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Departamento de Ciencias Biomédicas

Efecto de la melatonina sobre la proliferación, apoptosis y angiogénesis en un modelo in vitro de hepatocarcinoma

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Memoria presentada por la Licenciada en Biología Sara Carbajo Pescador para la obtención del grado de Doctor por la Universidad de León

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INFORME DE LOS DIRECTORES DE LA TESIS

(Art. 11.3 del R.D. 56/2005)

El Dr. D. Javier González Gallego y el Dr. D. José Luis Mauriz Gutiérrez, Directores de la Tesis Doctoral titulada "Efecto de la melatonina sobre la proliferación, apoptosis y angiogénesis en un modelo *in vitro* de hepatocarcinoma" realizada por la Lcda. Dña. Sara Carbajo Pescador, en el Departamento de Ciencias Biomédicas, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa

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El Departamento de Ciencias Biomédicas en su reunión celebrada el día ___ de _____ de 2013 ha acordado dar su conformidad a la admisión a trámite de lectura de la Tesis Doctoral titulada "Efecto de la melatonina sobre la proliferación, apoptosis y angiogénesis en un modelo *in vitro* de hepatocarcinoma", dirigida por los Dres. D. Javier González-Gallego y D. Jose Luis Mauriz Gutiérrez, y elaborada por la Lcda. Dña. Sara Carbajo Pescador, cuyo título en inglés es: "Effect of melatonin on proliferation, apoptosis and angiogenesis in an *in vitro* model of hepatocarcinoma".

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Dirección/ *Adress: Langenbeckstr. 1, 55101 Mainz, Germany*
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Tesis Doctoral por compendio de publicaciones

Melatonin modulation of intracellular signaling pathways in hepatocarcinoma HepG2 cell line: role of the MT1 receptor.

Carbajo-Pescador S, Garcia-Palomo A, Martin-Renedo J, Piva M, Gonzalez-Gallego J, Mauriz JL (2011).

Journal of Pineal Research, **51**: 463-471.

Factor de Impacto: 7.304, 4/79 Categoría: Fisiología

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British Journal of Cancer, **108**: 442-449.

Factor de Impacto: 5.082, 35/196 Categoría: Oncología

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British Journal of Cancer, doi: 10.1038/bjc.2013.285.

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Parte de los resultados expuestos en la presente memoria han sido objeto de las siguientes comunicaciones a congreso

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"Efecto antiangiogénico de la melatonina en células HepG2 por inhibición de Hif1 alfa y Stat3." Publicado en: Gastroenterología y Hepatología, 36(1), 39, 2013 (Poster).

S. Carbajo-Pescador , R. Ordoñez , J. González-Gallego, J.L. Mauriz.

XVIII Congreso de la Sociedad Española de Investigaciones Quirúrgicas (SEIQ) 18-19/10/2012 León (España)

"Actividad anti-angiogénica de la melatonina en un modelo in vitro de carcinoma hepatocelular humano." (Poster).

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FEPS (Federation of European Physiological Societies) and Spanish Physiological Society Scientific Congress 2012 Santiago de Compostela 8-11/9/2012 (España)

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XXIV European Congress on Surgical Infection. 13-16 /06/ 2012 Lund (Suecia)

"Melatonin induces apoptosis in HEPG2 Hepatocarcinoma cells though transcriptional regulation of BIM by FOXO3a." (Premio al mejor poster)

Sara Carbajo-Pescador, José Luis Mauriz, Javier Martín-Renedo, Maiara Piva, María Victoria García-Mediavilla,María José Cuevas, Andrés García-Palomo, Pilar S Collado, Susanne Strand, Javier González-Gallego.

XXXVII Congreso de la Asociación Española para el Estudio del Hígado (AEEH) 15-17/02/2012 Madrid (España)

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“La prueba más clara de sabiduría es una alegría continua.”

Michel De Montaigne

A mis padres y a Diego

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Departament of Biomedical Sciences

Effect of melatonin on proliferation, apoptosis and angiogenesis in an in vitro model of hepatocarcinoma

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PhD Dissertation

Sara Carbajo Pescador

León, 2013

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5-FU	5-Fluorouracil
AA-NAT	Arylalkylamine N-acetyltransferase
AFB1	Aflatoxin B1
AFP	α-Fetoprotein
AIPs	Inhibitors of apoptosis
AMPK	AMP-activated protein kinase
APAF-1	Apoptotic protease factor 1
APO-1	Apoptosis antigen-1
Bad	Bcl-2-associated death promoter
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bid	BH3 interacting-domain death agonist
Bim	B-cell lymphoma-2 (Bcl-2)-interacting modulator of cell death
Bok	Bcl-2 related ovarian killer
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
cGMP	Cyclic guanosine monophosphate
ChIP	Chromatin immunoprecipitation
COP1	Coat protein complex
CT	Computed tomography
DNA	Deoxyribonucleic acid
DUSPs	Dual-Specificity phosphatases
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FGF	Fibroblast growth factor
FIH	Factor Inhibiting Hif1
FoxO	Forkhead box proteins
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIF	Hypoxia-inducible factor
HIOMT	Hidroxindol O-metiltransferasa
HK2	Hexokinase II
HREs	hypoxic responsive elements
HUVECs	Human umbilical vein endothelial cells

IFN-alpha2b	Interferon-alpha2b
IGF-2	Insulin-like growth factor-2
IgG	Immunoglobulin G
IL-1β	Interleukin-1 beta
IL-23	Interleukin-23
IL-6	Interleukin-6
JNKs	Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase
MAPKKKs	MAPKK-kinases
MAPKKs	MAPK-kinases
MDM2	Murine double minute 2
MMPs	Matrix metalloproteinases
MOMP	Major outer membrane protein
MR	Magnetic resonance
Mrna	Messenger RNA
MT1	Melatonin membrane receptor 1
MT2	Melatonin membrane receptor 2
MT3	Melatonin membrane receptor 3
NF-kB	Nuclear factor kappa B
NGF	Nerve growth factor
NQO2	Quinone oxireductase 2
NSCLC	Non–small lung cancer
ODD	Oxygen-dependent degradation domain
OGT	O-glycoxylations performed by O-GlcNAc transferase
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PRMT1	Protein arginine methyltransferase 1
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROR	Retinoid orphan receptors
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
RZR	Retinoid Z receptors
SAPK	Stress-activated protein kinase
SGKs	Serum- and glucocorticoid-inducible kinases
siRNA	Small interfering RNA
Skp2	S-phase kinase-associated protein 2
STAT3	Signal transducer and activator of transcription 3
TGF-β1	Transforming growth factor beta 1
TNF-α	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand

US	Ultrasonography
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Introduction and Aims

Hepatocellular carcinoma (HCC) is the fifth most common tumor worldwide and the third cause of cancer-related deaths, with more than half a million new cases each year. It is a heterogeneous disease which occurs in more than 80% cirrhotic livers. At the moment, this disease has a very poor prognosis and patients with HCC and advanced cirrhosis have a 2-years survival rate lower than 32%. To date, only surgical resection and liver transplantation are curative therapies, nonetheless only 30% of the patients are potential candidates. Moreover, conventional chemotherapy is not very effective for patients with non-resectable HCC. There are also non-surgical treatments available, such as catheter ablation with alcohol or chemoembolization with cisplatin or doxorubicin, radiofrequency and cryotherapy, aimed to preventing tumor progression while on a waiting list for transplantation; but unfortunately in many cases results obtained are rather discreet.

A better understanding of the carcinogenesis process turns out to be crucial to identify potential targets for its treatment and prevention; hence, while there is no dominant pathway responsible for HCC development, there are evidences pointing to certain signaling cascades that appear most frequently altered and would be attractive targets to design effective drugs. Currently, basic studies to test drugs in HCC can be performed either *in vitro*, with primary cultures from patient samples, and established tumor cell lines; or *in vivo,* using animal models. In the present study we chose an *in vitro* model consisting in an established human HCC cell line. That model allowed us to deepen into some molecular mechanisms involved in HCC, and since these human cells had the same tumor origin to ensure homogeneity and reproducibility. However, we are aware of the limitations of an *in vitro* study and further *in vivo* models would be necessary before the potential development of clinical trials in the future.

Melatonin is a strong antioxidant molecule which has been found to be effective in several pathological situations, with widespread agreement of its apparent nontoxicity in normal cells. A number of *in vitro* and *in vivo* studies and some clinical trials support the anticancer and oncostatic beneficial effects of melatonin against a wide range of tumors. However, more mechanistic studies are required to confirm

these results. Our group has recently shown that melatonin not only inhibits proliferation, but also induces apoptosis of HepG2 human HCC cells, suggesting that this indole hormone might be useful at least as adjuvant in HCC therapy.

Considering these data, and based on the increasing pharmacological interest of melatonin, the main purpose of this thesis was to analyze the mechanisms involved on the oncostatic effects of melatonin treatment in the HepG2 cell derived from HCC.

The following specific objectives were proposed:

- To determine the contribution of the MT1 melatonin membrane receptor to melatonin effects on cell viability, mitogen-activated protein kinase (MAPKs) activation, and cAMP levels.

- To assess the existence of an internal regulation between the different melatonin receptors at membrane, cytosolic and nuclear level in HepG2 cells.

- To evaluate melatonin selectivity between healthy and tumor liver cells.

- To analyze whether melatonin administration can induce apoptotic cell death of the tumor cells, and elucidate the molecular pathway involved in its pro-apoptotic features.

- To investigate the potential anti-angiogenic effects of melatonin treatment in HCC, and the molecular mechanism responsible.

Literature review

1. Hepatocellular carcinoma (HCC)

The liver is the heaviest organ in the body and one of the largest, located in the upper right part of the abdomen. It has a wide range of vital functions such as nutrients process, bile formation, detoxification, and proteins synthesis, which make it essential for survival (Gumucio and Miller, 1981).

On the other hand, cancer is a major burden of disease worldwide. Each year, tens of millions of people are diagnosed with cancer around the world, and more than half of the patients eventually die of the disease (Ma and Yu, 2006). In this way, oncology research remains the most important initiative regarding the future of cancer, enabling people to know as much as possible about the causes, prevention, diagnosis, and treatment.

Both topics come together with the most common type of liver cancer: Hepatocellular carcinoma (HCC).

1.1. Global Epidemiology of HCC

There are striking variations in the cancer incidence between geographic areas, mainly associated with exposition to known or suspected risk factors such as lifestyle or environment. Data from the International Agency for Research on Cancer place HCC as the third most common diagnosed cancer and the fifth cause of cancer related death worldwide (Jemal *et al*, 2011).

Regarding to HCC, near 80% of cases occur in sub-Saharan Africa and in Eastern Asia, with more than 20 patients per 100,000 individuals. Southern European countries (such as Spain, Italy, and Greece) present mid-incidence levels (10.0 –20.0 per 100,000 individuals), while North America, South America, Northern Europe, and Oceania have a lower incidence of HCC (5.0 per 100,000 individuals). However, epidemiologic studies reflect constant changes in the HCC incidence rates; on the one hand a slight decrease in the HCC cases has been reported among Asian areas (such as China, Hong Kong, Shanghai, and Japan), associated with the new policies for the vaccination of all newborns against hepatitis B virus (HBV) (Chang *et al*, 1997), and with healthier alimentary habits which decreased the exposure to aflatoxin B1 (AFB1)

(Wong and Chan, 2012); on the other hand data emerging from epidemiologic studies demonstrated that HCC incidence rates are increasing in Western areas (United States, Europe and Canada) partially attributable to growing levels of HBV infection and alcohol consumption (El-Serag, 2012). Although it is hard to calculate future changes in HCC epidemiology, some experts suggested that global HCC incidence will continue rising in the next few years; despite of improvements in the control of HBV and hepatitis C virus (HCV) infection, risk factors such as diabetes and obesity become increasingly important drivers of future HCC incidence trends (Figure 1) (Venook *et al*, 2010).

Taking into account all current data on incidence and mortality of HCC, along with their potential tendency to increase, we consider it as an interesting and relevant topic for our PhD Thesis project.

1.2. Etiology, risk factors and HCC development

Similarly to other tumors, HCC etiology is very heterogeneous, while liver cirrhosis (Bruix *et al*, 2004), and infection by hepatitis viruses (Anzola, 2004) seems to be the major risk factors for its development. In addition, a variety of risk factors including tobacco, Aflatoxin B1 (AFB1), nonalcoholic fatty liver disease, diabetes, obesity, and hemochromatosis have been also associated with hepatocarcinogenesis (Wild and Hall, 2000) (Fig.1). These factors differ according to the geographical area; hence, while HBV chronic infection is prevalent in many Asian countries and Africa, HCV is dominant in Japan and the United States (El-Serag and Rudolph, 2007).

Although the precise histopathology and molecular features leading to HCC development and progression are still poorly understood, evidences suggest that, in order to repair liver damage caused by the previously mentioned factors, there is an increased cell turnover, and subsequent aberrant proliferation of preneoplastic hepatocytes with accumulated mutations (El-Serag and Rudolph, 2007, Farazi and DePinho, 2006). In recent years, significant attention has been drawn to study the tumor microenvironment, since a better understanding of the relationship between tumor cells and their microenvironment may be useful for the discovery of novel

molecular therapeutic targets. (Yang *et al*, 2011). It's widely accepted that, during the pre-neoplastic state, there are several interaction between the different tumor cell types in the stroma (such as fibroblasts, macrophages, leukocytes, endothelial cells, neutrophils, and dendritic cells) and components of the extracellular matrix, (collagen, fibronectin, laminin, or glycosaminoglycans) (Schrader and Iredale, 2011). Each of these cells produces growth factors, cytokines, free radicals, and other tumorigenic substrates that contribute to tumor initiation and progression (Wu *et al*, 2012).

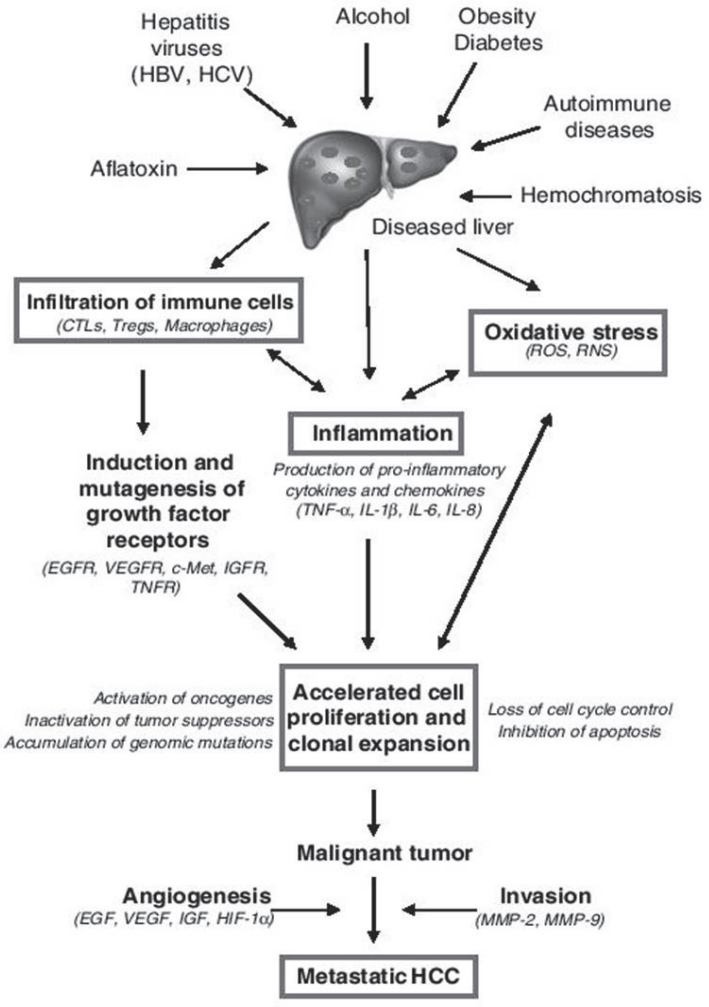

Figure 1. Risk factors and molecular mechanisms involved in hepatocarcinogenesis. (Aravalli et al, 2013).

Within the complex microenvironment where tumors start to develop, inflammatory reactions are considered highly important. Regarding to HCC, viruses, bacteria, alcohol and drugs metabolites cause liver damage and subsequent release

of pro-inflammatory signals like Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-23 (IL-23), Tumor necrosis factor alpha (TNF-α), and Transforming growth factor beta 1 (TGF-β1) (Hoshida, 2011). As a part of the immune response, tumor hepatocytes are known to express cell surface receptors for a number of cytokines and liver sinusoidal endothelial cells, both targets and a source of pro-inflammatory molecules (Leonardi *et al*, 2012). As previously mentioned, virus infection plays major role in HCC, and both chronic B and C hepatitis are recognized as risk factors for its development (Idilman *et al*, 1998). Several studies have demonstrated the existent correlation between chronic inflammatory status in liver, and hepatitis infection. Thus, chronic stimulation of antigen-specific immune response has been reported in HCV patients as a result of an immune system response to complete elimination of the viral infection (Budhu and Wang, 2006), been also associated with hepatocytes damages induction by a continued expression of cytokines and activated lymphocyte under anti-HCV antibodies production (Leonardi *et al*, 2012).

Not only HCC but cancers in general show a complex phenotype derived from the acquisition of multiple sequential genetic and epigenetic alterations. There are increasing evidences suggesting that carcinogenesis occurs through a multistep process resulting in the progression of normal cells to preneoplastic ones which derivate into invasive cancer cells (Boyd and Reade, 1988). This supposition is based in the fact that accumulation of a critical combination of alterations becomes sufficient to deregulate the normal homeostatic mechanisms, and develop cancer phenotypes. Cancer cells are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, aberrant angiogenesis, tissue invasion and metastasis, each of which are necessary for eventual cancer development (Boyd and Reade, 1988). Damaged liver tissue undergoes several mutations that cause genetic and epigenetic alterations, related with aberrant expression of cellular proteins, inhibition of tumor suppressors, oncogenes overexpression, and molecules that regulate these events including microRNAs and various cellular pathways (Aravalli *et al*, 2013).

1.3. Management of HCC

Presentation of HCC is considered to be non-specific; patients who develop HCC usually have no symptoms other than those related to their chronic liver diseases, and detection based on liver failure and/or physical deterioration reflects an advanced stage where cure is no longer an option (Kew *et al*, 1971). Therefore, surveillance of patients with cirrhosis and/or chronic hepatitis infection remains the best option to detect HCC at a curable stage (de Lope *et al*, 2012). Among the diagnostic techniques currently available, which include computed tomography (CT), magnetic resonance (MR), α-fetoprotein (AFP) quantification as serologic tumor marker and ultrasonography (US), the last one is the most recommended test for surveillance, based on its high sensitivity and specificity (Singal *et al*, 2009). Once the tumor is detected and having diagnostic confirmation of HCC, disease staging is needed in order to select the most accurate treatment regarding to improve life expectancy (de Lope *et al*, 2012).

Curative therapeutic strategies for HCC involve liver transplantation, surgical resection, and radio-frequency ablation (Bruix *et al*, 2011). Although liver transplantation may sound as the best option for HCC, only patients who have a single tumor ≤ 5 cm in diameter or 2-3 tumors each ≤ 3 cm in diameter, without macro vascular involvement, and extra hepatic disease are most likely to benefit from this procedure, providing 5-years survival rates of 70% and recurrence rate <10% of cases (Mazzaferro *et al*, 1996). Besides, the ongoing deficiency of liver donors remains as the principal limitation for transplant. When transplant is not an option, tumor resection becomes another curative approach for early HCC patients with similar criteria to those who are candidates for transplant; but unfortunately, this procedure has also high recurrence rates (up to 70% at 5 years and possibility of intrahepatic metastases within 2 years in 60-70% cases) (Imamura *et al*, 2003).

Due to the previously mentioned limitations of the surgery for HCC patients, at present, non-surgical treatments, including radiation therapy, interventional therapy, systemic and biological treatments are widely used in clinical practices (Cheng and Lv,

2013). Local tumor ablation therapies, achieved either by chemical substances (ethanol, acetic acid) or changes of the cells tumor temperature (radio frequency, laser, cryoablation or ultrasounds) are considered as minimal invasive procedures for HCC which have shown excellent efficacy in early HCC patients (Kudo, 2004). However, knowing that most of the HCCs develop from a cirrhotic liver which severely compromises hepatic functions, patients not only require tumor treatment, but also combined therapy to stabilize and prevent underlying liver damages (Huynh, 2010). To this respect, chemotherapy remains as a systemic approach for HCC treatment; nonetheless, meta-analysis studies to evaluate systemic and regional chemotherapies, hormonal therapy and immunotherapy in HCC patients demonstrated low efficiency and response rates (Llovet *et al*, 2003).

Considering the lack of survival benefits of the conventional drugs such as cisplatin, interferon-alpha2b (IFN-alpha2b), doxorubicin or 5-fluorouracil (5-FU) (Simonetti *et al*, 1997), new agents and novel therapeutic strategies are urgently needed. Understanding cancer at the genetic level had gained significant attention over the last years, and several genome-wide profiling studies has been published regarding to HCC pathogenesis, providing us with gene sets, to better understand tumor physiology. Although these studies have identified different signaling pathways and oncogenes which appear most frequently altered in HCC (Farazi and DePinho, 2006), it seems to be no a dominant pathway responsible for HCC development (Rossi *et al*, 2010).

HCCs are characterized as highly vascularized solid tumors with rapid growth, in which angiogenic pathways appear commonly hyper-activated. Receptor tyrosine kinases (RTKs), involved in tumoral processes like abnormal proliferation differentiation and apoptosis, have also a significant role in angiogenesis, reason by which are considered rational targets for innovative HCC therapies (Giannelli *et al*, 2007). In 2007, a phase III randomized controlled trial confirmed sorafenib, an inhibitor of tyrosine kinase receptors and vascular endothelial growth factor receptor (VEGFR), becoming the first systemic agent capable to increase survival time of patients with HCC (Simonetti *et al*, 1997). Sorafenib is now the standard of care for

patients with advanced-stage HCC and has paved the way for novel therapies. To this respect, many targeted agents such as monoclonal antibodies (e.g. bevacizumab and cetuximab), small molecule tyrosine kinase inhibitors and serine-threonine kinase inhibitors (temsirolimus and everolimus) have already been tested in several clinical trials and some of them have recently entered in phase III studies (Huynh, 2010).

2. The mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine-specific protein kinases, largely expressed in all cell types, and directly involved in signal transduction pathways (Figure 2). MAPKs pathways play important roles in cell modulating proliferation, gene expression, differentiation, mitosis, cell survival, or apoptosis in response to a variety of external stimuli such as mitogens, osmotic stress, heat shock or inflammation. Due to their roles in these processes, MAPKs have been found to play critical functions in embryonic development, tissue homeostasis and inflammation. Moreover, these pathways are often found to be deregulated in various types of human tumors, including HCC (Huang *et al*, 2010, Wagner and Nebreda, 2009).

MAPK signaling cascades are organized hierarchically into three-tiered modules as summarized in Fig 3. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKKs are in turn activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK module to cell surface receptors or external stimuli. Furthermore, MAPKs are dephosphorylated by a group of dual-specificity phosphatases (DUSPs), with phosphatase activity (Patterson *et al*, 2009). There are at least four subfamilies of MAPKs among which extracellular signal-regulated kinase 1 and 2 (ERK1/2), Jun N-terminal kinases (JNKs), and p38 have been better characterized (Pearson *et al*, 2001). In view of the common upstream kinases and phosphatases, as well as the shared downstream targets of these MAPK proteins, is not surprising the existence of multiple cross-talks between these subfamilies pathways under several circumstances (Min *et al*, 2011).

2.1 ERK

ERK1 and ERK2 are serine/threonine kinases which share high homology in sequences and regulation. Both are mainly activated by tyrosine kinases receptors like the epidermal growth factor receptor (EGFR), and Ras and GTPases proteins, leading to a phosphorylation cascade where MEK1 and MEK2 induce ERK1 and ERK2 activation to modulate the expression of more than 160 known nuclear and cytosolic substrates implicated in several cellular processes (Pearson *et al*, 2001). Despite of their common features, a number of studies with deficient cells, mice or zebrafishes confirm the distinct roles of these two kinases, regarding to functional aspects, and relating them with different processes like cell survival, proliferation or migration (Fischer *et al*, 2005, Krens *et al*, 2008, Mazzucchelli *et al*, 2002).

Due to the wide variety of cellular events in which ERK participates, and considering that some of them appear frequently altered in cancer development, it is not surprising their reported importance in carcinogenesis (Lee *et al*, 2006). As previously mentioned, HBV and HCV are major risk factors for human liver diseases including chronic hepatitis, cirrhosis, and HCC, and activation of the ERK pathway seems to be important in HBV- and HCV-infection-associated pathogenesis and liver tumorigenesis (Chin *et al*, 2007). Besides, phosphorylated ERK levels have been found significantly increased in human HCC samples and alterations of this pathway are well documented in human HCCs samples (Lee *et al*, 2006). Nonetheless, the analysis on liver tumorigenesis has not been reported in mice with conditional ERK1 or ERK2 alleles, which may be critical to understand the oncogenic functions of ERK *in vivo* (Min *et al*, 2011). Despite of the multiple studies reporting the role of ERK in the proliferation and survival, there is still some controversy concerning to its precise role in tumorigenesis. Curiously, ERK1/2 activation seems to be responsible of p53 phosphorylation and subsequent Bcl-2 mediated apoptosis observed in doxorubicin treatment (Bien *et al*, 2007, Chua *et al*, 2006). In addition, phosphor-ERK is required for cisplatin induced growth arrest and apoptosis in mouse H22 hepatoma cells (Qin *et al*, 2010), and paclitaxel, another mitotic inhibitor commonly used in cancer

chemotherapy, also depends on ERK activation to promote G2/M cell cycle arrest and cell death in human MCF7 breast carcinoma cells (Bacus *et al*, 2001).

2.2 JNK

Three different genes (MAPK8, MAPK8, MAPK8) encode for c-Jun NH2-terminal kinases or JNK, which can result in 10 different isoforms by alternative splicing, being JNK1 and JNK2 the best characterized (Gupta *et al*, 1996). Genetic studies, using newborn mice lacking JNK1 and/or JNK2, suggest that both kinases have complementary and essential functions during embryonic development (Sabapathy *et al*, 1999). Similarly to other MAPKs, there are several external stimuli able to activate the JNK pathway via dual-specificity protein kinases (MKK4 and MKK7) or inactivate it by dephosphorylation via DUSP proteins, suggesting their involvement on a number of cellular processes (Weston and Davis, 2007). This pathway has been shown to participate in cellular events closely associated with carcinogenesis, like apoptosis, proliferation and cell cycle progression (Weston and Davis, 2007). Activation of JNK pathway has been reported in human HCC samples and may initiate liver tumor development in mouse models (Xu *et al*, 2009), indicating that JNK cascade could play a role in tumorigenesis (Wu *et al*, 2010). However, there is certain controversy since some studies show opposite conclusions, remarking its role in induced apoptosis via Bcl-2 family proteins and mitochondrial pathway, and suggesting the existence of a crosstalk between JNK and the NF-κB pathway in JNK-mediated cell death (Ventura *et al*, 2004). Thus, similarly to the other MAPKs, JNK pathway cannot be restrictively defined as tumorigenic inductor or restrictor itself; must be carefully considered based on the great number of processes where its involved and additional characterization with *in vivo* models is required to clarify the discrepancy.

2.3 p38

The p38 subfamily consists of four different isoforms: p38α, p38β, p38γ and p38δ which mainly differ in their expression pattern along the body tissues. However, since most of the published studies on p38 MAPKs refer to p38α, from now on we will

focus on this one. p38 is activated by upstream MKK3, MKK4, and MKK6 via phosphorylation, although this auto phosphorylation has also been reported (Cuenda and Rousseau, 2007). Firstly identified for its role in regulating pro-inflammatory cytokines (IL-1, IL-6 and TNFα) production (Huang *et al*, 2010), these MAPKs are also known to be essential inhibitors of cell proliferation, though various molecular mechanisms largely dependent on the cell type (Keshet and Seger, 2010). The suppressive role of p38 in proliferation appears to be at least in part, mediated by its control on cell cycle transition, thus greater proliferation and regeneration ratios were observed in old mice carrying p38 dominant-negative allele with mutated activation sites (Wong *et al*, 2009). Besides, its functions on cell cycle have been closely linked with differentiation; p38α activation seems to be essential to restrain proliferation and to maintain the differentiation of lung progenitor cells (Ventura *et al*, 2007). Together these data point to p38, as a major regulator of cell cycle, proliferation and differentiation. As the other MAPKs, there are also studies relating p38 with apoptosis. Thus, has been reported that ROS enhances p38 activity inducing apoptosis, and mitochondrial ROS generated from hypoxia contributes to hypoxia-induced cell death (Kim *et al*, 2006).

Regarding to the role of p38 in cancer development, direct evidence using mice models, suggest that these kinase exert a negative regulation of tumorigenesis (Ventura *et al*, 2007). A number of studies indicate that p38 participates in several mechanisms during liver tumorigenesis (Min *et al*, 2011). In this way, a recent study described a new mechanism whereby p38 donwregulates cell proliferation by antagonizing the JNK-c-Jun pathway in several cell types and in liver cancer development (Hui *et al*, 2007). Furthermore, p38 it has been related to HBV- and HCV-associated hepatitis; thus, during the HBV infection, HBx viral protein contributes to p38 activation, critical for p53-dependent apoptosis (Mankouri *et al*, 2009). Additionally, HCV viral proteins induce p38 activation leading to pro-inflammatory chemokine expression and deregulation of the mitotic checkpoint during virus infection-associated hepatitis, which largely accounts for the initiation stage of hepatocarcinogenesis (Spaziani *et al*, 2006).

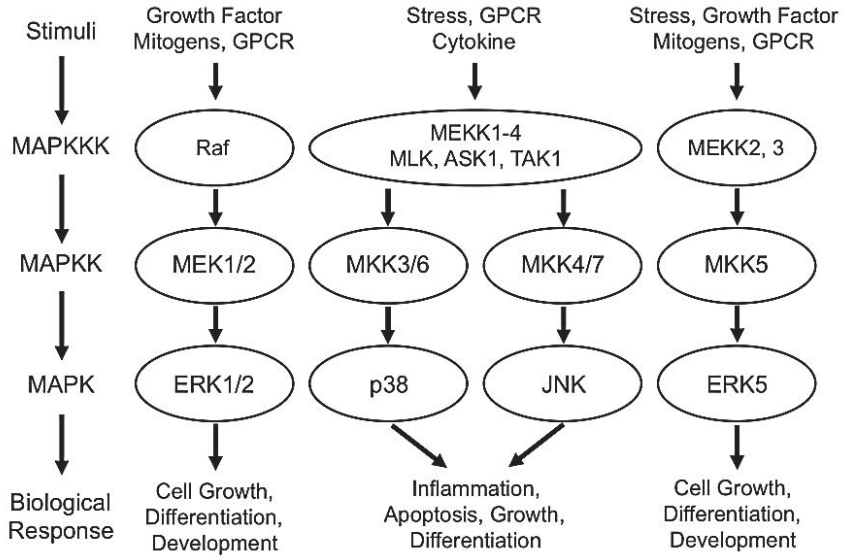

Figure 2. ***The MAPK modules***. MAPK are activated through a phosphorylation cascade or MAPK modules in which the MAPK is activated by a dual-specificity MAPK kinase (MAP2K), itself activated by a MAPK kinase kinase (MAP3K). Receptor activation of a module may involve the participation of small GTPases, such as Ras and Rac. The MAPK phosphorylate many substrates, including transcription factors (Huang *et al*, 2010).

3. Apoptosis

Apoptosis or programmed cell-death is a multi-step pathway firstly described by Kerr and colleagues in 1972 (Kerr *et al*, 1972). In last years, apoptosis has become a subject of increasing attention, in fact the 2002 Nobel Prize in Physiology or Medicine was awarded to Sydney Brenner, H. Robert Horvitz and John E. Sulston for their discoveries concerning to the genetic regulation of organ development and programmed cell death (Jaryal, 2003).

This controlled process takes place both under physiological and pathological situations, been inherent in every cell of the body. Since it does not typically induce adjacent cell death, inflammation or tissue damage, there are several growth-control mechanisms that coordinate cell proliferation and tissue homeostasis associated to apoptosis during development and aging. Although a number of conditions are able to trigger apoptosis, the response can be modulated depending on type and level of stimuli conducing to cell die by apoptosis or necrosis. This highly coordinated process

involves an energy dependent activation of caspases, a group of cysteine proteases that link the starting stimuli to the final cell death (Elmore, 2007).

In mammals, there are two different pathways regarding to the apoptosis initiation, briefly described in Figure 3. Although both pathways lead to caspase activation culminating in cell death, we can distinguish between an extrinsic or cytoplasmic pathway; and an intrinsic or mitochondrial pathway. The first one involves trans-membrane receptors from the tumor necrosis factor family (TNF) with cysteine-rich extracellular domains. After binding their specific ligand, external cell death signals induce pro-caspase 8 proteolytic cleavage and subsequent activation (Utz and Anderson, 2000). To date, the best-characterized ligands and corresponding receptors include FasL/FasR, TNF-a/TNFR-1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Elmore, 2007). Unlike this pathway, the intrinsic pathway can be initiated by non-receptor-mediated stimuli like chemotherapy, irradiation growth-factor depletion among others, leading to cytochrome-c release from the mitochondria into cytosol, where it binds to apoptotic protease factor 1 (APAF-1) activating essentially caspase-9, and subsequent death signals (Pei *et al*, 2003). Pro-apoptotic Bcl-2 family proteins like Bax, Bid, Bad and Bim are important mediators of these signals (Hanada *et al*, 1995).

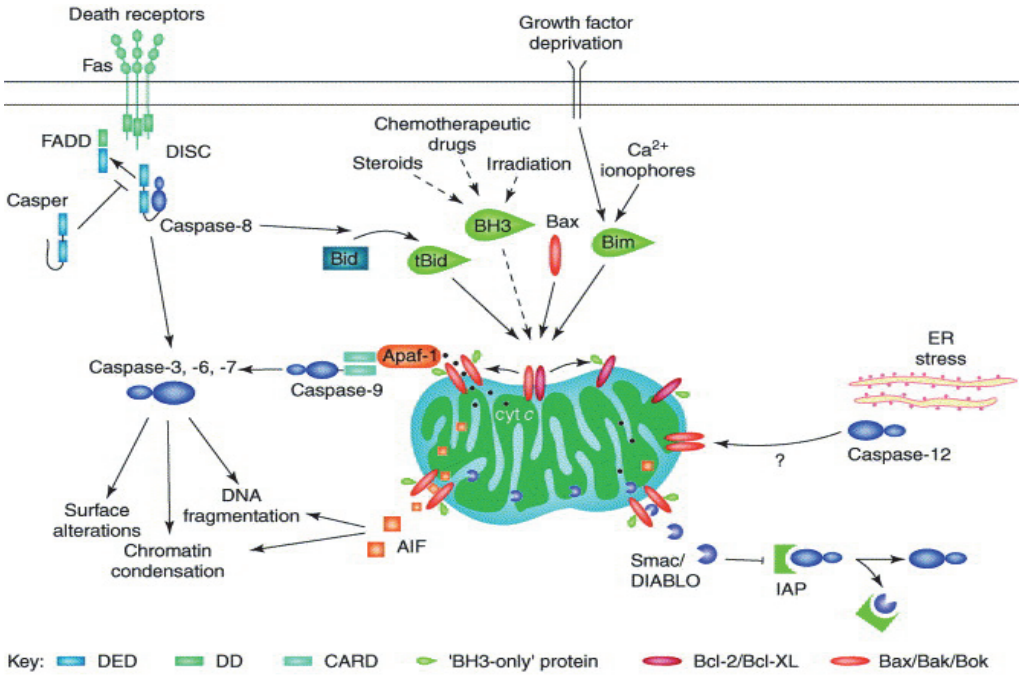

Figure 3. Overview of the apoptotic pathway in mammalian cells. Once death receptors are activated, initiate caspase-8 auto catalytically triggering and subsequent caspases-3, -6 and -7 activation. These cleave select intracellular targets leading to apoptosis. Moreover, other apoptotic stimuli induce mitochondrial dysfunction and intrinsic apoptosis initiation. BH3-only proteins subsequently translocate to mitochondrial surfaces, where they associate with multidomain Bcl-2-related proteins. Anti-apoptotic Bcl-2 family members (e.g. Bcl-2, Bcl-XL) are inactivated, while pro-apoptotic members (e.g. Bax, Bak) are activated. Release of cytochrome c induces the apoptosome formation in association with Apaf1 and caspase 9. AIF induces other hallmarks of apoptosis independent of caspases. Smac/DIABLO inactivates 'inhibitor of apoptosis proteins' (IAPs), thereby activating caspases (Joza *et al*, 2002).

Once the apoptotic program starts, apoptotic cells exhibit several biochemical modifications which include protein cross-linking and cleavage, DNA breakdown, cytoplasmic shrinkage, formation of cytoplasmic blebs, apoptotic bodies and exposure of cell surface markers to induce phagocytic recognition by the neighboring cells (Conradt, 2009). Most of these cellular alterations are mediated trough an energy dependent cascade of caspases. These family members can be mainly divided into apoptosis initiators (caspase-2, 8, 9 and 10) and executioners (caspase-3, 6 and 7). Once activated, the executioner's mission is to cleave mandatory proteins for the maintenance of homeostasis, leading to the collapse and demise of the cell (Hanada *et al*, 1995).

While the apoptotic machinery is tightly controlled by several proteins (Hanada *et al*, 1995), the previously mentioned Bcl-2 family members, coordinates apoptosis at the mitochondrial level, and deserves an especial attention. Proteins from this family have been found mainly located in mitochondrial, endoplasmic reticulum and nuclear membrane, and as summarized in Figure 4, can be divided into three groups based on functional and structural criteria. Group I composed mostly by anti-apoptotic members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/Bfl1, Boo/Diva, Nrf3, and Bcl-B), and two groups of pro-apoptotic proteins; groups II (Bax, Bak, and Bok/Mtd) and III, the most heterogeneous one that includes Bid, Bad, Bik, Bim, Blk, Bmf, Hrk, Bnip3, Nix, Noxa, PUMA, and Bcl-G (Zhang *et al*, 2005).

Through their BH3 domain, these Bcl-2 proteins can either interact with anti-apoptotic proteins inhibiting their function and/or act together with pro-apoptotic multidomain proteins stimulating their activity. Pro-apoptotic proteins are considered the master effectors of apoptosis; responsible of the mitochondrial outer membrane permeabilization protein (MOMP), a 'point of no return' event that leads to cytochrome c diffusion and subsequent activation of the initiator and executioner caspases (Elmore, 2007).

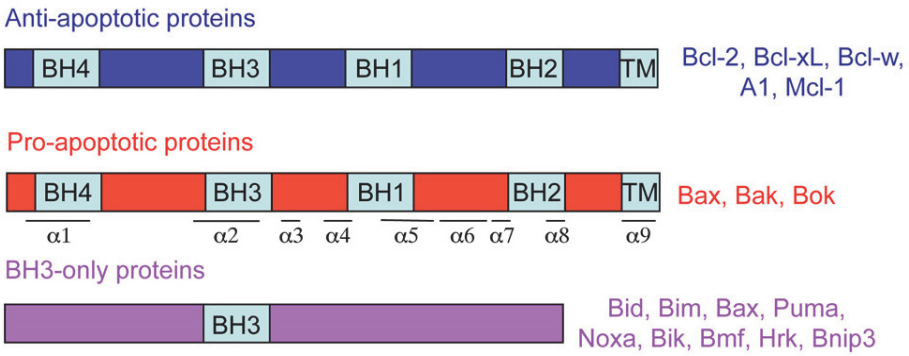

Figure 4. Bcl-2 family members and related proteins in apoptosis. Bcl-2 family plays a crucial role in apoptosis through their ability to regulate mitochondrial cytochrome c release. There are three subfamilies with BCL-2 homology (BH) domains. Most members of this subfamily also contain transmembrane domains (TM) to associate with membranes. The pro-apoptotic BAX-like subfamily lacks BH4 domains and promotes apoptosis by forming pores in mitochondrial outer membranes (Taylor *et al*, 2008).

3.1 Apoptosis in health and disease

As a major component of both health and disease, apoptosis has a critical role in maintenance of body homeostasis. For example, it has been estimated that, around 10 billion cells appear each day just to balance those dying by apoptosis (Renehan *et al*, 2001). Increased apoptosis is needed under certain physiological situations such as removal of inflammatory cells or evolution of granulation tissue into scar during wound healing (Greenhalgh, 1998); and is also required to abolish auto-aggressive immune cells during maturation in the central lymphoid organs (bone marrow and thymus) or in peripheral tissues (Osborne, 1996). Apoptosis deregulation has been associated with different pathologies, including developmental defects, autoimmune diseases, neurodegeneration, or cancer (Elmore, 2007).

Cancer is a clear example of deregulated cellular homeostasis, and aberrant apoptosis is thought to play a central role in the development and progression of a number of tumors (Kerr *et al*, 1994). While the precise mechanism whereby tumor avoid apoptosis remains unclear, it is well accepted that neoplastic cells show overexpression of anti-apoptotic proteins (like Bcl-2) while the pro-apoptotic ones (such as Bax) are down-regulated; in addition they seems to be able to synthetize nonfunctioning death receptor, and to secrete high levels of a soluble receptors to sequester the Fas ligand, and prevent apoptosis to occur (Elmore, 2007).

The development and progression of hepatic tumors, like HCC and biliary tumors has been associated with insufficient apoptosis. Thus, evidences suggest an aberrant apoptosis during hepatocarcinogenesis, especially in early stages and during tumor promotion (Guicciardi and Gores, 2005). Furthermore, several *in vitro* and *in vivo* studies analyze the contribution of apoptotic related pathways blockage with HCC development and progression (Mauriz and Gonzalez-Gallego, 2008). At a molecular level, Fas expression has been found to inversely correlate with tumor differentiation (Strand *et al*, 1996), and while its reduction is not sufficient to escape from apoptosis, some chemotherapy compounds (etoposide, camptothecin, norcantharidina, etc.) are capable to overexpress Fas, increasing apoptotic sensitivity (Guicciardi and Gores, 2005). Abnormal expression of apoptosis inhibitors like

survivine, directly correlated with tumor recurrence and a poor prognosis (Ye *et al*, 2007); furthermore, *in vitro* studies with antisense oligonucleotides against this protein have shown reduced cell proliferation rates (Sun *et al*, 2006).

Although significant progresses have been made to define and characterize the apoptotic death program, especially regarding to caspases, Bcl-2 and IAPs proteins, there are still several questions unsolved; whereby and a better understanding of the mechanisms controlling and executing apoptosis is an attractive challenger for the scientific community. Moreover, from a therapeutic point of view, this knowledge would permit the design and use of synthetic molecules that may act as tumor cells sensitizers, allowing more proficient treatments.

4. Forkhead transcription factors

FoxO proteins integrate extracellular signals from a wide variety of upstream signaling pathways, acting as protein transcriptional activators of genes involved in cellular processes such as proliferation, transformation, differentiation and longevity. Since 1989, when Weigel and Jäckle first discovered a fkh gen functioning as transcriptional regulator in *Drosophila melanogaster* (Weigel *et al*, 1989, Weigel and Jackle, 1990), more than 100 different forkhead genes have been identified in several species from yeast to humans. The current adopted nomenclature for this Fox family refers to Forkhead box proteins, characterized by 80-100 conserved amino-acids conforming a winged helix domain with a FoxO consensus binding sequence GTAAACA, which recognizes conserved DNA sequences functioning as transcription factors (Furuyama *et al*, 2000). The main mammalian Fox members FoxO1/FKHR, FoxO3/FKHRL1FoxO1, FoxO4/AFX and FoxO6 are, widely expressed throughout the body tissues, with different expression patterns depending on their functions (Yaklichkin *et al*, 2007). FoxO1 mainly controls body energy and metabolism, and therefore is highly expressed in insulin-associated tissues (adipose tissue, muscle, liver or pancreas) (Gross *et al*, 2009). FoxO3 and FoxO4 are significantly related with stress stimuli, cell cycle arrest and apoptosis (Furukawa-Hibi *et al*, 2005, Kops *et al*, 2002). FoxO6 recently identified as a novel member of the FoxO group, is predominantly expressed in the adult brain, and unlike the other FoxO members,

shows a high nuclear localization, and additional mechanisms besides phosphorylation seem to be involved in its regulation (Jacobs *et al*, 2003).

4.1 FoxO regulation

FoxO transcription factors are able to integrate cellular signals emanating from insulin, growth factors, cytokines, and oxidative stress, via numerous post-translational modifications (PTMs), to guarantee the transcription of specific target genes in response to environmental factors. These modifications summarized in Figure 5, include phosphorylation, acetylation, ubiquitination, methylation and glycosylation, catalyzed by enzymes by different mechanisms that finally allow FoxO cellular activities by changing their subcellular location, molecular half-life or DNA-binding activity (Zhao *et al*, 2011).

AKT/protein kinase B (PKB), and the serum- and glucocorticoid-inducible kinases (SGKs), are two of the best known serine-threonine kinases involved in FoxO phosphorylation. Activation of those proteins by the phosphoinositide 3-kinase (PI3K) pathway implies FoxO cytoplasmic retention and consequent inhibition of its nuclear translocation (Brunet *et al*, 1999, Pearce and Kleyman, 2007). When expressed at physiological levels, both AKT and SGK phosphorylate FoxO members at Thr32, but while SGK prefers Ser315, AKT shows a marked preference for phosphorylating Ser253 (Brunet *et al*, 2002). Moreover, phosphorylation at the Thr28 and Ser193 sites induce FoxOs structural changes, allowing association with the chaperone protein 14-3-3, which enhances FoxO nuclear export and decreases FoxO nuclear entry (Brunet *et al*, 2002). FoxO1 phosphorylation at Ser256 changes the positive to negative charges in the DNA-binding domain, modifying its binding activity (Zhang *et al*, 2002). Thus, AKT/PKB activation by insulin or by growth factors play a critical role in the inhibition of FoxO transcriptional activity, not only by inducing FoxO retention in the cytoplasm but also through its degradation, leading to decreased DNA-binding activity (Huang *et al*, 2005, Plas and Thompson, 2003).

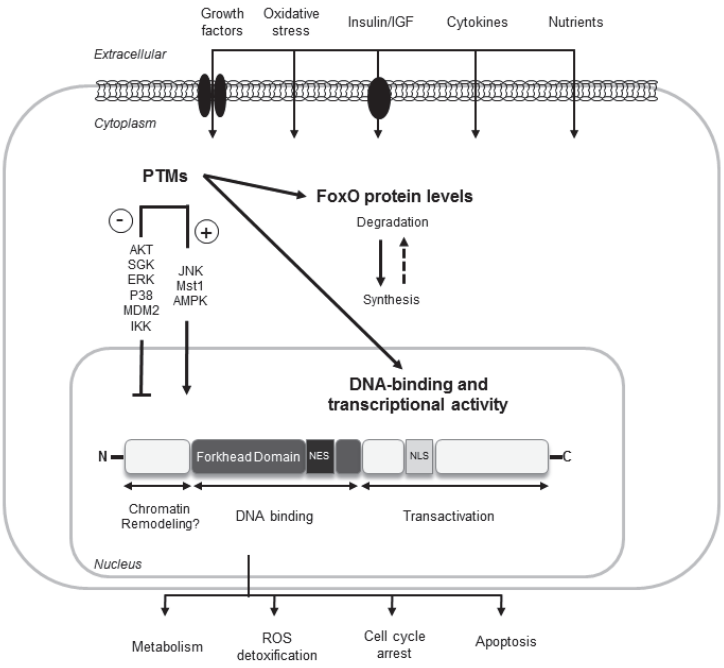

Figure 5. FoxO transcription factors regulation. The FoxO family of Forkhead transcription factors response to environmental stimuli, (insulin, growth factors, nutrients and oxidative stress) to regulate the expression of target genes involved critical cellular processes. The precise regulation of FoxOs is enacted by an intricate combination of post-translational modifications (PTMs), which alter their levels, subcellular localization, DNA binding and transcriptional activity. NLS, nuclear localization signal; NES, nuclear export sequence.(-),FoxO inhibition; (+), FoxO activation.

Under oxidative stress conditions, FoxO proteins are also phosphorylated by other different protein kinases, including JNK or Mst1, which induce the opposite AKT's action, causing FoxO translocation from the cytoplasm to the nucleus (Essers *et al*, 2004, Lehtinen *et al*, 2006). Furthermore, FoxO modulation has been related to other mitogen-activated protein kinases (MAPKs) such as extracellular signal regulated kinase (ERK), and p38. The RAS–ERK pathway is a direct down regulator of FoxO3a via a murine double minute 2 (MDM2) ubiquitin-proteasome pathway (Yang *et al*, 2008). The central NF-κB regulator, IkappaB kinase (IKK), inhibits FoxO3a independently of PI3K/AKT or ERK pathways, causing proteolysis of FoxO3a via ubiquitination and proteasomal degradation (Hu *et al*, 2004). Additionally, AMP-activated protein kinase (AMPK) directly phosphorylates human FoxO3 at six different

residues (Thr179, Ser399, Ser413, Ser439, Ser555, and Ser588) from those phosphorylated by other protein kinases (Calnan and Brunet, 2008). Mostly present in the C-terminal, its phosphorylation can impair FoxO3-dependent transcription and expression of a subset of target genes involved in oxidative stress and energy metabolism, without affecting FoxO3 localization (Oellerich and Potente, 2012).

FoxO proteins can be also post-translationally modified by acetylation/deacetylation to regulate their subcellular location, DNA-binding activity, stability and interaction with other proteins. The precise mechanism implicated on FoxO modulation by acetylation remains unclear; inhibition through acetylation of FoxO transcription factors seems to be functionally equivalent to the inhibition by kinases previously described, preventing their transcriptional and biological activities. In addition, ubiquitination processes (by proteins like Skp2, MDM2 or COP1, mainly from the E3 ligases family) constitute a critical step in FoxO degradation through the proteasome pathway (Huang *et al*, 2005, Matsuzaki *et al*, 2003, Yang *et al*, 2008). Finally, methylation and glycosylation of FoxO proteins are important events of FoxO regulation. Protein arginine methyltransferase (PRMT1) methylate FoxO at several arginine residues, blocking AKT-mediated phosphorylation. *O*-glycoxylations performed by *O*-GlcNAc transferase (OGT), quickly cycle on serine and threonine FoxO residues in an analogous manner to phosphorylation (Hart *et al*, 2007)

Based on the multiple FoxO regulation sites, as well as the several pathways involved and its complex intercross, the whole understanding of FoxOs regulation remains as an attractive challenge for future investigations.

4.2 FoxO in cancer

Due to their relevant role in cell cycle regulation, differentiation and apoptosis, FoxO proteins are considered as proper tumor suppressors, and the development of novel agents which specifically activate FoxO members, seems to be a promising approach for cancer treatment. Furthermore, cancer has a remarkable age-dependent onset, and FoxO factors may serve as molecular link between longevity and tumor development (Calnan and Brunet, 2008). FoxO factors deregulations as well as its chromosomal deletion have been found in leukemia and

in other tumor types (like prostate, breast cancer, and glioblastoma) (Arden, 2006). Increased FoxO3a nuclear levels inversely correlate with tumor size and growth inhibition in an orthotropic breast cancer mouse model, while silencing FoxO3a with FoxO3a-siRNA appears to be highly associated with tumourigenesis (Hu *et al*, 2004). Furthermore, direct interaction between FoxO and the major tumor suppressors p53, has also been reported, suggesting that while FoxO3 is not strictly necessary for p53-dependent cell cycle arrest, p53-related apoptosis its modulated by FoxO3 (Renault *et al*, 2011). Concerning to apoptosis as a remarkable cell process commonly altered in tumors, the pro-apoptotic Bcl-2 family member Bim, as well as Fas ligand are two of the best known pro-apoptotic genes containing FoxO responding elements (FRE) within their promoters (Hagenbuchner *et al*, 2012). Besides, FoxO family members have been shown to promote cell cycle arrest in different cell types via up regulation of the cyclin-dependent kinase inhibitor p27KIP1 and/or down-regulation of cyclin D (Huang *et al*, 2005). Some research has found a transcriptional activation of Fas ligand promoter by FoxO3a, whereas several other studies have shown cell cycle arrest and p27 activation by FoxO1a and FoxO4 in fibroblasts cells (Brunet *et al*, 1999, Dijkers *et al*, 2000). Activation of each member of this family in transformed and non-transformed cells results in up regulation of the cyclin-dependent kinase inhibitor p27KIP1 and/or down-regulation of D-type cyclins, thereby arresting cells at G1 (Huang *et al*, 2005).

Regarding to its role in HCC, significant increases in posttranscriptional degradation of FoxO1 and other cell cycle regulators (p21, p27, etc.) have been found during liver cancer development. Moreover, epigenetic mechanisms may induce FoxO1 silencing by aberrant methylation in 33% of HCC patients with poor prognosis (<3 years survival) versus 20% en patients with better prognosis (>3 years survival). Furthermore, highest values indicating FoxO1 destruction by ubiquitination were found in patients with poor diagnosis (Calvisi *et al*, 2009). *In vitro* experiments using HepG2 tumor hepatocytes have shown that FoxO4 is inactivated by p300/CBP acetylation, mitigating its transcriptional activity (Fukuoka *et al*, 2003). Moreover, experiments using other human tumor hepatocyte line, HuH7, demonstrated that saturated free fatty acids can induce cell death by replication of lipoapoptosis (a

critical feature of nonalcoholic steatohepatitis) via overexpression of the pro-apoptotic Bim protein mediated by FoxO3a activation (Barreyro *et al*, 2007).

4.3 FoxO, a good candidate for cancer treatment

Based on the anti-tumor properties of FoxO members, efforts to enhance its controlled transcriptional activation should be considered as a powerful tool for cancer therapy. Since, it has been widely accepted that both FoxO proteins and cell dead are mainly down-regulated by the pro-survival PI3K/AKT pathway; the use of specific inhibitors might be an attractive alternative. Some research groups have obtained worthy results when using small molecules to block the AKT pathway (such as LY294002 or wortmannin) (Cheng and White, 2011, Uddin *et al*, 2006). Chemical inhibitors and/or activators have strongly contributed to a better understanding of the FoxO pathway, with an ongoing effort to promote the clinical use of these substances. Based on this principle, several drugs currently employed in cancer treatment like paclitaxel, doxorubicin and imatinib have been shown to exert its anti-tumoral proprieties trough FoxO pathway (Boreddy *et al*, 2011a, Essafi *et al*, 2005, Sunters *et al*, 2003, van Gorp *et al*, 2006) Besides, the use of siRNA has become a powerful weapon in different experimental works, showing promising results in many *in vitro* studies and a few clinical trials (Chen and Zhaori, 2011). These new drugs should be able to block one or different molecular pathways involved in FoxO cytoplasmic location or decrease its acetylation status. Together these data suggest that drugs designed to increase or restore FoxOs activity may constitute a potential strategy in combination with other chemo- or radiotherapy treatments.

5. Angiogenesis

Angiogenesis is a physiological process which consists in the new blood vessels formation from pre-existing ones. This event differs from vasculogenesis, which imply *de novo* formation of endothelial cells from mesoderm cell precursors. Angiogenesis has a critical role in growth and development, as well as in wound healing and in the formation of granulation tissue, however, like happened with apoptosis, imbalances within this process contribute to several malignant, inflammatory, ischemic,

infectious and immune disorders, and has been also found to be a vital step in tumor malignance and spread (Carmeliet, 2005).

Since the early 1970s, when Judah Folkman first point out the importance of the high density of permeable blood vessels observed in most of solid tumors (Folkman, 1971), researches in the field of tumor angiogenesis have become critical for cancer management. Initially, tumors remain inactive in an avascular phase, where active proliferation and aberrant apoptosis are responsible for the tumor growth. It has been proposed that in this phase, tumors expansion is limited by an insufficient vascular supply. Therefore, Folkman hypothesized that within tumors, nutrients and waste products diffusion rate, limited its growth to a maximum diameter of 1-2 mm^3; and beyond this size, the development of new vessels is absolutely required for tumor enlargement. Accordingly, he postulated that if new blood vessels were indeed indispensable for tumor growth, then inhibiting angiogenesis should prevent tumor expansion, causing tumor mass regression to an avascular 1-2 mm^3 size (Folkman, 1972).

Nowadays, angiogenesis is a basic component for cancer progression, being widely accepted that tumor growth and metastasis depend on angiogenesis and lymphangiogenesis, initiated by chemical signals from tumor cells, for a phase of rapid growth. Based on the Folkman´s postulates and considering the complexity and the high variety of existing tumors, it is reasonable to believe that targeting the tumor vasculature may be a more effective therapeutic strategy than targeting the tumor itself. Unlike chemotherapeutics treatments effectively developed to inhibit the gene products aberrantly expressed at a particular stage of a specific cancer depending on the tumor type, anti angiogenic drugs have activated endothelial cells as unique targets, which clearly improves its specificity and selectivity. Besides, drug resistance may not be an issue for pharmaceuticals that target endothelial cells, because they are genetically stable. Moreover, activated endothelial cells express certain receptors, and adhesion molecules which make them different from not quiescent endothelial or other cells, which may also avoid adverse side effects. In addition, blocking tumor angiogenesis is a way of prevent metastasis (O'Byrne and Steward, 2001). In order to

designee the most effective tumor therapy, anti-angiogenesis drugs should be effectively combined with therapy directly aimed at killing tumor cells. Thus, successful preclinical data, using antiangiogenic agents, alone or in combination with conventional therapies, are now in clinical trials (Feldman and Libutti, 2000).

Angiogenesis itself can be summarized in a few basic steps, briefly: blood vessels suffer vasodilation and vascular permeability increases which permits extravasation of plasma proteins and subsequent formation of the scaffolding matrix network required for endothelial cells migration. This process is negatively regulated by inhibitors of vascular permeability, preventing excessive vascular leakage and circulatory collapse. Once the vessels are destabilized, and endothelial cells lose their cell contacts, been able to detach from supporting smooth muscle cells. As a consequence, endothelial cells migrate and proliferate to distant sites, responding to angiogenic chemotactic factors. Finally, pericytes (a type of contractile cells that wrap around the endothelial cells of capillaries) are attracted to produce the new basal lamina forming blood vessels (Liao and Johnson, 2007). The progression from a non-angiogenic to an angiogenic phenotype has been termed “angiogenic switch” and is mainly controlled by the balance between angiogenic activators and inhibitors (Folkman *et al*, 1989). Although exists many factors with an occasional role in promoting tumor angiogenesis, major attention has focused on members of the VEGF (vascular endothelial growth factor) and FGF (fibroblast growth factor) families as the most common tumor angiogenic ones (Dvorak *et al*, 1999, Fernig and Gallagher, 1994). VEGF is the best investigated angiogenic factor in HCC, and since the studies performed within the present doctoral thesis refer mainly to this factor, we will focus on this factor and its relation with cancer and angiogenesis.

5.1 VEGF and angiogenesis in HCC

Been one of the most vascular solid tumors, the rapid growth pattern of HCC, require a considerable amount of nutrient and oxygen from the circulation for tumor cell survival and expansion, and so angiogenesis plays an important role in HCC development, progression, and metastasis. Hence, arterialization and sinusoidal capillarization are the most frequent vascular changes associated with the

progression of dysplastic nodules to HCC development (Poon *et al*, 2003a). Accordingly, the expression of microvessel density markers (MVD) has been shown to correlates with poor prognosis in patients (Poon *et al*, 2002).

The general terms, 'VEGF' covers a number of proteins that result from the alternative splicing of the mRNA from the *VEGF* gen, thus, in humans 7 different isoforms ($VEGF_{121}$, $VEGF_{121}b$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165}b$, $VEGF_{189}$ and $VEGF_{206}$) have been characterized. VEGF is a growth factor with potent mitogenic activity, it feature was firstly described for endothelial cells derived from arteries, veins, and lymphatic vessels, and so it was named “Vascular Endothelial Growth Factor”, however, it also exerts an appreciable mitogenic activity for other cell types (Ferrara and Davis-Smyth, 1997). These members share overlapping capacities to interact with cell-surface receptor tyrosine kinases and regulate endothelial cell functions. At the moment, two homologous receptors for VEGF have been described: VEGF receptor-1 (VEGFR-1/Flt-1) and VEGF receptor-2 (VEGFR-2/Flk-1); both of which have critical roles during blood vessel formation (Liao and Johnson, 2007). VEGF induces the expression of several proteins intimately linked with angiogenesis, cellular invasion and tissue remodeling like metalloproteinases and collagenases which increase micro vascular permeability, inducing plasma protein leakage and subsequent vessels formation. Moreover, VEGF has been showed to stimulate the rate of hexose transport, which may have relevance for increased energy demands during endothelial cell proliferation or inflammation (Pekala *et al*, 1990). Besides, VEGF has regulatory effects on certain blood cells, promoting monocyte chemotaxis, inducing colony formation of mature granulocyte-macrophage progenitor cells, and having an inhibitory effect on the maturation of host professional antigen-presenting cells such as dendritic cells, which support the hypothesis that VEGF may allow the tumor to evade the induction of an immune response, and thus, facilitate its growth (Gabrilovich *et al*, 1996). Molecular studies by *in situ* hybridization have demonstrated that VEGF mRNA is markedly up-regulated in the majority of human tumors analyzed (liver, lung, thyroid, breast, gastrointestinal tract, kidney and bladder, ovary, angiosarcoma, among others) (Ferrara and Davis-Smyth, 1997).

VEGF expression has been showed to increases gradually from low-grade dysplastic nodules to high-grade and, small HCCs show a higher status of neoangiogenesis and proliferation activity than advanced HCCs while the highest VEGF levels appear in metastatic HCC patients. Accordingly, these levels correlate with micro vascular density and with elevated expression of CD34, a marker of sinusoidal capillarization (Park *et al*, 2000). Serum VEGF level in patients with HCC significantly correlates with mRNA and protein expression from tumor cells, which confirm basis for using circulating VEGF as a prognostic marker (Poon *et al*, 2003b, Semela and Dufour, 2004).

There is substantial evidence in pre-clinical studies suggesting that antiangiogenic therapy using different approaches inhibits the growth of HCC. Thus, tyrosine kinase inhibitors targeting VEGF receptors, suppress HCC tumor cell growth, both *in vitro* and *in vivo*, and effectively prevent revascularization in tumor tissues after hepatic artery ligation (Liu *et al*, 2005). Moreover, the use of drugs that directly inhibit the proliferation of endothelial cells could constitute another interesting approach on antiangiogenic therapy. TNP-470, a fumagillin analogue that can inhibit endothelial cell proliferation, has been shown to suppress the growth and metastasis of human HCC xenotransplant in an animal model Inhibitory effect of the angiogenesis inhibitor (Xia *et al*, 1997). Researches have also assayed agents that can prevent the degradation of extracellular matrix and basement membrane, a step essential for angiogenesis, for antiangiogenic treatment of HCC (Qin *et al*, 1999). Gene therapy based on delivery natural antiangiogenic factors, such as angiostatin and endostatin, can also achieve antiangiogenic effects in HCC xenograft in nude mice (Ishikawa *et al*, 2003). Taking together these studies and the pre-clinical data available, suggest that it is reasonable to conduct a clinical trial to test whether combined anti-VEGF therapy can enhance the efficacy HCC treatment.

5.2 HIF

To survive against decreases in the O_2 tension, higher eukaryotes have developed coordinated mechanisms, both at transcriptional and translational levels. Thereby transcription of genes controlling glycolysis, glucose transport, cell survival

and death, angiogenesis and erythropoiesis, are activated by the hypoxia-inducible factor (HIF) to restore O_2 homeostasis (Pages and Pouyssegur, 2005). In view of that, and based on the Folkman´s theories, it is reasonable to consider hypoxia, as a primary physiological regulator of the angiogenic switch. Summarizing, tumor cells hyper proliferation increases its metabolism and subsequent oxygen consumption, altering the local pH and leading to oxidative stress; together with decreased O_2 concentration due to increased diffusion distances within the expanding tumor mass, lead to a hypoxic microenvironment (North *et al*, 2005).

Hif proteins (Hif1α, Hif2α, Hif3α, and Hif1β) are $\alpha\beta$-heterodimers belonging to a family of transcription factors which shear a basic helix-loop-helix. Unlike the oxygen regulated Hif1α subunits, Hif1β is constitutively expressed but does not respond to oxygen. Hif1α is the most ubiquitously expressed Hif1α subunit, functioning as the major regulator of oxygen homeostasis in many cell types. Hif2α is predominantly expressed in endothelial cells, with not well known functions; and Hif3α, also known as iPAS is mostly present in the cornea, cerebellum and cerebrum, and negatively regulates Hif1α (Liao and Johnson, 2007).

Hif1α is controlled at diverse molecular levels, including mRNA expression, protein stability and transcriptional activity. In normoxic conditions, Hif1α mediates the hydroxylation of two proline residues in an oxygen-dependent degradation domain (ODD), and rapid proteosomal degradation by E3 ubiquitin-protein ligases. However, under hypoxia Hif1α protein escapes degradation, enabling heterodimeriation with Hif1β and subsequent binding to hypoxic responsive elements (HREs) within its target genes promoters (Liao and Johnson, 2007). Its activity is also regulated by activators and inhibitors; to this respect, Factor Inhibiting Hif1 (FIH), prevents the Hif1α binding to p300/CBP transcriptional co-activators, once Hif1α is hydroxylated. Contrary, the signal transducer and activator of transcription 3 (STAT3), also activated in response to hypoxia, and commonly up-regulated in solid tumors, function as a Hif1 activator able to increase its stability and transcriptional activity

(Shin *et al*, 2011). Besides, Hif1α regulation also occurs at the mRNA level, through the PI(3)K/AKT signal transduction pathway (Grunstein *et al*, 2000).

The implication of Hif on tumor growth has been well documented using animal models and *in vitro* studies. To date, more than 100 Hif1 downstream genes involved in angiogenesis, erythropoiesis, iron metabolism, glucose metabolism, cell proliferation, survival and apoptosis, have been identified (Ke and Costa, 2006). Among the large number of genes involved in angiogenesis which have been shown to increase under hypoxia, the vascular endothelial cell growth factor (VEGF) seems the major Hif1 target gene (Ke and Costa, 2006). Experimental evidences reported that, deficient mice in Hif1α gene showed reduced tumor mass and increased apoptosis of teratocarcinomas (Ryan *et al*, 1998), and the positive role for Hif1 in angiogenesis and growth has been also proved in solid subcutaneous tumors using xenograft mouse models, were hypoxic regions showed elevated Hif-1 activation and subsequent VEGF expression (Maxwell *et al*, 1997). Moreover, recent studies with human samples from malignant breast and gliomas tumors found elevated levels of VEGF and Hif1α suggesting their clinical relevance as tumor markers (Bos *et al*, 2001, Jensen *et al*, 2006). The role of hypoxia in hepatocarcinogenesis has been also widely reported and is explained within the Figure 6; therefore, low oxygen availability seem to induce the expression of proteins involved in the energy metabolism, like the hexokinase II (HK2) or the insulin-like growth factor-2 (IGF-2), to directly stimulates the growth of HCC cells. Besides, recent data propose the ability of Hif1α to block apoptosis in HCC by up regulation of anti-apoptotic proteins such as myeloid cell factor-1, or decrease of the ratio between Bax/Bcl-2 (Baek *et al*, 2000).

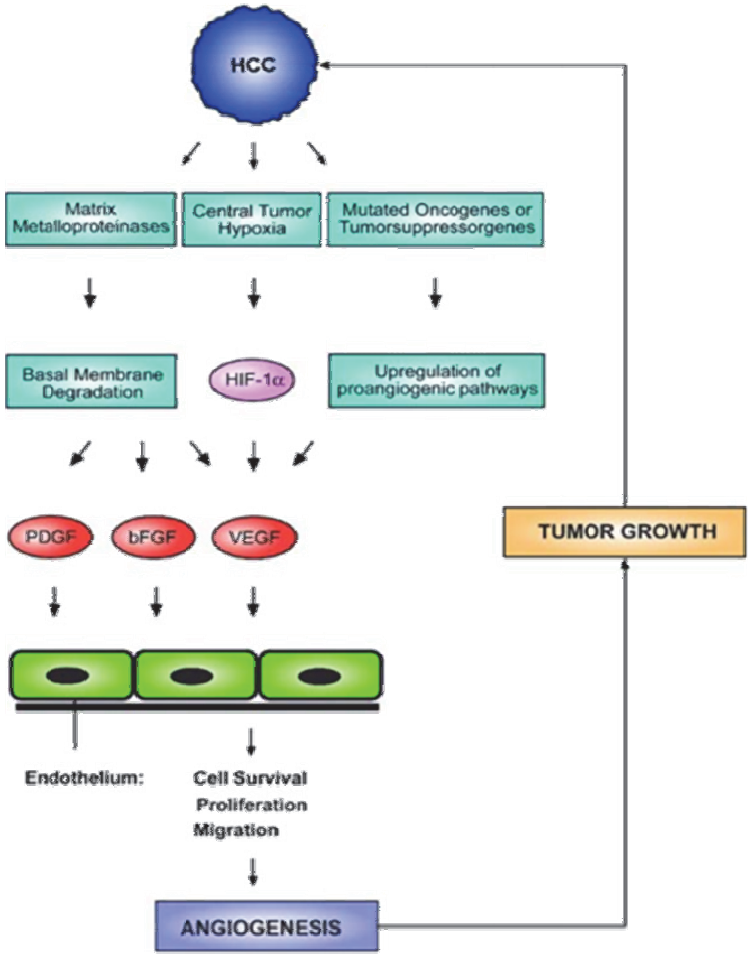

Figure 6. Role of hypoxia in HCC angiogenesis. Hypoxia, as a tumor growth inductor, leads to intracellular stabilization of hypoxia-inducible factor Hif1α, which acts as key transcription factor inducing the expression of several hypoxia response genes, such as VEGF, promoting cell survival, proliferation and migration of endothelial cells needed for the formation of new blood vessels in the tumor periphery or angiogenesis (Semela and Dufour, 2004).

6. Melatonin

Melatonin is the main synthesis product of the pineal gland; the first notions of this gland as a distinct cerebral organ appeared in the III and IV centuries BC (Hoffman and Reiter, 1965). Galen of Pergamon (130–200 AD) named it *'konareion'* placed in the human brain for its pine cone shape, and characterized it as a gland based in its analogy with lymph glands, providing a first description of this organ (Hoffman and Reiter, 1965). As other anatomical structures, this gland has received several names, often depending on the contemporary state of knowledge about its

structure and functions, and the development of new technologies. Hence, 'pineal body', 'epiphysis cerebri', in reference to its position at the top of the brain in many animals, 'epiphysis', or the 'third eye', based on the supposition that the mammalian pineal was evolutionarily linked to photosensory organs are some examples found in the literature (Macchi and Bruce, 2004). In the sixteenth century, philosophy and anatomy made great progresses and René Descartes mentioned the pineal gland in two of his main books (*The passions of the soul* in 1649 and *Treatise of man* in 1664). While Descartes was neither the first nor the last philosopher who wrote about the pineal gland, and many of Descartes' basic anatomical and physiological assumptions were erroneous, he tried to explain most of our mental life in terms of processes involving the pineal; thus he claimed that it was involved in sensation, imagination, memory and bodily movements, and regarded it as the principal seat of the soul and the place in which all our thoughts are formed (Macchi and Bruce, 2004).

Due to its tiny size (5–8 mm) and its previously unknown functions, the pineal gland was originally thought to be a vestigial remnant of a larger organ; nowadays we know that it is indeed an endocrine gland, nearly present in the brain's center of all vertebrates. The pinealocytes are the major cell type of the human pineal parenchyma, surrounded by connective tissue spaces, and the gland's surface is covered by a pial capsule (Kleinschmidt-DeMasters and Prayson, 2006). Whereas most of the mysteries in terms of physiology and anatomy of this gland have been decrypted; the last 40 years have seen a proliferation of research focused on its main product, melatonin.

The identical melatonin molecular structure has been found in all major taxa of organisms tested, including bacteria, unicellular eukaryotes, algae, plants, fungi and invertebrate animals, defining it as a ubiquitous compound (Hardeland, 2009). First mentioned by McCord and Allen in 1917, it was not until 1958 when Lerner and colleagues finally reported the isolation of a small amount of an active factor from more than 2500 beef pineal glands. This compound was chemically known as ´*N*-acetyl-5-methoxytryptamine´ but familiarly called ´*melatonin*´ based on its ability to

lighten the melanin producing cells, and for being a serotonin derived compound (Lerner *et al*, 1960).

6.1 Melatonin structure and synthesis

Melatonin is a highly soluble indoleamine primarily synthesized and secreted by the pineal gland. Darkness is a required condition for melatonin production and release, displaying a circadian rhythmicity (Jimenez-Jorge *et al*, 2005). Its synthesis can be summarized in tree major steps (Fig. 9), starting with the transformation of l-tryptophan from the cerebral vessels, into serotonin, followed by serotonin subsequent metabolization by the arylalkylamine N-acetyltransferase (AA-NAT) to N-acetyl-5-hydroxytryptamine, a rate-limiting enzyme in the melatonin synthesis which activity increases from 30-fold to 70-fold at night (Klein *et al*, 1997).The final step is the conversion of N-acetyl-5-HT into melatonin by hidroxindol O-metiltransferasa (HIOMT) (Ekmekcioglu, 2006).

It is important to mention that in vertebrates, melatonin is not only formed in the pineal gland, but also in other organs and cells such as the Harderian gland, retina, the membranous cochlea, mononuclear leukocytes, skin, and the gastrointestinal tract, which implies a great amount of extrapineal melatonin. Owing to its ubiquitous occurrence from bacteria to plants and animals, we must assume that in a normal diet, melatonin represents a daily component (Hardeland, 2012, Pandi-Perumal *et al*, 2013). The melatonin amounts differ greatly between species; it is a molecule with a short half-life of in circulation (∼10 min) and since is a highly lipophylic substance, easily cross any membrane reaching all fluids, tissues and cellular compartments thus, it is hard provide the exact melatonin concentration in circulating blood. Moreover, apart from the melatonin levels fluctuations associated with the day-night switch, melatonin synthesis has been reported to be significantly age depend on age be quite different depending on the life state.

Melatonin is metabolized primarily in the liver by hydroxylation to 6-hydroxymelatonin, which is then converted to a sulfate or a glucuronide, while there are alternatively melatonin degradation pathways in in the retina, brain, choroid

plexus and in the pineal. Once degraded, 90% of the circulating melatonin is excreted in the urine (Vanecek, 1998).

6.2 Melatonin functions

When Lerner and his colleagues first isolate melatonin in 1958, it is unlikely that they anticipated the future implications of their work. While it has to be probably disappointing that the melatonin skin-lightening properties in fish and amphibians were not applicable to mammals, failing to constitute an effective treatment for vitiligo; a few years later, this hormone would receive considerably more attention as the major regulator of the circadian rhythms (Redman *et al*, 1983). Soon thereafter, investigators observed, in studies on laboratory animals, that melatonin could affect the brain and, thereby, the gonads and other components of neuroendocrine systems (Wurtman RJ, 1985).

Five and a half decades later, evidence begins to accumulate that this indolamine has significantly broader actions; thus melatonin has been identified as a powerful direct free radical scavenger with high efficiency to protect against reactive oxygen (ROS) and reactive nitrogen species (RNS) (Hardeland, 2008). Besides, several papers have reported its anti-inflammatory functions and its relation to the immune system (Guerrero and Reiter, 2002) by mechanism that include the inhibition of NFκB and subsequent down regulation of pro-inflammatory cytokines (Carrillo-Vico *et al*, 2005, Reiter *et al*, 2000). Moreover, melatonin administration at pharmacological dosage has been shown to effectively control complications of diabetes (Peschke, 2008). Regarding to liver, a number of studies have demonstrated its protective role in different pathophysiologic situations such as nonalcoholic (Tahan *et al*, 2009), hepatic reperfusion injury (Ambriz-Tututi *et al*, 2009, Pandi-Perumal *et al*, 2013), oxidative stress induced damage (Mauriz *et al*, 2007) and apoptotic liver changes associated with aging (Molpeceres *et al*, 2007).

6.3 Melatonin receptors

Perhaps the first and one of the best established functions of melatonin is its ability to regulate circadian rhythms and seasonal responses. However, a number of

research papers suggest its role in many other different physiological and biochemical functions (Pandi-Perumal *et al*, 2013). Some of these actions seem to depend on the melatonin antioxidant and free radicals scavenge properties. Nonetheless, interactions with either intracellular proteins such as calmodulin, or binding to specific receptors, have been described within the melatonin mechanisms of action in mammalian (Ekmekcioglu, 2006). For these reasons, melatonin receptor agonists and antagonists are very useful compounds to define the multiple mechanisms by which melatonin modulate physiological and pathological processes. Since 1994, when Reppert and colleagues firstly reported the expression of a high-affinity melatonin receptor from *Xenopus* melanocytes (Ebisawa *et al*, 1994), membrane, cytosolic and nuclear melatonin receptors have been described, cloned and studied in detail.

MT1 and MT2, formerly designated as Mel1a and Mel1b, are the two classes of plasma membrane melatonin receptors identified in several human tissues (central nervous system, gastrointestinal tract, lung, skin, adrenal gland, gonads kidney, heart, blood vessels, adipose tissue, neutrophils, lymphocytes and lymphoid tissues) (Dubocovich and Markowska, 2005). Besides, its expression has been reported in human cancer cell lines of different origin (Chan *et al*, 2002, Imbesi *et al*, 2008, Ramracheya *et al*, 2008). MT1 and MT2 belong to a G protein–coupled receptor superfamily consisting seven transmembrane domains, which share high homology in their amino acid sequence, and mainly differ in their distribution profile and gene localization within chromosomes. Activation of these receptors promotes dissociation of G proteins into α and βγ dimmers, and inhibits adenylate cyclase; reducing the cAMP levels and activating phospholipase C (Dubocovich and Markowska, 2005). Moreover, activation of the MT1 receptor increases phosphorylation of MEK1-2 and ERK1/2 (Pala *et al*, 2013), potentiates ATP and prostaglandin F2α (Barrett *et al*, 2003), and regulates functional responses of melatonin in calcium and potassium ion (Vanecek, 1998). MT2 receptor is associated with activation of similar second messenger pathways; while appears to be highly related with cGMP inhibition rather than cAMP (Dubocovich and Markowska, 2005).

In addition to these plasma membrane melatonin receptors, a novel putative MT3-receptor has been identified and studied hamster organs, presenting lower melatonin affinity (nanomolar range), and very rapid ligand association/dissociation kinetics (Nosjean *et al*, 2000). This MT3-protein, shows 95% homology to the human Quinone Oxireductase 2 (NQO2), a cytosolic enzyme involved in free radicals, toxins and heavy metals detoxification; nevertheless its physiological role is still not entirely known (Volkova *et al*, 2012).

Due to its ability to cross lipid membranes, melatonin is able to directly reach the nuclei, mediating its actions through a group of hormone nuclear receptors ROR/RZR (retinoid orphan receptors/retinoid Z receptors) (Becker-Andre *et al*, 1994). Their structure consists of an N-terminal domain, a DNA binding sequence, a hinge region, and a ligand-binding part in the C-terminal domain (Becker-Andre *et al*, 1994). ROR/RZR are differently distributed according to their subtype, with RZRβ found in neuronal tissues, and RZRα in adipose tissue, skin, testes, cartilage, and liver (Kobayashi *et al*, 2003). While the question of whether melatonin interacts directly with these nuclear receptors has been a source of controversy, the discovery of a nuclear signaling pathway for melatonin contribute to the understanding of the diverse and profound effects of this hormone. Therefore, the activation of this nuclear sites may induces their bind to specific regions of certain genes promoting the transcription of target genes involved in survival, proliferation and cell differentiation (Sanchez-Barcelo *et al*, 2003). In addition, some studies establish a correlation between the nuclear melatonin receptors and the melatonin roles in the immune system (Steinhilber *et al*, 1995).

Summarizing, while several melatonin actions are mediated through its specific receptors, some others are receptor independent (Figure 7). Thus, all these binding sites are attractive pharmacological targets for immunomodulation, regulation of endocrine functions, anti-cancer activity, circadian activity, cardiovascular activity, skin pigmentation, lipid and glucose metabolism, and aging; consistently, a better knowledge of their expression, regulation, signaling and

functions may have an impact on the pharmacotherapy of a wide array of diseases (Jung and Ahmad, 2006).

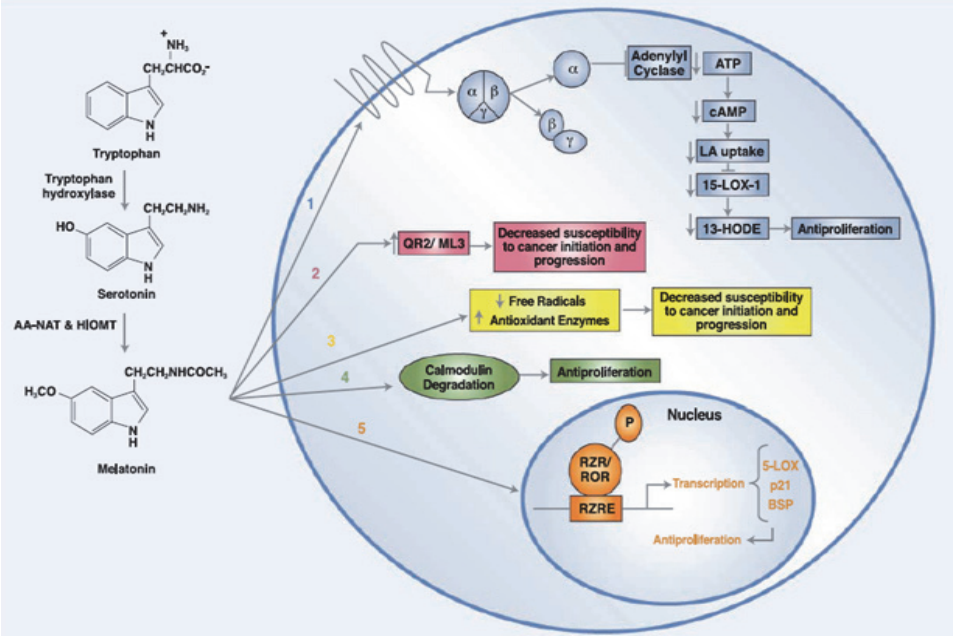

Figure 7. Synthesis and mechanism of action of melatonin. Melatonin is synthesized from tryptophan under the control of tryptophan hydroxylase, AA-NAT, and HIOMT. Five potential mechanisms are proposed for the anti-proliferative effects of melatonin. *1*, (blue), melatonin binds to its receptor(s), ML1 and ML2; *2*, (red), melatonin can also bind its ML3 receptor, which, decreases the susceptibility to cancer initiation and progression; 3, (yellow), melatonin has been reported to be a powerful scavenger of ROS as well as an inducer of many antioxidant enzymes; 4, (green), melatonin has been shown to increase calmodulin degradation to result in anti-proliferative effects; 5, (gold), melatonin bind to its nuclear receptors, RZR/RORα and RZRh, altering the transcription of several genes related with cellular proliferation and apoptosis (Jung and Ahmad, 2006).

6.4 Melatonin and cancer

The oncostatic and/or cytotoxic effects of melatonin against several cancer cells have been investigated *in vitro* (Cabrera *et al*, 2010, Carbajo-Pescador *et al*, 2009, Cini *et al*, 2005, Cui *et al*, 2012, Farriol *et al*, 2000, Futagami *et al*, 2001, Garcia-Navarro *et al*, 2007, Garcia-Santos *et al*, 2006, Hill *et al*, 2009), showing in general promising results. Based on the effectiveness observed in cell culture models, the anti-proliferative effects of melatonin have also been examined in tumor animal models such as mammary and prostate carcinomas, pituitary tumors, melanomas, colon

cancer, and malignant gliomas (Martin *et al*, 2006, Vijayalaxmi *et al*, 2002). A common finding of these *in vitro* and *in vivo* studies was that melatonin does not have any unfavorable side effects, which support its use in preclinical studies. Epidemiologic studies have suggested a correlation between melatonin and cancer risk. For instances, increased in breast cancer and colorectal cancer risk appears in subjects who frequently did not sleep during the peak of melatonin synthesis (Reed, 2011, Schernhammer *et al*, 2003). Patients with breast tumors have a decrease in circulating melatonin blood level (Stevens, 1987).Moreover, low melatonin plasma levels seems to be an indicator of endometrial cancer (Grin and Grunberger, 1998).

At the moment clinical trials, in non–small lung cancer (NSCLC) patients, which often does not respond well to cisplatin therapy, shown mean survival time significantly higher in melatonin treated, than those receiving supportive care alone (Lissoni *et al*, 1992). Melatonin has been shown to protect skin cells from UV radiation–mediated damage via its antioxidant property. In one study conducted in 40 patients with metastatic malignant melanoma, melatonin showed an overall response rate of 30% and no adverse effects were found (Gonzalez *et al*, 1991). Melatonin has also been tested in advanced solid tumors with brain metastases in a trial with 50 patients; from those, 9 of the 24 who received melatonin survived 1 year compared with 3 of 26 who did not receive melatonin (Lissoni *et al*, 1992). It's interesting to mention the Lissoni et colleges research performed in 250 metastatic solid tumor patients from lung, breast, gastrointestinal tract neoplasms, head and neck cancers, were melatonin administration as adjuvant was found to decrease patient suffering, prolonging their survival, specially advanced cancer patients with poor clinical status (Lissoni *et al*, 1999).

As described, several studies have high light benefits of combined therapies with melatonin and chemotherapeutic agents, increasing efficacy and decreasing side effects (Blask *et al*, 2002). Despite of all these data, additional studies are required to confirm the melatonin use in cancer patients; besides, more cancer types, different dosage, administration timing, and combinations of melatonin with other anticancer

agents should be investigated in appropriate animal tumor models, which possess relevance to human disease.

Based on the lack of available data regarding the oncostatic role of melatonin in human hepatocarcinoma, and the successful results of its administration in other tumor types, we consider this area as a challenger field for study.

1st publication

Melatonin modulation of intracellular signaling pathways in hepatocarcinoma HepG2 cell line: role of the MT1 receptor.

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Melatonin modulation of intracellular signaling pathways in hepatocarcinoma HepG2 cell line: role of the MT1 receptor

Abstract: Melatonin reduces proliferation in many different cancer cell lines. However, studies on the oncostatic effects of melatonin in hepatocarcinoma are limited. We have previously demonstrated that melatonin administration induces cycle arrest, apoptosis, and changes in the expression of its specific receptors in HepG2 human hepatocarcinoma cells. In this study, we used the receptor antagonist luzindole to assess the contribution of MT1 melatonin membrane receptor to melatonin effects on cell viability, mitogen-activated protein kinase (MAPKs) activation, and cAMP levels. Additionally, effects of MT1 inhibition on mRNA levels of cytosolic quinone reductase type-2 (NQO2) receptor and nuclear retinoic acid-related orphan receptor alpha (RORα) were tested. Melatonin, at 1000 and 2500 μM, significantly reduced cell viability. Pre-incubation with luzindole partially inhibited the effects of melatonin on cell viability. Melatonin at 2500 μM significantly reduced cAMP levels, and this effect was partially blocked by luzindole. Both melatonin concentrations increased the expression of phosphorylated p38, ERK, and JNK. ERK activation was completely abolished in the presence of luzindole. NQO2 but not RORα mRNA level significantly increased in luzindole-treated cells. Results obtained provide evidence that the melatonin effects on cell viability and proliferation in HepG2 cells are partially mediated through the MT1 membrane receptor, which seems to be related also with melatonin modulation of cAMP and ERK activation. This study also highlights a possible interplay between MT1 and NQO2 melatonin receptors in liver cancer cells.

Sara Carbajo-Pescador[1], Andrés García-Palomo[2], Javier Martín-Renedo[1], Maiara Piva[1], Javier González-Gallego[1] and José L. Mauriz[1]

[1]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd) and Institute of Biomedicine, University of León, León, Spain; [2]Service of Oncology. Hospital of León. Complejo Asistencial Universitario de León, León, Spain

Key words: cAMP, hepatocarcinoma, melatonin, mitogen-activated protein kinases

Address reprint requests to José L. Mauriz, Institute of Biomedicine, University of León, 24071-León, Spain.
E-mail: jl.mauriz@unileon.es

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Introduction

Hepatocellular carcinoma (HCC) is the most common malignant hepatobiliary disease and one of the leading causes of cancer mortality worldwide [1]. Risk factors for HCC development include infection with hepatitis virus B or C, cirrhosis, nonalcoholic steatohepatitis, and the presence of genetic metabolic diseases [2]. In the early stages of HCC, surgical resection, local ablation, or liver transplantation is able to cure a proportion of patients [3]. In advance stages, treatments are limited, but the identification of signaling pathways and the recognition of their role in the pathogenesis of the disease could result in the development of drugs directed at specific therapeutic targets [4].

It is commonly accepted that melatonin (*N*-acetyl-5-methoxytryptamine), the most relevant pineal secretory product, has oncostatic properties in a wide variety of tumors processes, such as breast, prostate, colon, ovarian, and bone cancers, among others [5–11]. Some of the physiological effects of melatonin are mediated by its interaction with two membrane receptors, MT1 and MT2, which are coupled to the inhibition of cAMP through the heterotrimeric guanine nucleotide-binding proteins (G proteins) [12, 13]. In vitro studies have proven the effectiveness of the melatonin analogues S23219-1 and S23478-1 at suppressing the growth of MCF-7 human breast cancer cells through MT1/MT2 activation [14, 15]. Research also supports a potential role for MT2 receptor in the antiproliferative effects of melatonin in human choriocarcinoma cells and in Ishikawa endometrial cancer cells [16, 17]. However, studies in HT-29 human cancer cells and Colon 38 murine cancer cells have shown that membrane receptors are not indispensable to the antiproliferative effect of melatonin [18, 19]. Thus, implication of membrane receptors on the oncostatic action of melatonin may depend on the type of tumor cell assayed.

Members of mitogen-activated protein kinases (MAPKs) superfamily including JNK, ERK, and p38 are serine and threonine kinases, which transduce external stimuli into a wide range of cellular responses, such as proliferation, survival, differentiation, and migration. Owing to their essential roles in these cellular functions, deregulated MAPKs are often found to contribute to the development of many cancers, including HCC [20]. Several studies have shown that modulation of MAPKs could be involved in the melatonin-induced cell growth inhibition of tumoral cells, such as human LNCaP prostate cancer cells, MCF-7 breast cancer cells, and melanoma SK-MEL-1 cells [21–23]. Melatonin stimulatory effect on MAPKs links to G

protein-coupled receptors in MT1/MT2 transfected Cos-7 cells [24].

Although there is an increasing interest in determining the pharmacological effects of melatonin in cancer treatment, mechanisms of this indole on human HCC are still not clearly elucidated. We have recently shown that melatonin administration induces cell cycle arrest and apoptosis in hepatocarcinoma HepG2 cells [25]. Antiproliferative effect of melatonin is driven through MT1 but not MT2, whose expression is absent in HepG2 cells, and the cytosolic quinone reductase type-2 (NQO2) receptor and nuclear retinoic acid-related orphan receptor alpha (RORα) are also overexpressed [26]. In this study, we focused on the contribution of MT1 melatonin receptor to the antiproliferative effect of melatonin, looking to its potential capacity to modulate cAMP levels and MAPKs activation. We additionally tested the effects of MT1 inhibition on the expression of NQO2 and RORα receptors.

Materials and methods

Cell culture and melatonin treatment

Human HepG2 hepatocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Stock cells routinely were grown as monolayer cultures in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), glutamine (4 mM), and pyruvate (100 μg/mL) in a humidified 5% CO_2 atmosphere at 37°C, and the medium was changed every other day. Cell culture reagents were from Gibco (Invitrogen Life Technologies, Barcelona, Spain). Melatonin was obtained from Sigma (St Louis, MO, USA), and luzindole (*N*-Acetyl-2-benzyltryptamine) was purchased from Tocris Bioscience (Ellisville, MO, USA).

Confluent HepG2 cells growing in complete media were replated in 9.6-cm^2 culture dishes, at a density of 150,000 cells/plate, in a total volume of 2 mL of complete medium. After 24 hr, the plating medium was replaced with fresh medium containing the appropriate concentrations of melatonin (1000–2500 μM) dissolved in DMSO (0.2% DMSO final concentration in the plates) for 12, 24, and 48 hr. Control-untreated cells were incubated with culture medium, and vehicle-treated cells received 0.2% DMSO. Four different experiments were performed by triplicate.

Cell viability assay

For the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tertazolium bromide (MTT) cell viability assays, confluent HepG2 cells growing in complete media were replated in 24-well culture plate, at a density of 20,000 cell/well in a total volume of 1 mL of complete medium. After 24 hr, the plating medium was replaced with fresh medium. Appropriate concentrations of melatonin (1000–2500 μM) dissolved in 0.2% dimethyl sulfoxide (DMSO) were added to the medium for 12, 24, and 48 hr. Control-untreated cells were incubated with culture medium, and vehicle-treated cells received 0.2% DMSO (in culture medium) for 48 hr. To investigate the inference of MT1 melatonin receptor on our further studies, some groups of HepG2 confluent cells were pre-incubated for 1 hr with 1 μM luzindole (a competitive melatonin MT1/MT2 antagonist), before addition of 1000–2500 μM melatonin. The experiment was performed by triplicate.

The MTT assay was carried out as described by Deniziot and Lang [27]. Briefly, after exposure of cells to melatonin, culture media was changed by free serum culture media. MTT dissolved in phosphate-buffered saline (PBS) was added to each well. MTT in free serum media was incubated for 3 hr at 37°C. After this interval, free serum culture media containing MTT was discarded and DMSO was added to each well dissolving the precipitate. The optical densities were measured at 560 nm spectral wavelength using microtiter plate reader (Synergy™ HT Multi-Mode Microplate Reader; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Western blot analysis

After treatments, cultured cells were washed twice with ice-cold PBS and lysed by adding ice-cold lysis buffer containing 25 mM HEPES, 1% Triton X-100, 2 mM EDTA, 0.1 M NaCl, 25 mM NaF, 1 mM Sodium Orthovanadate, and protease cocktail inhibitor (Roche, Basel, Switzerland) and scraped off the plate. The extract was transferred to a microfuge tube and centrifuged for 10 min at 15,000 g. The supernatants were collected and stored at −20°C.

For Western blot analysis, equal amounts of protein (20μg) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis for 1.5 h at 100 V and the membranes were blotted by wet transfer (100 V 2h 4°C) on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 4% nondry fat milk in phosphate-buffered saline containing 0.05% Tween 20 (Bio-Rad, Hercules, CA, USA) (PBST) for 30 min at 37°C. The membranes were probed with a primary antibody overnight at 4°C in PBST containing 4% nonfat dry milk [28]. Primary antibodies for phospho p38, phospho ERK phospho JNK, p38, ERK, and JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and those for JNK and p38 from Cell Signaling (Beverly, MA, USA).

Equal loading of protein was demonstrated by probing the membranes with a rabbit anti β-actin polyclonal antibody (Sigma, 1:2000 dilution). After washing with PBST bound, primary antibody was detected with horseradish peroxidase-conjugated antigoat antibody (Dako, Glostrup, Denmark) and blots were developed using an enhanced chemiluminescence detection system (GE Healthcare, Chalfont St Giles, UK). Densitometry analysis of specific bands was performed by Scion Image software (Scion Corporation, Frederick, MD, USA).

Real-time reverse transcriptase–polymerase chain reaction

For real-time reverse transcriptase–polymerase chain reaction (RT–PCR), confluent HepG2 cells growing in complete media were replated in 6-well culture plate, at a density of

150,000 cell/well in a total volume of 2 mL of complete medium. After treatment, total RNA was obtained using a Trizol reagent (Applied Biosystems, Carlsbad, CA, USA) and quantified by spectrophotometry (Nanodrop 1000; Thermo Scientific, Waltham, MA, USA). Residual genomic DNA was removed by incubating RNA with RQ1 RNase-free DNase (Promega, Madison, WI, USA). First-standard cDNA was synthesized using M-MLV RT (Roche Molecular Systems), and the negative control (no transcriptase control) was performed in parallel. cDNA was amplified using FastStart TaqMan Probe Master (Roche) on an StepOnePlus Real-Time PCR Systems (Applied Biosystems). TaqMan primers and probes for MT1 (GenBank accession no.: NM_005958 and Hs00195567_m1), NQO2 (NM_000904.3 and Hs00168552_m1), RORα (NM_134260.2 and Hs00931968_m1), and beta-actin (NM_001101.2 and Hs99999903_m1) genes were derived from the commercially available TaqMan Gene Expression Assays (Applied Biosystems). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method [29]. The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of beta-actin detection, referred to as ΔCT.

Cyclic AMP immunoassay

The amount of cAMP was measured using the cAMP Direct Immunoassay Kit from Abcam (Cambridge, UK) as described by the manufacturer. Briefly, cells were grown in 9.6-cm^2 dishes. After the treatment, media was removed and 270 μL of 0.1 M HCl was added to the plate, incubating cells for 20 min at room temperature. Then, cells were scraped off the surface with a cell scraper, transferred to a centrifuge tube, and centrifuged at top speed for 10 min. All samples and standards were acetylated before the assay to increase sensitivity of detection. The optical density of samples was measured at 450 nm, and the concentration of cAMP was calculated from a standard curve (approximately 0.1–10 pmol), which was assayed along with the samples.

Statistical analysis

Results are expressed as mean values ± S.E.M. of the indicated number of experiments. Comparisons between the different treatments were performed by a two-way ANOVA with repeated measures for treatment (control, vehicle, luzindole, melatonin, and melatonin + luzindole) and time, followed by Newmann–Keuls *post hoc* test when appropriate. $P < 0.05$ was considered significant. Values were analyzed using the statistical package Statistica 10.0 (Statsoft Inc, Tulsa OK, USA).

Results

To assess the contribution of MT1 melatonin receptor to the effects of melatonin on cell viability, MTT test was performed in HepG2 cells after melatonin administration with and without 1 μM pre-incubation with luzindole, an antagonist of MT1 and MT2 melatonin receptors. The growth inhibition of HepG2 was dose and time dependent for both 1000 and 2500 μM concentrations. As shown in Fig. 1, melatonin administration at 1000 μM reduced cell viability by about 50% between 12 and 48 hr, and pre-incubation with luzindole recovered cell viability up to 31% after 24 hr of treatment. HepG2 cells incubated with melatonin in a concentration of 2500 μM showed a 70–80% decrease in cell growth, which was also partially abolished by luzindole treatment, recovering cell viability values by about 50% at 24 hr.

Melatonin affects several second messengers via the G-coupled MT1 receptor, being a main intracellular target adenylate cyclase, whose inhibition in turn leads to cAMP suppressed production in various cell types [30]. To confirm the coupling of MT1 to the inhibition of cAMP, levels were measured in HepG2 cells treated with melatonin in the presence/absence of luzindole in the culture medium. Using a cAMP direct immunoassay, we observed that luzindole administration alone and melatonin 1000 μM did not significantly alter cAMP levels compared with the controls. However, a higher 2500 μM melatonin dose was able to decrease cAMP level to about 78% of control (Fig. 2). Simultaneous treatment with 2500 μM melatonin plus 1 μM luzindole inhibited by about 31% the effect of melatonin on cAMP levels.

Because MAPK signaling pathways are dysregulated in various types of human tumors, including HCC [31], we tried to clarify the involvement of MT1 in melatonin-induced activation of MAPKs. Western blot analyses were performed using antibodies against the phosphorylated and total forms of ERK, p38, and JNK. Following melatonin administration at 1000 or 2500 μM, ERK phosphorylation significantly increased versus control-untreated cells (values between +62% and +93%, Fig. 3). ERK activation decreased after incubation with 1 μM luzindole and melatonin for 48 hr (−33% at 1000 μM and −71% at 2500 μM, Fig. 3), reaching values that did not significantly differ from the controls. Levels of phosphorylated p38 also increased after melatonin administration, and this effect was partially

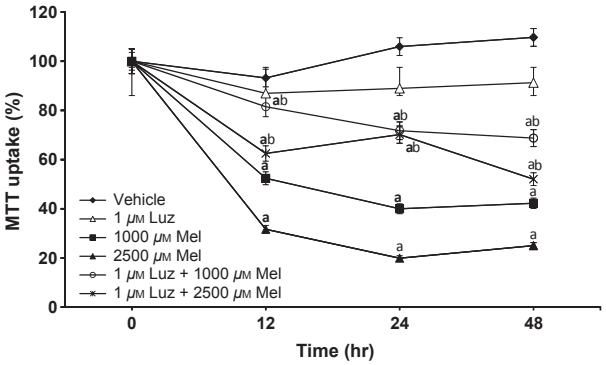

Fig. 1. Effect of melatonin treatment on HepG2 cell viability. When indicated, HepG2 cells were treated with melatonin with and without 1 μM luzindole pre-incubation. Viability was assessed by MTT assay. Data are expressed as a percentage of mean values ± S.E.M. of four different cell batches from four different cultures. Experiments were performed in triplicate. [a]$P < 0.05$, compared with control cells at 0 h; [b]$P < 0.05$, compared with melatonin-treated cells.

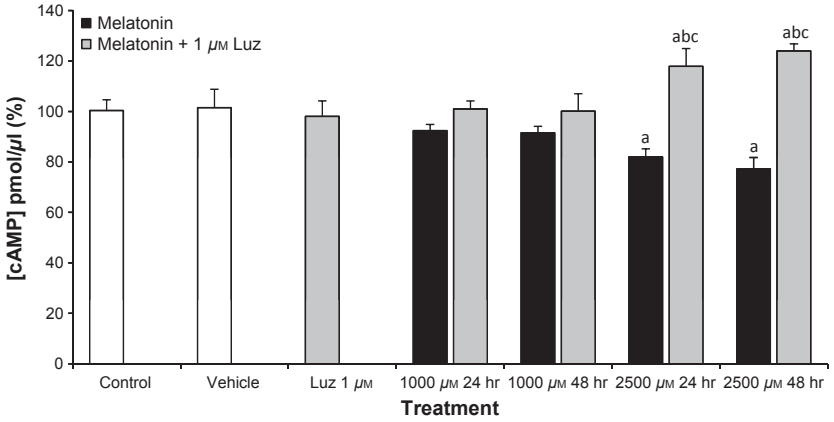

Fig. 2. Effect of melatonin treatment on cAMP levels in HepG2 cells. When indicated, HepG2 cells were treated with melatonin with and without 1 μM luzindole pre-incubation. Concentration of cAMP was measured using the cAMP Direct Immunoassay Kit. Data are expressed as a percentage of mean values ± S.E.M. of four different cell batches from four different cultures. Experiments were performed in triplicate. $^{a}P < 0.05$, compared with control cells; $^{b}P < 0.05$, compared with melatonin-treated cells; $^{c}P < 0.05$, compared with 24 hr, same treatment.

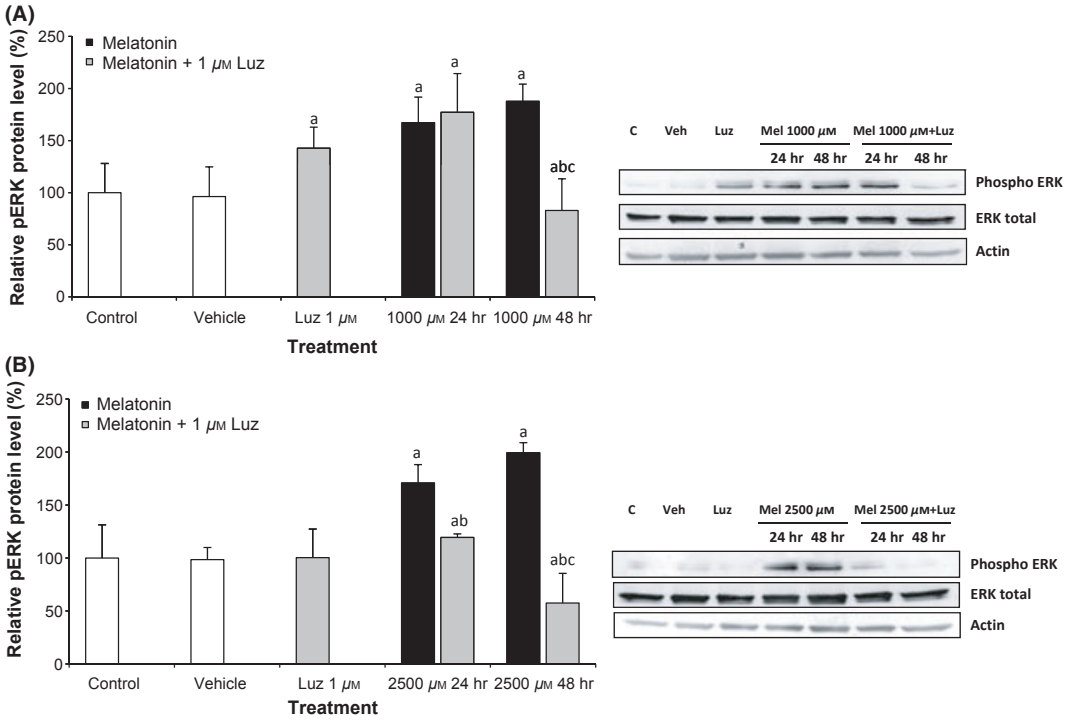

Fig. 3. Effect of melatonin treatment on ERK expression in HepG2 cells. When indicated, HepG2 cells were treated with melatonin with and without 1 μM luzindole pre-incubation. (A) 1000 μM melatonin treatment. (B) 2500 μM melatonin treatment. Left: densitometry; right: Western blot analysis. Equal loading of proteins is illustrated by β-actin bands. Protein was separated on 15% SDS–polyacrylamide and blotted with ERK and phospho ERK antibodies. Data are expressed as a percentage of mean values ± S.E.M. of four different cell batches from four different cultures. Experiments were performed in triplicate. $^{a}P < 0.05$, compared with control cells; $^{b}P < 0.05$, compared with melatonin-treated cells; $^{c}P < 0.05$, compared with 24 hr same treatment.

abolished by luzindole in comparison with melatonin-treated cells. However, as shown in Fig. 4, luzindole was not able to completely return p38 to control levels. JNK phosphorylation was significantly increased during exposure to melatonin at both 1000 and 2500 μM. Values did not significantly decrease in cells treated with luzindole (Fig. 5).

We have previously reported that protein levels of both the cytosolic NQO2 receptor and the nuclear RORα increase in melatonin-treated HepG2 cells [26]. To know whether MT1 blockage by luzindole could result in transcriptional changes not only of MT1 but also of the cytosolic and nuclear melatonin receptors, mRNA levels

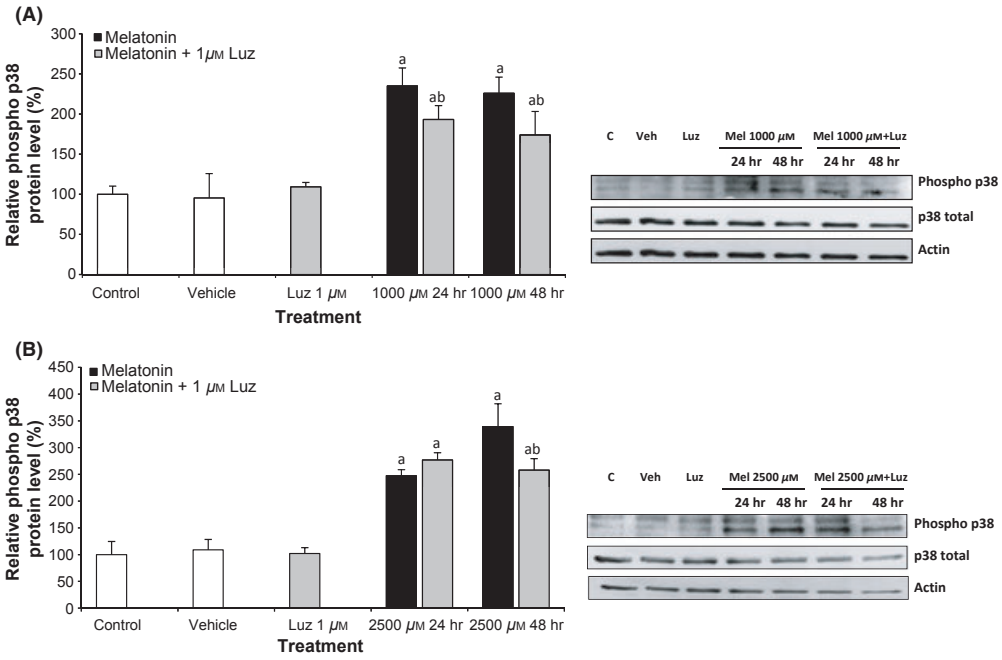

Fig. 4. Effect of melatonin treatment on p38 expression in HepG2 cells. When indicated, HepG2 cells were treated with melatonin with and without 1 μM luzindole pre-incubation. (A) 1000 μM melatonin treatment. (B) 2500 μM melatonin treatment. Left: densitometry; right: Western blot analysis. Equal loading of proteins is illustrated by β-actin bands. Protein was separated on 15% SDS–polyacrylamide and blotted with p38 and phospho p38 antibodies. Data are expressed as a percentage of mean values ± S.E.M. of four different cell batches from four different cultures. Experiments were performed in triplicate. $^{a}P < 0.05$, compared with control cells; $^{b}P < 0.05$, compared with melatonin-treated cells; $^{c}P < 0.05$, compared with 24 hr same treatment.

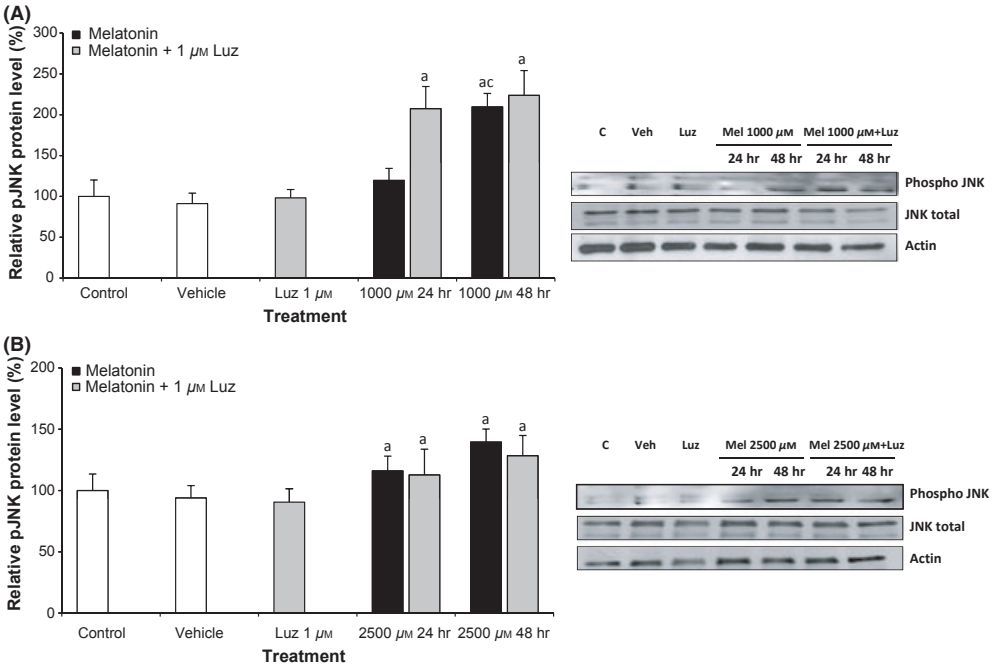

Fig. 5. Effect of melatonin treatment on JNK expression in HepG2 cells. When indicated, HepG2 cells were treated with melatonin with and without 1 μM luzindole pre-incubation. (A) 1000 μM melatonin treatment. (B) 2500 μM melatonin treatment. Left: densitometry; right: Western blot analysis. Equal loading of proteins is illustrated by β-actin bands. Protein was separated on 15% SDS–polyacrylamide and blotted with p38 and phospho p38 antibodies. Data are expressed as a percentage of mean values ± S.E.M. of four different cell batches from four different cultures. Experiments were performed in triplicate. $^{a}P < 0.05$, compared with control cells; $^{b}P < 0.05$, compared with melatonin-treated cells; $^{c}P < 0.05$, compared with 24 hr same treatment.

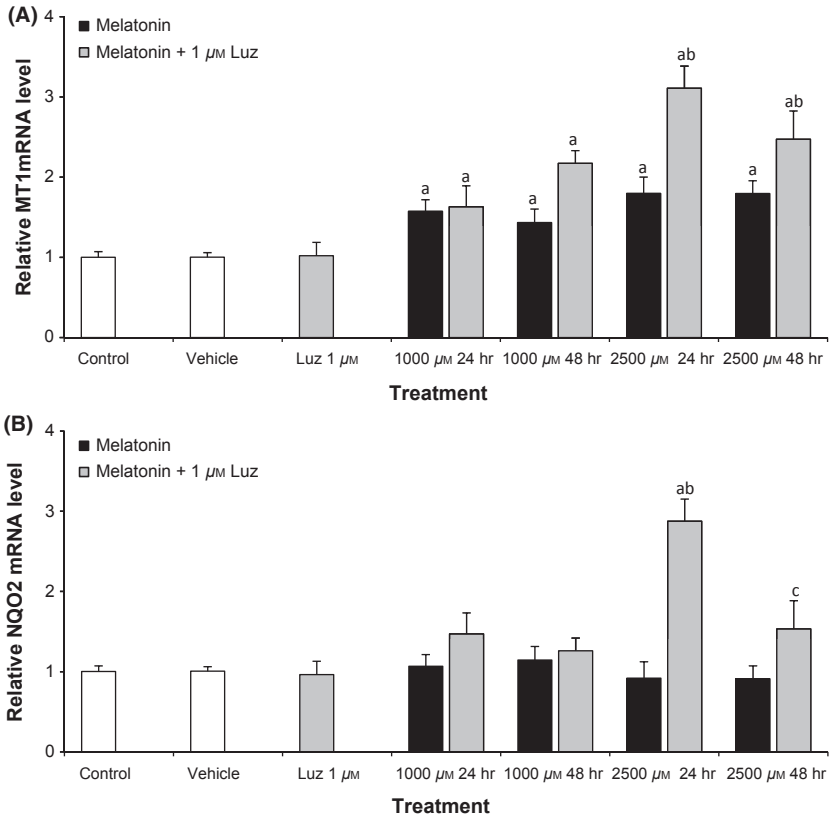

Fig. 6. Real-time reverse transcriptase–polymerase chain reaction (RT–PCR) of melatonin receptors. (A) MT1 mRNA expression. (B) NQO2 mRNA expression. When indicated, HepG2 cells were treated with melatonin with and without 1 μM luzindole pre-incubation. Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method. Data normalized against β-actin are presented as percentage versus control group. Data are expressed as mean values ± S.E.M. of four different cell batches from four different cultures. Experiments were performed in triplicate. $^{a}P < 0.05$, compared with control cells; $^{b}P < 0.05$, compared with melatonin-treated cells; $^{c}P < 0.05$, compared with 24 hr same treatment.

were measured by real-time RT–PCR. A significant increase in MT1 mRNA at both 1000 and 2500 μM was observed in cells pre-incubated with luzindole (Fig. 6). No significant change was induced by luzindole treatment in RORα mRNA levels (data not shown). However, analysis of the cytosolic receptor NQO2 revealed that mRNA level was significantly higher in melatonin plus luzindole co-treated cells compared with that treated with melatonin (Fig. 6).

Discussion

Even when the antiproliferative effects of melatonin have been widely reported in different tumor cells [32–34], the mechanisms by which this indole exerts its pharmacological effects remain controversial and seem to be largely dependent on the dose and cell type evaluated. It is widely accepted that the melatonin effects could be mediated by the G protein-coupled high-affinity receptors MT1 and MT2, localized on the plasma membrane [30]. While functional MT1 receptors have been described in many tissues, including liver [35, 36], we have previously reported an absence of MT2 expression in hepatocarcinoma HepG2 cells [26], which agrees with the fact that this receptor is more restrictively expressed than MT1, and no evidences for liver MT2 expression in humans have been previously published. Currently, the use and development of melatonin antagonist molecules such as luzindole, a competitive MT1 and MT2 antagonist, has contributed to a better understanding of the mechanisms of action of these receptors [19, 37]. Luzindole treatment could therefore help to elucidate the role of MT1 expression to the antiproliferative effect of melatonin in HepG2 cells.

We observed a dose-dependent antiproliferative effect of melatonin on cell viability at both 1000 and 2500 μM when administered for 12, 24, and 48 hr, which supports our previous findings in this cell line [25, 26]. The fact that pre-incubation with luzindole significantly increased cell viability suggests that melatonin growth inhibition properties in liver cancer cells could be, at least in part, mediated through the G protein-coupled MT1 receptor. Our findings are consistent with similar results reported in other cancer cell lines. Thus, it is known that melatonin, via activation of its MT1 receptor, suppresses the development and growth of breast cancer [38]. Furthermore, melatonin inhibits pancreatic cancer growth by an interaction with MT1 and MT2 membrane receptors; this effect has been observed both in vivo and in vitro experimental studies [39]. In addition, a high expression of MT1 mRNA has been detected in patients with bone pathology [40].

Although other signal transduction pathways are also brought into play according to the cell type studied (protein kinase C, Ca^{2+}, or K^{+} channel) [41], it is well known that melatonin regulates cell functions by affecting several second messengers through its specific membrane receptors [42, 43]. Thus, inhibition of the production of cAMP by a G_i/G_o protein is one of the most important signalling pathways involving MT1 and MT2 receptors [12, 13]. Here, we further investigated whether melatonin was able to induce changes in basal cAMP levels and whether these

effects were mediated through the MT1 receptor. As determined by cAMP direct immunoassay, the higher 2500 μM melatonin dose was able to decrease cAMP concentration, and this effect was abolished by luzindole, which increased cAMP in the presence of melatonin but not when administered alone. It has been shown that melatonin inhibits activity of adenylyl cyclase in a dose-dependent manner [44]. Previous studies have indicated that the coupling of both MT1 and MT2 receptors seem to be necessary to inhibit adenyl cyclase when melatonin is administered at low doses [45]. This could help to explain our results, and the fact that melatonin at 1000 μM melatonin did not significantly change cAMP levels in HepG2, in which MT2 is not present and only MT1 is expressed. Concerning luzindole, a similar effect, attributed to the fact that it acts as an inverse agonist at the MT1 receptor in the presence of melatonin, has been already observed in CHO-mt1 transfected cells [46].

Although the precise mechanisms by which melatonin downregulates cell proliferation in cancer cells are not fully understood, it has been suggested that modulation of MAPKs pathways may be involved [47–49]. We found a significantly increase in ERK, JNK, and p38 phosphorylation following treatment with both doses of melatonin, which confirms the previous data from our laboratory [25]. When melatonin was co-administered with 1 μM luzindole, only ERK activation decreased markedly and did not significantly differ from control values. However, luzindole co-administration was not able to return phospho-p38 to control levels and did not significantly diminished phospho-JNK expression. These results clearly suggest a connection between MT1 and ERK activations. The use of transfected cells that only express MT1 has demonstrated that melatonin stimulates specifically ERK, whereas in cells expressing both MT1 and MT2 receptors, melatonin inhibits ERK phosphorylation [24]. Previous studies have shown that ERK is mainly activated by cell surface receptors such as G protein-coupled receptors [50]. Our observations in HepG2 cells confirm the previous finding of ERK1/2 pathway activation by melatonin treatment through MT1-dependent mechanisms in ovary CHO cells [51]. Even though ERK has been associated with cell proliferation and survival, evidence has been provided that ERK activation is required for cisplatin-induced growth arrest and apoptosis in H22 hepatoma cells [52]. In addition, it has been reported that paclitaxel also depends on ERK activation to promote G2/M cell cycle arrest and cell death in MCF7 human breast carcinoma cells [53].

Not only melatonin membrane G protein-coupled receptors but also other cytosolic and nuclear melatonin-binding sites have been characterized. Thus, the enzyme quinone reductase type 2 (NQO2), which belongs to a group of reductases involved in the protection against oxidative stress, has been identified as the cytosolic melatonin receptor MT3 [54]. RORα, a member of the steroid hormone receptor ROR/RZR superfamily expressed in many tissues and cells, acts as a nuclear receptor of melatonin [55]. Interplay between membrane and nuclear melatonin receptors has been reported in human lymphocytes, where activation of MT1 or MT2 receptor by melatonin would lead to inhibitory responses on the cAMP signal transduction cascade, resulting in decreased activity of protein kinase A and affecting RORα expression levels in the immune system [56]. We have previously found that melatonin is able to increase the protein expression of both NQO2 and RORα s in HepG2 cells [26]. In the current study, concomitant administration of 2500 μM melatonin and luzindole resulted in a significant increase in both MT1 and NQO2 mRNA levels. Transcriptional upregulation of NQO2 when MT1 is competitively blocked by luzindole, could be explained as a cellular response aimed to bind melatonin present in the media. Thus, our results highlight an interesting potential connection between MT1 and NQO2 melatonin receptors in HepG2 cells. Considering the previous reports that NQO2 knockout mice are far more prone to develop skin tumors [57] and that inhibition of different tumor cell growth correlates with upregulation of NQO2 [58, 59], the present findings could be of relevance for the oncostatic effects of melatonin in HepG2 cells.

In conclusion, the present results provide evidence that melatonin effects on cell viability and proliferation in HepG2 cells are partially mediated through the MT1 membrane receptor, which seems to relate also to melatonin modulation of cAMP and ERK activations. While melatonin could activate ERK in a MT1 receptor-dependent manner, the precise mechanism by with melatonin modulates p38 and JNK pathways needs further elucidation. This study also highlights a possible interplay between MT1 and NQO2 melatonin receptors in liver cancer cells.

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Melatonin induces transcriptional regulation of Bim by FoxO3a in HepG2 cells.

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Melatonin induces transcriptional regulation of Bim by FoxO3a in HepG2 cells

S Carbajo-Pescador[1], C Steinmetz[2], A Kashyap[2], S Lorenz[2], J L Mauriz[1], M Heise[3], P R Galle[2], J González-Gallego[1] and S Strand*[,2]

[1]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd) and Institute of Biomedicine, University of León, León, Spain; [2]Department of Internal Medicine, University Medical Center, Johannes Gutenberg University, Mainz, Germany and [3]Department of Transplantation and Hepatobiliary Surgery, University Medical Center, Johannes Gutenberg University, Mainz, Germany

Background: Melatonin induces apoptosis in many different cancer cell lines, including hepatocellular carcinoma cells. However, the responsible pathways have not been clearly elucidated. A member of the forkhead transcription factors' family, FoxO3a, has been implicated in the expression of the proapoptotic protein Bim (a Bcl-2-interacting mediator of cell death). In this study, we used human HepG2 liver cancer cells as an *in vitro* model to investigate whether melatonin treatment induces Bim through regulation by the transcription factor FoxO3a.

Methods: Cytotoxicity of melatonin was compared in HepG2 hepatoblastoma cells and primary human hepatocytes. Proapoptotic Bim expression was analysed by reverse transcriptase–polymerase chain reaction and western blot. Reporter gene assays and chromatin immunoprecipitation assays were performed to analyse whether FoxO3a transactivates the Bim promoter. Small interfering RNA (siRNA) was used to study the role of FoxO3a in Bim expression. Immunofluorescence was performed to analyse FoxO3a localisation in HepG2 cells.

Results: Melatonin treatment induces apoptosis in HepG2 cells, but not in primary human hepatocytes. The proapoptotic effect was mediated by increased expression of the BH3-only protein Bim. During melatonin treatment, we observed increased transcriptional activity of the forkhead-responsive element and could demonstrate that FoxO3a binds to a specific sequence within the Bim promoter. Furthermore, melatonin reduced phosphorylation of FoxO3a at Thr^{32} and Ser^{253}, and induced its increased nuclear localisation. Moreover, silencing experiments with FoxO3a siRNA prevented Bim upregulation.

Conclusion: This study shows that melatonin can induce apoptosis in HepG2 hepatocarcinoma cells through the upregulation of proapoptotic Bim mediated by nuclear translocation and activation of the transcription factor FoxO3a.

Each year, hepatocellular carcinoma (HCC) is diagnosed in more than half a million people worldwide, being the most common malignant hepatobiliar disease (El-Serag, 2011; Jemal *et al*, 2011). Viral hepatitis B and C, as well as alcohol abuse, are the main risk factors for its development (Cornella *et al*, 2011). Dysregulation of cellular proliferation and apoptosis are frequent events related with malignant phenotype and poor responsiveness of HCC towards chemotherapy (Müller *et al*, 1997). For this reason, advances in understanding these processes are needed for developing effective pharmacological therapies for HCC.

Melatonin, the hormone of the pineal gland, controls circadian rhythms, and it has been reported to exert additional functions in other organs. A large number of studies have demonstrated the protective role of melatonin in different pathophysiological situations in the liver, showing antioxidant and antiapoptotic proprieties (Pan *et al*, 2006; Subramanian *et al*, 2007; Thong-Ngam *et al*, 2007; Tahan *et al*, 2009). On the other hand, *in vitro* studies with different cancer cell lines have provided evidence for melatonin induction of apoptosis in tumour cells (Hill and Blask, 1988; Farriol *et al*, 2000; Futagami *et al*, 2001; Cini *et al*, 2005;

*Correspondence: Dr S Strand; E-mail: sstrand@uni-mainz.de

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Garcia-Santos *et al*, 2006; Garcia-Navarro *et al*, 2007; Cabrera *et al*, 2010; Chiu *et al*, 2010; Gonzalez *et al*, 2010). We have recently reported that melatonin administration induces cell cycle arrest and apoptosis in hepatocarcinoma HepG2 cells through MT1 melatonin receptor by modulation of cAMP basal levels and ERK kinase activation (Carbajo-Pescador *et al*, 2009, 2011). Furthermore, melatonin-induced apoptosis was related with enhanced caspase-3 and caspase-9 activity, cytosolic cytochrome *c* release and upregulation of the proapoptotic protein Bax (Martin-Renedo *et al*, 2008). Nevertheless, the molecular pathways that underlie melatonin-induced apoptosis in human HCC are not fully elucidated.

The FoxO subfamily of forkhead transcription factors (FoxO1/FKHR, FoxO3/FKHRL1 and FoxO4/AFX identified in mammals) plays an important role in tumour suppression by upregulating target genes involved in cell cycle arrest and apoptosis. Interestingly, low levels of FoxO3 have been reported to confer chemotherapy resistance in human cancers, being significantly associated with poor prognosis in cancer patients (Jin *et al*, 2004; Fei *et al*, 2009; Su *et al*, 2011). Moreover, enhanced activity and expression of active forms of FoxO transcription factors is required for tumour chemosensitisation (Sunters *et al*, 2003; Paroni *et al*, 2011). FoxO proteins are activated in response to a wide range of external stimuli. Regulation of its activity depends mainly on changes in the subcellular localisation, achieved via post-translational modifications, including phosphorylation, acetylation and ubiquitination (Calnan and Brunet, 2008). Several genetic and biochemical studies indicate that the FoxO family is a key downstream target of the PI3K-Akt pathway in development and longevity (Lin *et al*, 1997; Brunet *et al*, 1999). Thus, phosphorylation of FoxO factors in specific serine and/or threonine sites modulates their subcellular localisation (Rena *et al*, 2002; Barthel *et al*, 2005; Anton *et al*, 2007). Once placed in the nucleus, they play tumour suppressor roles through enhanced transcription of proapoptotic genes, such as *BCL6*, a Bcl-2-interacting mediator of cell death (Bim), and Fas ligand (Dijkers *et al*, 2000; Yang *et al*, 2006).

Bim is a proapoptotic member of the Bcl-2 family, and is one of the main downstream targets of FoxO3a. After transcription, Bim mRNA undergoes an alternate splicing, giving three isoforms (BimS, BimL and BimEL) with different length (Ewings *et al*, 2007). Interestingly, there are *in vivo* and *in vitro* evidence demonstrating an essential role of Bim proteins in Bax activation (Ren *et al*, 2010). Based on this information, we focused this study on the FoxO3a regulation of Bim expression after treatment with pharmacological concentrations of melatonin, in an attempt to gain further mechanistic insights on the molecular pathways leading to melatonin-induced apoptosis in HepG2 liver cancer cells.

MATERIALS AND METHODS

Cell culture. HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM). Primary human hepatocytes were isolated from healthy liver tissue of patients undergoing partial hepatectomy by two-step collagenase perfusion. Cells were seeded on collagen-coated culture dishes in Williams medium supplemented with 10% fetal bovine serum, 15 mmol l^{-1} HEPES (pH 7.4), 2 mmol l^{-1} glutamine, 100 U ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin. LY294002 was from Tocris (Bristol, UK). Melatonin and epidermal growth factor (EGF) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Viability assays. HepG2 cells or primary human hepatocyte were seeded in 96-well plates. Melatonin dissolved in dimethyl sulphoxide (DMSO) was added to the cells at the concentrations as indicated in the figures. Apoptosis was induced in HepG2 cells with 200 ng ml^{-1} of the monoclonal antibody (Ab) to human (APO-1/Fas) anti-APO-1, kindly provided by Peter H Krammer. Cell viability was determined using the CellTiter-Glo (Promega, Fitchburg, WI, USA) and 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays (Sigma-Aldrich). CellTiter-Glo assay was performed according to the manufacturer's instructions (Promega). Luminescence was determined in a Saphire luminometer (Tecan Austria, Grödig, Austria). The MTT assay was carried out as described by Denizot and Lang (1986). Briefly, after exposure of cells to melatonin, culture media were changed by serum-free culture media. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide dissolved in phosphate-buffered saline (PBS) was added to each well for 3 h. After this interval, the culture media containing MTT were discarded and DMSO was added to each well, dissolving the precipitate. The optical densities were measured at 560 nm spectral wavelength using microtitre plate reader (Synergy HT Multi-Mode Microplate Reader; Bio-Tek Instruments Inc., Winooski, VT, USA).

Transfection and luciferase reporter assay. Transient transfection of HepG2 human hepatocytes was performed using the TransFectin reagent (Bio-Rad, Hercules, CA, USA). Constructs used were the FHRE-Luciferase reporter (Addgene plasmid 1789 kindly provided by M Greenberg's lab) (Tran *et al*, 2002) and the FoxO3a expression construct (Addgene plasmid 8355 kindly provided by A Brunet's lab) (Brunet *et al*, 1999). Inducible activation of FoxO3a was performed through transfection of the HA-FoxO3a-WT-ER plasmid. The HA-FoxO3a-WT-ER fusion protein is constitutively expressed but is inhibited unless exposed to a modified ligand for the oestrogen receptor (ER), 4-hydroxytamoxifen (4-OHT). HepG2 cells were transfected using the TransFectin reagent (Bio-Rad, Munich, Germany) with 1 μg of HA-FoxO3a-WT-ER plasmid (Tran *et al*, 2002). Activation of the accumulated FoxO3a protein was induced by treatment with the ER ligand 4-OHT 1 h before melatonin treatment. The luciferase reporter activity was measured using a commercially available luciferase assay system (Promega). Transfection efficiency was normalised by β-galactosidase activity.

Western blot analysis. After treatments, cultured cells were washed two times with ice-cold PBS and lysed by adding ice-cold RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 10% sodium deoxycholate, 10% SDS, 1 mM NaF and protease cocktail inhibitor (Roche, Basel, Switzerland), and scraped off the plate. The extracts were transferred to a microfuge tube and centrifuged for 10 min at 15 000 g. Equal amounts of the supernatant protein (20 μg) were separately subjected to SDS–PAGE and transferred to a PVDF membrane (Bio-Rad). Primary Abs were diluted in blocking solution and incubated overnight at 4 °C with polyclonal Ab to Bim (rabbit, 1 : 1000 dilution; eBioscience, San Diego, CA, USA), phospho-FoxO3a Thr^{32} and FoxO3a Ser^{253}(rabbit, 1:1000 dilution; Cell Signalling Technology, Beverly, MA, USA) and FoxO3a (rabbit, 1:1000 dilution; Abcam, Cambridge, UK). Equal loading of protein was demonstrated by probing the membranes with a rabbit anti-β-actin polyclonal Ab (1:2000 dilution; Sigma), anti-lamin B1 (H-90) (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-tubulin (Sigma-Aldrich). After washing with PBST, membranes where incubated with phosphatase-conjugated anti-rabbit secondary Ab from Sigma-Aldrich diluted in blocking solution and incubated for 1 h at room temperature. The proteins were detected and visualised by chemiluminescence using the CDP star detection system (Tropix Applied Biosystems, Carlsbad, CA, USA). Densitometry analysis of specific bands was performed by the Scion Image software (Scion Corporation, Frederick, MD, USA).

Real-time reverse transcriptase–polymerase chain reaction. For real-time reverse transcriptase–polymerase chain reaction (RT–PCR), confluent HepG2 cells growing in complete media were replated in six-well culture plates, at a density of 150 000 cell per well in a total volume of 2 ml of complete medium. After treatment, total RNA was obtained by using a Trizol reagent (Applied Biosystems, Carlsbad, CA, USA) and quantified by spectrophotometry (Nanodrop 1000; Thermo Scientific, Waltham, MA, USA). The iScript cDNA Synthesis Kit (Bio-Rad) was used to reverse transcribe RNA into cDNA. Real-time PCR was performed using the iCycler Absolute QPCR SYBR Green Mix (ABgene, Waltham, MA, USA). Bim mRNA levels were normalised to RNA polymerase II (RpII) using the $2^{-\Delta Ct}$ method based on the threshold cycle (CT) value. Primer sequences were as follows: Bim, 5′-AACCACTATCTCAGTGCAAT-3′ and 5′-GGTCTTCGGCTG CTTGGTAA-3′; RPII, 5′-GCACCACGTCCAATGACAT-3′ and 5′-GTGCGGCTGCTTCCATAA-3′.

Small interfering RNA transfection. HepG2 cells (0.5×10^6 cells per ml) were seeded in DMEM medium without antibiotics overnight. After washing the cells with PBS, 1 ml of media without antibiotics were added. Thereafter, 200 μl of Lipofectamine 2000 complex was added into each plate. The cells were transfected with FoxO3a small interfering (siRNA) (FKHRL1 siRNA sc-37887) and Bim siRNA (sc-29802) (Santa Cruz Biotechnology) for 48 h according to the manufacturer's instructions. A non-targeting siRNA-A sc-37007 was used as a negative control. At 48 h after transfection, medium was replaced for complete DMEM and cells were treated with or without melatonin.

Chromatin immunoprecipitation assays. Chromatin-immunoprecipitation (ChIP) assays were performed using chromatin immunoprecipitation kit (Upstate Cell Signaling, Lake Placid, NY, USA) according to the manufacturer's instructions. Samples treated with the 1000 μM melatonin concentration were immunoprecipitated with anti-FoxO3a Ab (Abcam) or rabbit IgG (Sigma). Polymerase chain reaction was performed using primers specific for the Bim promoter: forward, 5'-CCTTCGCGAGGACCAACC-CAGTC-3' and reverse, 5'-CCGCTCCTACGCCCAATCACTGC-3'.

Immunofluorescence. HepG2 cells were seeded in eight-well chamber slides and then treated with melatonin as indicated. After treatment, they were fixed in 4% paraformaldehyde and stained with Abs to FoxO3a (rabbit, 1:100 dilution; Abcam). Alexa Fluor 488-labelled anti-rabbit Ab (Invitrogen, Carlsbad, CA, USA) was used as a secondary Ab. Counterstaining of nuclei was performed with Hoechst 33342 (Invitrogen). The cells were imaged directly in the chambers using a Zeiss LSM 710 NLO confocal laser scanning microscope and image analysis was performed using the Zeiss Zen-2009 software (Carl Zeiss Microimaging GmbH, Jena, Germany).

Statistical analysis. Results are expressed as mean values ± s.e.m. of the indicated number of experiments. A *t*-test was used to determine differences between pairs of treatments, as indicated in Results. One-way ANOVA followed by Student–Newmann–Keuls *post hoc* test was used to determine differences between the mean values of the different treated groups. $P<0.05$ was considered significant. Values were analysed using the statistical package Statistica 10.0 (Statsoft Inc., Tulsa, OK, USA).

RESULTS

Effect of melatonin treatment on cell viability. Most types of antitumour therapy result in a certain amount of damage to healthy tissues with associated side effects. Previously, we have shown that melatonin has oncostatic effects in HepG2 liver cancer cells, and in this study, we used healthy primary human hepatocytes to investigate the selectivity of melatonin between healthy and cancerous cells. In our experiments, 48 h melatonin treatment from 50 to 2000 μM did not significantly affect the viability of primary human hepatocytes (Figure 1A). In contrast, growth inhibition of HepG2 cancer cells under melatonin treatment was dose-dependent (40% reduction *vs* control), becoming even higher following preincubation with the human apoptosis inductor anti-APO-1 (60% reduction *vs* control) (Figure 1B). The melatonin concentration that exerted the strongest growth inhibition (1000 and 2000 μM) in HepG2 cells was used in further experiments. These results suggest that melatonin selectively protects normal primary human hepatocytes from injury during apoptosis induction. Next, we focused on elucidating the molecular pathway leading to the proapoptotic effects of melatonin in liver cancer cells.

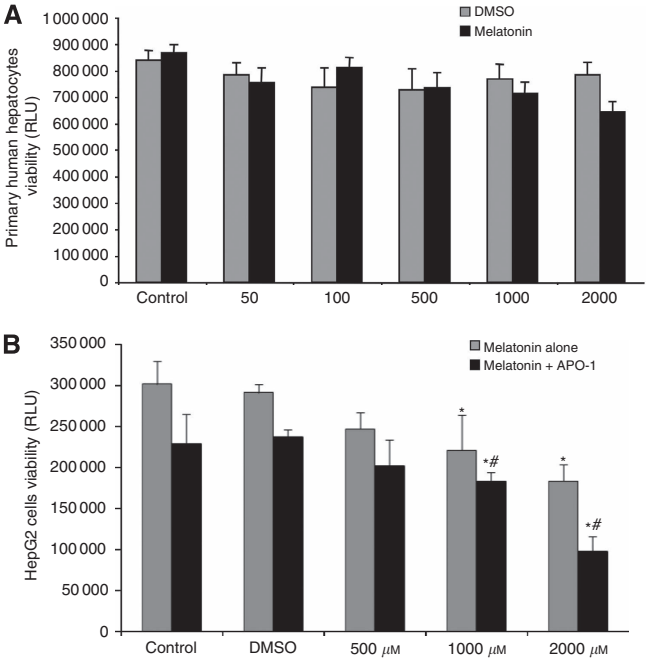

Figure 1. Effect of melatonin treatment on cell viability in primary human hepatocytes (**A**) and HepG2 cells (**B**). Data are expressed as a percentage of mean values ± s.e.m. of four experiments performed in triplicate. *$P<0.05$ significant differences *vs* control. #$P<0.05$ significant differences between melatonin and melatonin + APO-1-treated cells. Abbreviations: RLU = relative light unit.

Effect of melatonin treatment on FoxO3a transcriptional activity. FoxO transcription factors play an important role in tumour suppression. To determine whether FoxO3a was activated by melatonin treatment, HepG2 cells were transfected with a luciferase reporter constructs containing FoxO3a response element. Following a kinetic experiment from 1 to 48 h, 1000 μM melatonin incubation increased FoxO3a activity with values that represent approximately 150% of control after 24 and 48 h. Moreover, luciferase activities were more elevated with a higher concentration of 2000 μM, reaching a maximum of 173% at 48 h compared with control (Figure 2A).

Effect of melatonin treatment on Bim expression. After analysing transactivation of FoxO3a-responsive promoter elements after melatonin treatment, we studied the BH3-only protein of the Bcl-2 family Bim, a known downstream target of FoxO3a, required for the initiation of apoptosis. Both melatonin concentrations tested increased Bim EL protein level, with maximum values reached at 24 and 48 h. Western blot results were consistent with

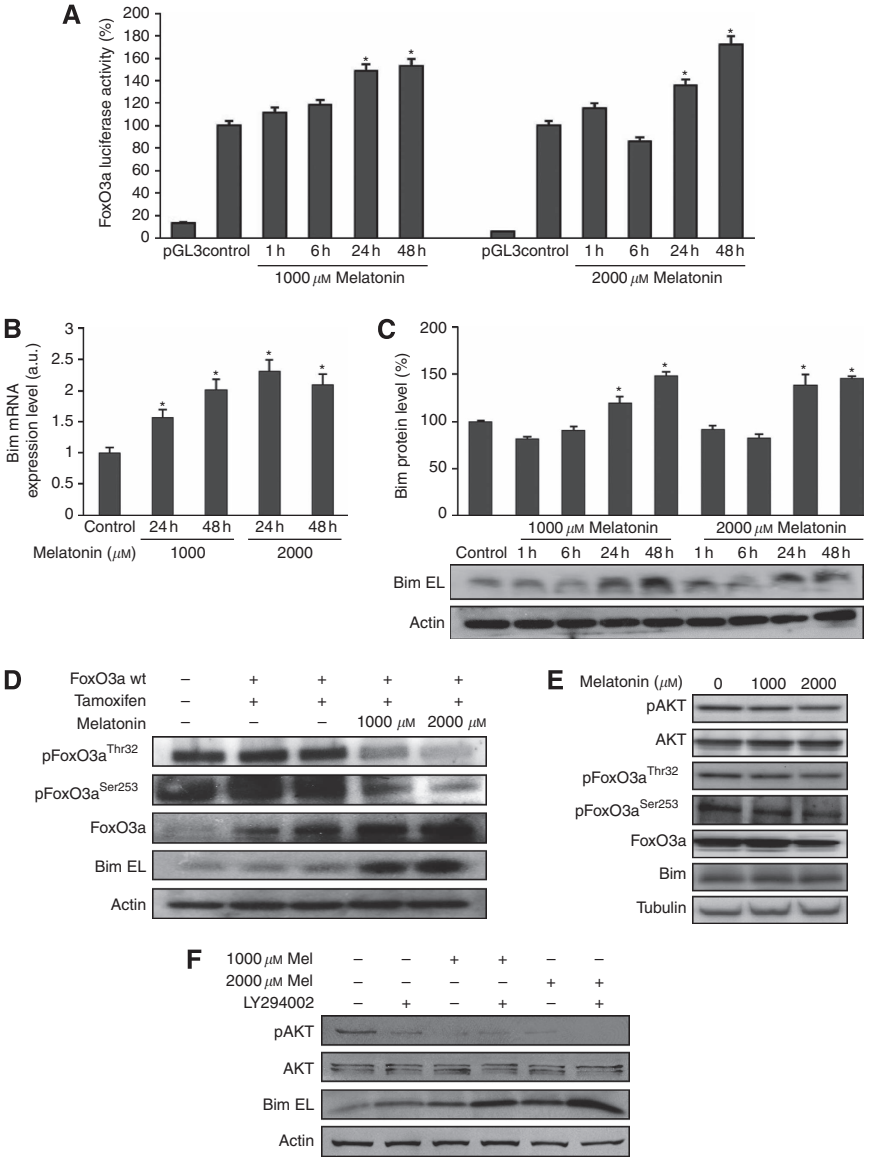

Figure 2. Consistent relation between PI3K, FoxO3a and Bim transcriptional regulation induced by melatonin administration in HepG2 cells. (**A**) Effect of melatonin treatment on FoxO3a luciferase activity. (**B**) Effect of melatonin treatment on Bim expression in HepG2 cells by real-time RT–PCR. (**C**) Melatonin induces the expression of Bim in time-dependent manner analysed by western blot. (**D**) Effect of melatonin treatment on phosphorylation status of FoxO3 and Bim EL expression. (**E**) Effect of melatonin treatment on PI3K/FoxO3/Bim EL pathway in primary human hepatocytes. (**F**) Effect of the inhibition of PI3K pathway on phospho-AKT, AKT and Bim EL expression in HepG2 cells. *$P<0.05$ significant differences vs control.

RT–PCR data, demonstrating that melatonin treatment increases Bim expression both at transcriptional and translational levels (Figures 2B and C).

Effect of melatonin treatment on the PI3K/FoxO3/Bim EL pathway. The FoxO transcription factors are targets of the PI3K signalling pathway, which regulates their activity via phosphorylation on multiple threonine and serine residues. Dephosphorylation of these specific sites is associated with FoxO3a nuclear translocation, needed for its transcriptional activity (Burgering and Kops, 2002). Having observed FoxO3a transactivation of promoter elements and Bim induction by melatonin treatment, we next investigated whether Bim protein expression correlates with the phosphorylation status of FoxO3a. HepG2 cells transfected with the FoxO3a construct were treated with 1000 and 2000 μM melatonin for 24 h. Immunobloting assays showed a reduction of the dephosphorylated forms of FoxO3a at the critical phosphorylation sites (Thr^{32} and Ser^{253}) after melatonin treatment. It is notable that, while melatonin induced hypophosphorylation of FoxO3a, it was also accompanied by an increase in the protein level of the FoxO3a total form and Bim EL proapoptotic protein (Figure 2D). Moreover, as shown in Figure 2E, melatonin treatment induced a decrease in AKT phosphorylation in the basal state. Using the PI3K inhibitor LY294002 in combination with melatonin, enhanced expression of Bim EL protein was observed (Figure 2E). No changes in PI3K/FoxO3/Bim EL pathway were observed in primary human hepatocytes when treated with melatonin (Figure 2F). Additionally, we examined the effect of melatonin on cell viability and the PI3K-Akt pathway when stimulated by EGF. As shown in Figures 3A and B melatonin treatment led to decreased Akt phosphorylation and cell viability, suggesting a consistent relation between PI3K, FoxO3a and Bim transcriptional upregulation.

Induction of FoxO3a nuclear translocation and Bim promoter occupancy after melatonin treatment. As melatonin treatment

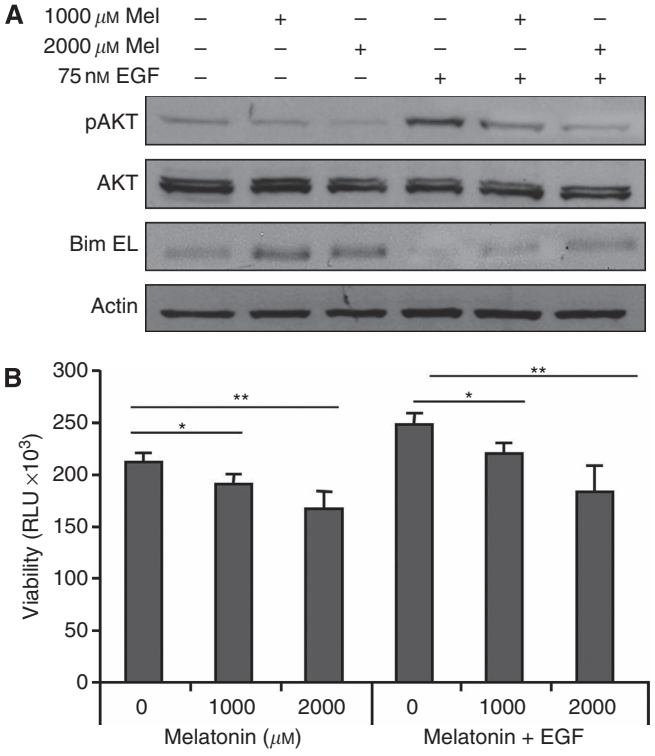

Figure 3. Melatonin is effective in cells stimulated with EGF. (**A**) Effect of melatonin on PI3K-Akt pathway stimulated by EGF. (**B**) Effect of melatonin on cell viability stimulated by EGF. Data are expressed as a percentage of mean values ± s.e.m. of three experiments. *$P<0.05$, **$P<0.01$ significant differences *vs* control. Abbreviations: p = phosphorylated; RLU = relative light unit.

enhanced FoxO3a dephosphorylation in liver cancer cells, we next examined whether changes on FoxO3a subcellular location were also induced by melatonin. By using fluorescence microscopy of HepG2 cells, we visualised the dynamic translocation of FoxO3a to the nucleus after melatonin treatment (Figure 4A). These results were consistent with FoxO3a nuclear localisation studies analysed by western blot of cytoplasmic and nuclear extracts after melatonin treatment (Figure 4B).

To explore whether FoxO3a is directly responsible for Bim induction after melatonin treatment, we performed gene silencing experiments transfecting HepG2 cells with siRNA specific for FoxO3a. As shown in Figure 4C, 1000 and 2000 μM melatonin treatment for 24 h increased Bim EL protein level, while silencing of FoxO3a abrogated the melatonin-induced expression of Bim protein as determined by western blot. Next, we investigated whether the FoxO3a occupancy of the Bim promoter was affected by melatonin through ChIP assays (Figure 4D). Our results showed that upon melatonin treatment increased levels of FoxO3a could be detected binding to the promoter of Bim. Moreover, to functionally link FoxO3a and Bim with melatonin-induced apoptosis, we examined the effect of melatonin in HepG2 cells after FoxO3a and Bim knockdown with siRNA. As shown in Figure 4E, observed melatonin effects on cell viability were partially abolished when FoxO3a and Bim EL were silenced. Taken together, these results support a functional correlation between FoxO3a transcriptional activity and the levels of Bim expression in melatonin-induced apoptosis.

DISCUSSION

Hepatocellular carcinoma is the most common liver cancer and effective therapy is still lacking (Cornella *et al*, 2011). In this study, we tested the effects of pharmacological doses of melatonin, a natural compound synthesised in the pineal gland, which has been shown to inhibit growth of different tumours (Srinivasan *et al*, 2011). The role of melatonin in increasing apoptotic cell death in cancer has been widely documented (Martin-Renedo *et al*, 2008; Cabrera *et al*, 2010; Leja-Szpak *et al*, 2010; Cutando *et al*, 2011). However, there is a wide controversy about the melatonin oncostatic concentration; thus, while melatonin oncostatic effects have been reported in ME-180 and HELA human uterine neck cancer cells, OAW-42 ovarian cancer cells, HT-29 human colon cancer cells or CT-26 mouse colon cancer cells, at a concentration range 1000–6000μM (Papazisis *et al*, 1998; Petranka *et al*, 1999; Farriol *et al*, 2000), human breast cancer MCF-7 cells or human choriocarcinoma Jar cells seem to be much more melatonin sensitive, responding to nanomolar doses (Hill and Blask, 1988; Shiu *et al*, 1999). In this respect, and having previously demonstrated that melatonin has antiproliferative and proapoptotic properties in an *in vitro* model of HCC (Carbajo-Pescador *et al*, 2009, 2011), we used non-tumour primary human hepatocytes and the human liver cancer cell line HepG2 to analyse melatonin effects on the PI3K/FoxO3/Bim EL pathway. In our experiments, melatonin treatment had no negative effects on either this pathway or cell viability in human primary hepatocytes. Meanwhile, melatonin inhibited HepG2 cell viability, and the combination of the human CD95 agonistic Ab, anti-APO-1, with melatonin enhanced the growth inhibitory effect. Similarly to our results, melatonin has been shown to exhibit protective effects against doxorubicin-induced liver toxicity in rats (Oz and Ilhan, 2006), while synergistic effects on apoptosis induction of melatonin and doxorubicin have been reported in hepatoma cells (Fan *et al*, 2010), highlighting the selectivity and beneficial properties of melatonin based on the cell type and its features.

Dysregulation of apoptosis and cellular proliferation are clearly associated with the malignant HCC phenotype; therefore, advances in understanding these signalling pathways are necessary to develop an effective pharmacological therapy for this disease (Müller *et al*, 1997). We have previously demonstrated that melatonin oncostatic effects in liver cancer are partially mediated through the MT1 membrane receptor, modulation of cAMP and ERK activation (Carbajo-Pescador *et al*, 2011). However, the precise mechanisms whereby melatonin influences apoptosis remain unclear. FoxO transcription factors play an important role in tumour suppression by upregulation of proapoptotic genes, such as *Bim* (Zanella *et al*, 2010; Tzivion *et al*, 2011). While FoxO pathways have been extensively studied in different tumour cell lines (Roy *et al*, 2011; Hong *et al*, 2012), little is known about its role in HCC. The present data show for the first time that melatonin-dependent apoptosis in HepG2 cells may be mediated, at least in part, by FoxO3a activation and subsequently increased Bim expression.

Apoptotic cell death is a complex programme mainly controlled by the Bcl-2 family proteins. We have previously reported, using HepG2 cells, an extrinsic apoptosis induction after melatonin treatment, associated with upregulation of one of these proapoptotic proteins, Bax, cytochrome *c* release and caspases activation (Martin-Renedo *et al*, 2008). However, it is known that the presence of BH3-only molecules like Bid, Bim and Puma is required for direct activation of Bax at the mitochondria. In this study, we observed an increase in Bim expression, both at mRNA and at protein levels after melatonin treatment in HepG2 cells. Upregulation and activation of Bim protein is involved in the oncostatic effect of many other chemotherapeutic drugs in liver cancer, pointing to its role in critical steps of the apoptosis initiation (Schneider-Jakob *et al*, 2010). It has been reported that Bid, Bim and Puma triple-knockout mice present developmental defects associated with deficiency of Bax. Moreover, genetic deletion in neurons and T lymphocytes prevents the

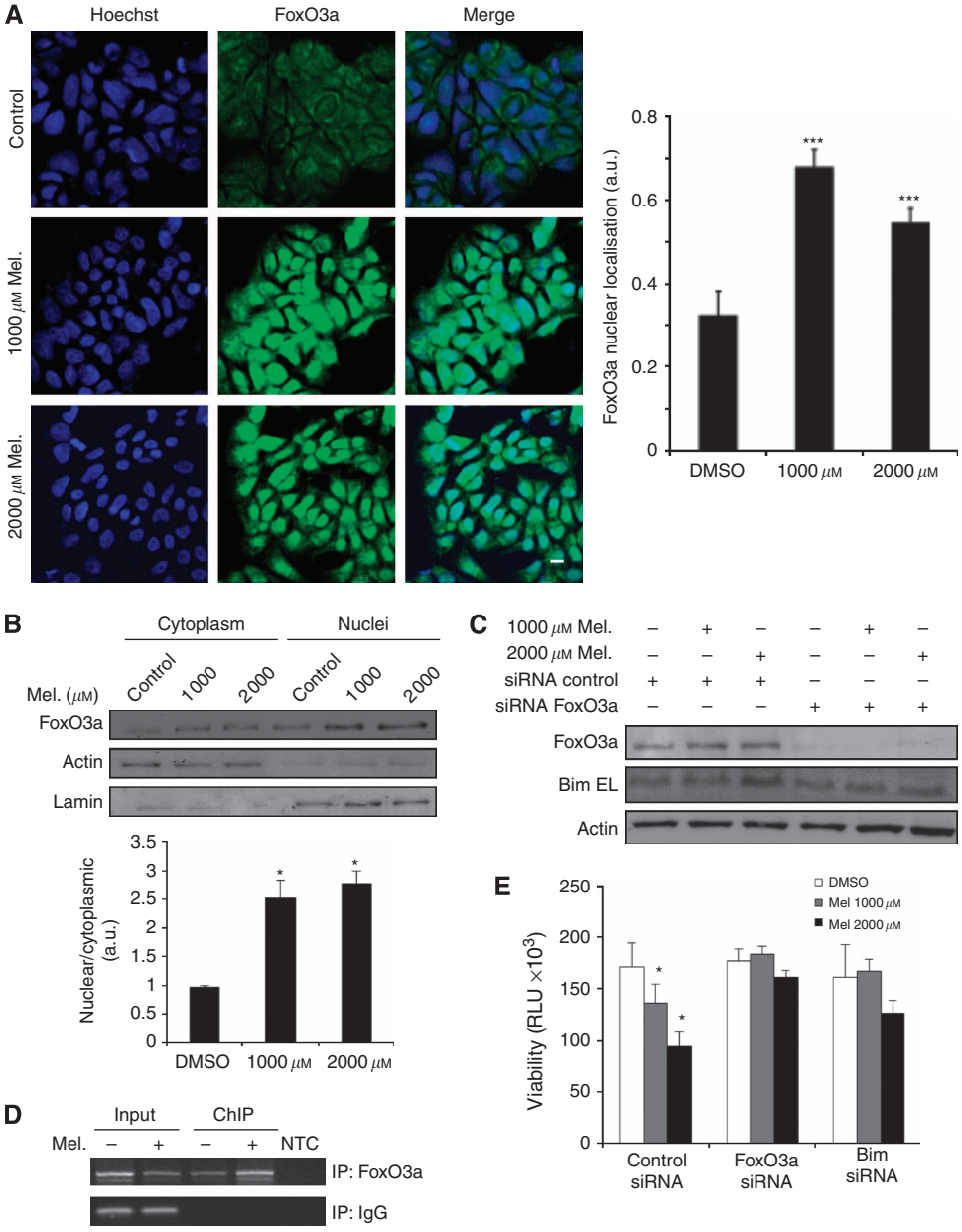

Figure 4. Induction of FoxO3a nuclear translocation and Bim promoter occupancy after melatonin treatment. (**A**) FoxO3a nuclear translocation. ***$P<0.001$ significant differences in nuclear localisation of FoxO3a in control *vs* melatonin-treated cells. Data points represent mean ± s.d. from separate high-power field images. Bar = 10 μM. (**B**) Effect of melatonin on FoxO3a cytoplasmic and nuclear protein expression. Lower panel is nuclear to cytoplasmic ratios of FoxO3a of western blot samples. (**C**) Effect of FoxO3a silencing and melatonin treatment on Bim expression. (**D**) Melatonin enhances binding of FoxO3a to Bim promoter region as analysed by ChIP. (**E**) Effect of FoxO3a and Bim silencing on HepG2 cell viability. Representative results of three individual experiments. *$P<0.05$. Abbreviations: a. u. = arbitrary units; IgG = rabbit control immunoglobulin G; NTC = non-template control; RLU = relative light unit.

homo-oligomerisation of Bax and Bad, and thereby cytochrome *c*-mediated activation of caspases in response to diverse death signals (Ren *et al*, 2010).

Although we observed an induction of Bim expression both at RNA and at protein level after melatonin treatment, FoxO3a transcriptional activity was also significantly increased from 1 to 48h with 1000 and 2000 μM melatonin incubation, suggesting a possible correlation between FoxO3a as a transcription factor of Bim in HepG2 liver cancer cells. Moreover, further experiments focused on understanding the molecular mechanism that underlay FoxO3a activation, showed a decrease in the dephosphorylated forms of FoxO3a at Thr^{32} and Ser^{253}, complemented by an increase of total FoxO3a and Bim EL in response to melatonin treatment. FoxO3a accumulation and Bim protein expression were greatly reduced upon silencing of FoxO3a, providing evidences to suggest that FoxO3a is functioning as a transcriptional regulator of Bim expression in HepG2 cells after melatonin treatment. It has been previously demonstrated that FoxO3a pathway can induce Bim expression and subsequently cell death in several cancer models, like MCF-7 breast cancer cell line (Sharma *et al*, 2011), mice xenografts model of pancreas tumour (Boreddy *et al*, 2011) and lymphoma cells (Bhalla *et al*, 2011), among others. It is generally accepted that the FoxO family is a key downstream target of the PI3K pathway (Weidinger *et al*, 2011). While phosphorylation of FoxO factors by Akt causes relocalisation of FoxO proteins from the nucleus to the cytoplasm, dephosphorylated FoxO forms

activate target genes (Hong *et al*, 2012). Therefore, our work provides clear evidence of melatonin-induced activation of FoxO3 in cells, promoting changes in its phosphorylation status. Moreover, translocation of FoxO3a to the nucleus was also confirmed by fluorescence microscopy experiments as well as by FoxO3a western blot in nuclear and cytoplasmic extracts. In this study, melatonin caused an inhibition of AKT phosphorylation even after EGF stimulation, and the PI3K inhibitor LY294002, combined with melatonin, resulted in a synergic effect enhancing Bim protein expression. Our knowledge of the mechanisms by which melatonin induces apoptosis in human HepG2 hepatoma cells are limited; however, the FoxO3a/Bim pathway has been shown to participate in apoptotic processes in response to other chemotherapeutic agents like cisplatin (Fernandez de Mattos *et al*, 2008; Yuan *et al*, 2011). Moreover, resveratrol, another antioxidant molecule, has been reported to behave as melatonin, exerting an oncostatic and proapoptotic activity in different tumour cells, including HepG2 (Hsieh *et al*, 2005; Notas *et al*, 2006). Although little is known about the resveratrol effect on the FoxO3a pathway, several groups have reported FoxO3a dephosphorylation, nuclear translocation and Bim induction after resveratrol treatment in *in vitro* cancer models (Chen *et al*, 2010; Roy *et al*, 2011), helping us to support our hypothesis.

Our study provides important information regarding the mechanisms by which melatonin regulates apoptosis through the activation of FoxO transcription factors. Taken together, all these results demonstrate that Bim plays a significant role in melatonin-induced apoptosis in HepG2 liver cancer cells, most likely through the activity of FoxO3a. Thereby, while this work could represent a significant advance for the understanding of the melatonin oncostatic pathway *in vitro*, further *in vivo* experiments are required to bridge the gap between clinical applications and to investigate whether this indol could be safely used as a therapeutic drug in HCC treatment, perhaps as an adjuvant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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3rd publication

Inhibition of VEGF expression through blockade of Hif1α and STAT3 signaling, mediates the anti-angiogenic effect of melatonin in HepG2 liver cancer cells.

FULL PAPER

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Inhibition of VEGF expression through blockade of Hif1α and STAT3 signalling mediates the anti-angiogenic effect of melatonin in HepG2 liver cancer cells

S Carbajo-Pescador[1], R Ordoñez[1], M Benet[2,3], R Jover[2,3,4], A García-Palomo[5], J L Mauriz[1,2] and J González-Gallego[1,2]

[1]Institute of Biomedicine (IBIOMED), University of León, Campus Universitario, 24071 León, Spain; [2]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Spain; [3]Experimental Hepatology Unit, Hospital La Fe, Valencia, Spain; [4]Department of Biochemistry and Molecular Biology, University of Valencia, Valencia, Spain and [5]Service of Oncology, Hospital de León, Complejo Asistencial Universitario de León, León, Spain

Background: Hepatocellular carcinoma (HCC) growth relies on angiogenesis via vascular endothelial growth factor (VEGF) release. Hypoxia within tumour environment leads to intracellular stabilisation of hypoxia inducible factor 1 alpha (Hif1α) and signal transducer and activator of transcription (STAT3). Melatonin induces apoptosis in HCC, and shows anti-angiogenic features in several tumours. In this study, we used human HepG2 liver cancer cells as an *in vitro* model to investigate the anti-angiogenic effects of melatonin.

Methods: HepG2 cells were treated with melatonin under normoxic or $CoCl_2$-induced hypoxia. Gene expression was analysed by RT–qPCR and western blot. Melatonin-induced anti-angiogenic activity was confirmed by *in vivo* human umbilical vein endothelial cells (HUVECs) tube formation assay. Secreted VEGF was measured by ELISA. Immunofluorescence was performed to analyse Hif1α cellular localisation. Physical interaction between Hif1α and its co-activators was analysed by immunoprecipitation and chromatin immunoprecipitation (ChIP).

Results: Melatonin at a pharmacological concentration (1 mM) decreases cellular and secreted VEGF levels, and prevents HUVECs tube formation under hypoxia, associated with a reduction in Hif1α protein expression, nuclear localisation, and transcriptional activity. While hypoxia increases phospho-STAT3, Hif1α, and CBP/p300 recruitment as a transcriptional complex within the VEGF promoter, melatonin 1 mM decreases their physical interaction. Melatonin and the selective STAT3 inhibitor Stattic show a synergic effect on Hif1α, STAT3, and VEGF expression.

Conclusion: Melatonin exerts an anti-angiogenic activity in HepG2 cells by interfering with the transcriptional activation of VEGF, via Hif1α and STAT3. Our results provide evidence to consider this indole as a powerful anti-angiogenic agent for HCC treatment.

Over half a million new cases of hepatocellular carcinoma (HCC) are diagnosed each year, being the main malignant hepatobiliary disease and the third cause of cancer-related death worldwide (Jemal *et al*, 2011). Hepatocellular carcinoma is associated with cirrhosis and hepatic dysfunction in 80% of patients, which makes its prognosis and treatment more difficult than in many other cancers (Sengupta and Siddiqi, 2012). Dysregulation of cellular proliferation, apoptosis, and angiogenesis is frequently associated with HCC development and progression, and understanding these molecular pathways becomes essential to the development of

*Correspondence: Professor J González-Gallego, E-mail: jgonga@unileon.es

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effective therapies (Cornella *et al*, 2011). Like some other solid tumours, HCC growth relies on new vessels formation, required for nutrients and oxygen supply (Hanahan and Weinberg, 2011). Incipient success of anti-angiogenic therapy in similar tumours (Hurwitz *et al*, 2004; Johnson *et al*, 2004) suggests that adjuvant anti-angiogenic agents combined with chemotherapy or radiotherapy may be particularly attractive approaches to target tumour and endothelial cells, and thus, to enhance treatment efficacy (Sun and Tang, 2004).

Deficit of oxygen availability within the tumour microenvironment is highly associated with tumour progression (Albini *et al*, 2012). Oxygen homeostasis is directly regulated via hypoxia inducible factor 1 (Hif1α). Whereas normoxia leads to its ubiquitination and subsequent proteasomal degradation, under hypoxia, Hif1α is stabilised and able to translocate to the nucleus, where it induces the expression of several genes such as erythropoietin (EPO), nitric oxide synthases, and vascular endothelial growth factor (VEGF) (Vaupel, 2004). Vascular endothelial growth factor is a secreted protein that acts as a potent mitogen for vascular endothelial cells and some other cell types, being one of the main regulatory factors in angiogenesis and neovascularisation (Ferrara *et al*, 2003). Moreover, VEGF appears frequently hyperexpressed in HCC tissues, which consistently correlates with tumour size and histologic tumour grade (Tischer *et al*, 1991). Signal transducer and activator of transcription 3 (STAT3) is a well-known oncogene in HCC and in some other tumour types (Ji and Wang, 2012). Besides its participation in normal physiological processes, it has been found constitutively activated in cancers, transcriptionally activating oncogenes encoding for apoptosis inhibitors and cell-cycle regulators, such as Bcl-x(L), cyclin D1, and c-Myc (Wang *et al*, 2012). Many studies suggest that, like Hif1α, STAT3 also behaves as an angiogenesis inductor involved in VEGF expression; activated via phosphorylation, it enhances Hif1α stability and acts as a co-activator under hypoxia (Jung *et al*, 2005). Interestingly, both STAT3 and Hif1α have been associated in mediating VEGF transcription, and the presence of regulatory sites located in high proximity within its promoter suggests their close cooperation in the transcriptional regulation of this growth factor (Niu *et al*, 2008).

Nowadays melatonin, the main product of the pineal gland, has attracted increasing attention because of its protective role in several pathophysiological situations, including different cancer types, where it exerts oncostatic effects (Hill and Blask, 1988; Farriol *et al*, 2000; Futagami *et al*, 2001; Cini *et al*, 2005; Garcia-Santos *et al*, 2006; Garcia-Navarro *et al*, 2007; Mauriz *et al*, 2007; Cabrera *et al*, 2010; Chiu *et al*, 2010; Gonzalez *et al*, 2010; Carbajo-Pescador *et al*, 2011, 2013). Our group has shown the melatonin oncostatic activity in HepG2 liver tumour cells, highlighting its pro-apoptotic properties through upregulation of cell death-related processes (Martin-Renedo *et al*, 2008; Carbajo-Pescador *et al*, 2009, 2011, 2013). In addition, it has been recently reported a mechanism by which melatonin sensitises human hepatoma cells to endoplasmic reticulum stress and induces apoptosis by downregulating COX-2 expression, increasing the levels of CHOP and decreasing the Bcl-2/Bax ratio (Zha *et al*, 2012). Melatonin anti-angiogenic properties have been reported both in *in vivo* (Cho *et al*, 2011; Cui *et al*, 2012) and *in vitro* models, implementing the existing knowledge regarding to its oncostatic role (Kim *et al*, 2013). Moreover, a significant decline in the serum levels of VEGF has been found in metastatic cancer patients with different histotypes, including HCC, when treated with melatonin, which suggests that its ability to control tumour growth could be related, at least in part, to its anti-angiogenic features (Lissoni *et al*, 2001). However, the precise mechanism that underlies melatonin anti-angiogenic effects in HCC has not been fully elucidated. Herein, the present research was aimed to assess melatonin action on tumour angiogenesis in an *in vitro* model of HCC, focusing on its ability to interfere with the transcriptional activation of VEGF via Hif1 and STAT3.

MATERIALS AND METHODS

Cell culture. Human HepG2 hepatocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Stock cells routinely were grown as monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), glutamine (4 mM), and pyruvate (100 μg ml^{-1}) in a humidified 5% CO_2 atmosphere at 37 °C and the medium was changed every other day. Cell culture reagents were from Gibco (Life Technologies, Madrid, Spain). Melatonin was obtained from Sigma (St Louis, MO, USA). Confluent HepG2 cells growing in complete media were replated in 9.6 cm^2 culture dishes, at a density of 150 000 cells/plate, in 2 ml of complete medium. After 24 h, the plating medium was replaced with fresh medium containing melatonin dissolved in DMSO (0.2% DMSO final concentration in the plate). Two melatonin concentrations were tested, 1 nM (within the 0.3–1.2 nM physiological range), and 1 mM as a supraphysiological/pharmacological concentration (Cui *et al*, 2012). $CoCl_2$ was added at a final concentration of 100 μM to mimic hypoxia. Among all the divalent metals that act as hypoxic mimetics, $CoCl_2$ has shown to induce hypoxia, increasing Hif1α stability by antagonising Fe^{+2}, an essential cofactor required for oxygen to interact with prolyl hydroxylases (PHDs) and degradate Hif1α (Bansal, 2009). Besides, our dose of cobalt has been previously used to induce hypoxia with non-toxic effects (Dai *et al*, 2008). For inhibition studies, hepatocytes were incubated in the presence or absence of 10 μM Stattic (Tocris, Bristol, UK) for 1 h before treatment.

Western blot analysis. After treatments, cultured cells were washed twice with ice-cold PBS and lysed by adding ice-cold RIPA buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 10% sodium deoxycholate, 10% SDS, 1 mM NaF and protease cocktail inhibitor (Roche, Basel, Switzerland) and scraped off the plate. The extract was transferred to a microfuge tube and centrifuged for 10 min at 15 000 **g**. Equal amounts of the supernatant protein (20 μg) were separately subjected to SDS–PAGE and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Primary antibodies were diluted in blocking solution and incubated overnight at 4 °C with polyclonal antibody to VEGF (rabbit, 1 : 1000 dilution; Abcam, Cambridge, UK), Hif1α (rabbit, 1 : 1000 dilution; Abcam), Phospho-Stat3 (Tyr705) (D3A7) from mouse and Stat3 from rabbit 1 : 100 dilution (Santa Cruz, Santa Cruz, CA, USA). Equal loading of protein was demonstrated by probing the membranes with a rabbit anti β-actin polyclonal antibody (1 : 2000 dilution; Sigma). After washing with TBST, the membranes were incubated for 1 h at room temperature with secondary horseradish peroxidase (HRP)-conjugated antibody (1 : 4000; Dako, Glostrup, Denmark) and visualised using ECL detection kit (Amersham Pharmacia, Uppsala, Sweden). The density of the specific bands was quantified with an imaging densitometer (Scion Image, Maryland, MA, USA).

Real-time reverse transcriptase PCR. For real-time reverse transcriptase PCR (RT–PCR), confluent HepG2 cells growing in complete media were replated in 6-well culture plate, at a density of 150 000 cell/well in a total volume of 2 ml of complete medium. After treatment, total RNA was obtained by using a Trizol reagent (Applied Biosystems, Carlsbad, CA, USA) and quantified by spectrophotometry (Nanodrop 1000; Thermo Scientific, Waltham, MA, USA). Residual genomic DNA was removed by incubating RNA with RQ1 RNase-free DNase (Promega, Madison, WI, USA). First-strand cDNA was synthesised using M-MLV RT (Roche Molecular Systems), and the negative control (no transcriptase

control) was performed in parallel. cDNA was amplified using FastSt art TaqMan Probe Master (Roche) on a StepOnePlus Real-Time PCR Systems (Applied Biosystems). TaqMan primers and probes for Hif1α (NM_001530 and Hs00153153_m1) and VEGF (NM_001025366 and Hs00900055_m1) genes were derived from the commercially available TaqMan Gene Expression Assays (Applied Biosystems). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Crespo *et al*, 2008). The cycle number at which the transcripts were detectable (CT) was normalised to the cycle number of β-actin detection.

Tube formation assay. Basement membrane extracellular matrix (Matrigel, BME, BD Bioscience, San Jose, CA, USA) was thawed at 4 °C overnight. A 96-well plate and 200 μl pipette tips were also kept at 4 °C overnight and both the plate and tips were placed on ice during the entire experiment. In all, 50 μl of Matrigel was loaded in each well of the 96-well plate and the plate was incubated at 37 °C in a tissue culture incubator for 30 min to allow the matrix to polymerise. Trypsinised human umbilical vein endothelial cells (HUVECs) from a primary culture with rare or non expression of endogenous VEGF were mixed with EBM-2 supplemented with VEGF (20 ng ml^{-1}) or the conditioned media (CM) from HepG2 cells incubated under hypoxia ($CoCl_2$) with or without the pharmacologic concentration (1 mM) of melatonin for 24 h, making the appropriate cell density (25 000 cells/well) and added on top of the gel in the 96-well plate. The plate was then incubated at 37 °C in a tissue culture incubator and the formation of the capillary-like tubes was observed after 6 h. Wells were imaged using a Nikon microscope. Quantification of tube formation was assisted by S.CORE, a web-based image analysis system (S.CO BioLifescience, Munich, Germany). Tube formation indices represent the degree of tube formation. The indices were calculated using the following equation: (tube formation index) = (mean single tube index)^2 × (1 − confluent area) × (number of branching points/total length skeleton). The values of the variables used in the equation were obtained automatically by S.CORE.

Quantitation of VEGF production. Media were collected from 200 000 cells in 6-well culture plates and centrifuged at 800 r.p.m. for 4 min at 4 °C to remove cellular debris and then stored at 70 °C. Vascular endothelial growth factor in the medium was measured by using the Quantikine human VEGF ELISA kit from R&D Systems (Minneapolis, MN, USA) according to manufacturer's instruction.

Hif1α transcription activity assay. The Hif1α transcriptional activity was analysed by Hif1α transcription factor assay using TransAM HIF-1 transcription factor assay kit (Active Motif, Carlsbad, CA, USA) according to manufacturer's instructions. Briefly, nuclear extracts were added onto 96-well microplate coated with oligonucleotides containing hypoxia response element (HRE) (5′-TACGTGCT-3′) from the EPO gene. The HIF dimers present in nuclear extracts bind with high specificity to this response element and are subsequently detected with an antibody directed against Hif1α. Addition of a secondary antibody conjugated to HRP provides a sensitive colorimetric readout that is easily quantified by spectrophotometry. The HeLa-$CoCl_2$ nuclear extracts provided with the commercial kit were used as a positive control of Hif1α transcriptional activity. Values are expressed as optical density (OD) at 450 nm with a reference wavelength of 655 nm.

Co-immunoprecipitation. HepG2 cells were washed with ice-cold PBS and lysed in buffer containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% NP-40, 1 mM NaF, 1 mM Na_3VO_4, and EDTA-Free Halt Protease Inhibitor Cocktail (Thermo Scientific Pierce, Rockford, IL, USA) for 30 min on ice. Total cell lysates (1 mg of protein) were subjected to immunoprecipitation with 2 μg of anti-phospho0-STAT3 (Santa Cruz), anti-Hif1α (Abcam), and anti-Acetyl-CBP (Lys1535)/p300 (Lys1499) (Cell Signaling Technology, Danvers, MA, USA) for 1 h at 4 °C. Protein G Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was added and incubation continued overnight at 4 °C. Precipitates were washed three times with ice-cold lysis buffer, resuspended in Laemmli buffer, and boiled for 10 min. Bound proteins were separated on an SDS–polyacrylamide gel and analysed by western blotting using the indicated antibodies.

ChIP assay. Chromatin of cultured cells was fixed and immunoprecipitated according to Benet *et al* (2010). Cells were incubated with 100 μM $CoCl_2$, 1 mM melatonin and both together for 24-h period. Then, cells were treated with 1% formaldehyde in PBS buffer by gentle agitation for 10 min at room temperature to crosslink proteins to DNA. Next, cells were washed, resuspended in lysis buffer and sonicated on ice for 8 × 15 s steps at a 20% output in a Branson Sonicator. Sonicated samples were centrifuged to clear supernatants. DNA content was quantified by picogreen (Life Technologies) and properly diluted to maintain an equivalent amount of DNA in all the samples (input DNA). For the immunoprecipitation of protein–DNA complexes, 4 μg of specific antibody (anti-HIF1α NB100-134; NOVUS Biologicals, Littleton, CO, USA) and rabbit pre-immune IgG (sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (background DNA fraction) were added. Samples were incubated overnight at 4 °C on a 360° rotator (antibody-bound DNA fraction). Immunocomplexes were affinity absorbed with 60 μl of protein G agarose/Salmon Sperm DNA (Millipore, Billerica, MA, USA) (pre-washed with lysis buffer for 1 h at 4 °C by gentle rotation), and collected by centrifugation (1000 r.p.m., 1 min). The antibody-bound and background DNA fractions were washed as described in Benet *et al* (2010). Crosslinks were reversed by adding 100 μl of 10% Chelex (Bio-Rad Laboratories) and boiling for 10 min. The Chelex/protein G bead suspensions were incubated with proteinase K (20 mg ml^{-1}) for 30 min at 55 °C while shaking, followed by another 10 min boiling. Suspensions were centrifuged and supernatants were collected. The eluates were used directly (input 1/5) as a template for Q-PCR with a LightCycler 480 instrument. Amplification was real-time monitored, stopped in the exponential phase of amplification and analysed by agarose gel electrophoresis. Amplifications of the VEGF gene sequences among the pull of DNA were performed with specific primers flanking from −1041 to −750 region, forward 5′-CAGGAACAAGGGCCTCTGTCT-3′, reverse 5′-TGTCCCTCTGACAATGTGCCATC-3′. The PCR conditions for the VEGF promoter region were 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C. The amplification of the VEGF promoter region was analysed after 35 cycles.

Immunofluorescence analysis. To study the localisation of Hif1α, staining of Hif1α was performed on HepG2 cells. Cells were grown on coverslips for 24 h. Thereafter, cells were treated with melatonin as indicated. Cells were washed with PBS and fixed with 4% paraformaldehyde, permeabilised with 0.5% Triton X-100 in PBS, and pre-treated with blocking solution. After fixation and after blocking the non-specific binding, the coverslips were incubated with rabbit anti Hif1α (HIF1α NB100-134, NOVUS Biologicals) antibodies at 4 °C overnight. Thereafter, the secondary antibodies donkey anti-rabbit conjugated with FITC (Jackson Immuno Research, Baltimore, PA, USA). After washing, the coverslips were mounted on DakoCytomation Fluorescent Mounting Medium (DAKO). The preparations were analysed with an inverted fluorescence microscope (Nikon Eclipse Ti, Melville, NY, USA). Image analysis was performed using the ImageJ software v3.91 (http://rsb.info.nih.gov/ij). To quantify Hif1α nuclear translocation, results from fluorescence colocalisation studies were represented graphically in scatterplots where the intensity of one colour was plotted against the intensity of the second colour for each pixel. Nuclear regions were defined as 'region of interest' (ROI) to determine Hif1α nuclear translocation, and the Pearson's

correlation coefficient (PCC) was used as a statistic for quantifying colocalisation.

Statistical analysis. Results are expressed as mean values ± s.e.m. of the indicated number of experiments. A *t*-test was used to determinate differences between pairs of treatments, as indicate in results. One-way ANOVA followed by Student–Newmann–Keuls *post hoc* test was used to determine differences between the mean values of the different treated groups. $P<0.05$ was considered as significant. Values were analysed using the statistical package Statistica 10.0 (Statsoft Inc, Tulsa OK, USA).

RESULTS

Effect of melatonin on VEGF levels and hypoxia-induced angiogenesis. Oxygen deficiency is a hallmark of solid tumours that drives VEGF production and angiogenesis. To determine the effect of oxygen levels on angiogenesis-related factors in our *in vitro* model of HCC, HepG2 cells were incubated in normoxia or exposed to $CoCl_2$ (100 μM) as a hypoxia mimetic in a kinetic experiment from 2 to 24 h.

As shown in Figure 1A, there was a hypoxia-dependent VEGF induction from the first 2 h of treatment, and an increase in the protein levels of Hif1α and phospho-STAT3 which reached a maximum at 24 h, time at which the inhibitory effect of the pharmacological melatonin concentration (1 mM) resulted more effective. Once our experimental conditions were set up and the use of $CoCl_2$ as an effective hypoxia inductor confirmed, the 24-h time point was chosen for further experiments.

Beside growth factors and interleukins, hypoxia is the major VEGF inductor, which stimulates new capillary vessels formation to counteract low oxygen tension. Thus, in our experiments, 24 h of 100 μM $CoCl_2$ treatment effectively enhanced VEGF mRNA levels (Figure 1B). Significant increases in VEGF protein expression and in the amount of VEGF released into the culture medium were also observed (Figures 1C and D). Moreover, as shown in Figure 1, melatonin did not exert a significant effect on protein levels in normoxia, only affecting the amount of secreted VEGF under these conditions. By contrast, melatonin treatment did clearly reduce hypoxia-induced VEGF expression and release to the medium.

To further confirm melatonin anti-angiogenic activity suggested by the induced decrease of VEGF levels under hypoxia, HUVECs tube formation assay was performed. HepG2 cells were cultured under conditions of $CoCl_2$-induced hypoxia with or without the pharmacological concentration of melatonin (1 mM) for 24 h, and CM were applied to HUVECs in a series of angiogenic assays. As show in Figure 1E, hypoxia induced HUVECs to display their typical morphology and phenotype of endothelial cells, whereas this effect was prevented by melatonin treatment.

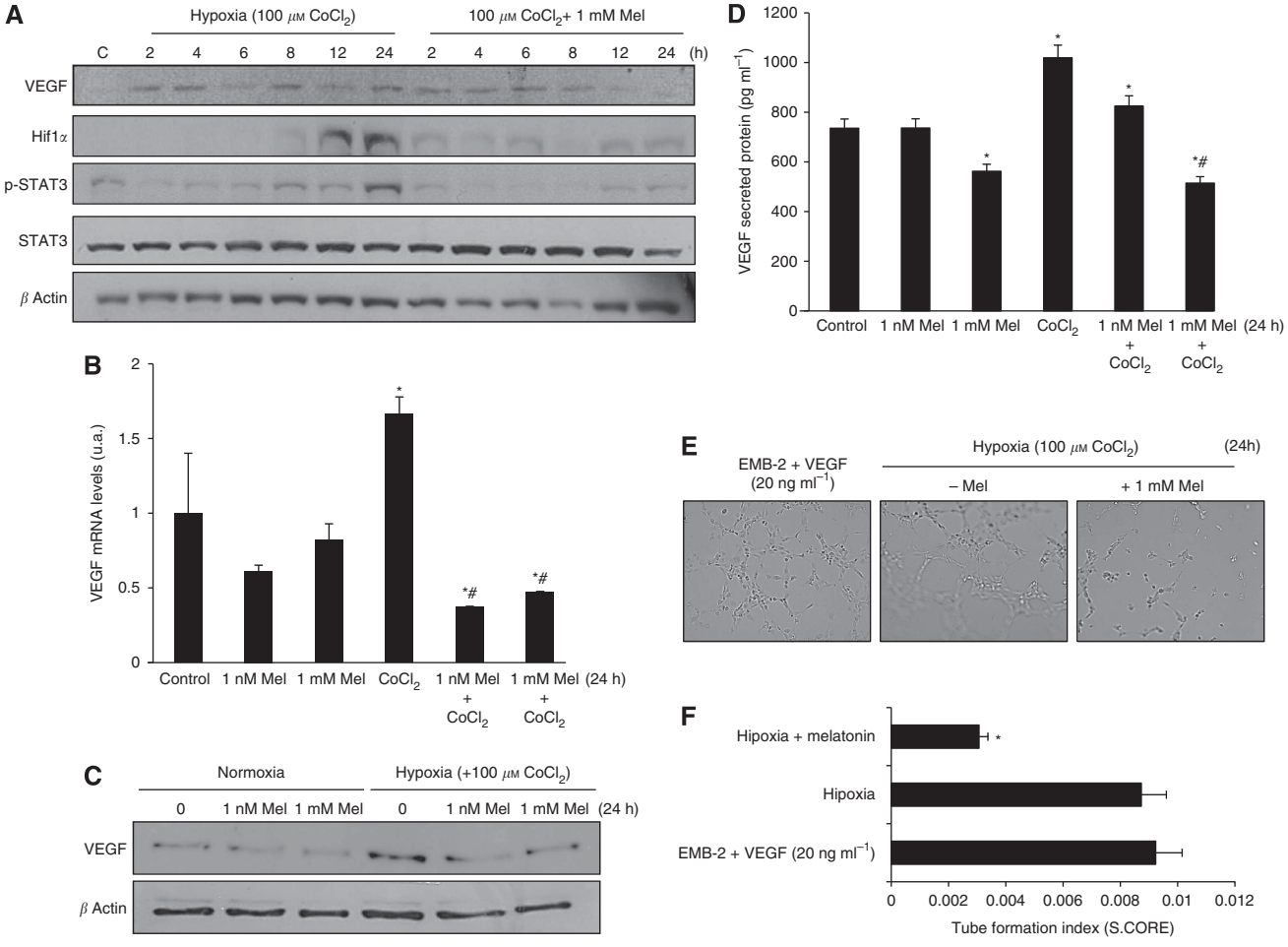

Figure 1. Effect of melatonin on VEGF levels and hypoxia-induced angiogenesis. HepG2 cells were incubated in normoxia or exposed to $CoCl_2$ (100 μM) as a hypoxia mimetic in a kinetic experiment from 2 to 24 h, and VEGF, Hif1α phospho-STAT3, and STAT3 protein expression was analysed by western blot (**A**). Effect of normoxia/hypoxia and melatonin treatments on VEGF mRNA levels (**B**), VEGF cell protein levels (**C**) and VEGF secreted to the culture media (**D**). HUVECs tube formation assay (**E**). Tube formation index (S.CORE) (**F**). Data are expressed as a percentage of mean values ± s.e.m. of four experiments performed in triplicate. *$p<0.05$ significant differences *vs* control. #$P<0.05$ significant differences between $CoCl_2$ and melatonin + $CoCl_2$-treated cells.

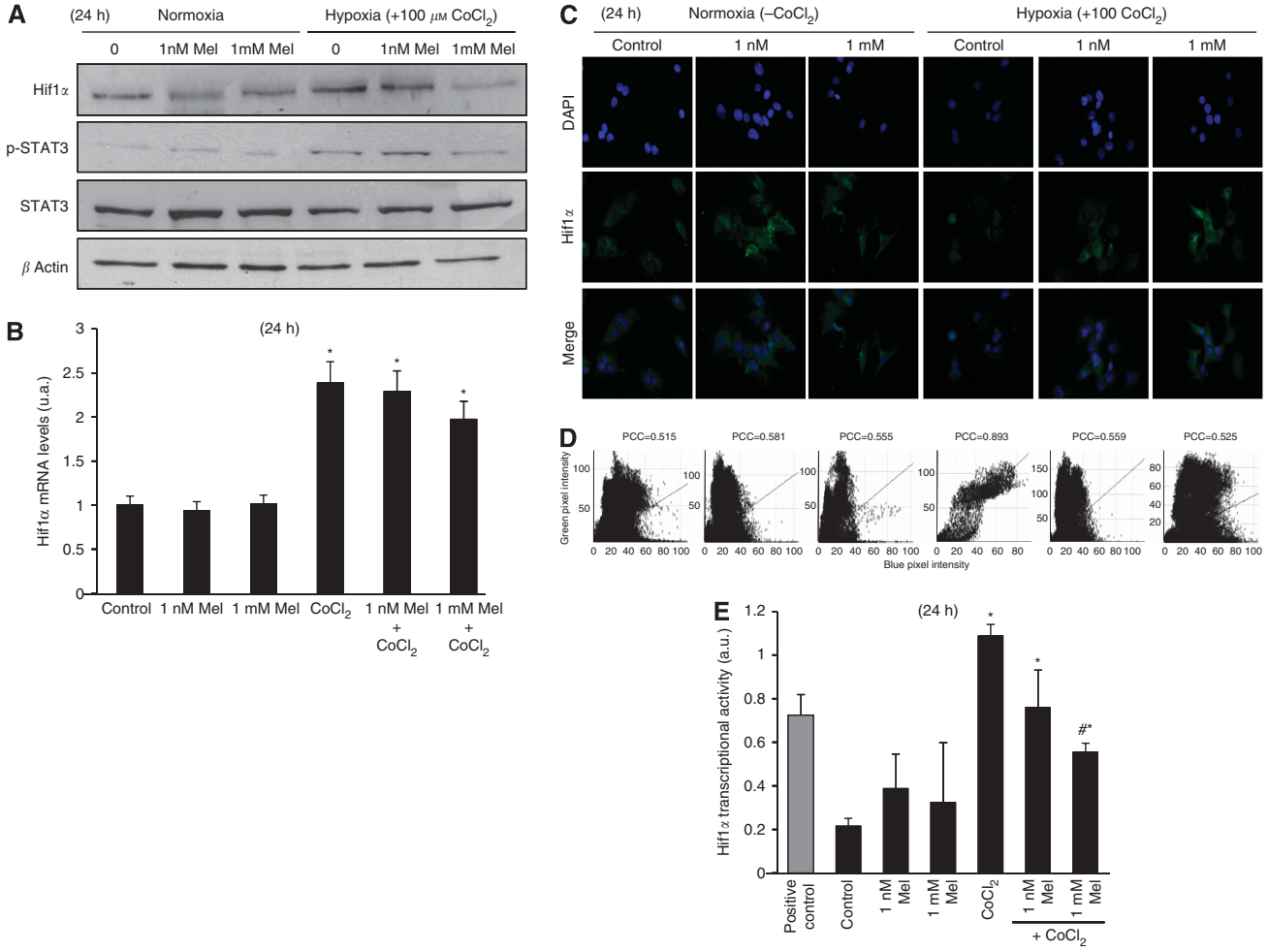

Figure 2. Melatonin inhibits hypoxia-induced Hif1α activation. Effect of normoxia/hypoxia and melatonin treatments on protein levels (**A**), Hif1α mRNA levels (**B**), and Hif1α nuclear translocation (**C**). Scatterplots of green and blue green pixel intensities and Pearsońs correlation coefficient (PCC) (**D**). Effect of melatonin treatment on Hif1α transcriptional activity. HeLa cell nuclear extracts treated with $CoCl_2$ provided with the kit were used as a positive control for Hif1α activation (**E**). Data are expressed as a percentage of mean values ± s.e.m. of four experiments performed in triplicate. *$p<0.05$ significant differences *vs* control. #$P<0.05$ significant differences between $CoCl_2$ and melatonin + $CoCl_2$-treated cells.

Melatonin inhibits hypoxia-induced Hif1α activation. Once shown that melatonin anti-angiogenic activity is related with its ability to modulate VEGF levels, we next focused on elucidating the molecular mechanisms involved. Assuming that Hif1α is the major regulator of this endothelial growth factor, Hif1α mRNA levels and protein expression were measured in HepG2 cells exposed to normoxia or hypoxia and melatonin treatment. As expected, Hif1α transcription was induced by $CoCl_2$ treatment, as shown by the increases in both mRNA and protein level. Similarly to the effects found on VEGF, only the 1 mM melatonin dose exerted an inhibitory effect on Hif1α-induced expression. However, while Hif1α protein expression was inhibited (Figure 2A), Hif1α mRNA levels did not decrease significantly after melatonin treatment (Figure 2B), even under hypoxia, suggesting that melatonin effects takes place at a post-transcriptional level.

It is widely accepted that to transcriptionally activate its target genes, Hif1α needs to translocate into the nucleus. Thus, we visualised its dynamic translocation by using fluorescence microscopy of HepG2 untreated or treated cells under normoxia or induced hypoxia. As show in Figure 2C, under normoxia, Hif1α was always located in the cytosol, and melatonin treatment did not affect its location. However, $CoCl_2$ treatment induced Hif1α nuclear translocation, an effect that was prevented by melatonin 1 mM. Furthermore, results were consistent with those observed when measuring Hif1α ability to specifically bind the HRE. As shown in figure 2D, hypoxia significantly enhanced Hif1α transcription, while melatonin inhibited its activation in a dose-dependent manner.

Melatonin prevents hypoxia recruited STAT3/Hif1α/CBP/p300 within VEGF promoter. STAT3 is an essential mediator of VEGF transcription by direct binding to its promoter (Niu *et al*, 2008). Furthermore, it induces Hif1α stability and enhances its transcriptional activity, behaving as a co-activator and conforming a transcriptional complex together with CBP/p300 (Gray *et al*, 2005). Considering the close relationship between Hif1α and STAT3, we decided to analyse whether effects on Hif1α and VEGF expression could be mediated via STAT3 inhibition. As shown in Figure 2A, $CoCl_2$ treatment resulted in STAT3 activation by phosphorylation at Tyr 705 residue, without affecting total STAT3 levels. However, this effect was prevented by incubation with melatonin 1 mM. Furthermore, melatonin effects under hypoxia were similar to those observed when HepG2 cells were exposed to the selective STAT3 inhibitor, Stattic (Figures 3A and B), and combined treatment with both melatonin and Stattic resulted in a synergic inhibitory effect on Hif1α and phospho-STAT3 activation and VEGF expression.

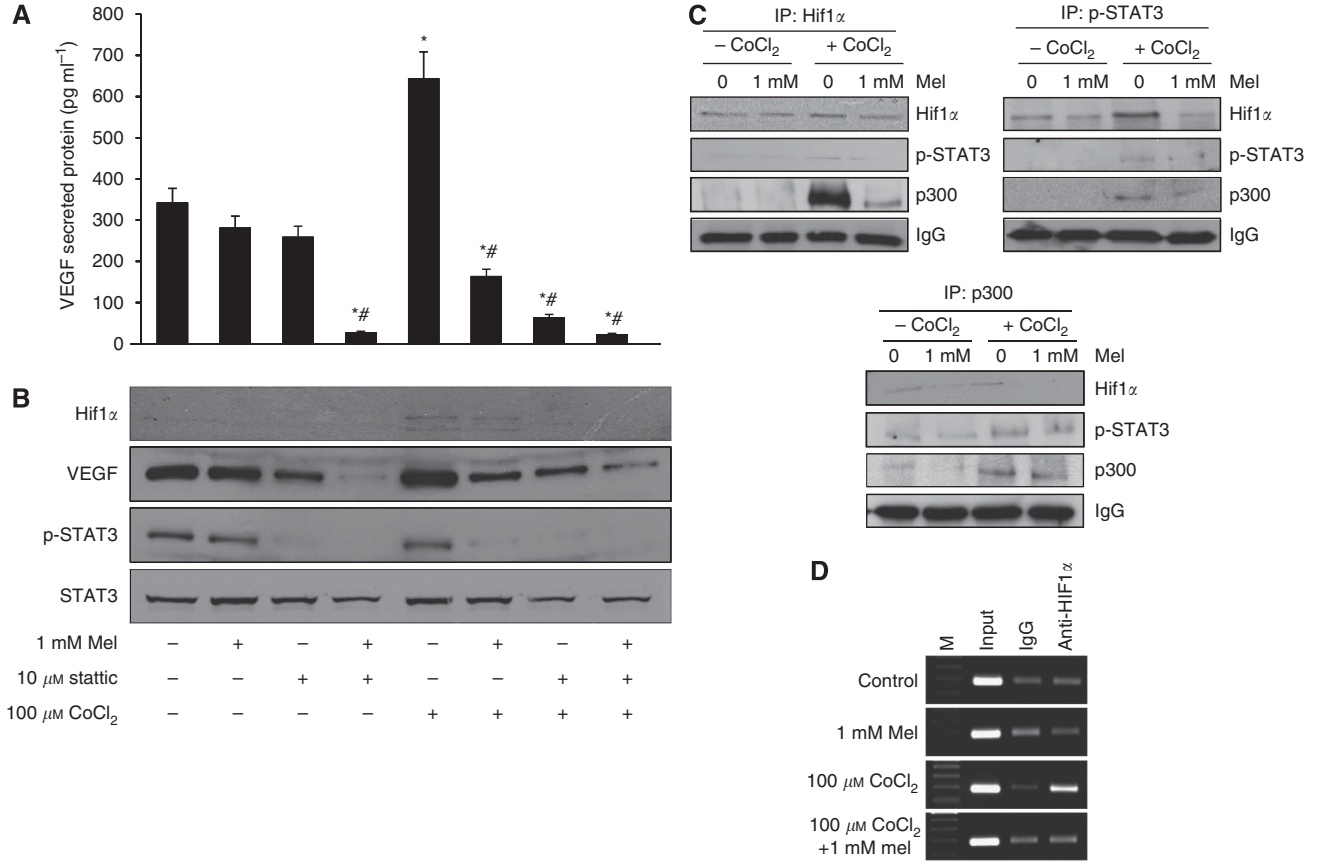

Figure 3. Melatonin prevents hypoxia recruited STAT3/Hif1α/CBP/p300 within VEGF promoter. VEGF secreted protein from HepG2 cells under normoxia/hypoxia, with or without melatonin treatment and exposed to the selective STAT3 inhibitor, Stattic (**A**). Hif1α, phospho-STAT3, STAT3, and VEGF protein expression in HepG2 cells under normoxia/hypoxia, with or without melatonin treatment and exposed to the selective STAT3 inhibitor, Stattic (**B**). Effect of hypoxia and/or melatonin treatment on Hif1α, phospho-STAT3, and CBP/p300 association measured by co-immunoprecipitation (**C**). Effect of melatonin on Hif1α binding to VEGF promoter region analysed by ChIP. M: marker; IgG: Rabbit control IgG (**D**). Data are expressed as a percentage of mean values ± s.e.m. of four experiments performed in triplicate. $^{*}p<0.05$ significant differences *vs* control. $^{\#}P<0.05$ significant differences between $CoCl_2$ and melatonin + $CoCl_2$-treated cells.

Since both functional Hif1α and phospho-STAT3 are known to be required for high VEGF activity (Jung *et al*, 2005), and owing to the proximity of their binding sites within VEGF promoter, they are assumed to share a transcriptional complex together with CBP/p300. To analyse melatonin ability to disrupt the stability of this complex, we performed co-immunoprecipitation assay. As shown in Figure 3C $CoCl_2$ treatment resulted in increased association between Hif1α, phospho-STAT3, and CBP/p300, confirming that they are likely linked. Furthermore, our results suggest that melatonin treatment inhibited this association and thus, VEGF transcriptional activation.

Although our data support the idea that melatonin prevents VEGF synthesis by blocking its transcription in HepG2 cells, a chromatin immunoprecipitation (ChIP) assay was performed under normoxia and hypoxia to investigate whether Hif1α occupancy of the VEGF promoter was affected by melatonin. As shown in Figure 3D. $CoCl_2$-induced hypoxia resulted in enhanced promoter binding activity *vs* normoxia, and this effect was suppressed by 1 mM melatonin treatment.

DISCUSSION

HCC is the most common liver cancer, and even been the third-leading cause of cancer-related deaths worldwide, effective therapy is currently lacking (Midorikawa *et al*, 2010). This type of cancer is considered as a hypervascular tumour, and angiogenesis has a critical role in HCC growth and progression (Wu *et al*, 2007). The high proliferation rate of tumour cells enhances local hypoxia within the HCC microenvironment; increasing new vessels formation for tumour oxygen and nutrients supply (Li *et al*, 2011), and allowing metastatic spreading by connection to the pre-existing vessels (Chekhonin *et al*, 2012). Although melatonin's ability to suppress angiogenesis mainly through VEGF down-regulation has been shown in other tumour types (Lissoni, 2002; Kim *et al*, 2013), there are no reports about its anti-angiogenic activity in liver cancer. In the present study, we used the human liver cancer cell line HepG2 and HUVEC to analyse the potential anti-angiogenic activity of melatonin in HCC.

As indicated above, VEGF is an important growth factor implicated in cancer angiogenesis and can also be used as a tumour marker (Kammerer *et al*, 2012; Marton *et al*, 2012). Different studies have suggested that targeting the VEGF pathway could be even more effective strategy that targeting the tumour itself, based on the fact that angiogenesis and hypervascularisation is a common denominator of solid tumours, regardless their etiological origin (Tabernero, 2007). In HCC, increased VEGF expression has been reported to correlate with tumour progression, microvessel invasion and metastasis of HCC (Sun and Tang, 2004), and VEGF serum measurement are accepted to indirectly estimate tissue values, providing useful prognostic information for HCC management (Poon *et al*, 2003). Although VEGF expression is driven by many factors, hypoxia seems to be the main regulator of its

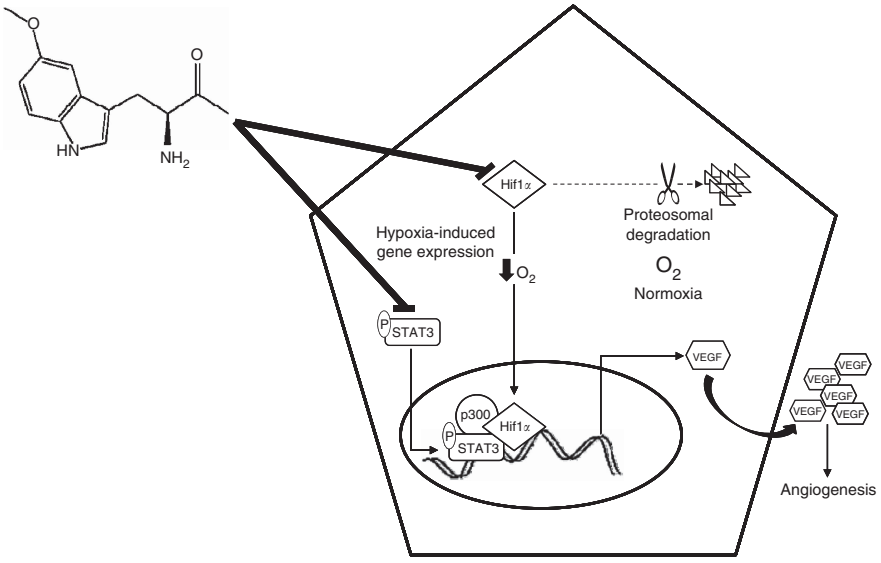

Figure 4. Model of melatonin inhibition of hypoxia-induced angiogenesis in HCC. Hif1 is the major regulator of oxygen homeostasis. Whereas normoxia induces Hif1α proteosomal degradation, under hypoxia is stabilised and translocates to the nucleus where it forms a complex with phospho-STAT3 and CBP/p300 to upregulate VEGF expression, and thus hypoxia-induced angiogenesis. Melatonin pharmacological doses exert anti-angiogenic effects through inhibition of the previously described molecular mechanism.

production (Ferrara, 2004). Results from the present study verified that incubation of cells with $CoCl_2$ mimics the hypoxia situation, as confirmed by the observed increase of VEGF protein expression and secretion *vs* normoxia. Moreover, VEGF levels were effectively reduced by melatonin treatment, mainly at the tested pharmacological dose. Thus, and since microvessels density reflects tumour angiogenicity, and is considered vital for tumour prognostic (Kitamura *et al*, 2012; Sun *et al*, 2012), we performed *in vitro* tube formation assay, as a widely accepted approach to measure the reorganisation stage of angiogenesis, under our experimental conditions. Results showed that melatonin treatment decreased VEGF secretion by HepG2 cells; therefore, HUVECs cultured with CM from the melatonin treated group were unable to display its endothelial features. Similarly to our findings, melatonin has been previously shown to inhibit human pancreatic carcinoma cell PANC-1-induced HUVECs proliferation and migration by inhibiting VEGF expression (Cui *et al*, 2012).

Once confirmed the anti-angiogenic properties of melatonin, we focused on elucidating the responsible molecular mechanism. Results showed a hypoxia-dependent activation of VEGF transcriptional regulators, Hif1α and phospho-STAT3, which were also inhibited by melatonin at the pharmacologic concentration, 1 mM. The present data suggest that melatonin anti-angiogenic activity in liver cancer cells may be mediated, at least in part, by inhibition of Hif1α nuclear translocation, required for its transcriptional activation and subsequent VEGF expression. Moreover, results reported in other tumour types also point to melatonin ability to modulate Hif1α activity (Dai *et al*, 2008; Park *et al*, 2010; Cho *et al*, 2011; Alvarez-Garcia *et al*, 2012, 2013).

STAT3 has been shown to be a potential modulator of Hif1α-mediated VEGF expression, and the development of STAT3 inhibitors may be of interest for clinical treatment, especially of solid tumours (Jung *et al*, 2007). In this regard, molecules like betulinic acid, with anti-cancer and anti-inflammatory properties similar to melatonin, have been reported to suppress angiogenesis via STAT3 and Hif1α inhibition in PC-3 prostata cancer cells (Shin *et al*, 2011). Micro RNAs like miR-20b, which are known to regulate cellular processes such as proliferation and angiogenesis, have been documented to modulate VEGF expression by targeting HIF-1α and STAT3 in MCF-7 breast cancer cells (Cascio *et al*, 2010). Consistently, in the present study we observed a decrease of STAT3 activation after melatonin treatment. Combined treatment with the STAT3 inhibitor Stattic, known to prevent STAT3 activation, dimerisation and nuclear translocation (Schust *et al*, 2006); resulted in a synergic effect, with a decrease in VEGF production, via inhibition of the activation of Hif1α and STAT3 required for optimal VEGF synthesis. This suggests that melatonin anti-angiogenic effects may due to its ability to prevent STAT3 activation, which normally increases Hif1α stability and enhances its transcriptional activity.

Although previous studies reported that either Hif1α or STAT3 alone transcriptionally activate VEGF expression (Matsumura *et al*, 2012; Riddell *et al*, 2012), some evidence suggest that a maximal induction is reached when both transcription factors bind to the VEGF promoter, where they are presumably linked within the same transcriptional complex together with CBP/p300 co-activator (Gray *et al*, 2005; Rathinavelu *et al*, 2012). Considering the importance of this active complex for an efficient VEGF production, we hypothesised that melatonin effects could be related with its capacity to interrupt this transcriptional complex stability. Thus, while hypoxia induced by $CoCl_2$ treatment resulted in increased association between Hif1α, phospho-STAT3, and CBP/p300, melatonin was able to prevent the physical interaction between these proteins, as confirmed by the immunoprecipitation assays. Moreover, our ChIP experiments revealed that Hif1α occupancy of the VEGF promoter was affected by melatonin treatment, providing final evidence to support a hypothetic model of melatonin inhibition of hypoxia-induced angiogenesis in HCC, which is depicted in Figure 4. Results observed at the higher melatonin dose may be, in some aspect, related with the exceptionally high physiological concentrations of melatonin in the bile of mammals (Tan *et al*, 1999), suggesting that hepatocytes may have a particular ability to concentrate melatonin, and so pharmacologic doses may be especially effective in inhibiting liver cancer.

Summarising, this is the first report showing that Hif1α and STAT3 transcription factors promote VEGF production in hypoxia-related angiogenesis in HCC. Considering the results from the current study and previous research data (Carbajo-Pescador *et al*, 2009, 2013), as well as the lack of toxicity of

melatonin even at high doses, it seems reasonable to recommend further research to test the usefulness of the indole for the prevention and treatment of liver cancer in patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Results and Discussion

HCC is the main hepatic tumor worldwide, and unfortunately its incidence is increasing in recent times (El-Serag, 2012). Prevention of chronic liver disease such as cirrhosis or hepatitis remains the first line of action; however, once established, HCC development is silent and asymptomatic (Mazzaferro *et al*, 1996). Once detected, HCC can be treated mainly by surgery, chemotherapy, radiation therapy, or immunotherapy; essentially depending upon the grade of the tumor and the stage of the disease. Complete removal of the cancer without damage the surrounding tissues is the goal of treatment (de Lope *et al*, 2012). However, the propensity of HCC to invade adjacent tissue or to spread to distant sites by metastasis often limits the effectiveness of available treatments; and chemotherapy and radiotherapy unfortunately exerts negative effect on normal cells.

In recent years, therapies addressed against specific molecular targets are proposed as a promising alternative in oncology. Either administered alone or in combination with other treatments, these therapies are aimed to enhance effectiveness and selectivity, and reduced harmful effects on non-tumor cells (Rossi *et al*, 2010). However, since "cancer" means a class of diseases, it is unlikely that there will be a single "cure for cancer".

As we previously mentioned, melatonin is a ubiquitous indoleamine highly conserved throughout evolution. Because of its chemical structure and its hydro-and lipophilic characteristics, it is a very versatile molecule, able to interact with different intracellular pathways to modulate several physiological processes (Hardeland, 2009, Leja-Szpak *et al*, 2010). Lately, its antioxidant, immunomodulatory and anti-inflammatory features have been extensively documented; reaching increasing interest in several fields of the biomedical research (Carrillo-Vico *et al*, 2005, Guerrero and Reiter, 2002, Reiter *et al*, 2000). In addition, several studies both *in vivo* and *in vitro* have shown its antitumor properties (Martin *et al*, 2006, Vijayalaxmi *et al*, 2002). Moreover, a number of clinical trials have addressed the beneficial effects of melatonin on solid tumors, with none or rare toxicity, even at high dosages (Mills E *et al*, 2005, Wang YM *et al*, 2005). Despite of its reported antitumor effects, the precise

mechanism by which melatonin may act in this way have not been fully elucidate, and there are only a few data regarding to its effects in HCC.

Previous analyses from our group showed that melatonin decreased tumor hepatocytes viability, and suggest that this effect may be associated with an induction in the expression of their receptors (Carbajo-Pescador *et al*, 2009, Martin-Renedo *et al*, 2008). However, we need to understand the scientific basis of these results in order to confirm the potential properties of melatonin in HCC.

In line with those data, the studies achieved within this PhD Thesis focused on analyze the molecular mechanisms involved in the melatonin antitumor effects in HepG2 derived tumor hepatocytes as an *in vitro* model of HCC.

While melatonin receptors have been described at the three cellular levels (plasma membrane, cytosol and nucleus), membrane receptors MT1 and MT2 seem to play a very important role in the oncostatic effects attributed to this indole. As cited previously, MT2 is expressed narrower than MT1, and to date has not been detected in human liver tissue (Dubocovich and Markowska, 2005); besides, previous results from our group reported the absence of MT2 in HepG2 (Carbajo-Pescador *et al*, 2009), confirming that the melatonin antitumor effect cannot be associated with a MT2 dependent mechanisms. Having shown that melatonin was able to significantly increase, MT1 expression (Carbajo-Pescador *et al*, 2009), we decided to analyze the contribution of this receptor on the antitumor melatonin features on HepG2.

As indicated in the materials and methods section from the first article, we used the MT1 and MT2 melatonin competitive antagonist, luzindole, to inhibit MT1 activation. As show in the results, luzindole pre-treatment effectively blocked melatonin effects, restoring the cell viability of tumor hepatocytes (Figure 1 from Article 1). Our results are in concordance with similar data from breast tumors, where the antitumor effect of melatonin was associated with over-expression of MT1 (Dubocovich and Markowska, 2005, Yuan *et al*, 2002). Moreover, in patients with bone tumor, which generally exhibit reduced melatonin levels, over-expression of MT1 appears to reduce tumor progression when administered exogenous melatonin

(Toma *et al*, 2007); and has also been reported that the anti-proliferative effect of melatonin on endometrial tumors could be mediated at least in part by MT2 (Kobayashi *et al*, 2003). Furthermore, in association with the changes on cell viability, we detected a cAMP reduced production after melatonin treatment (Figure 2 from Article 1), followed by subsequent activation of the main MAPKs (ERK, JNK and p38). On the opposite side, luzindole pre-treatment efficiently restored the cAMP values, and blocked ERK phosphorylation without significant changes on JNK and p38 (Figures 3, 4 and 5 from Article 1) These data seem to confirm that, on these signaling cascades, melatonin effects were MT1 receptor dependent, and suggest that these membrane receptors may play a role in melatonin effects on HepG2 viability.

The melatonin capacity to modulate MAPKs proteins has been also verified by several authors (Esposito *et al*, 2009, Joo and Yoo, 2009, Nah *et al*, 2009), and our results agree with those of studies where melatonin activates ERK through a MT1 dependent mechanism (Bondi *et al*, 2008). Furthermore, it has been also described that other drugs commonly used in cancer treatment like cisplatin or paclitaxel, behaves as melatonin inducing ERK phosphorylation to counteract tumor cells proliferation (Qin *et al*, 2010, Bacus *et al*, 2001). Although ERK activation has been commonly associated with cell proliferation, an exhaustive analysis of the scientific literature shows that in cells genetically modified to express only MT1, as our HepG2 cells, melatonin specifically stimulates ERK activation; whereas in the presence of both receptors, the indole exerts the opposite effect, reducing its phosphorylation (Chan *et al*, 2002). Given the above, it appears that ERK activation through MT1 receptors could play a significant role on the observed melatonin antitumor effect in HepG2 cells derived from HCC.

In order to complement the study of the molecular pathways affected by MT1 melatonin membrane receptors, and having previously reported melatonin administration was able to increase the protein expression of both NQO2 and RORα in HepG2 cells (Carbajo-Pescador *et al*, 2009); we questioned whether MT1 blockage by luzindole could result in transcriptional changes on the MT1 mRNA, but also on the cytosolic and nuclear melatonin receptors mRNA. As show in the figure 6 from the

first article, concomitant administration of melatonin and luzindole resulted in a significant increase in both MT1 and NQO2 mRNA levels, while no significant changes where observed on RORα mRNA levels (data not shown). In view of these results, the transcriptional upregulation of NQO2 when MT1 is competitively blocked by luzindole, could be explained as a cellular response aimed to bind melatonin present in the media. Similarly to our observations, the existence of an interplay between membrane and nuclear melatonin receptors has been reported in human lymphocytes (Iskander K *et al*, 2004). Thus, while additional experiments may be required, this study highlights a possible interplay between MT1 and NQO2 melatonin receptors in liver cancer cells.

Most of antitumor therapy currently used are not selective enough, and damage healthy tissues with associated side effects. In our work, once we reported the melatonin oncostatic effects in HepG2 liver cancer cells, we investigate its selectivity between healthy and cancerous cells using healthy primary human hepatocytes. As shown in the results from the second paper, the pharmacological doses tested did not negatively affect the cell viability of healthy hepatocytes; while effectively decreased HepG2 cell viability and increasing the anti-APO-1 effects, a well-known inducer of apoptosis, when administered together (Figure 1 from Article 2). These results confirmed once again the melatonin oncostatic properties, and show the high selectivity of this molecule between healthy and tumor cells, a desired feature for potential molecular therapies. In line with that, it is worth mentioning that, while dosage are often a controversial topic, the 20-40 mg/day melatonin doses have been shown to be effective in reducing cancer, even being much higher than the 1,5-5 mg recommended for insomnia and jet lag (Mills E *et al*, 2005). Moreover, besides the higher levels intake, the most likely melatonin side effects are related to sleepiness and head ache (Seely D *et al*, 2012, Mills E *et al*, 2005), which may be considered almost harmless compared to the chemotherapy side effects.

Previous studies conducted during this PhD Thesis related the melatonin effects on cell viability with its capability to induce apoptosis, showing an increase in the expression of pro-apoptotic protein Bax, mitochondrial cytochrome c release, and

caspases 9 and 3 activation (Martin-Renedo *et al*, 2008). Based on these results, and given the complex regulation that underlies this process, we decided to delve deeper into the possible pro-apoptotic pathways induced by melatonin in HepG2.

As indicated in more detail within the introduction, although proteins Bax and Bak are necessary for the initiation of the intrinsic apoptosis pathway, BH3-only molecules like Bid, Bim and Puma are required for direct activation of Bax at the mitochondria (Ren *et al*, 2010). After analyzing the effects of melatonin on Bim and PUMA protein levels, we observed increased expression of both proteins, though only Bim mRNA levels were significantly affected by our treatment. According to our results, it has been reported that certain chemotherapeutic agents used in HCC, such as cisplatin or doxorribicin, induce Bim expression in liver tumor cells (Schneider-Jakob *et al*, 2010).

In order to understand the molecular pathway responsible for the observed increase on Bim expression, we decided to study the FoxO family of Forkhead transcription factors. Although these proteins were firstly discovered just two decades ago, in the lastly they have received considerable interest within the scientific community due to its association with longevity and tumor suppression. In this way, some authors agree that these proteins provide the most compelling example for a conserved genetic pathway at the interface between ageing and cancer (Greer EL and Brunet A, 2008). Consequently, it has been reported that low level of certain FoxOs might be involved in resistance to chemotherapy in some tumors, and are significantly associated with a poor prognosis in cancer patients (Hu *et al*, 2004), suggesting that the activation of FoxO pathway may be a potential therapeutic target for several diseases including cancer (Cheng and White, 2011, Uddin *et al*, 2006).

In this study, we observed a melatonin inductor effect on Bim expression both at RNA and at protein level, accompanied by FoxO3a translocation to the nucleus and subsequent transcriptional activation. Our, melatonin treatments induced a decrease in the dephosphorylated forms of FoxO3a at Thr32 and Ser253, complemented by an increase of total FoxO3a and Bim (Figures 2 from Article 2). Besides, melatonin

caused an inhibition of AKT phosphorylation even after EGF stimulation; and the PI3K inhibitor LY294002, combined with melatonin, resulted in a synergic effect enhancing Bim protein expression (Figures 3 from Article 2). In addition, FoxO3a accumulation and Bim protein expression were greatly reduced upon silencing of FoxO3a (Figures 4 from Article 2), providing evidences to suggest a consistent relation between PI3K, FoxO3a and Bim transcriptional regulation induced by melatonin in HepG2 cells.

According with our results, it has been previously reported that FoxO3a pathway can induce Bim expression and subsequent cell death in several *in vitro* and *in vivo* cancer models (Sharma et al, 2011; Boreddy et al, 2011a; Bhalla et al, 2011). The FoxO3a/Bim pathway has been shown to participate in apoptotic processes in response to other chemotherapeutic agents like cisplatin (Fernandez de Mattos et al, 2008; Yuan et al, 2011). Moreover, resveratrol, another antioxidant molecule, seems to behave as melatonin, exerting oncostatic and proapoptotic activities in different tumor cells, including HepG2 (Hsieh et al, 2005; Notas et al, 2006); and several groups have reported FoxO3a dephosphorylation, nuclear translocation and Bim induction after resveratrol treatment in in vitro cancer models (Chen et al, 2010; Roy et al, 2011), helping us to support our hypothesis.

Summarizing, the results compelled within the second paper provides important information regarding the mechanisms by which melatonin regulates apoptosis, demonstrating that Bim plays a significant role in melatonin induced apoptosis in HepG2 liver cancer cells, most likely through the activity of FoxO3a. Thereby, while this work could represent a significant advance for the understanding of the melatonin oncostatic pathway in vitro, further in vivo experiments are required to bridge the gap between clinical applications and to investigate whether this indol could be safely used as a therapeutic drug in HCC treatment, perhaps as an adjuvant.

Tumors generally present a high degree of disorder and complexity, where fundamental cellular processes such as proliferation, differentiation and angiogenesis are mutated or improperly regulated; the efficient molecular therapy require molecules capable of modulate not only one, but several of these altered processes,

while maintaining the integrity of non-tumor cells (Sun and Tang, 2004). For this reason, and based on the reported pro-apoptotic properties of melatonin in HepG2 cells and its almost nonexistent toxicity in normal hepatocytes, we investigated the potential use of this indole as anti-angiogenic substance in HepG2 cells.

According to the literature, the ability of melatonin to inhibit angiogenesis and has been shown by different groups both *in vivo* and *in vitro* in non HCC models (Cho *et al*, 2011, Cui *et al*, 2012, Kim *et al*, 2012). Furthermore, a study in cancer patients with metastases in various organs, including the liver, demonstrated that melatonin administration significantly correlates with a decrease in VEGF blood levels, but have not explore the molecular mechanism involved (Lissoni *et al*, 2001).

The HCC is a highly vascularized tumor, where oxygen and nutrients availability limit the tumor size, forcing the new vessels formation and allowing the subsequent growth and spreading to other tissues. As previously mentioned, hypoxia is one of the major inductor of the angiogenic switch, through Hif1α and STAT3 activation, which leads to VEGF release.

Once confirmed the melatonin oncostatic effects, we decided to analyze its potential antiangiogenic features focused on the route of STAT3/Hif1α/VEGF; in an *in vitro* model with HepG2 and HUVEC endothelial cells.

As shown in the third article of this PhD Thesis, in response to oxygen deficiency induced by $CoCl_2$ treatment, we observed an increase in VEGF, Hif1α and phospho-STAT3 protein expression, which was efficiently counteracted by the pharmacological dosage of melatonin (1 mM). Thus, we observed a hypoxia induction of VEGF transcription, secretion and activity; when evaluated the tubes formation from HUVEC cell when cultured with conditioned media from HepG2 cell. Melatonin at a pharmacological concentration (1 mM) decreases cellular and secreted VEGF levels, and prevents HUVECs tube formation under hypoxia (Figures 1 from Article 3), associated with a reduction in Hif1α protein expression, nuclear localization, and transcriptional activity (Figures 2 from Article 3). Moreover, while hypoxia increases phospho-STAT3, Hif1α, and CBP/p300 recruitment as a transcriptional complex within

the VEGF promoter, melatonin 1 mM decreases their physical interaction. To this respect, some studies have reported that either Hif1α or STAT3 alone transcriptionally activate VEGF expression (Matsumura et al, 2012; Riddell et al, 2012), it has been reported that maximal induction is reached when both transcription factors bind to the VEGF promoter, presumably linked within the same transcriptional complex together with CBP/p300 co-activator (Gray et al, 2005; Rathinavelu et al, 2012). In line with that, our results suggest that melatonin anti angiogenic effects could be related with its capacity to interrupt this transcriptional complex stability. Besides, melatonin co-treatment with the selective STAT3 inhibitor, Stattic, showed a synergic effect on Hif1α, STAT3, and VEGF expression (Figures 3 from Article 3).

As previously mentioned, VEGF is an important pro angiogenic (Kammerer et al, 2012; Marton et al, 2012) which increased expression has been shown to correlate with tumour progression, microvessel invasion and metastasis of HCC (Sun and Tang, 2004). Moreover, since angiogenesis and hypervascularisation is a common denominator of solid tumours, regardless their etiological origin, several studies pointed out that targeting the VEGF pathway could be even a more effective strategy that targeting the tumour itself (Tabernero, 2007).

Similarly to our findings, melatonin has been shown to inhibit human pancreatic carcinoma cell PANC-1-induced HUVECs proliferation and migration by inhibiting VEGF expression (Cui *et al*, 2012); and accordingly, the melatonin capacity to modulate Hif1α has been also observed in other tumour types (Dai et al, 2008; Park et al, 2010; Cho et al, 2011; Alvarez-Garcia et al, 2012, 2013). Moreover, molecules like betulinic acid, with anti-cancer and anti-inflammatory properties similar to melatonin, have been reported to suppress angiogenesis via STAT3 and Hif1α inhibition in PC-3 prostate cancer cells (Shin et al, 2011).

Summarizing, this is the first report showing that Hif1α and STAT3 transcription factors promote VEGF production in hypoxia-related angiogenesis in HCC. Furthermore being widely accepted that Hif1α and STAT3 activation is required

condition for VEGF transactivation and subsequent angiogenic activity (Gray MJ *et al*, 2005; Jung JE et al, 2005), these data show for the first time the existent relationship between Hif1α, STAT3 and VEGF in a hypoxia-model in tumor hepatocytes; describing the molecular model responsible for the anti-angiogenic capacity of melatonin in this *in vitro* model.

Conclusions

First conclusion

Melatonin effects on cell viability and proliferation in HepG2 cells are partially mediated through the MT1 membrane receptor, which seems to relate also to melatonin modulation of cAMP and ERK activation.

Second conclusion

The transcriptional upregulation of NQO2 induced by melatonin when MT1 is competitively blocked by luzindole, could be explained as a cellular response aimed to bind melatonin present in the media, which highlights a novel interplay between MT1 and NQO2 melatonin receptors in liver cancer cells.

Third conclusion

Our results confirm melatonin oncostatic properties, and show the high selectivity of this molecule between healthy and tumor hepatocytes.

Fourth conclusion

Melatonin can induce apoptosis in HepG2 cells through the upregulation of proapoptotic Bim mediated by nuclear translocation and activation of the transcription factor FoxO3a.

Fifth conclusion

Melatonin exerts an anti-angiogenic activity in HepG2 cells by interfering with the transcriptional activation of VEGF, via Hif1α and STAT3.

General conclusion

Melatonin effects on proliferation, apoptosis and angiogenesis in an *in vitro* model of HCC, together with its reported beneficial properties in other tumors and its lack of toxicity in normal cells, indicate that the indol could be of interests useful at least as adjuvant in HCC therapy. However, further research to test the usefulness of this molecule for HCC prevention and treatment is required.

Resumen en español

universidad de león

Departamento de Ciencias Biomédicas

Efecto de la melatonina sobre la proliferación, apoptosis y angiogénesis en un modelo in vitro de hepatocarcinoma

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Memoria presentada por la Licenciada en Biología Sara Carbajo Pescador para la obtención del grado de Doctor por la Universidad de León

León, 2013

Índice

Introducción

Hepatocarcinoma

El Hepatocarcinoma (HCC) es el principal tipo de cáncer hepático, constituyendo el 80-90% de los tumores primarios de hígado, siendo el quinto más frecuente en hombres y el séptimo en mujeres. Su prevalencia varía según el área geográfica, presentando los ratios de incidencia más elevados (>80% de los casos) en regiones del sudeste asiático y el África Sub-Sahariana (El-Serag, 2011).

Con una etiología variada, las hepatitis por los virus B y C (VHB y VHC respectivamente), junto con la cirrosis se definen como las causas más comunes del HCC (Anzola, 2004). Durante su desarrollo o hepatocarcinogénesis, a fin de compensar el daño hepático, se produce un incremento en el recambio celular, asociado a procesos inflamatorios, depósitos de tejido conectivo, y daños en el DNA. Todos estos cambios alteran la fisiología y el microambiente hepático, y componen un escenario perfecto para el desarrollo de la neoplasia (Ji and Wang, 2012). Durante la hepatocarcinogénesis, aumenta la tasa de división celular, y con ello la posibilidad de mutaciones en oncogenes y genes supresores de tumores, alterándose así la proliferación, diferenciación y apoptosis celular (Leonardi *et al*, 2012).

Dado que en la mayoría de los casos el HCC está ligado a enfermedades hepáticas crónicas subyacentes, la principal medida en la lucha contra el HCC radica en la prevención y el uso de antivirales o vacunas frente al VHB. Sin embargo, este tipo de medidas no resultan útiles para bloquear el desarrollo del HCC una vez que la cirrosis se ha establecido. Desgraciadamente, los primeros estadios son asintomáticos, y por ello muchos pacientes son diagnosticados en fases avanzadas donde las terapias resultan ya menos efectivas (Llovet *et al*, 2003). La extirpación y trasplante de hígado son los tratamientos quirúrgicos más eficaces, especialmente cuando el tamaño de los tumores es pequeño. Sin embargo, aunque no todos los pacientes son candidatos óptimos para el trasplante, existe una escasez relativa de donantes de hígado y las listas de espera son largas, por lo que en la mayoría de las

veces la enfermedad se va agravando por la progresión tumoral. Si el tumor mide más de 5 cm, los tratamientos no quirúrgicos más empleados son la ablación percutánea con alcohol, la quimioembolización y la radiofrecuencia; aunque por desgracia, no siempre se obtienen los resultados esperados (Bruix *et al*, 2004). A día de hoy no existen tratamientos quimioterapéuticos completamente específicos y efectivos contra el HCC, por lo que el desarrollo de nuevas terapias dirigidas contra dianas moleculares que puedan hacer vulnerable a este tipo de tumor se plantea como una alternativa necesaria (Lachenmayer *et al*, 2010).

Con todo lo anteriormente expuesto, parece claro que es necesario llevar a cabo más investigaciones para aumentar el conocimiento de la hepatocarcinogénesis, reducir la recurrencia del tumor y desarrollar fármacos capaces de alargar y mejorar la calidad de los pacientes.

MAPKs

La vía de las proteínas MAPK consiste en la activación secuencial de quinasas, y funciona como un mecanismo de transducción intracelular de señales altamente conservado a lo largo de la evolución. Hasta la fecha han sido descritas cuatro cascadas principales dentro de las MAPK, denominadas con el nombre de su enzima clave: ERK (concretamente ERK1 y ERK2), quinasa N-terminal c-Jun 1/2/3 (JNK, existiendo JNK1-3), p38 y ERK5 (Huang *et al*, 2010). Gracias a la activación de estas cascadas de señalización, distintos estímulos externos se transmiten al interior de la célula para controlar procesos fisiológicos, en muchos casos opuestos entre sí, como la proliferación, diferenciación, supervivencia, apoptosis, desarrollo o respuesta al estrés (Wagner and Nebreda, 2009) (Figura 3). Dada su importancia, la desregulación de estas MAPKs ha sido relacionada con diversas patologías como el cáncer y la diabetes, habiéndose descrito su contribución a la hepatocarcinogénesis (Min *et al*, 2011).

ERK1 fue la primera MAPK caracterizada en mamíferos, originalmente descrita por su respuesta a factores de crecimiento (PDGF, EGF, NGF o insulina) su activación está asociada a receptores tirosín quinasa y receptores acoplados a proteínas G. Esta

ruta se relaciona frecuentemente con la proliferación celular; y se sabe por ejemplo, que ERK1/2 es inducido por agentes mitogénicos, y su activación parece ser necesaria para la progresión de la fase G_1 a S durante el ciclo celular (Bacus *et al*, 2001). Por otro lado, se ha destacado su importancia en el desarrollo del HCC a partir inducidas por VHB y VHC (Chin *et al*, 2007), y se ha visto que las formas activas de esta proteína aparecen significativamente aumentadas en HCC humanos (Lee *et al*, 2006). Sin embargo, existe cierta controversia en cuanto al papel de ERK en carcinogénesis, habiendo estudios que la relacionan con la activación del gen supresor de tumores p53 y la proteína pro-apoptótica Bcl-2 en respuesta a quimioterapia (Bien *et al*, 2007, Chua *et al*, 2006).

La cascada de quinasas JNK, también conocidas como "proteín-quinasas activadas por estrés" (SAPK), consta de tres isoformas (JNK1, JNK2 y JNK3) que comparten más de un 85% de homología en sus secuencias. Mientras JNK1 y JNK2 están distribuidas por prácticamente todos los tejidos, JNK3 aparecen principalmente en neuronas, testículos y cardiomiocitos (Sabapathy *et al*, 1999). Se activan en respuesta a varios tipos de estrés celular (estrés oxidativo, térmico, radiación ionizante, daños en el DNA, citoquinas, radiación ultravioleta, etc.) y va asociada a la fosforilación dual en residuos de treonina y tirosina (Weston and Davis, 2007). Al igual que ocurre con ERK1/2, las proteínas JNK activas pueden translocarse al núcleo donde inducen la transcripción de genes implicados en proliferación, apoptosis y diferenciación. JNK aparece hiperactivada en muestras de HCC humanos, y se ha relacionado con el inicio de tumores hepáticos en ratones (Xu *et al*, 2009). Sin embargo, algunos estudios muestran conclusiones opuestas, remarcando su papel en la apoptosis inducida a través de Bcl-2 y NF-κB (Ventura *et al*, 2004). Así, y dada la variedad de procesos celulares en los que participa la ruta JNK, debe ser cuidadosamente considerada en cuanto a sus funciones, tal y como sucede con las demás MAPKs.

Por último, destacaremos la ruta de la quinasa p38 también conocida como SAPK2, que presenta un 50% de homología con ERK2. Hasta la fecha se han identificado tres isoformas (p38β, p38γ y p38δ) con distintos patrones de expresión

(Cuenda and Rousseau, 2007). En la mayoría de los mamíferos estudiados, la ruta de p38 es intensamente activada por citoquinas inflamatorias (TNF-α, IL-1), estrés oxidativo, radiación UV, hipoxia o isquemia. Se ha descrito que p38 presenta además capacidad de autofosforilación. Al igual que ocurre con otras MAPK, p38 puede encontrarse tanto en el núcleo como en el citoplasma, y se sabe que se acumula en el núcleo en situaciones de estrés (Tung *et al*, 2012). Además de su papel en la respuesta inflamatoria, donde modula la producción de citoquinas, se ha descrito su implicación en otros procesos típicos de quinasas como la proliferación, la supervivencia celular y la inducción de apoptosis (Huang *et al*, 2010).

Apoptosis

La apoptosis o muerte celular programada, es un proceso ordenado por el que la célula muere en respuesta a estímulos extra o intracelulares. Se trata de una secuencia de eventos, que se producen tanto en situaciones fisiológicas como patológicas, caracterizada por cambios bioquímicos y morfológicos como la condensación nuclear o la contracción y fragmentación celular (Elmore, 2007). La presencia de rutas apoptóticas aberrantes es común a la mayoría de los tumores; además hay evidencias que indican insuficiente apoptosis en los estadios iniciales de la hepatocarcinogénesis (Guicciardi and Gores, 2005). Por otro lado, existen múltiples estudios tanto *in vitro* e *in vivo*, analizando la contribución de la inhibición o bloqueo de la apoptosis al desarrollo y progresión del HCC (Mauriz and Gonzalez-Gallego, 2008). En base a esto, parece razonable suponer que la inducción o restauración de la apoptosis en células tumorales podría constituir una interesante estrategia en el tratamiento del HCC.

Las caspasas son una familia de serín-treonin proteasas, que procesan y lisan distintos sustratos citosólicos y nucleares, dando lugar a la mayoría de los cambios morfológicos típicos de la apoptosis (Elmore, 2007). Existen dos tipos de rutas o vías apoptóticas que difieren principalmente en la procedencia de los estímulos que las desencadenan. Así, podemos hablar de una *vía extrínseca,* activada a través de los receptores de muerte como CD95 (APO-1/Fas) o TRAIL; que tras unirse a sus ligandos,

activan a la caspasa 8 y está a la caspasa 3 capaz de lisar diversos componentes celulares (Utz and Anderson, 2000); y de una *vía intrínseca o mitocondrial* en la que proteínas proapoptóticas de la familia Bcl-2 (Bax, Bak y Bok) inducen la permeabilización de la membrana mitocondrial y la liberación del citocromo C y otras proteínas activadoras de caspasas (Hanada *et al*, 1995).

FoxO

La familia de proteínas *Forkhead* o Fox, se compone de una serie de factores de transcripción, todos ellos caracterizados por la presencia de un dominio conservado de unión a DNA llamado "forkhead box" (Weigel *et al*, 1989, Weigel and Jackle, 1990). Dentro de ésta, existen cuatro miembros de la clase FoxO (Forkhead, clase O) en mamíferos: FoxO1 (FKHR), FoxO3a (FKHRL1), FoxO4 (AFX) y FoxO6, que difieren en su distribución tisular y en algunas de sus funciones. Todos ellos reconocen una misma secuencia consenso (GTAAACA) en el promotor de ciertos genes (Furukawa-Hibi *et al*, 2005, Kops *et al*, 2002). Estas proteínas responden a estímulos externos, regulando la expresión génica de proteínas implicadas en el desarrollo, el metabolismo, la longevidad o la supresión tumoral. Así por ejemplo, inducidas por insulina, modulan los niveles de enzimas gluconeogénicas como la glucosa-6-fosfatasa (G6Pasa) y la fosfoenolpiruvato-carboxiquinasa (PEPCK); contrarrestan deños celulares, promoviendo la parada de ciclo celular vía p21, p27, o ciclina D1 (Kops *et al*, 2002), o inducen la expresión de la superóxido dismutasa dependiente de manganeso (MnSOD) y a la catalasa en situaciones de estrés oxidativo (Zhao *et al*, 2011).

Al igual que sucede con otros factores de transcripción, las proteínas de la familia FoxO se encuentran altamente reguladas por ciertas rutas moleculares que determinaran la localización subcelular, los niveles proteicos, la unión al DNA y su actividad transcripcional. Diversas modificaciones post-traduccionales (fosforilaciones, acetilaciones, metilaciones y ubiquitinaciones) determinan principalmente su "secuestro" en el citoplasma o su paso al núcleo; y juegan un papel fundamental en su activación (Brunet *et al*, 1999). Destaca la vía de señalización PI3K-

AKT/SGK, que inhibe la activación de FoxO en respuesta a insulina y a factores de crecimiento. Así, la fosforilación de FoxO por proteínas quinasas de estas rutas promueve su rápida relocalización desde el núcleo al citoplasma, impidiendo la transactivación de sus genes diana (Brunet *et al*, 1999, Pearce and Kleyman, 2007). Además, la fosforilación de FoxO por AKT induce su degradación proteasomal. Sin embargo, la fosforilación de FoxO no siempre supone inhibición; ya que cuando es mediada JNK y MST1 en respuesta a estrés, promueve la localización nuclear de FoxO y su activación (Essers *et al*, 2004, Lehtinen *et al*, 2006).

La acetilación/deacetilación de FoxO por parte de proteínas acetilasas–CBP, p300 y PCAF, afecta tanto a su localización subcelular como a su capacidad de unión al DNA (Matsuzaki *et al*, 2003). Se sabe que en respuesta al estrés oxidativo, FoxO se acetila y se transloca al núcleo donde es deacetilado por enzimas SIRT, que favorecen su activación transcripcional y la expresión de genes de resistencia a estrés (Brunet *et al*, 1999, Pearce and Kleyman, 2007).

Dada la gran variedad de procesos celulares en los que participan, y su aparente importancia en la protección frente al envejecimiento, algunos autores han apuntado a estos factores como posible vínculo molecular entre longevidad y desarrollo tumoral (Calnan and Brunet, 2008). De acuerdo con esto, un aumento en los niveles nucleares de FoxO3a parece estar inversamente relacionado con el tamaño tumoral y la inhibición del crecimiento en un modelo murino de cáncer de mama (Hu *et al*, 2004). El papel de éste factor ha sido descrito, además, en la inducción de p53 incrementando la apoptosis y provocando parada del ciclo celular en respuesta a daños en el DNA de fibroblastos y timocitos (Renault *et al*, 2011). Además, se ha observado que el efecto pro-apoptótico de FoxO está relacionado con su capacidad para inducir la expresión de Bim y Fas ligando, que presentan en su promotor elementos de respuesta para dicho factor de transcripción (Hagenbuchner *et al*, 2012). Respecto al papel de FoxO en HCC, un estudio con muestras humanas encontró FoxO1 silenciado en un 33% de los pacientes, coincidiendo con tasas de supervivencia <3 años (Calvisi *et al*, 2009); y, en experimentos *in vitro* con hepatocitos tumorales, FoxO4 aparecía acetilado e inactivo en citoplasma (Fukuoka *et al*, 2003).

Angiogénesis

La angiogénesis es un proceso fisiológico que consiste en la formación de nuevos vasos sanguíneos a partir de los vasos preexistentes. Se trata de un fenómeno de vital importancia durante distintas etapas del desarrollo embrionario, el crecimiento, la menstruación o la cicatrización de las heridas; sin embargo, también aparece frecuentemente ligado a situaciones patológicas como isquemias, enfermedades infecciosas y del sistema inmune, o cáncer (Carmeliet, 2005). La angiogénesis puede ser resumida en los siguientes pasos: estimulación de células endoteliales (CE) por factores de crecimiento, liberación de metaloproteasas de matriz (MMPs), degradación de la matriz extracelular; y por último migración y asentamiento de las CE para formar nuevos capilares (Liao and Johnson, 2007).

Existen diversos estímulos inductores de angiogénesis, entre los que podríamos resaltar la hipoxia, distintos estreses metabólicos, cambios en el pH, o la rápida división celular. Uno o varios de estos estímulos inducen lo que se conoce como "switch angiogénico", alterando el balance entre factores pro- y anti-angiogénicos en el microambiente de las células endoteliales (Folkman *et al*, 1989). Los factores pro-angiogénicos mejor caracterizados son FGF (Factor de Crecimiento Fibroblástico) y VEGF (Factor de Crecimiento del Endotelio Vascular), si bien, este último merece especial atención por su papel en la proliferación y diferenciación, no sólo de células endoteliales si no de diversos tipos celulares. No en vano, la ruta de señalización de VEGF es considerada como uno de los blancos más interesantes en la inhibición de la angiogénesis tumoral (Pekala *et al*, 1990).

La hipoxia, entendida como un descenso acusado en los niveles de oxígeno, estimula la angiogénesis mediante la activación del factor transcripcional inducible por hipoxia-1 (Hif1). En normoxia, el oxígeno induce la hidroxilación de Hif1 en residuos específicos de prolina, que sirve como marca para su degradación en el proteosoma. En cambio, en condiciones de hipoxia dicha hidroxilación no tiene lugar, por lo que Hif1 escapada a la degradación y se transloca al núcleo donde se une a elementos de respuesta de genes como VEGF, VEGFR1 y Tie2. Además, se ha descrito que la hipoxia es capaz de activar proteínas estabilizadoras de Hif1 como STAT3 y de

sus co-activadores transcripcionales como el complejo p300/CBP, a fin de asegurar un incremento en la expresión de proteínas para restaurar la homeostasis de oxígeno y el suplemento de nutrientes (Shin *et al*, 2011).

Como ya hemos mencionado, la proliferación descontrolada y la apoptosis insuficiente conducen a la formación de una masa tumoral que sin embargo, verá restringido su crecimiento en ausencia de oxígeno y nutrientes. De acuerdo con esto, la angiogénesis es condición indispensable para el crecimiento sostenido del tumor, su dispersión y posterior establecimiento en otros tejidos (Folkman, 1972). Por tratarse de un tumor sólido, el HCC se caracteriza por su alta vascularización, factor empleado en su diagnóstico como sinónimo de agresividad tumoral. Además, este tipo de tumores hepáticos suelen presentar elevados niveles de los factores anteriormente mencionados (VEGF, EGF, Hif1 etc) (Poon *et al*, 2003b, Semela and Dufour, 2004). Finalmente, cabe destacar que algunos fármacos antiangiogénicos como el sorafenib, se presentan como una de las pocas terapias dirigidas al tratamiento del HCC (Sun and Tang, 2004).

Melatonina

La melatonina (*N-acetil-5-metóxitriptofano)*, es una hormona de naturaleza indólica presente en gran variedad de seres vivos desde bacterias y protozoos, hasta plantas, hongos e invertebrados. En mamíferos, la melatonina se sintetizada principalmente en la glándula pineal, aunque existen otras fuentes como son la retina, la médula ósea y el tracto gastrointestinal (Jimenez-Jorge *et al*, 2005). Su síntesis, que tiene lugar en oscuridad, parte del triptófano y es directamente dependiente de la disponibilidad del mismo. El triptófano es transformado en serotonina y posteriormente en N-acetilserotonina en una reacción catalizada por la enzima aril-alquilamina N-acetiltransferasa (AA-NAT), enzima limitante de este proceso. No existe un lugar concreto de almacenamiento para esta hormona, por lo que los niveles detectados en plasma son indicativos de la actividad de la glándula pineal. La melatonina se caracteriza por una elevada solubilidad tanto en agua como en lípidos, propiedad que facilita el paso través de las membranas celulares de forma directa o a través de receptores, llegando a todos los fluidos, tejidos y

compartimentos celulares (Klein *et al*, 1997). Su degradación se produce principalmente a nivel hepático, para ser excretada por orina en forma de sulfato, o en menor medida, conjugada con glucurónico (Vanecek, 1998).

Entre los diversos efectos atribuidos a la melatonina, podemos señalar su capacidad antioxidante (Hardeland, 2008), sus efectos antiinflamatorios e inmunomoduladores (Carrillo-Vico *et al*, 2005, Reiter *et al*, 2000). Además, y de mayor interés para esta tesis doctoral, cabe resaltar sus propiedades oncostáticas, observadas tanto *in vitro* (Cabrera *et al*, 2010, Carbajo-Pescador *et al*, 2009, Cini *et al*, 2005, Cui *et al*, 2012, Farriol *et al*, 2000, Futagami *et al*, 2001, Garcia-Navarro *et al*, 2007, Garcia-Santos *et al*, 2006, Hill *et al*, 2009) como *in vivo* (Blask *et al*, 2002) en distintos tipos de tumores. De acuerdo con la literatura, dichos efectos podrían estar mediados por un gran número de elementos como la unión a receptores, a través de la señalización Ca^{2+}-calmodulina, canales iónicos y segundos mensajeros (Ekmekcioglu, 2006).

A día de hoy se han descrito tres tipos de receptores para la melatonina, presentes tanto a nivel de membrana, en el citosol y en el núcleo celular. Los primeros, MT1 y MT2, pertenecen a familia de receptores acoplados a proteína G, con siete dominios transmembrana, y si bien mantienen una alta homología en su secuencia de aminoácidos, difieren en su distribución tisular, y en la localización génica dentro de los cromosomas (Dubocovich and Markowska, 2005). La proteína G a la que van ligados inhibe la adenilato ciclasa, y activa la fosfolipasa C, lo que conduce a un descenso en los niveles de AMP cíclico (cAMP) y un aumento de diacilglicerol. Estas moléculas actúan como segundos mensajeros, induciendo cascadas de señalización intracelulares en respuesta a estímulos externos (Dubocovich and Markowska, 2005).

Muchos autores proponen la existencia de MT3, una flavoproteina citosólica, también conocida como NQO2, perteneciente a una familia de quinonas reductasas, que parece estar implicada en los efectos antioxidantes de la melatonina (Volkova *et al*, 2012).

Además, teniendo en cuenta sus características lipofílicas, la melatonina puede atravesar directamente las membranas celulares e interaccionar con receptores nucleares. Se sabe que la melatonina se une a receptores de hormonas esteroideas de la familia RZR/ROR; y si bien existe cierta controversia sobre el papel que juegan estos receptores en los mecanismos de acción del indol, algunos estudios sugieren que una vez unida la melatonina, los RZR/ROR podrían actuar como factores de transcripción de genes implicados en la supervivencia, proliferación y diferenciación celular (Steinhilber *et al*, 1995).

Objetivos

- Determinar la contribución del receptor de membrana MT1 en los efectos de la melatonina sobre la viabilidad celular, la activación de proteín quinasas activadas por mitógeno (MAPKs), y los niveles de cAMP.

- Investigar la existencia de una posible inter-regulación entre los receptores de melatonina a nivel de membrana, citosol y núcleo en las células HepG2.

- Evaluar la selectividad de la melatonina entre hepatocitos sanos y tumorales.

- Analizar el mecanismo molecular implicado en sus características pro-apoptóticas de la melatonina.

- Investigar los potenciales efectos anti-angiogénicos de la melatonina en el tratamiento de HCC, así como el mecanismo molecular responsable.

Material y métodos

Ámbito de trabajo

El trabajo experimental se ha desarrollado en el Instituto Universitario de Biomedicina (IBIOMED) de la Universidad de León, el Departamento de Medicina Interna de la Universidad Johannes Gutenberg de Mainz (Alemania), y el Departamento de Bioquímica y Biología Molecular de la Universidad de Valencia.

Línea celular y cultivo primario de hepatocitos

Se emplearon células HepG2 de una línea celular establecida de HCC humano procedentes de la Colección Americana de Cultivos Tipo (ATCC, Manassas, Va, EE.UU.). Las células fueron cultivadas en monocapa y mantenidas en medio de cultivo *Dulbecco's Modiffied Eagle's Medium* (DMEM), suplementado con 10% de suero bovino fetal (SBF), penicilina (100 U/mL), estreptomicina (100 µg/mL), glutamina (4 mM) y piruvato (100 µg/mL). Además se utilizaron cultivos primarios de hepatocitos humanos aislados de tejido hepático de pacientes sometidos a hepatectomía parcial. Estos hepatocitos se sembraron en placas de cultivo recubiertas con colágeno y se mantuvieron en medio *Williams* suplementado con 10% de suero fetal bovino (SBF), HEPES (15 mM, pH 7,4), penicilina (100 U/mL), estreptomicina (100 µg/mL), glutamina (2 mM). Las células fueron mantenidas en un incubador con una atmósfera con un 95% de humedad, 5% de CO_2 y 95% de aire a 37ºC.

Reactivos utilizados

La melatonina, el factor de crecimiento epidérmico (EGF) y el $CoCl_2$ se obtuvieron de Sigma-Aldrich (St Louis, MO, EE.UU.), los inhibidores luzindol, LY294002 y Stattic se obtuvieron de Tocris Bioscience (Bristol, UK).

Ensayos de Viabilidad Celular

Con el fin de analizar el efecto de las distintas concentraciones de melatonina sobre la viabilidad celular se utilizó el test del *3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide* (MTT) siguiendo la técnica previamente descrita por

Denizot y Lang (Denizot and Lang, 1986). La acción de la melatonina sobre la apoptosis inducida por los anticuerpos anti-APO-1/Fas (en HepG2) y APO-1 (en los cultivos primarios de hepatocitos humanos), se determinó utilizando el quit comercial *Cell Titer-Glo* (Promega, Fitchburg, WI, EE.UU.) siguiendo las recomendaciones del fabricante.

Western Blot

El efecto de nuestros tratamientos sobre la expresión proteica de las distintas proteínas analizadas se determinó mediante Western Blot; tras finalizar los tratamientos, las células fueron lavadas dos veces con PBS frío y posteriormente lisadas con buffer de lisis (25 mM HEPES, 1% Triton X-100, 2 mM EDTA, 0,1 M NaCl, 25 mM NaF, 1 mM ortovanadato sódico e inhibidores de proteasas y fosfatasas o buffer RIPA (50mM Tris-HCl (pH 7.4), 150mM NaCl, 2mM EDTA, 0.1% Triton X-100, 10% desoxicolato sódico, 10% SDS, 1mM NaF e inhibidores de proteasas y fosfatasas dependiendo de los experimentos. Los lisados se centrifugaron durante 10 minutos a 15000 g a 4ºC. Para la cuantificación proteica se utilizó el reactivo comercial Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, EE.UU.) siguiendo la técnica espectrofotométrica previamente descrita por M. M. Bradfrod (Bradford, 1976). La separación de cantidades iguales de proteína se llevó a cabo mediante electroforesis en geles de poliacrilamida y condiciones desnaturalizantes con dodecil sulfato sódico (SDS-PAGE), a 100 V durante 1,5 horas. Tras separar las proteínas, estas fueron transferidas a membranas de fluoruro de polivinidideno (PVDF) (Millipore, Billerica, MA, EE.UU.) posteriormente bloqueadas con leche desnatada en PBS con 0,05% de Tween 20 (PBST) durante 30 minutos a 37ºC. Tras el bloqueo, las membranas se incubaron toda la noche con el anticuerpo primario pertinente en cada caso, con diluciones entre 1:200 y 1:2000 en leche desnatada con PBST. Los anticuerpos primarios utilizados fueron los siguientes: fosfo-p38, fosfo-ERK, ERK, fosfo-JNK, fosfo-AKT, AKT, lamin B1, fosfo-STAT3 (Tyr705) (D3A7), STAT3 (Santa Cruz Biotechnology, CA, EE.UU.); JNK, p38, fosfo-FoxO3a Thr32, fosfo-FoxO3a Ser253, FoxO3a (Cell Signaling, Beverly, MA, EE.UU.); VEGF, Hif1α (Abcam, Cambridge, Reino Unido); b-actina y tubulina (Sigma-Aldrich, St Louis, MO, EE.UU.). Tras 3 lavados con PBST, las

membranas se incubaron con los anticuerpos secundarios (anti conejo, ratón o cabra, según el anticuerpo primario) conjugados con peroxidasa (Dako, Glostrup, Dinamarca y Sigma-Aldrich, St Louis, MO, EE.UU.) durante 1 hora a temperatura ambiente A continuación, las membranas fueron lavadas con PBST y posteriormente incubadas con ECL (GE Healthcare, Chalfont St Giles, Reino Unido) o *CDP star detection system* (Tropix Applied Biosystems, Carlsbad, CA, EE.UU.). Finalmente se realizó una autorradiografía, exponiendo las membranas a películas fotográficas Fujifilm (Fujifilm Super RX, Tokyo, Japon).

PCR a tiempo real

El análisis de la expresión del mRNA de los distintos genes estudiados se llevó a cabo por RT-qPCR. Tras los tratamientos, se procedió a la extracción de mediante Trizol (Applied Biosystems, Carlsbad, CA, EE.UU.) y a su posterior cuantificación por espectrofotometría usando un Nanodrop 1000 (Thermo Scientific, Waltham, MA, EE.UU.). A fin de eliminar los restos de ADN genómico, el RNA se incubó con RNasa RQ1 libre de DNAsas (Promega, Madison, WI, EE.UU.). Para la síntesis de cDNA se utilizó el kit High-Capacity cDNA (Applied Biosystems). Para la amplificación de dicho cDNA se empleó el kit comercial FastStart TaqMan Probe Master (Roche) siguiendo el procedimiento de StepOnePlus Real-Time PCR (Applied Biosystems). Los primers utilizados fueron los siguientes MT1 (NM_005958, Hs00195567_m1), NQO2 (NM_000904.3, Hs00168552_m1), RORa (NM_134260.2, Hs00931968_m1), Hif1α (NM_001530, Hs00153153_m1), VEGF (NM_001025366, Hs00900055_m1) y b-actina (NM_001101.2, Hs99999903_m1) procedentes de TaqMan Gene Expression Assays (Applied Biosystems). Los niveles de mRNA de Bim y RNA Polimerasa II (RPII) se analizaron con reactivos SYBR Green Mix (ABgene, Waltham, MA, EE.UU.) utilizando cebadores con las siguientes secuencias: Bim, 5´-AACCACTATCTCAGTGCAAT-3´ y 5´-GGTCTTCGGCTGCTTGGTAA-3´; RPII, 5´-GCACCACGTCCAATGACAT-3´ y 5´-GTGCGGCTGCTTCCATAA-3´. Los cambios en la expresión de dichos genes fueron detectados mediante el método de 2^{-DDCT} previamente descrito por Livak y Schmittgen (Livak and Schmittgen, 2001).

Determinación de la cantidad de AMP cíclico por inmunoensayo

Para evaluar el efecto de nuestros tratamientos sobre los niveles celulares de AMP cíclico se utilizó el kit comercial *Direct Immunoassay Kit Abcam* (Cambridge, Reino Unido) de acuerdo con las instrucciones del fabricante.

Determinación de la actividad transcripcional de FoxO3a

A fin de valorar los efectos de nuestros tratamientos sobre la activación transcripcional de FoxO3a, células HepG2 fueron transfectadas con TransFectin (Bio-Rad, Hercules, CA, EE.UU.) introduciéndoles plásmidos que contenían constructos del gen de interés FoxO3a unidos al gen de una luciferasa; la transcripción conjunta de ambas proteínas se indujo con 4-hydroxytamoxifen (4-OHT), 1h antes del tratamiento con melatonina. La emisión de luz por parte de la luciferasa se determinó mediante el kit comercia *Liciferase Assay* (Promega, Fitchburg, WI, EE.UU.) y se correlacionó positivamente con la actividad de FoxO3a. La eficacia de transfección fue normalizado por la actividad b-galactosidasa.

Silenciamiento con RNA de interferencia (siRNA)

Con objeto de confirmar la implicación de FoxO3a y Bim en los efectos pro-apoptóticos de la melatonina en HepG2; disminuimos su expresión génica transfectando las células con los RNAs de interferencia FKHRL1 siRNA sc-37887 Bim siRNA sc-29802 y siRNA control sc-37007 (Santa Cruz Biotechnology, CA, EE.UU.) con *Lipofectamine 2000* (Invitrogen CA, EE.UU.) de acuerdo con las instrucciones del fabricante. Tras 48h el medio fue reemplazado por DMEM completo y las células fueron tratadas.

Inmunofluorescencia

Para analizar cambios en la localización celular de los dos factores de transcripción estudiados (FoxO3a y Hif1α) las células HepG2 fueron sembradas y tratadas en portas de inmunofluorescencia. Tras el tratamiento, se lavaron con PBS y se fijaron con 4% paraformaldehído-PBS, se permeabilizaron con 0,5% Triton X-100 en

PBS, y pre-tratado con solución de bloqueo. Posteriormente fueron incubadas con anti-FoxO3a (Invitrogen, Carlsbad, CA, EE.UU.) o anti-Hif1α (HIF1α NB100-134, Novus Productos Biológicos, Biologicals, Littleton, CO, EE.UU.) a 4ºC durante la noche. A continuación se lavaron con PBS y se incubaron con anticuerpos secundarios Alexa Fluor 488 anti-conejo (Invitrogen, Carlsbad, CA, EE.UU.) y FITC conjugado de burro anti-conejo (Jackson Immuno Investigación, Baltimore, PA, EE.UU.) respectivamente. Para el contraste de núcleos se utilizó Hoechst 33342 y DAPI (Invitrogen Carlsbad, CA, EE.UU.) Una vez montados, los cubres se visualizaron en microscopio de fluorescencia (Nikon Eclipse Ti, Melville, NY, EE.UU.).

Inmunoprecipitación de Cromatina (ChIP)

Esté método se utilizó para confirmar la unión física de los factores de trascripción estudiados (FoxO3a y Hif1α) a regiones concretas del promotor de nuestros genes de interés (*bim* y *vegf* respectivamente). Para el ChIP FoxO3-*bim* se empleó un kit comercial de inmunoprecipitación Upstate Cell Signaling, Lake Placid, NY, EE.UU.), de acuerdo con las instrucciones del fabricante. Las muestras tratadas con melatonina fueron inmunoprecipitadas con anti-FoxO3a Ab (Abcam, Cambridge, Reino Unido) o IgG de conejo (Sigma-Aldrich, St Louis, MO, EE.UU.). Las secuencias del promotor de *bim* se amplificaron usando los siguientes cebadores: F-5'-CCTTCGCGAGGACCAACCCAGTC-3'y R-5'-CCGCTCCTACGCCCAATCACTGC-3'. El ChIP Hif1α-*vegf* se realizó de acuerdo con el protocolo descrito por Benet *et al.* (Benet *et al*, 2010) utilizando anti-HIF1α NB100-134 (Novus Productos Biológicos, Littleton, CO, EE.UU.) e IgG sc-2027 (Santa Cruz Biotechnology, CA, EE.UU.) como anticuerpos de inmunoprecipitación, y cebadores F-5'-CAGGAACAAGGGCCTCTGTCT-3', R-5' TGTCCCTCTGACAATGTGCC ATC-3', para amplificar la región de unión al promotor de *vegf*.

Ensayo de formación de tubos sobre Matrigel

Para determinar visualmente la capacidad anti-angiogénica de la melatonina se emplearon cultivos primarios de células HUVEC, sembradas en placas de 96 pocillos sobre una superficie de Matrigel (Matrigel, BME, BD Bioscience) siguiendo las

instrucciones del fabricante y el protocolo de Shin, J et colls. (Shin *et al*, 2011). Tras la siembra, las HUVECs fueron mantenidas en medio procedente de HepG2 tratadas con melatonina bajo normoxía y/o hipoxia. Como control positivo se trató un grupo con medio EBM-2 suplementado con VEGF (20 ng/ml). La placa se mantuvo en el incubador (95% de humedad, 5% de CO2 y 95% de aire a 37ºC) y tras 6 horas, se tomaron fotografías de la formación de los capilares con un microscopio de Nikon Eclipse Ti, Melville, NY, U.S.A.). El número de capilares, así como la estabilidad de sus uniones se determinó utilizando el sistema de análisis de imágenes S.CORE (S.CO BioLifescience, Munich, Alemania).

Cuantificación de la producción de VEGF

El efecto de nuestros tratamientos sobre los niveles de VEGF secretado al medio de cultivo de realizó mediante un kit comercial *Quantikine human VEGF ELISA* de R&D Systems (Minneapolis, MN, EE.UU.). Tras los tratamientos, los medios de cultivo fueron recogidos y centrifugados a 800 rpm y 4ºC durante 4 min para eliminar los desechos celulares; posteriormente fueron analizados con el kit siguiendo las instrucciones del fabricante.

Determinación de la actividad transcripcional de Hif1α

La actividad transcripcional de Hif1α se determinó mediante el kit comercial *TransAM HIF-1 transcription factor assay kit* (Active Motif, Carlsbad, CA, EE.UU.) de acuerdo con las instrucciones del fabricante. Tras tratar las células se aislaron extractos nucleares que fueron añadidos a una placa de 96 pocillos recubierta con oligonucleótidos de elemento de respuesta a la hipoxia (HRE) Los dímeros de HIF presentes en los extractos nucleares se unieron a dichos elementos y pudieron ser detectados mediante la adicción de un anticuerpo dirigido contra Hif1α y un anticuerpo secundario conjugado con peroxidasa de rábano picante (HRP), dando lugar a una reacción colorimétrica detectable a 450/655 nm.

Inmunoprecipitación de las proteínas del complejo transcripcional Hif1α/STAT3/CBP/p300

A fin de analizar el efecto de nuestros tratamientos sobre la interacción física de Hif1α y sus co-activadores STAT3 y anti-acetil-CBP/p300, las células HepG2 fueron tratadas y sus extractos celulares fueron inmunoprecipitados tal y como se describe el protocolo de García-Mediavilla *et al.* (Garcia-Mediavilla *et al*, 2012). Los anticuerpos utilizados para la inmunoprecipitación fueron: anti-fosfo-STAT3 (Santa Cruz), anti-Hif1α (Abcam), y anti-acetil-CBP (Lys1535)/p300 (Lys1499) (Cell Signaling, Technology, Danvers, MA, EE.UU.). Tras los lavados pertinentes las muestras separaron en un gel de SDS-poliacrilamida y se analizada por Western Blot utilizando los anticuerpos anteriormente indicados.

Análisis Estadístico

Todos los análisis estadísticos se realizaron utilizando el software Statistica 7.0 (Statsoft Inc, Tulsa OK, EE.UU.). Los resultados fueron expresados como valores de la media ± error estándar de la media (EEM). Para la comparación de datos se utilizó un análisis de varianza (ANOVA). En los casos en que se encontraron diferencias significativas de $p<0,05$, las medias se compararon mediante el test Newmann Keuls.

Resultados y Discusión

El HCC constituye el principal tumor hepático a nivel mundial, y desgraciadamente su incidencia está sufriendo un incremento preocupante en los últimos tiempos (El-Serag, 2012). La prevención de enfermedades hepáticas crónicas como la cirrosis o la hepatitis supone la primera línea de actuación; sin embargo, una vez establecidas, su evolución hacía el HCC es silenciosa y asintomática, lo que implica un diagnóstico tardío y resta eficacia a las terapias actuales (Mazzaferro *et al*, 1996). La resección o el trasplante constituirían las mejores opciones curativas, aunque se limitan a unos pocos pacientes. Existen además tratamientos no quirúrgicos para reducir el tamaño de la masa tumoral como la ablación, la quimioembolización o la radiofrecuencia, que sin embargo se asocian con un alto índice de recurrencia, con lo que resultados distan de ser completamente satisfactorios (Llovet *et al*, 2003). En los últimos años, las terapéuticas dirigidas contra dianas moleculares y cambios específicos de células tumorales se plantean como una esperanzadora alternativa en oncología, pudiendo ser administradas solas o en combinación con otros tratamientos, a fin de aumentar su efectividad y la selectividad para reducir en lo posible los efectos dañinos sobre células no tumorales.

Tal y como hemos citado al principio de esta Tesis Doctoral; la melatonina es una indolamina ubicua y altamente conservada a lo largo de la evolución (Hardeland, 2009, Leja-Szpak *et al*, 2010). Dada su estructura química y sus características hidro- y lipofílicas es una molécula muy versátil, capaz de interaccionar con diversas vías de señalización intracelulares, afectando a distintos procesos fisiológicos. En los últimos años su capacidad antioxidante, inmunomoduladora y antiinflamatoria ha sido ampliamente documentada por lo que ha despertado un creciente interés en distintos campos de investigación biomédica (Carrillo-Vico *et al*, 2005, Guerrero and Reiter, 2002, Reiter *et al*, 2000). Además, son varios los estudios tanto *in vivo* como *in vitro* que ponen de manifiesto su actividad antitumoral, relacionándola con la parada del ciclo celular, la inducción de la apoptosis o la inhibición de la angiogénesis en

distintos tipos de tumores; si bien no existen demasiados datos sobre sus efectos en el HCC.

Estudios previos realizados en nuestro grupo, demostraron que la melatonina disminuía la viabilidad celular de hepatocitos tumorales, efecto que se parecía estar relacionado con inducción en la expresión de sus receptores (Carbajo-Pescador *et al*, 2009, Martin-Renedo *et al*, 2008). Continuando con esta línea de investigación, los estudios llevados a cabo dentro de la presente Tesis Doctoral se han centrado en analizar los mecanismos moleculares implicados en dichos efectos antitumorales, utilizado la línea celular establecida HepG2 derivada de HCC humano.

Si bien se han descrito receptores para la melatonina en los tres niveles estructurales de la célula (membrana plasmática, citosol y núcleo), los receptores de membrana MT1 y MT2 parecen jugar un papel bastante importante en los efectos oncostáticos atribuidos a este indol. Como hemos mencionado con anterioridad, MT2 se expresa de forma más restringida que MT1, y hasta la fecha no se ha detectado en tejido hepático humano (Dubocovich and Markowska, 2005); además, en análisis anteriores, no detectamos expresión de este receptor ni a nivel proteico ni del mRNA en HepG2 (Carbajo-Pescador *et al*, 2009), corroborando la ausencia de dicho receptor en nuestra línea celular; y descartando que el efecto antitumoral de la melatonina, estuviera mediado por MT2.

Sabiendo que la melatonina era capaz de incrementar, de forma significativa, la expresión de MT1 (Carbajo-Pescador *et al*, 2009), decidimos analizar la contribución de dicho receptor a los efectos antitumorales del indol. Como se indica en el material y métodos del primer artículo, para la inhibición de MT1, empleamos el antagonista competitivo contra la unión de la melatonina en MT1 y MT2, luzindol. El pre-tratamiento con esta sustancia bloqueó eficazmente el efecto de la melatonina, restaurando la viabilidad celular de los hepatocitos HepG2 (Figura 1 en el Artículo 1). Nuestros resultados en HCC, coinciden con resultados similares en tumores de mama, donde el efecto antitumoral de la melatonina se relaciona con la sobre-expresión de MT1 (Dubocovich and Markowska, 2005, Yuan *et al*, 2002). Además, en pacientes con

tumores óseos, que generalmente presentar niveles de melatonina reducidos, la sobre-expresión de MT1 parece inhibir la progresión tumoral cuando se administra melatonina exógena (Toma *et al*, 2007), y parte del efecto anti proliferativo de la melatonina en tumores de endometrio podría estar mediado por MT2 (Kobayashi *et al*, 2003).

Asociados a los cambios en la viabilidad celular, observamos cómo la administración de melatonina, reducía la producción de AMPc, e inducía la activación de las principales MAPKs ERK, JNK y p38. De forma totalmente opuesta, el pre-tratamiento con luzindol restauraba los valores celulares de AMPc y bloqueaba mayoritariamente la fosforilación de ERK, sin alterar significativamente las rutas de las otras quinasas (JNK y p38) (Figuras 2, 3, 4 y 5 en el Artículo 1); confirmando la implicación directa de MT1 en los efectos de la melatonina sobre estas cascadas de señalización celular, y la viabilidad de nuestras células tumorales.

La capacidad de la melatonina para modular proteínas MAPKs ha sido puesta de manifiesto por distintos autores (Esposito *et al*, 2009, Joo and Yoo, 2009, Nah *et al*, 2009), y nuestros resultados concuerdan con los de otros trabajos donde la melatonina activa ERK mediante un mecanismo dependiente de MT1 (Bondi *et al*, 2008). Además, se ha observado que otros fármacos utilizados en el tratamiento del cáncer inducen mecanismos moleculares similares. Así por ejemplo, el tratamiento con cisplatino estimula la fosforilación de ERK en células H22 derivadas de HCC (Qin *et al*, 2010), y el paclitaxel activa ERK para interrumpir el ciclo entre las fases G_2/M en células MCF7 derivadas de tumores de mama (Bacus *et al*, 2001). Aunque la activación de ERK ha sido comúnmente asociada con proliferación celular, un exhaustivo análisis de la literatura científica indica que en células modificadas genéticamente para expresar sólo MT1, tal como ocurre en nuestros hepatocitos, la melatonina estimula más específicamente la activación de ERK; mientras que en presencia de ambos receptores, el indol ejerce el efecto contrario, reduciendo la su fosforilación (Chan *et al*, 2002).

Por todo lo anterior, parece que el receptor MT1 podría jugar un papel determinante, a través de ERK, en el efecto antitumoral de la melatonina observado en las células HepG2 derivadas de HCC.

Como hemos indicado previamente, además de los receptores de membrana MT1 y MT2, la melatonina presenta receptores específicos a nivel citosólico (NQO2) y a nivel nuclear. Estudios previos realizados en nuestro grupo confirmaron que la administración de melatonina inducía un aumento en la expresión proteica de ambos receptores en células HepG2 (Carbajo-Pescador et al, 2009), de acuerdo con estos datos y a fin de complementar el estudio de las vías moleculares afectadas por los receptores de membrana de melatonina MT1, analizamos el efecto del bloqueo de MT1 sobre la transcripción de los restantes receptores. Como se muestra en la figura 6 del primer artículo, la administración conjunta de la melatonina y luzindol indujo significativamente los niveles de mRNA de MT1 y NQO2, si bien no observamos cambios destacable en el mRNA del receptor nuclear RORα. En base a estos resultados, parece razonable pensar que en ausencia de un receptor funcional a nivel de membrana, existe un mecanismo celular por el cual se induce la expresión de NQO2 para captar la melatonina presente en el medio. Otros autores han observado relaciones similares entre receptores de membrana y receptores nucleares de melatonina en linfocitos humanos (Iskander K et al, 2004). Así, aunque son necesarios más experimentos, nuestro estudio pone de manifiesto la existencia de una posible inter-regulación entre MT1 y NQO2 en células HepG2.

Como complemento a nuestros estudios con HepG2, analizamos los efectos de distintas dosis farmacológicas de melatonina sobre la viabilidad de hepatocitos no tumorales. Lejos de ejercer efectos negativos, la melatonina se mostró inocua frente los hepatocitos sanos; si bien disminuyó eficazmente la viabilidad de HepG2. Además, la nuestro tratamiento resulto ejercer un efecto sinérgico cuando se administró conjuntamente con un inductor de apoptosis (anti-APO-1) (Figura 1 en el Artículo 2). Estos resultados confirmaron una vez más las propiedades antitumorales de la melatonina; poniendo de manifiesto además, la elevada selectividad de esta molécula

entre células sanas y tumorales, característica deseada en cualquier tratamiento oncológico.

Estudios previos a los realizados durante esta Tesis Doctoral relacionan los efectos de la melatonina sobre la viabilidad celular, con su capacidad para inducir apoptosis, observándose un incremento en la expresión de la proteína pro-apoptótica Bax, la exclusión mitocondrial del citocromo C y la activación delas caspasas 9 y 3 (Martin-Renedo *et al*, 2008). En base a estos resultados, y dada la compleja regulación que subyace tras este proceso, decidimos profundizar aún más en las posibles rutas pro-apoptóticas inducidas por la melatonina en HepG2.

Tal y como se ha indicado con mayor detalle dentro de la introducción, aunque las proteínas Bax y Bak son necesarias para la iniciación de la apoptosis a nivel mitocondrial, las proteínas de domino BH3-*only* (Bid, Bim y PUMA) resultan indispensables para la activación directa de las primeras (Ren *et al*, 2010). Tras analizar los efectos de la melatonina sobre los niveles proteicos de Bim y PUMA, observamos un aumento en la expresión de ambas, si bien los niveles de mRNA sólo se vieron afectados por el tratamiento en el caso de Bim (Figura 2 en el Artículo 2), razón por la que continuamos con la ruta de Bim como proteína pro-apoptótica. De acuerdo con nuestros resultados, se ha visto que ciertos fármacos quimioterapéuticos empleados en HCC, como la doxorribicina o el cisplatino inducen la expresión de Bim (Schneider-Jakob *et al*, 2010).

Con el fin de entender el mecanismo molecular responsable del aumento observado en la expresión de Bim, se decidió estudiar la familia FoxO de factores de transcripción Forkhead. A día de hoy, cada vez son más los trabajos que presentan a estas proteínas como nexo entre longevidad y cáncer, apuntando a la activación de la ruta de FoxO como una potencial diana terapéutica para distintas enfermedades entre las que destaca el cáncer (Cheng and White, 2011, Uddin *et al*, 2006). Además, se ha descrito que bajos niveles de ciertas proteínas FoxO podrían estar implicados en la resistencia a la quimioterapia en varios tipos de tumores, asociándose significativamente con un mal pronóstico en pacientes con cáncer (Hu *et al*, 2004).

Los resultados de nuestro estudio mostraron que el efecto inductor de la melatonina sobre la expresión de Bim, se acompañaba de la translocación de FoxO3a desde el citoplasma al núcleo, y su posterior activación transcripcional. Como mecanismo responsable de tal activación, la melatonina disminuía las formas fosforiladas de FoxO3a en Thr32 y Ser253, al tiempo que aumentaba la forma total de FoxO3a y Bim (Figura 2 del Artículo 2). Tal y como hemos mencionado, la ruta PI3K/Akt, es una de las principales cascadas celulares responsable de la regulación de FoxOs, por lo que su inhibición se relaciona inversamente con la activación de las proteínas FoxO. De acuerdo con esto, la melatonina interfería en la fosforilación de AKT, incluso después de la estimulación de EGF; además el tratamiento de combinado de melatonina con el inhibidor de PI3K LY294002, inducía un aumento aún mayor en la expresión de Bim (Figura 3 del Artículo 2). Por otro lado, mediante técnicas de silenciamiento con siRNA específicos para FoxO3a y Bim confirmamos que: si FoxO3a estaba inhibido, el incremento en Bim no se producía a pesar de tratar con melatonina; y además, la presencia de Bim era condición necesaria para los efectos pro-apoptóticos de nuestro indol en HepG2 (Figura 4 del Artículo 2).

De acuerdo con nuestros resultados, otros autores han observado que la activación de FoxO3a induce la expresión de Bim, mediando la apoptosis tanto en modelos *in vitro* como *in vivo* (Sharma y otros, 2011; Boreddy et al, 2011a; Bhalla et al, 2011). Además, la inducción de esta vía ha sido descrita en el mecanismo molecular agentes quimioterapéuticos como el cisplatino (Fernández de Mattos et al, 2008; Yuan et al, 2011) o de sustancias antioxidantes y con propiedad oncostáticas similares a la melatonina, como el resveratrol (Chen et al, 2010; Roy et al, 2011).

Con lo anteriormente expuesto, los resultados del segundo artículo de esta memoria nos indican claramente que la melatonina es capaz de inducir FoxO3a y Bim, para así reducir la viabilidad de hepatocitos tumorales. Si bien son necesarios más experimentos *in vitro* e *in vivo*, estos datos sugieren el potencial uso de la melatonina, al menos como adyuvante.

Teniendo en cuenta que los tumores en general presentan un alto grado de desorden y complejidad, donde procesos celulares fundamentales como la proliferación, la diferenciación o la angiogénesis aparecen mutados o erróneamente regulados; el camino hacia una terapia molecular eficaz y selectiva requiere de sustancias capaces de modular no sólo uno, si no varios de estos procesos alterados, respetando en el mayor grado posible a las células no tumorales adyacentes. Con este objetivo en mente, una vez comprobadas las propiedades pro-apoptóticas de la melatonina en HepG2 y su casi inexistente toxicidad incluso en dosis altas en hepatocitos sanos, nos planteamos analizar los potenciales efectos antiangiogénicos de este indol en HCC.

De acuerdo con la literatura previamente publicada, la capacidad de la melatonina para inhibir angiogénesis ha sido ya demostrada por distintos grupos tanto *in vitro* como *in vivo* (Cho *et al*, 2011, Cui *et al*, 2012, Kim *et al*, 2012), aunque por desgracia los datos disponibles sobre HCC son muy escasos. Merece la pena destacar el estudio de Lissoni y sus colaboradores, que demostraron una reducción de los niveles plasmáticos de VEGF tras la administración de melatonina a pacientes con cáncer y metástasis en distintos órganos incluyendo al hígado, aunque dichos autores no profundizaron en el mecanismo molecular implicado (Lissoni *et al*, 2001).

Como ya hemos explicado, el HCC se caracteriza por ser un tumor altamente vascularizado, donde su propio crecimiento induce hipoxia y limita la disponibilidad de oxígeno y nutrientes. Esta situación requiere de la formación de nuevos vasos para continuar creciendo y diseminarse a otros tejidos. Por todo ello, los niveles de VEGF se emplean como marcadores tumorales en HCC (Poon *et al*, 2003b, Semela and Dufour, 2004), siendo la administración de sorafenib, un inhibidor de tirosina quinasas que actúa sobre receptores de factores angiogénicos, una de las pocas estrategias quimioterapéuticas disponibles para el tratamiento de este tipo de tumor (Sun and Tang, 2004).

Es necesario destacar que durante el desarrollo tumoral, la hipoxia induce la liberación de factores pro-angiogénicos, principalmente VEGF, estimulando la actividad transcripcional de Hif1α y su co-activador STAT3 (Ke Q and Costa M, 2006).

Así, una vez confirmados los efectos oncostáticos de nuestra hormona, decidimos analizar el potencial efecto antiangiogénico de la melatonina sobre la ruta STAT3/Hif1α/VEGF; continuando con nuestro modelo in vitro HepG2 y empleado además células endoteliales HUVEC comúnmente empleadas en estudios angiogénicos *in vitro*.

Tal como se indica en el tercer artículo de esta Tesis, en respuesta al déficit de oxígeno inducido por el tratamiento con $CoCl_2$, un mimético de hipoxia (Dai *et al*, 2008, Shrivastava *et al*, 2008); observamos un incremento en la expresión de VEGF, Hif1α y fosfo-STAT3. La administración de melatonina en una dosis farmacológica (1mM) resultó eficaz en la inhibición de tales niveles. Además, el efecto inductor del $CoCl_2$, y la capacidad inhibidora de la melatonina sobre VEGF, se observaron a nivel de mRNA, en los valores de VEGF secretado al medio de cultivo, y sobre la actividad de VEGF como inductor de angiogénesis, estimulando la formación de tubos por parte de células HUVEC (Figura 1 del Artículo 3).

Con estos datos, y apoyándonos en resultados similares de otros autores con melatonina (Cui *et al*, 2012, Dai *et al*, 2008), quisimos analizar los efectos de la melatonina sobre el principal factor de respuesta a hipoxia, Hif1α. Como cabía esperar, la hipoxia estimulaba la expresión de Hif1α, su translocación nuclear y su actividad transcripcional; si bien, en dosis farmacológica, la melatonina reducía los niveles proteicos de tal factor, inhibiendo su localización en el núcleo, y por consiguiente su actividad sobre el promotor de VEGF (Figura 2 del Artículo 3). No se observaron cambios significativos a niveles de mRNA, por lo que los efectos antiangiogénicos del indol parecían ser posteriores a la traducción de Hif1α. Además, el tratamiento con melatonina, inhibía la fosforilación de STAT3 y la interacción física de éste con Hif1α y sus activadores CBP/p300 dentro del complejo transcripcional de unión al promotor de VEGF (Figura 3 del Artículo 3).

De acuerdo con nuestros resultados, otros autores han demostrado que a inhibición de Hif1α y STAT3 y por consiguiente VEGF, es la responsable del efecto anti angiogénico de distintas moléculas en distintos modelos *in vivo* e *in vitro* (Boreddy *et al*, 2011b, Dal Monte *et al*, 2012, Matsumura *et al*, 2012, Mei *et al*, 2012). Sin embargo, los datos presentados dentro de esta Tesis, documentan por primera vez la relación entre Hif1α, STAT3 y VEGF, en un modelo de hipoxia en hepatocitos tumorales; y lo que es aún más interesante, describen el modelo molecular que parece ser responsable de los capacidad anti angiogénica de la melatonina en tales hepatocitos.

Aunque parece clara la necesidad de realizar más experimentos tanto *in vitro* como *in vivo;* los resultados de la presente Tesis Doctoral, analizando los efectos antitumorales de la melatonina en un modelo *in vitro* de HCC, junto con la aparente falta de toxicidad de ésta sobre los hepatocitos sanos, parecen indicar el potencial interés de la melatonina, al menos como adyuvante, en la terapia de HCC.

Conclusiones

CONCLUSIÓN PRIMERA

Los efectos de la melatonina sobre la viabilidad celular y la proliferación en las células HepG2 están parcialmente mediados por el receptor de membrana MT1, que actúa como modulador de los niveles de AMP cíclico y activador de la ruta de protein quinasas ERK.

CONCLUSIÓN SEGUNDA

La sobre-expresión de NQO2 inducida por la melatonina cuando se bloquea MT1 competitivamente por luzindol, podría explicarse como una respuesta celular que permite la unión de la melatonina presente en el medio de cultivo, y pone de relieve una nueva interacción entre los receptores MT1 y NQO2 en células tumorales hepáticas.

CONCLUSIÓN TERCERA

Nuestros resultados confirman las propiedades oncostática de la melatonina, y muestran la alta selectividad de esta molécula entre los hepatocitos sanos y tumorales.

CONCLUSIÓN CUARTA

La melatonina puede inducir la apoptosis en células HepG2 induciendo la expresión de la proteína pro-apoptótica Bim, a través de la translocación nuclear y la activación del factor de transcripción FoxO3a.

CONCLUSIÓN QUINTA

La melatonina ejerce una actividad anti-angiogénica en células HepG2, inhibiendo la activación transcripcional de VEGF, a través de Hif1α y STAT3.

CONCLUSIÓN GENERAL

Los efectos de la melatonina sobre la proliferación, la apoptosis y la angiogénesis en un modelo *in vitro* de HCC, junto con sus propiedades beneficiosas ya

documentadas en varios tumores, y su falta de toxicidad, demuestran el potencial interés de esta molécula, al menos como adyuvante, en la terapia del HCC. Sin embargo, se requiere más investigación para confirmar la utilidad de esta molécula en la prevención y el tratamiento de pacientes con cáncer.

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