as a cellular housekeeper enzyme, overexpression of EEF1A1 is found in melanomas and tumors of the pancreas, breast, lung, prostate and colon [59,60]. It has been demonstrated that EEF1A expression is related to increased cell proliferation [61,62], oncogenic transformation [63], delayed cell senescence [64] and metastasis [65]. Moreover, increased EEF1A1 expression has been related to cisplatin [66], doxorubicin [67] and MTX resistance [68], maybe due to its ability to inhibit apoptosis [69]. It has been proposed that EEF1A overexpression promotes cell growth and replication by contributing to an overall increase in protein translation. Antisense-mediated abrogation of *EEF1A1* expression inhibits tumorigenesis and anchorage-independent cell replication in prostate tumor cells [70]. Our functional analyses using siRNA technology against *EEF1A1* are in keeping with these results, and show a chemosensitization of MIA PaCa-2 cells, thus stating a role for EEF1A1 in MTX resistance in this cell line.

In summary, our results provide evidence that node genes can be identified by constructing BANs with lists of genes differentially expressed in common between cell lines resistant to MTX. RNA interference technology has enabled us to demonstrate a role for DKK1, UGT1As and EEF1A1 in MTX resistance.

Conclusions

BANs were constructed using genes differentially expressed in common between cells resistant to MTX from seven human cancer cell lines representative of five tissues. We have been able to identify important node genes in the BANs, namely *DKK1* in colon cancer cells, *UGT1A*s in breast cancer cells and *EEF1A1* in pancreatic cancer, leukemia and osteosarcoma cells. These three genes were functionally validated using siRNAs against their respective mRNAs, which resulted in increased sensitivity to MTX.

Abbreviations

APRT = Adenine Phosphoribosyltransferase; BAN = biological association network; DHFR = dihydrofolate reductase; DKK1 = Dikkopf homolog 1; EEF1A1 = eukaryotic translation elongation factor 1 alpha 1; MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTX = methotrexate; NLP = Natural Language Processing; SE = standard error; siRNA = small interfering RNA; TCF = T-cell factor; UGT = UDP glucuronosyl transferase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ES participated in microarray data analyses, BAN generation and cell treatment with siRNAs. CO carried out the determination of *DHFR* mRNA levels, *dhfr* copy number and protein levels. SR performed the luciferase experiments. CA generated MDA-MB-468 cells resistant to MTX. VN helped with data interpretation and drafting the manuscript, critically revising it. CJC conceived the study, participated in microarray data analyses and in BAN generation. All authors read and approved the final manuscript.

Additional data files

The following additional data are available with the online version of this paper: a table listing of genes differentially expressed at least twofold in HT29 MTX-resistant cells (Additional data file 1); a table listing genes differentially expressed at least twofold in Caco-2 MTX-resistant cells (Additional data file 2); a table listing genes differentially expressed at least twofold in MCF-7 MTX-resistant cells (Additional data file 3); a table listing genes differentially expressed at least twofold in MDA-MD-468 MTX-resistant cells (Additional data file 4); a table listing genes differentially expressed at least twofold in MIA PaCa-2 MTX-resistant cells (Additional data file 5); a table listing genes differentially expressed at least twofold in K562 MTX-resistant cells (Additional data file 6); a table listing genes differentially expressed at least twofold in Saos-2 MTX-resistant cells (Additional data file 7).

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