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Post-transcriptional control of HIF-1α gene expression: role of the natural antisense transcript "aHIF"

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SUMMARY

INTRODUCTION

THE HYPOXIA-INDUCIBLE FACTOR (HIF)-1α

The Hypoxia-Inducible Factor (HIF)-1 was identified in 1992 as a nuclear factor whose binding to sequences necessary for transcriptional activation of the erythropoietin (EPO) gene could be induced by hypoxia and required de novo protein synthesis (Semenza G.L. and Wang G.L., 1992). HIF-1 can activate EPO gene transcription in Hep3B cells treated with the hypoxia mimetic cobalt chloride ($CoCl_2$) and, subsequently, participates in the hypoxic induction of several genes involved in cellular adaptation to low oxygen conditions such as vascular endothelial growth factor (VEGF) (Shweiki D., 1992), tyrosine hydroxylase (Norris M.L. and Millhorn D.E., 1995), phosphoglycerate kinase 1, lactate dehydrogenase (Firth J.D., 1994), aldolase A, pyruvate kinase M (Semenza G.L., 1994), and the glucose transporters 1 and 3 (GLUT1/3) (Ebert B.L., 1995). The biochemical purification of HIF-1 revealed that this factor is composed by two different subunits, HIF-1α (120 kDa) and HIF-1β (91-94-kDa), both of which directly bind DNA (Wang G.L. and Semenza G.L., 1995). Both HIF-1α and HIF-1β subunits are basic-helix-loop-helix proteins containing a PAS (Per-ARNT-AHR-Sim) domain, defined by its presence in the Drosophila PERiod and SIngle-Minded proteins and in the mammalian Aryl Hydrocarbon Receptor Nuclear Translocator and Aryl Hydrocarbon Receptor proteins (Wang G.L., 1995). Immunoblot analysis of nuclear and cytoplasmic extract from Hep 3B cells exposed to hypoxia (1% O_2) for various times revealed that both subunits are induced by hypoxia and their expression in nuclear extracts reached maximum at 4-8 hours. However, when cells were exposed to hypoxia for 4 hours and returned to normoxic conditions, both HIF-1α and HIF-1β levels rapidly decreased, indicating that these proteins are unstable in post-hypoxic cells. Analysis of mRNA levels revealed that both *hif-1α* and *hif-1β* transcript were upregulated when Hep 3B cells were exposed to 1% O_2,

or to hypoxia-mimetics $CoCl_2$ or desferrioxamine, reaching peak of expression at 1-2 hours, and declined to near basal levels at 8 hours, thus suggesting a transcriptional and post-transcriptional regulation of HIF-1 subunits. However, it was later demonstrated that HIF-1β is constitutively expressed while HIF-1α is tightly regulated by cellular O_2 tension. Although many mechanisms of control of HIF-1α protein expression have been identified, the most notable of these is the HIF-1α post-translational degradation pathway which involves many factors such as the proteins prolyl hydroxylase, the von Hippel Lindau (VHL) tumour-suppressor protein and the proteasome. In normoxia, *HIF-1α* is constitutively transcribed and translated in cells, but it has an extremely short half-life (less than 5 minutes). Prolyl hydroxylase proteins (PHDs) belong to a conserved family of enzymes that bind oxygen, Fe^{2+} and 2-oxoglutarate and that were originally designated EGLN1–3 on the basis of sequence homology to EGL9, the HIF-1 prolyl hydroxylase in *Caenorhabditis elegans*. Three PHDs proteins have been identified so far (Epstein A.C., 2001): although *in vitro* all the three isoforms hydroxylate HIF-1α (Bruick R.K. and McKnight S.L., 2001), PHD2 plays a pivotal role in normoxic HIF-1α regulation *in vivo* (Appelhoff R.J., 2004; Berra E., 2003). PHDs modify prolines (Pro)-402 and -564 of HIF-1α within an Oxygen-Dependent Degradation (ODD) domain that consist of approximately 200 amino acid residues located in the central region of HIF-1α. (Bruick R.K. and McKnight S.L., 2001): PHDs hydroxylation of Pro-402 and Pro-564 is required for interaction of HIF-1α with VHL (Jaakkola P., 2001) which is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation (Cockman M.E., 2000). The prolyl hydroxylases use molecular O_2 and 2-oxoglutarate as substrates in a reaction that generates prolyl-hydroxylated HIF-1α and succinate. In normoxic conditions, O_2 is a limiting substrate, therefore providing a mechanism for O_2-dependent regulation of HIF-1α expression. VHL binds hydroxylated HIF-1α and recruits the elongin- C/ elongin-B/cullin-2 E3-ubiquitin-ligase complex, thus targeting HIF-1α for

degradation by the 26S proteasome (Maxwell P.H., 1999). Under low O_2, or in case of the lack of cofactors Fe^{2+} or 2-oxoglutarate, HIF-1α is no longer modified by PHDs (Loboda A., 2010), what prevents binding of pVHL. Therefore, HIF-1α escapes ubiquitination and proteasomal degradation and can migrate in the nucleus where dimerizes with ARNT/HIF-1β (Chilov D., 1999) and recruits numerous coactivators such as CBP/p300 (Fig. 1).

HIF-1 heterodimer bind hypoxia response elements (HREs), within the promoter regions of target genes and enhances adaptive gene transcription. HRE has a core five-nucleotide sequence RCGTG (R: A/G), which is well conserved among numerous hypoxia responsive genes (Semenza G.L., 1996). HIF-1 heterodimer in association with transcriptional cofactors drives the transcription of more than 60 genes such as those involved in cell proliferation (CYCLIN G2, IGF2, WAF1) erythropoiesis (EPO), angiogenesis (VEGF), glucose metabolism (GLUT-1, Hexokinase), pH regulation (Carbonic Anhydrases) and many others (Semenza G.L., 2003).

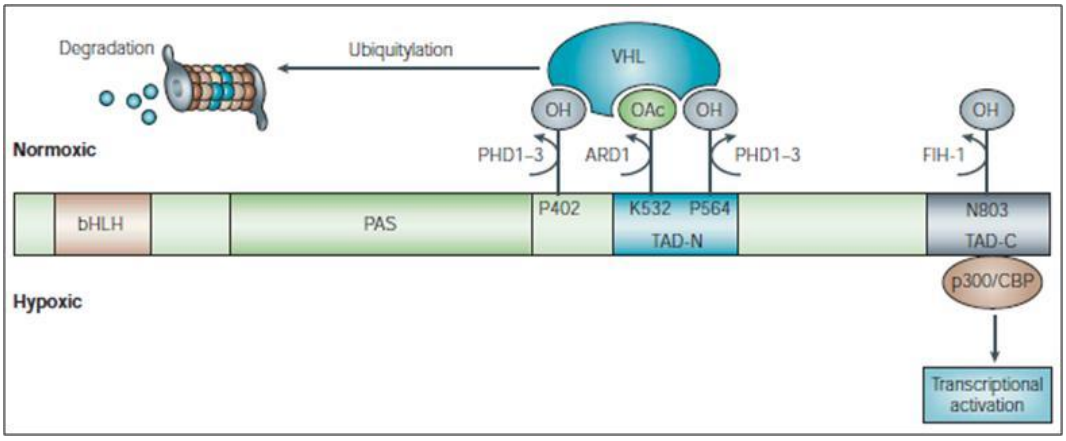

Fig. 1. O_2-dependent regulation of HIF-1α expression and activity. In normoxia, HIF-1α is hydroxylated by Prolyl hydroxylases, bound by VHL which mediates HIF-1α ubiquitylation and proteasomal degradation. Under low O_2, or in case of the lack of cofactors Fe^{2+} or 2-oxoglutarate, HIF-1α escapes proteasomal degradation and migrates in the nucleus where dimerizes with HIF-1β, recruits coactivators such as CBP/p300 and promotes of target genes transcription (from Semenza G.L., 2003).

HIF-1α plays an important role in embryogenesis, chondrogenesis, adipogenesis, B lymphocyte development, osteogenesis, hemopoiesis, T-lymphocyte differentiation, and innate immunity (Semenza G.L., 2003). Furthermore, HIF-1α mediates protective effects in wound healing and in many pathologies such as heart diseases and gastrointestinal inflammatory diseases. It is known that hypoxia promotes self-renewal and influences the differentiation state of several stem cell populations including embryonic (hESCs), hemopoietic (HSCs), and neural stem cells (NSCs) (Mohyeldin A., 2010). Recently it was demonstrated that hypoxia regulates HSCs function *in vivo* via HIF-1α which is highly expressed in HSCs at both mRNA and protein levels. A fine regulation of HIF-1α level is crucial for maintaining of HSCs function: it has been demonstrated that knockout of HIF-1α in the HSCs compartment leads to a decreased quiescence of HSCs, subsequent exhaustion of HSCs, and defects in bone marrow reconstitution capacity during serial transplantation. Furthermore, over-stabilization of HIF-1α by pVHL elimination increases HSC quiescence, but results in loss of reconstitution ability upon transplantation and HSCs exhibit defects in homing and increased apoptosis (Takubo K., 2010).

HIF-1α plays key roles in many crucial aspects of cancer biology including angiogenesis, metabolic reprogramming, autocrine growth factor signaling, epithelial-mesenchymal transition, invasion, metastasis, and resistance to radiation therapy and chemotherapy (Semenza G.L., 2012). HIF-1α protein is overexpressed in several common human cancer and in regional and distant metastases in relative to normal tissues and is overexpressed in pre-neoplastic and pre-malignant lesions such as colonic adenoma, breast ductal carcinoma *in situ*, and prostate intraepithelial neoplasia (Zhong H., 1999). Furthermore, hypoxia signaling has been proposed to regulate self-renewal and multipotency of CSCs (Lee K.E. and Simon M.C., 2012). In addition, many aggressive tumors display gene expression signatures characteristic of hESC and HIF- 1α can drive an hESC-like transcriptional program, activating the expression of the induced

pluripotent stem cell (iPSC) inducers, OCT4, NANOG, SOX2, KLF4, cMYC, and microRNA-302 in 11 cancer cell lines (from prostate, brain, kidney, cervix, lung, colon, liver, and breast tumors) (Mathieu J., 2011).

REGULATION OF *HIF-1a* EXPRESSION

It is known that HIF-1α is under control of hypoxia and the PHDs-VHL-proteasome-mediated post-translational regulation plays a pivotal role in HIF-1α balance. However, in recent years, many studies reported a transcriptional and post-transcriptional control of *HIF-1a* expression that greatly influence HIF-1α balance.

Transcriptional regulation of *HIF-1a* expression

Hif-1a mRNA is constitutively expressed in many cell types, both in normoxic and hypoxic conditions. *HIF-1a* gene promoter sequence analysis in endothelial HMEC-1 cells, which express *hif-1a* in both hypoxia and normoxia, revealed that in different positions of the promoter sequence there are several putative binding sites for Sp1, AP-1, AP-2, HIF-1, c-Ets-1, NF-kB, and NF-1. (Minet E., 1999). Sp1 is a transcription factor that activates transcription of housekeeping genes and other TATA-less genes: its target gene products include factors involved in cell cycle progression and arrest (e.g. cyclins), both pro- and anti-angiogenic factors involved in invasion and metastasis, pro- and anti-apoptotic factors involved in genomic stability, proto-oncogenes (e.g. *c-myc*) and tumor suppressors (e.g. p53 gene). Interestingly, Sp1 protein level is augmented in cancer cells in comparison with normal cells. (Li L. and Davie J.R., 2010).

Post-transcriptional control of HIF-1α expression: regulation of *hif-1a* mRNA stability and translation

The regulation of mRNA stability and translation are essential in the control of gene expression: since their transcription, RNAs can be regulated at various levels: many factors (i.e. proteins and non-coding RNAs) control pre-mRNA splicing and maturation, mRNA export to the cytoplasm and cytoplasmic transit, storage, stability, and translation (Moore M.J., 2005). Post-transcriptional regulation of gene expression occur via specific *cis* elements on the mRNA (i.e. sequences and secondary structures in its 5'- and 3'-untranslated regions (UTRs), or in its coding region) that interact with *trans* binding factors. *Trans*-acting factors can be divided in two main classes: RNA-binding proteins (RBPs), which include 'housekeeping' RBPs that associate universally with mRNAs and RBPs that bind to unique sequences present in subsets of mRNAs and regulatory non-coding RNAs, which include microRNAs (miRNAs) and antisense RNAs (Galbán S. and Gorospe M., 2009). *Cis*- and *trans*-acting factors can control the half-life of a mRNA either by enhancing or inhibiting its degradation. In mammalian cells the sequence elements rich in adenosine and uridine, called AU-rich elements (AREs), are very important in mediating a rapid regulation of a mRNA. AREs are sequence elements of 50–150 nt located in the 3'UTRs of many but not all mRNAs that have a short half-life. Depending on the cellular type and the stimulus, AREs can either provoke destabilization or enhance the stability of a target mRNA (Barreau C., 2006). The minimal sequence motif capable to increase the turnover of a mRNA is the nonamer UUAUUUA(U/A)(U/A) (Zubiaga A.M., 1995), but two or four copies of the nonamer are more efficient in promoting instability than a single copy of this motif.

AREs have been grouped into three classes based on the number and the distribution of AUUUA pentamers. Class I AREs contain many dispersed copies of the AUUUA pentamer within U-rich regions. Class II AREs possess at least 2 overlapping UUAUUUA(U/A)(U/A) nonamers.

Bakheet and others constructed a database of mRNAs containing class II AREs and these regulatory elements were divided into five groups (Bakheet T., 2001): the classification in this ARE database is based on the repetition pattern of the AUUUA pentamer. Class III AREs are much less well defined: they are U-rich regions but contain no AUUUA motif (Barreau C., 2006).

The *hif-1a* 3'UTR contains a class II ARE which contains eight AUUUA instability pentamers (positions 2570, 2658, 2733, 2783, 2787, 3140, 3158, 3205; GenBank accession: U22431) co-localized with three UUAUUUAUU nonamers (positions 2781, 2785, 3138; GenBank accession: U22431) (Rossignol F., 2002). Computer analysis of the 3'UTR and secondary structure prediction for this area and for the whole *hif-1a* mRNA demonstrates that these motifs are inside a hairpin structure (Fig. 2). As reported here below, the *hif-1a* ARE plays an important role in controlling *hif-1a* mRNA stability and translation.

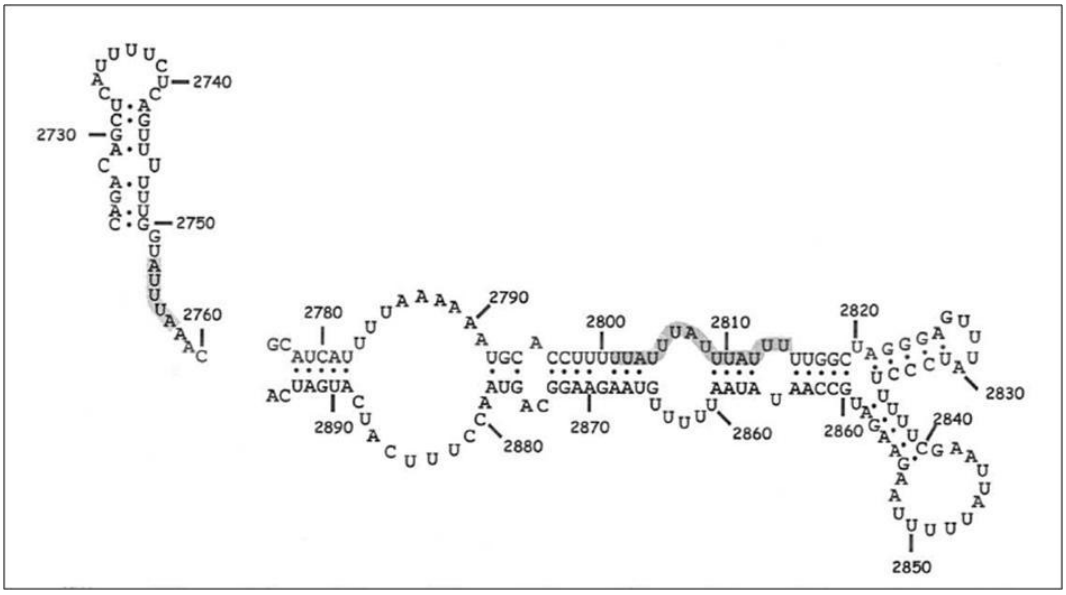

Fig. 2. Structure prediction of *hif-1a* mRNA ARE. *hif-1a* possess a class II ARE which plays an important role in post transcriptional control and is bound by many RNA-binding proteins and, possibly, non-coding RNAs (from Rossignol F., 2002).

Regulation of hif-1α *mRNA stability*

Many transcript are stabilized by exposure to hypoxia, such as VEGF, EPO, and GLUT1 mRNAs. These transcripts possess long 3'UTRs which are recognized by a family of *t*urnover and *t*ranslation *r*egulatory (TTR)-RBPs (Abdelmohsen K., 2008). TTR-RBPs recognize sequences that can mediate mRNA stabilization or destabilization. The *hif-1a* ARE can be bound by many RNA regulators (Fig. 3): among the AUBPs, Tristetraprolin (TTP) has been charachterize to enhance *hif-1a* mRNA decay. TTP family members are double-zinc finger proteins that bind to AREs located in the 3'UTR of many mRNAs encoding growth factors, cytokines, and inflammatory response proteins (Baou M., 2009). Overexpression of TTP in HeLa cells in hypoxic condition determines downregulation of HIF-1α protein: TTP binds to the 3'UTR of *hif-1a* mRNA promoting its degradation and reducing HIF-1α protein expression thereby lowering its transcriptional activity (Kim T.W., 2010). These data were confirmed in endothelial cells in which *hif-1a* mRNA underwent strong downregulation in hypoxia (Chamboredon S., 2011): while silencing of TTP did not affect *hif-1a* mRNA half-life in normoxia, downregulation of TTP in hypoxia completely abolished hypoxia-mediated decay of *hif-1a* mRNA. TTP specifically binds *hif-1a* 3'UTR and, interestingly, the most distal AU-rich (composed of two hexamers AUUUUA) elements present in *hif-1a* 3'UTR a short 187-base-long fragment within the 3'UTR (from b989 to b1175) is sufficient for TTP-mediated HIF-1α repression: mutations of these motifs into AUgUgA partially altered TTP repressive activity, whereas splitting led to a complete loss of TTP action.

Hif-1α *translational control*

Hypoxia provokes a general reduction of protein synthesis by more than 60% mammalian and human cell. However, the association of *hif-1a* mRNA with polysomes is unchanged in hypoxia compared to normoxia,

suggesting the HIF-1α mRNA is translated equally well under both conditions. The 5'UTR of *hif-1a* mRNA is 286 nt long and GC-rich (72%) but is efficiently translated under both normoxia and hypoxia because it contains an Internal Ribosome Entry Site (IRES). However, not only *hif-1a* mRNA is equally translated in normal and low oxygen conditions: a number of proteins that function during periods of cellular stress are translated from mRNAs that contain an IRES which is a crucial factor to maintain the levels of these proteins during the stress condition (Lang K. J. D., 2002).

It has been demonstrated that overexpression of the HER2 receptor tyrosin kinase increases HIF-1α protein and *vegf* mRNA levels in NIH/3T3 cells cultured both in normoxic and hypoxic conditions. Moreover, stimulation of MCF-7 breast cancer cells with heregulin, a ligand of HER2, specifically induces HIF-1α protein level. HIF-1α upregulation is triggered by the PI3K-AKT-FRAP pathway which activity promotes 5'UTR-mediated protein upregulation at the translational level without affecting *hif-1a* mRNA level (Laughner E., 2001).

An intriguing mechanism of regulation of the *hif-1a* mRNA has been elucidated by the research group of Myriam Gorospe who found that treatment of HeLa cells with either hypoxia (1% O_2) or the hypoxia mimetic $CoCl_2$ for 2.5 hours resulted in a marked increase of HIF-1α protein level but did not change *hif-1a* mRNA levels significantly. Incubation of HeLa with L-[35S]methionine and L-[35S]cysteine prior to exposure to $CoCl_2$ demonstrated that there is a marked increment of *de novo* translation of *hif-1a* in $CoCl_2$-treated cells compared to untreated cells; in addition, fractionation of polysomes on sucrose gradients revealed a significant enrichment in the distribution of *hif-1a* mRNA in polysomes in cell treated with $CoCl_2$, indicating that *hif-1a* mRNA is more engaged with the translational apparatus in hypoxia with respect to normoxia. Interestingly, increased translation of *hif-1a* during $CoCl_2$ treatment is not mediated by *hif-1a* IRES activity: instead, AUBPs PTB and HuR can bind to the 3'UTR and 5'UTR of *hif-1a* mRNA, respectively, and their association with the endogenous *hif-1a* mRNA increases

following $CoCl_2$ treatment which also promotes a PTB and HuR-dependent *hif-1a* translation (Galbán S., 2008).

Wang and Lin further demonstrated that PTB binds a region of 61 nucleotides within the *hif-1a* mRNA 5'UTR named D5. Inhibition of PTB expression in CL1 lung adenocarcinoma cells decreased the formation of complex I, which is the protein complex that bind the D5, whereas overexpression of PTB in CL1-5 (CL1-derived lung adenocarcinoma cells) increased the level of complex I, thereby decreasing the stability of *hif-1a* and D5-containing chimeric mRNAs, and decreased cell invasiveness. In summary, we have identified in lung adenocarcinoma cells a mechanism that regulates HIF-1α expression by modulating *hif-1a* mRNA stability (Wang M.J. and Lin S., 2009)

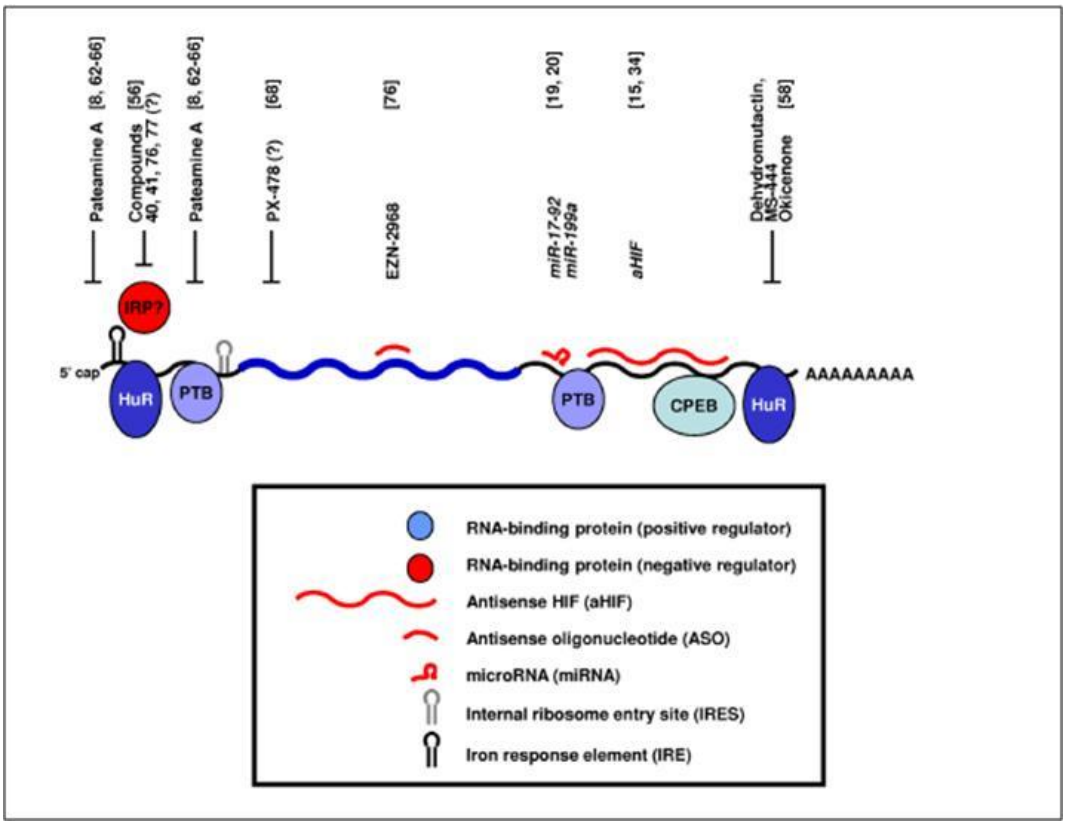

Fig. 3. Schematic representation of factors interacting with *hif-1a* mRNA. HIF-1α expression is regulated at post transcriptional-level by many RNA-binding proteins and non-coding RNAs. The blue line represent the coding region (from Galbán S. and Gorospe M., 2009).

Cytoplasmic polyadenylation-element-binding proteins (CPEBs) are the key factors for regulating mRNA translation: they bind cytoplasmic polyadenylation element (CPEs) in the 3'UTR of specific target mRNAs and promote polyadenylation. The newly elongated tail is then bound by the poly(A)+-binding protein, which promotes general translation by increasing the assembly of the eIF4F initiation complex and thus the recruitment of the 40S ribosomal subunit to the 5' end of the mRNA. The *hif-1a* 3'UTR contains a functional CPE which is bound by CPEB1 and CPEB2: it has been demonstrated that while simple overexpression of CEPB1 or CPEB2 decreases HIF-1α protein levels in unstimulated SK-N-MC neuroblastoma cells by repressing its translation, stimulation with insulin activates CPEB, elevated HIF-1α expression levels, and promote the translation of a heterologous reporter bearing the *hif-1a* 3'UTR (Hagele S., 2009).

HIF-1α post-translational regulation

Beside the classic PHDs-VHL-proteasome-mediated degradation, HIF-1α is regulated at post-translational level via several mechanisms. An alternative mechanism of degradation of HIF-1α depending on VHL but not on PHDs 1 and 3 was recently discovered: mutation of HIF-1α prolines Pro-402 and Pro-563 to alanine, which are crucial for degradation of HIF-1α in normoxia, generates a protein that is still degraded in normoxic condition and is transiently stabilized in response to hypoxia (André H. and Pereira T.S., 2008).

The c-Jun NH2 terminal kinase 1 (JNK1) plays an important role in the regulation of HIF-1α protein accumulation, HIF-1–dependent transactivation, and its downstream target gene transcription in cellular response to either hypoxia or hypoxia mimetic nickel chloride ($NiCl_2$) (Zhang D., 2010.). In mouse embryonic fibroblasts, JNK1 regulates HIF-1α protein stability in a VHL-independent manner protecting Hsp90/Hsp70 chaperones from proteasomal degradation, which results in HIF-1α stabilization in cellular response to nickel exposure.

Interestingly, JNK1 promotes the translation of acetyltransferase HDAC6, which is responsible for removing Hsp90 acetylation thereby facilitating its chaperon activity.
Overexpression of the anti-apoptotic gene bcl-2 in melanoma cells exposed to hypoxic conditions modulates HIF-1α expression at a post-translational level through the stabilization of the HIF-1α protein. bcl-2 interacts with HIF-1α protein in the nucleus and regulates its expression independently from prolyl hydroxylation. bcl-2 may promote stabilization of HIF-1α by increasing its ability to interact with the HSP90 chaperone complex, probably affecting its folding and maturation (Trisciuoglio D., 2010.

Regulation of HIF-1α transcriptional activity

The factor inhibiting HIF-1 (FIH-1) is a non-heme Fe^{2+}, α-ketoglutarate dependent dioxygenase that inhibits HIF-1α transcriptional activity. FIH-1 hydroxylates an asparagines residue (Asn803 in human HIF-1α) within the C-terminal transactivation domain (CTAD) of HIF-1α thereby blocking the recruitment of the co-activator p300. These hydroxylation reactions use O_2 and a-ketoglutarate as substrates and enzyme activity is inhibited under hypoxic conditions, leading to increased HIF-1α stability and transcriptional activity (Mahon P.C., 2001).

NON-CODING REGULATORY RNAs (ncRNAs)

Although approximately 90% of the Human genome is transcribed (Wilhelm B.T., 2008) only a small percentage of RNAs encodes protein: it has been revealed that approximately 2% of the eukaryotic genome encodes messenger RNAs (mRNA) and the majority of transcripts are non-protein coding RNAs (ncRNAs). Traditionally, while mRNAs belongs to the “coding transcripts” cathegory, “non-coding transcripts” are viewed to be ribosomal RNA (rRNA) and transfer RNA (tRNA), both of which play an essential function in protein translation. Although for

long time other ncRNAs (non-r and non-t RNAs), which can be either long (>200 nucleotides) or short, have been considered as transcriptional “thrash”, there is large evidence that these ncRNAs play an important role in cellular function, particularly in gene expression (Costa F.F., 2010; Knowling S. and Morris K.V., 2011). The ENCODE project identified several functional elements in the Human genome and demonstrated that RNA can be processed to yield both short and long RNAs, which can overlap the 5′ and 3′ ends of protein coding regions [Affymetrix ENCODE Transcriptome Project, Cold Spring Harbor Laboratory ENCODE Transcriptome Project, 2009). Classically gene expression has been considered to be in large part under control of transcription factors: nonetheless there is growing evidence that ncRNAs can control gene expression at different levels, i.e. transcriptional gene silencing through the targeted recruitment of epigenetic silencing complexes to particular loci, post-transcriptional gene silencing via degradation of transcriptionally active mRNAs and so on.

Interestingly, the FANTOM-3 mouse transcriptome sequencing consortium performed a large-scale mouse cDNA sequencing (FANTOM Consortium and RIKEN Genome Exploration Research Group and Genome Science Group (Genome Network Project Core Group), 2005) and revealed that more than 72% of all genome-mapped transcriptional unit (a group of expressed sequence tags or mRNAs, usually with alternative splice patterns, in which they share exonic overlap of at least one nucleotide and are in the same chromosomal orientation) seem to contain antisense transcripts.

Non-coding RNAs (ncRNAs) can be roughly divided on the basis of size into short ncRNAs (less than 200 nt in length) and long ncRNAs (lncRNAs). Short ncRNAs include microRNAs, endogenous short interfering RNAs, PIWI-interacting RNAs, small nucleolar RNAs and small nuclear RNAs, among others; long ncRNAs include promoter associated transcripts, long intergenic non-coding RNAs and natural antisense transcripts (Röther S. and Meister G., 2011) (Fig. 4).

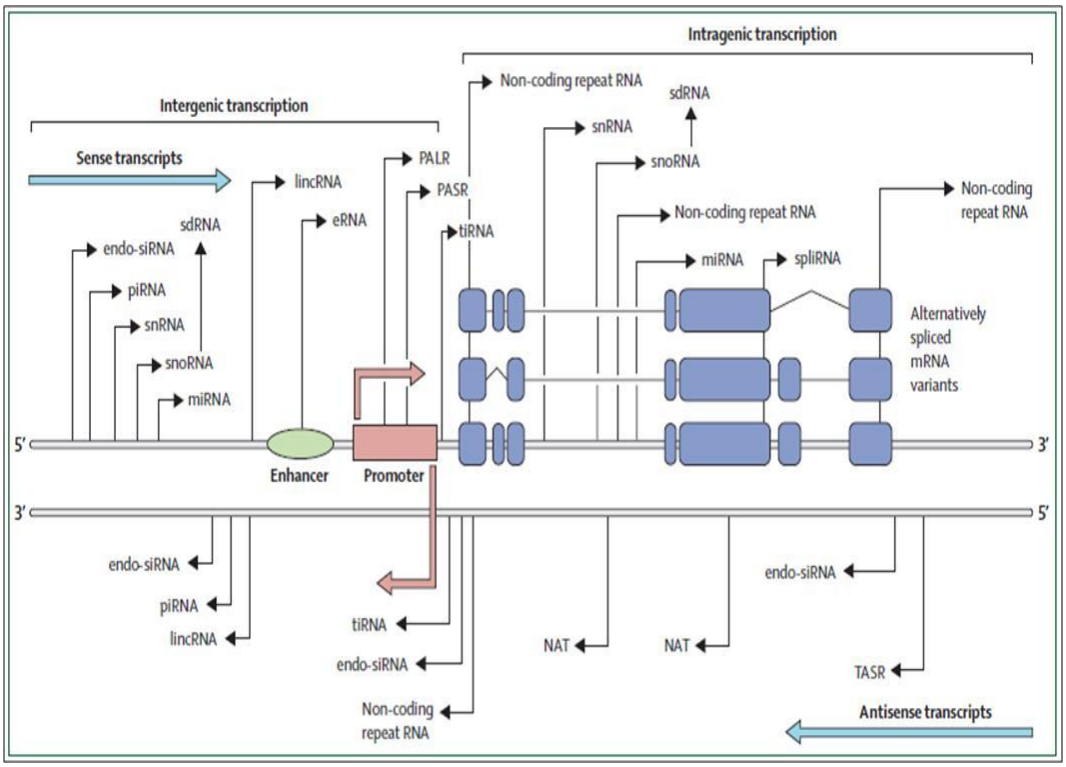

Fig. 4. Complexity of transcription patterns of non-coding RNAs. Non-coding RNAs can be transcribed by RNA polymerase I, II or III from intergenic or intragenic regions. Long non-coding RNAs can undergo modification similar to coding mRNAs (i.e. poly-adenylation, capping, splicing) or can be processed to generate short non-coding RNAs (from Salta E. and De Strooper B., 2012).

Short RNAs

MicroRNAs

MicroRNAs (miRNAs) were discovered in *Caenorhabditis elegans* (Lee R.C., 1993). miRNAs derive from primary miRNAs (pri-miRNAs) which genes are transcribed by RNA polymerase II or III; a pri-miRNA (0.5–7kb) folds into a single or a cluster of multiple hairpin structure(s) and is processed in the nucleus by a complex composed of the Rnase III Drosha and the RNA binding protein cofactor Pasha (microprocessor complex): cleavage of pri-miRNA results in the production of short hairpin RNAs (60–70 nucleotides) named precurson miRNAs (pre-miRNAs) that are transported across the nuclear membrane by Exportin

5; in the cytoplasm pre-miRNA is cleaved once more by the RNase Dicer1 which remove the hairpin loop from the double-stranded RNA, leaving a short miRNA duplex that is unwound by a helicase, cleaved into a mature miRNA (18–25 nucleotides), and incorporated into an RNA-induced silencing complex (RISC), with an Argonaute (Ago) protein as the catalytic component. The miRNA-RISC complex negatively regulates post-transcriptional gene expression by hybridizing to complimentary sequences in the 3'UTR of a target mRNA and inhibiting protein translation or degrading the mRNA itself. (Jeffrey S.S., 2008).

Endogenous small interfering RNAs

Endogenous small interfering RNAs (endo-siRNAs) have been found in many species. Endo-siRNAs are similar to miRNAs in their binding to the Ago subfamily proteins. siRNA processing from long double stranded RNA to 21 nt long siRNAs depends on Dicer activity but not on Drosha. Similar to miRNAs, a short double stranded intermediate RNA is formed and one strand, referred to as the guide strand is incorporated into the Argonaute-containing RNA-induced silencing complex. As for miRNAs, perfect sequence complementary between the guide strand loaded on RISC and the target mRNA leads to endonucleolytic cleavage of the mRNA and subsequent degradation endo-siRNAs are mainly implicated in small RNA-mediated silencing of transposable elements.

PIWI-interacting RNAs

PIWI-interacting RNAs (piRNAs) are 26-31 nt long transcripts, characterized by the presence of a 5’ uridine and their association with PIWI proteins, the germline specific Argonaute subfamily and are generated from single-stranded precursors in a RNase III-independent manner (Portnoy V., 2011). piRNAs are transcribed from intergenic repetitive elements in the genome called piRNA clusters that span a

wide region of the genome, occasionally consisting of more than 100 kilobases, and are mostly comprised of antisense to transposons and suppress transposon activity by transcriptional silencing. piRNAs seem to play a role in gametogenesis. (Ishizu H., 2012).

Small nucleolar RNAs and small nuclear RNAs

Small nucleolar RNAs (snoRNAs) are a highly evolutionary conserved class of non-coding RNAs concentrated in nucleoli where they are involved either in ribosomal RNA (rRNA) and U6 spliceosomal small nuclear RNA (snRNA) modification or in the processing of rRNA during ribosome subunit generation (Röther S. and Meister G., 2011). Two main classes of snoRNAs are known: C/D box snoRNAs and H/ACA box snoRNAs, both of which cooperate with a conserved pattern of proteins in controlling target RNAs processing at sites determined by RNA/RNA antisense interactions. In addition to rRNA processing and site-specific modification of ncRNAs there are many evidence of the existence of snoRNA-derived sRNAs that function similarly to miRNA in mRNA processing and silencing. The first snoRNA-derived sRNAs were described in the unicellular protozoan Giardia lamblia and in HEK293 T cells.

Long non-coding RNAs

Promoter associated transcripts

Promoter associated transcripts (promoter-associated RNAs, or paRNAs) are short-lived RNAs of different length which maps to gene promoter regions (Kapranov P., 2012): these transcript can originate from transcription start sites [PASRs (promoter-associated short RNAs)], terminator sites [TASRs (termini-associated short RNAs)] or promoter regions [PROMPTs (promoter upstream transcripts)]. PASRs and TASRs are between 20 and 90 nucleotides in lenght, whereas PROMPTs are

usually longer than 200 nucleotides (Werner A. and Swan D., 2010). Interestingly, all of the different RNAs occur in sense and antisense orientations and this pervasive divergent promoter activity suggests a lack of definite 5' and 3' boundaries for the transcribed genes. Although for the majority of paRNAs the mechanism of action is still unknown, there are evidences that some of these transcripts can exert either positive or negative regulation on the target gene by triggering heterochromatic histone modifications or promoting DNA hypermethylation via formation of an RNA/DNA triplex.

Long intergenic non-coding RNAs

An interesting class of lncRNAs is that of long intergenic non-coding RNAs. Long intergenic non-coding RNAs (lincRNAs) are a heterogeneous group of transcripts of 300 to thousands nucleotides in size that are involved in epigenetic control of the cell (Knowling S., 2009). The analysis of chromatin signatures revealed a group of over a thousand highly conserved lincRNAs, that contain sense and antisense transcripts which may have important regulatory functions. To date the human catalog of lincRNAs is estimated to be around 3,300 although the true number may be closer to 4,500. Interestingly, and unlike most NATs, lincRNAs acts as trans factors recruiting chromatin-modifying complexes and alter gene expression at distant loci. The 17,000 nucleotide transcript Xist is most studied and well understood lincRNA: it is involved in X chromosome inactivation by recruiting repressive regulatory complexes and resulting in chromatin silencing of the corresponding locus or X allele respectively. The lincRNA HOTAIR is encoded in antisense orientation in the HOX-C cluster on chromosome 12 and it is necessary for the correct expression of the HOX-D cluster of genes on chromosome 2. HOTAIR creates a scaffold for the recruitment of Polycomb Repressive Complex2 (PRC2), a complex composed by several histone methyltransferases, that silence the expression of HOX-D gene cluster. An impaired expression of HOTAIR is a predictor of

metastasis and poor prognosis in primary breast tumors. Furthermore, inhibition of HOTAIR expression in tumor cells determines reduction of invasiveness and metastatic potential. lincRNAs plays an important role in maintaining the pluripotency and repress lineage-specific gene expression in mouse Embryonic Stem Cells via trans-acting mechanisms of global gene expression regulation (Guttman M.,2011).

Natural antisense transcripts (NATs)

Natural antisense transcripts (NATs) are a particularly intriguing group of ncRNAs which are generally non-proteincoding transcribed from the opposite strand of protein-coding sense transcripts: however, diversely from other ncRNAs, they are usually fully processed, i.e. capped and poly-adenylated and partially or totally overlap transcripts originating from the opposite strand. NATs can regulate their corresponding sense mRNAs at different levels, including transcriptional interference, imprinting, alternative splicing, translation or RNA editing. Recently, it has been demonstrated that the *PU.1* locus encodes both mRNA and natural noncoding antisense transcripts that are originating from an intronic promoter (Ebralidze A.K., 2008). The transcription factor PU.1 expression is crucial for normal hemopoiesis and for determining cell fate: if deregulated, even modest decreases in PU.1 can lead to the onset of leukemias and lymphomas. The *PU.1* locus encodes both mRNA and natural noncoding antisense transcripts that are originating from an intronic promoter: this NAT have a regulatory function and control *PU.1* gene expression via the regulation of translation.
A bcl-2 antisense transcript was discovered in the t(14;18) DOHH2 and SU-DHL-4 cell lines but not in the t(14;18)-negative Raji and Jurkat lymphoid cells, suggesting that its expression is linked to the bcl-2/IgH fusion. This antisense transcript is a hybrid bc1-2/IgH RNA, that originates in the IgH locus, encompasses the t(14;18) fusion site and spans at least the complete 3' UTR region of the bcl-2 mRNA. Treatment of t(14;18) DOHH2 cell line with oligonucleotides (ODNs) by specifically

targeting the bc1-2/IgH antisense strand resulted in lowering bcl-2 gene expression and inhibition of neoplastic cell growth by inducing apoptosis. The bc1-2/IgH antisense transcript may contribute, by an unknown mechanism, to upregulation of bcl-2 gene expression in t(14;18) cells. The possibility has been considered that the hybrid antisense transcript mask AU-rich motifs present in the 3' UTR of the bcl-2 mRNA characterized in other genes as mRNA destabilizing elements (Capaccioli S., 1996).

HIF-1α is a crucial factor for cellular adaptation to hypoxia: in 1999 was discovered a natural antisense encoded by the antisense strand of *HIF-1a* and named aHIF. aHIF was strongly overexpressed in nonpapillary clear-cell renal tumors hallmarked by the inactivation of the tumor suppressor gene von Hippel–Lindau (VHL). aHIF is expressed in many human adult, fetal and tumor tissues and it is conserved in rodents. It has been speculated that this transcript could exert a negative regulation of *hif-1a* stability and, interestingly, aHIF expression is, at least in part, under the transcriptional control of HIF-1, thus suggesting existence of a HIF-1α autoregulatory negative loop.

How do natural antisense transcripts regulate RNA expression?

It is still unclear how NATs regulate sense RNAs expression: here, we report four mechanisms of action that have been proposed:

DNA replication interference

NATs can interfere with and inhibit DNA replication. This was demonstrated elegantly in the *E. coli* plasmid ColE1. The ColE1 primer transcript (RNA II) binds to the template DNA near replication origin. The plasmid replication is dependent on the primer formation (RNA II). Hybridization of RNA II to DNA is inhibited when a second RNA molecule RNA 1 (NAT RNA) binds to RNA II, thereby altering the secondary structure of RNA II.

RNA/DNA interactions: DNA and chromatin remodeling

NATs have been proposed to modulate sense RNA expression via interacting with chromatin: NATs can recruit many enzymes such as DNA methyltransferase causing suppression of transcription by DNA and chromatin modification at the promoter region of the sense strand. It is interesting to note that, although the abundance of a natural antisense RNA is very low compared to its sense pair, there are two copies of DNA for any given gene in cells; therefore, theoretically only two molecules of antisense RNA per cell are sufficient to bind to the corresponding DNA strand and to exert a regulatory function. Some of the antisense-mediated epigenetic changes are reported to be independent of Dicer, arguing against a role for small RNA mediators. Therefore, antisense RNA processing to small RNA, which may be mediated by Dicer in the cytoplasm, is not necessarily required for epigenetic modifications. In addition, NATs are also associated with genomic imprinting.

Transcriptional interference (transcription-related modulation)

In the "transcriptional interference model" the antisense RNA is not per se involved in sense RNA transcription interference or in altering its stability. This model is based on the assumption that RNA polymerases bind to the promoters of convergent genes on opposing strands of DNA and then proceed towards the 3' end of sense and antisense genes. Clash of RNA polymerase complexes elongating in convergent direction provokes a block of transcription: in other words, antisense transcription halts transcription of the sense mRNA determining a reduction of its level, but apparently this is not the main mechanism of NATs-mediate gene regulation.

RNA duplex formation

Natural antisense transcript can exert their regulatory role via direct interaction with sense RNAs either in the nucleus and in cytoplasm: due to a perfect complementary matching with target mRNAs, NATs can form duplex RNA that may result in several outcomes, all of which modulate sense mRNA expression. In the nucleus antisense RNA may bind to the sense RNA, masking the splice sites and thereby altering the balances between splice variants: similarly, antisense transcripts can potentially cause alternative polyadenylation and termination of transcription. In addition, NATs may modulate mRNA editing and mRNA nuclear transport: it has been observed that several cell stress inductors such as hypoxia, serum starvation and hydrogen peroxide, can alter the nuclear retention of antisense transcripts, thereby altering the levels of their sense partners (Faghihi M.A., 2008). It is then likely that antisense RNAs can interact with nuclear proteins or other nuclear RNAs. In the cytoplasm, the sense–antisense duplex RNA formation may affect sense mRNA stability or translation. The overlapping between a natural antisense and a sense RNAs can alter sense RNAs stability, reducing or promoting its decay: this could be due to alteration in the secondary or tertiary structure of the target RNA. (Zuker, M., 1994; Mathews, D.H., 1999). AU-rich elements (AREs) are sequence elements of 50–150 nt that are rich in adenosine and uridine bases and that represent a very important class of regulatory elements (Barreau C., 2005). They are located in the 3'UTRs of many but not all mRNAs that have a short half-life and have been identified by their capacity to cause stabilization or destabilization of target RNAs. AU-rich elements can be recognized by many AU-binding proteins (AUBP) that binds both sequence and secondary structure of ARE: disruption of the secondary structure by antisense RNAs can prevent RNA binding by AUBP, thus leading to an alteration of target RNA half-life. NATs can also modulate gene expression by inhibiting mRNA translation without affecting its level as in the case of *PU.1* mRNA.

THE NATURAL *hif-1a* ANTISENSE (aHIF)

The natural *hif-1a* antisense (aHIF) was discovered in 1999 by Catherine Thrash-Bingham and Kenneth D. Tartof in nonpapillary clear-cell renal carcinoma (Thrash-Bingham C.A. and Tartof K.D., 1999). Nonpapillary renal carcinomas have a distinct morphology from papillary renal carcinomas and are characterized by inactivation of the tumor suppressor gene von Hippel–Lindau (VHL) (Kovacs G. 1993; Gnarra J.R., 1994). VHL plays a fundamental role in mediating HIF-1α post-translational degradation trough the ubiquitine-proteasome pathway. Thrash-Bingham and Tartof identified a distinct antisense transcript that is strongly overexpressed in clear-cell renal tumors but not in normal kidney nor in renal oncocytoma. This antisense, named "aHIF", is 1577 nucleotides long (GenBank accession: U85044) and is not polyadenylated. The authors assessed the relative abundance of aHIF and *hif-1a* mRNA and demonstrated that in nonpapillary clear-cell renal tumors there is a 10- to 100-fold increase in aHIF expression compared to normal kidney tissue and a statistically significant increased expression of aHIF in nonpapillary tumors compared with papillary disease. However, although aHIF was markedly overexpressed in nonpapillary clear-cell tumors, its abundance was very modest: there was, in fact, about 250-fold more β-actin transcript than aHIF.

In order to determine if aHIF could be induced by hypoxia the authors exposed a nonpapillary renal cell line derived from one patient (named RCC22) to varying oxygen conditions (21%, 0.3%, 0.1%, and 0%) for 2 hours and found that aHIF transcript remained elevated and at about the same level, both in normoxic and in hypoxic conditions. Conversely, when an EBV-transformed lymphocyte cell line estabilished from the same patient (RCC22) was exposed to varying oxygen conditions (21%, 0.3%, 0.1%, and 0% oxygen) for 2 hours aHIF expression was strongly induced (8-fold) under conditions of 0.3% oxygen or less. Furthermore, in lymphocytes exposed to hypoxic conditions (0.1% oxygen) for various time, aHIF level tends to increase after two hours and this increase is

sustained over the next six hours of incubation while *hif-1a* mRNA level appeared to be reduced about twofold. This inverse relationship suggested to the authors that aHIF expression may negatively affect the abundance of *hif-1a* mRNA.

In 2002, Rossignol and coworkers reported that aHIF is expressed in many human adult tissues (muscle, liver, kidney, thymus, brain, leukocyte, and bone marrow) and fetal tissues (muscle, liver, thymus and lung but not in kidney). aHIF was expressed also in mammary carcinoma, in bone marrow presenting a chronical myeloid leukaemia and in neuroblastoma (Rossignol F., 2002). Measurement of the *hif-1a* mRNA/aHIF transcript ratio by real time RT-PCR revealed that the three fetal tissues tested have a higher level of aHIF transcript compared to their corresponding adult tissue and a lower *hif-1a* mRNA/aHIF transcript ratio, although an up-regulation of *hif-1a* messenger at the fetal stage in liver and muscle could be noted.

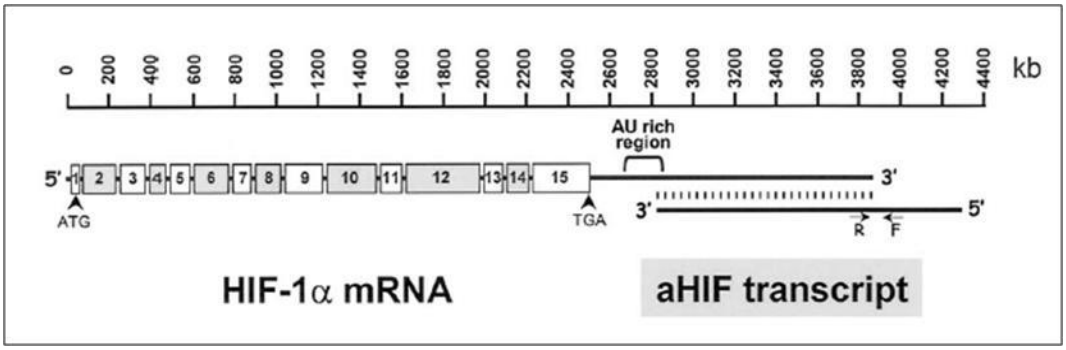

Fig. 5. Schematic representation of *hif-1a* mRNA and the natural antisense aHIF. aHIF could bind the 3'UTR of *hif-1a* mRNA and interfere with the AU-rich elements thereby disrupting secondary structures involved in *hif-1a* mRNA regulation (from Rossignol F., 2002).

Computer analysis of *hif-1a* and aHIF sequences revealed that aHIF transcript overlaps the *hif-1a* mRNA at least for 1027 bases (Fig. 5): thus, Rossignol and collaborators suggested that aHIF could form RNA duplex with the *hif-1a* mRNA thereby disrupting secondary structures within the 3′UTR. In particular, aHIF could interfere with AU-rich

element in the 3'UTR increasing *hif-1a* mRNA instability via exposing it to potential destabilizing proteins.

Uchida and collaborators studied the role of HIF-1α and HIF-2α regulation and speculated on the role of aHIF in prolonged hypoxia in A549 lung adenocarcinoma cell line. HIF-1α was almost undetectable in both whole cell and nuclear lysates while HIF-2α was detectable in whole cell lysates, but not in nuclear extracts. Exposure of cells to hypoxia (0.5% O_2) or with 250 μM $CoCl_2$ for 4 hours induced both HIF-1α and HIF-2α accumulation in both whole cell and nuclear extracts. Treatment with hypoxia for 4 hours, however, did not affected *hif-1a* and *hif-2a* mRNA levels. Interestingly, however, A549 cells showed a different HIF-1α and HIF-2α kinetics when exposed to hypoxia or $CoCl_2$ for more than 4 hours: HIF-1α protein level dramatically increase after 4 hours of hypoxia but decreases after 6 h while, after a strong induction after 4 hours of hypoxia, HIF-2α protein levels remained unchanged. Furthermore, treatment with $CoCl_2$ dramatically decreased the half-life of *hif-1a* mRNA compared with normoxia while, in contrast, the half-life of *hif-2a* mRNA did not change upon $CoCl_2$ treatment (17.2 h versus 18.2 h). Interestingly, aHIF transcript expression was strongly upregulated after 4 hours of hypoxia, and its expression remained elevated until 12 h. Furthermore, the authors transfected cells with a truncated HIF-2α protein (phEP-1-(1–485)) that functions as a dominant-negative mutant for both HIF-2α and HIF-1α subunits inhibiting their transcriptional role: transfection with phEP-1-(1–485) versus control prevented aHIF hypoxic upregulation indicating that aHIF expression is, at least in part, under the transcriptional control of HIF-1α.

aHIF as a prognostic marker

In recent years, long noncoding RNAs have been shown to have critical regulatory roles in cancer biology and some of them are prognostic marker. The long noncoding *malat-1* RNA has been indicated as a poor prognostic marker in non-small cell lung cancer (Schmidt L.H., 2011) and the lncRNA-HEIH has been found to be an oncogenic lncRNA that promotes tumor progression and a poor prognostic marker in hepatitis B virus (HBV)-related hepatocellular carcinoma (Yang F., 2011). Because of its overexpression in nonpapillary clear cell renal carcinoma, aHIF was considered as a possible tumor marker to be investigated. Rossignol and collaborators reported the expression of aHIF in breast carcinoma (Rossignol F., 2002). Furthermore, HIF-1α protein is frequently overexpressed in primary and metastatic human tumours and specifically in in ductal in situ and invasive breast carcinomas (Zhong H., 1999; Bos R., 2001). Cayre and collaborators examined 110 non metastatic primary breast cancers for the presence of *hif-1a* and aHIF transcripts, and studied their correlations *in vivo* to determine whether the concentrations of these transcripts are associated with prognosis in human breast cancer, the most common cancer in women (Cayre A., 2003). Expression of *hif-1a* and aHIF in breast tumours was highly variable: *hif-1a* and aHIF were detected in 85% and 72% of the tumours, respectively. A weak positive correlation was observed between *hif-1a* and aHIF. *hif-1a* was more highly expressed in lymph node-positive tumours than in lymph node-negative tumours and in estrogen receptors-negative receptors. Authors also observed a positive relation between HER2 and *hif-1a* whose expression in invasive ductal carcinoma was 4.5-fold that in invasive lobular carcinoma but found no relation between histological grade and either *hif-1a* or aHIF expression. In addition, aHIF is not related with clinical lymph node status, histological type, oestrogen receptors, HER2 or p53. aHIF expression was higher in tumours of more than 5 cm (T3–T4) and in those with a high proliferation rate. Pathological responses of patients were followed

after surgical resection but neither HIF-1α nor aHIF was predictive of treatment to neoadjuvant chemotherapy. Furthermore, authors compared expression of *hif-1a* and aHIF with disease-free survival: while there was no relation between *hif-1a* and survival of breast cancer patients, aHIF was found to be a strong independent poor prognostic marker. Association between aHIF expression and disease-free survival revealed that the 5-year survival rate was 80% in patients with low aHIF levels and 45% in patients with high aHIF levels; furthermore, the death risk for patients with high aHIF espression was 2.8-fold that in patients with espression of aHIF.

More recently, Span and collaborators investigated for the first time the role of the natural antisense transcript aHIF in Paragangliomas (PGLs) (Span P.N., 2011). PGLs are catecholamine-producing tumors deriving from chromaffin cells of the adrenal medulla or from sympathetic neuronal tissue in extra-adrenal locations of the abdomen or chest and that give rise to metastases in 10–15% of patients. Two different types of PGLs can be defined by expression profiling: cluster 1 PGLs exhibit VHL and/or succinate dehydrogenase mutations and a pseudohypoxic phenotype while cluster 2 PGLs exhibit mutation of RET and neurofibromatosis type 1 and are characterized by deregulation of the RAS/RAF/MAP kinase signaling cascade; sporadic PGLs can exhibit either profile. aHIF levels were significantly higher in cluster 1 compared with cluster 2 PGLs; sporadic and apparently sporadic tumors exhibited expression levels that were not significantly different from clusters 1 and 2 tumors. Furthermore, the authors assessed aHIF transcript expression in non-metastatic PGLs in comparison with primary tumors of metastatic PGLs and found that aHIF expression was significantly higher in the primary tumors of PGLs that metastasized during follow-up than in the non-metastatic PGLs. Finally, the authors found that high expression of aHIF is associated with decreased metastasis-free survival.

aHIF is conserved and plays a role in *hif-1a* regulation in mammalians

In 2002, Rossignol and coworkers detected aHIF in several human adult normal and tumor tissues as well as in fetal tissue and in 2004 they investigated if the natural antisense could be detected in rodent cell and tissues in order to understand if its sequence and its role could be conserved in other mammalian species (Rossignol F., 2004). The authors cloned mouse and rat aHIF sequences and found that each of those two sequences was complementary to the 3'UTR of its respective *hif-1a* mRNA; moreover, the 3' portions of mouse and rat aHIF that are complementary to *hif-1a* were very similar to the human aHIF sequence, while the 5' portions of aHIF sequences were not conserved between species. Both aHIF and *hif-1a* are expressed in all tested tissues although *hif-1a* mRNA levels were lower in liver than in the other tissues in both species.

In order to study the effect of hypoxia on aHIF and *hif-1a* expression in normal rat cells, the authors used three different oxygen conditions (normal, mild and severe hypoxia) using a rat proximal tubules cell model, and found that after 4 hours, *hif-1a* mRNA was more expressed in mild and in severe hypoxia than in normoxia and aHIF expression was upregulated in hypoxia.

Furthermore, the authors compared aHIF and *hif-1a* expression in two types of skeletal muscles, in heart and in brain of rats adapted to high altitude (3700 m) and tissues of rats bred at sea level. At sea level, aHIF transcript espression was the highest in fast, glycolytic-type skeletal muscle while brain espressed intermediate levels of aHIF and in slow, oxidative-type skeletal muscle and in heart aHIF transcript level was low. Interestingly, in fast muscle *hif-1a* mRNA amount was lower than brain or slow skeletal muscle. In high altitude-adapted rats, aHIF level was lowered in fast muscle, while *hif-1a* level was increased. In slow muscle and heart, though, there was a strong increase of aHIF transcript. However, *hif-1a* mRNA was not changed in slow skeletal

muscle and increased in heart and in brain, *hif-1a* and aHIF transcript levels remained stable in the two conditions of oxygenation. In addition to these datas, the authors found that in the rat NB104 and PC12 cell lines treatment with cobalt chloride for 6 h provoked an increase of aHIF transcript amounts with reduction of HIF-1α mRNA levels while in the mouse CT-26 colon cancer cell line, both *hif-1a* and aHIF were downregulated.

Maistrovski and coworkers studied HIF-1α regulation in mammalian hibernators (Maistrovski Y., 2012). Many small mammals use hibernation to survive winter conditions with subzero environmental temperatures and a lack of food. The hibernation period is characterized by periods of deep torpor during which metabolic rate is strongly reduced, often to just 1–5 % of the normal euthermic rate and body temperature falls to near ambient values.

Entrance into torpor is tightly regulated by strong reversible controls (e.g. protein phosphorylation) that alter protein function and reprogram metabolic processes. While transcriptional and translational processes are suppressed, some genes important for stress survival, are up-regulated and among them is HIF-1α. During torpor, breathing rate and blood flow are strongly reduced and blood flow: thus, the demand for oxygen is also strongly reduced because metabolic rates are 20- to 100-folds lower than in euthermia. The organs of hibernating animals are considered ischemic when compared to the active animal at 37°C and HIF-1 plays an important role in protecting and reprogramming cellular metabolism (Dirnagl U., 2009; Loor G. and Schumacker P.T, 2008; Chun Y.S., 2002). Because recent studies have characterized some elements of the HIF-1α and microRNA responses to hibernation in thirteen-lined ground squirrels (Ictidomys tridecemlineatus) (Morin P., 2008) , Maistrovski and collaborators investigated HIF-1α regulation in ground squirrels and little brown bats (Myotis lucifugus) during hibernation and changes in antisense aHIF mRNA expression.

HIF-1α protein expression increased in skeletal muscle, but not in liver, of hibernating squirrels (3 days in torpor) as compared to controls.

Conversely, both liver and skeletal muscle from hibernating little brown bats showed higher HIF-1α levels during torpor, compared to euthermic controls. Interestingly, *hif-1a* mRNA level remained unchanghed in either liver or skeletal muscle of hibernating thirteen-lined ground squirrels, compared with controls while it increased in both liver and skeletal muscle in hibernating versus euthermic little brown bats.
aHIF was not detected in the liver of either squirrels and bats. aHIF was present in skeletal muscle of ground squirrel muscle but its level remained unchanged both in euthermic and in hibernating group of animals. Conversely, in hibernating little brown bats aHIF levels were 13% of control group. These results suggest that increases of HIF-1α protein in selected tissues during hibernation could be due, in part, to the combined influence of non-coding RNAs on the translation of *hif-1a* mRNA.

5'HIF-1α antisense: a novel anti-*hif-1a* lnc-RNA

Recently, the group of Giovanni Capranico identified and charachterize a new lnc-RNA covering the 5' region of the *HIF-1a* gene locus (5'aHIF-1α) (Bertozzi D., 2011). Treatment of HeLa and HCT-116 cells with campthotecin, an analogue of Topotecan, induces transcription of 5' antisense RNA which is also expressed in human kidney cancers.The 5'aHIF-1α RNA is polyadenylated and 5'capped, it is localized mainly in the nucleus and accumulates at the perinuclear compartment. Its transcription may be dependent from RNA polymerase II and, apparently, 5'aHIF-1α antisense possess no obvious ORF. This antisense transcripts might be involved in mRNA degradation or chromatin inactivation of the *HIF-1a* gene locus. The finding of a new transcript encoded by the *HIF-1a* gene may shed a new light on HIF-1α ncRNAs-mediated regulation.

Anti- *hif-1a* antisenses in cancer therapy

Since its discovery, HIF-1 and its role in cancer biology have been widely studied (Semenza G.L., 2003). The fine regulation of HIF-1α expression, through transcriptional, post-transcriptional, and post-translational events, evidences the importance of modulating the abundance of HIF-1α both gradually and acutely (Galbán S. and Gorospe M., 2009). Many small molecules have been shown to interfere with HIF-1 expression and with its activity at different levels. Topoisomerase I inhibitors block HIF-1α expression via interfering with its mRNA translation. Disruption of microtubule polymerization by 2-methoxyoestradiol (2ME2) has also been shown to result in decreased HIF-1α levels and decreased *vegf* mRNA expression in cultured cells. Pateamine A, an anti-proliferative, pro-apoptotic, and immunosuppressive compound, can block protein translation by preventing cap-dependent and eIF4G-dependent as well as IRES-driven translation at concentrations lower than 0.1 μM. Many drugs such as inhibitors of the PI3K pathway (rapamycin, wortmannin, LY294002) and ERK inhibitors (PD98059) can inhibit Cap-dependent HIF-1α translation. The small molecule inhibitor PX-478 was shown to reduce the transcriptional activity of HIF-1α, in turn inhibiting angiogenesis and glycolysis. PX-478 was further shown inhibit HIF-1α deubiquitination and lower *hif-1a* mRNA levels. It should be noted, though, that the anticancer effects of these agents might be due, in part, to their inhibition of HIF-1, but, on the other hand, it seems that none of these drugs specifically target HIF-1. Conversely, RNA Inhibitors such as Antisense Specific Oligonucleotides (ASO), siRNAs, short hairpin RNAs (shRNA) and locked nucleic acids (LNAs), because of their complementarity to the target mRNAs, could be intriguing and specific drugs capable to lower HIF-1α. HIF-1α knockdown by RNAi in a glioma model slowed tumor progression (Li L., 2005). EZN-2968, an LNA designed to target the HIF-1α mRNA, which is currently being evaluated in phase I clinical trials, significantly decreased HIF-1α expression both

in cultured cancerous cells and in animals. After a single injection of EZN-2968, the levels of endogenous *hif-1a* and *vegf* mRNAs were reduced for several days. Moreover, EZN-2968 was also highly effective in downregulating HIF-1α levels and slowing down the growth of human prostate tumor xenografts in nude mice (Greenberger L.M., 2008).

Since the discovery of aHIF, only few attempts have been made to modulate *in vitro* or *in vivo* expression of HIF-1α through an anti-*hif-1a* antisenses therapy. In 2006, Chang at al. produced through PCR a 721-base pair cDNA fragment using the pCR3.1-FLAG-HIF-1α plasmid as a template and the fragment was cloned into pcDNA3.1(+) at BamHI and XbaI sites. Plasmid transfection of BxPc3 pancreatic tumor cell line exposed to severe hypoxia (0,5% oxygen) for 4 hours resulted in a strong diminuition of *hif-1a* mRNA and protein levels. Furthermore, the antisense-*hif-1a* plasmid synergized with chemotherapeutic drugs 5-Fluorouracyl, Gemcytabine and Doxorubicin in inhibiting cell proliferation, adhesion and invasion as well as enhancing apoptosis, i.e. enhances the overall chemosensitivity of pancreatic cancer cells (Chang Q., 2006). A different antisense, The antisense-*hif-1a* expression vector (aHIF-pcDNA3.1) containing a 320-bp antisense cDNA fragment of HIF-1α, was used by Sun and coworkers (Sun X., 2001; Sun X., 2003) and by Liu and collaborators in 2008. Sun and collaborators demonstrated that transfection of small EL-4 tumors, 0.1 cm in diameter, established in the right flank of C57BL/6 mice, with a DNA/liposome transfection vehicle containing 100 mg of aHIF-pcDNA3.1 plasmid determines downregulation of *hif-1a* and *vegf* and a complete eradication of tumors with respect of those transfected with control plasmid:. However, while aHIF-pcDNA3.1 treatment was able to eradicate small tumors, it could only delay the growth of larger 0.4 cm diameter tumors. Nevertheless, combined transfection of 0.4 cm diameter tumors with 100 mg of each of the aHIF-pcDNA3.1 and a plasmid overexpressing VHL (VHL-pcDNA3.1) resulted in a complete regression of tumors within 15 days of plasmid injection. Furthermore, injection of either aHIF-cDNA3.1or VHL-pcDNA3.1 into 0.4 cm tumors provoked an inhibition of inhibited

tumor angiogenesis and a reduction in tumor blood vessel density: not surprisingly, the combination of aHIF-cDNA3.1 and VHL-pcDNA3.1 was the most effective of all. Finally, transfection of tumors with aHIF-pcDNA3.1 dramatically increased the number of apoptotic cells in tumors as detected using the TUNEL method with respect of control vector-transfected tumors.

Liu and coworkers cultured HepG2 hepatocellular carcinoma cell line in the presence of $CoCl_2$ and transfected it with aHIF-pcDNA3.1: this provoked the lowering of HIF-1α protein expression in HepG2 cells as there was no significant difference in the rate of proliferation of HepG2 cells transfected with aHIF-pcDNA3.1 and control vector pcDNA3.1, when the cells were cultured in normoxia. However, when the latter cells were exposed to $CoCl_2$-induced hypoxia, the cells transfected with aHIF-pcDNA3.1 grew significantly more slowly than those transfected with pcDNA3.1. Antisense-*hif-1a* role was confirmed in tumors established by subcutaneous injection of 1 × 106 HepG2 cells into the flanks of mice: tumors were transfected with aHIF-pcDNA3.1 and the animals were sacrified after 4 days. Immunoblotting and immunohystochemical analysis revealed that gene transfer of aHIF-pcDNA3.1 led to downregulation of *hif-1a* and *vegf*. In addition, the combination of aHIF-pcDNA3.1 and doxorubicin further suppressed tumor growth with respect than the tumors treated with the aHIF-pcDNA3.1 and doxorubicin monotherapies. Combined therapy (aHIF-pcDNA3.1 and doxorubicin) strongly inhibits cell proliferation, assessed by proliferation marker Ki-67, as demonstrated by fewer Ki-67-positive cells in tumors treated with aHIF-pcDNA3.1 and doxorubicin compared with pcDNA3.1-treated tumors and with tumors treated with the monotherapies. Finally, antisense- *hif-1a* synergizes with doxorubicin to induce the apoptosis of HepG2 cells in situ as proved by the TUNEL staining: few apoptotic cells were detected in tumors injected with pcDNA3.1, while tumors treated with the combination of aHIF-pcDNA3.1 and doxorubicin contained the greatest number of apoptotic

cells, more than apoptotic cells detected in tumors treated with either aHIF-pcDNA3.1 or doxorubicin (Liu F., 2008).

The aHIF transcript appears to play a role in regulating *HIF-1a* expression. aHIF is expressed in many human and fetal tissues and in some tumors; furthermore, it is conserved in rodents, indicating that it may be interesting also from an evolutionary point of view. aHIF is overexpressed in nonpapillary clear-cell renal carcinomas but it is inducible by hypoxia treatment in lymphocytes and this increase correlates with *hif-1a* mRNA downregulation. Furthermore, aHIF is under the transcriptional control of HIF-1 thereby suggesting that aHIF may be involved in a HIF-1α negative auto-regulatory loop. Administration of different long antisense RNAs targeting *hif-1a* to tumor cells downregulates *hif-1a* expression suggesting that aHIF may effectively play an important role in HIF-1α control. In addition, treatment with antisenses-*hif-1a* synergized with chemotherapeutic drugs to induce apoptosis in cancer cells. To date, however, the functional role of aHIF has not been elucidated and this is the aim of our work.

MATERIAL AND METHODS

Cell lines and reagents

Human Burkitt's lymphoma Raji, human acute T cell leukemia Jurkat, human acute promyelocytic leukemia HL-60 and human cervical adenocarcinoma HeLa cell lines were purchased from ATCC and human lung adenocarcinoma cell line A549 was a kind gift of Prof. Enrico Mini of University of Florence. Cells were cultured in DMEM high glucose (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Lonza), 1% L-glutamine (Lonza) and 1% penicillin/streptomycin (Sigma). Hypoxia mimetic cobalt chloride was purchased from Sigma-Aldrich (St. Louis, Missouri, USA); topoisomerase I inhibitor Hycamtin (Topotecan Hydrochloride, 1 mg/ ml solution) was a kind gift of Farmacia della Azienda Ospedaliero-Universitaria di Careggi.

Antisense HIF-1α Plasmid Construction and Gene Transfer

Full-length aHIF cDNA (2051bp) was amplified with specific primers FW (5'- ctaGCTAGCTGTCCTCCATTGTAAGATAAAAAGAGC-3') and RV (5'-atagtttaGCGGCCGCTACTGCAGGGTGAAGAATTACTCAG -3') bearing a restriction site for NheI and NotI, respectively. Resulting fragment was run on a agarose gel and cDNA was subjected to clean-up with NucleoSpin Extract II (Macherey-Nagel, Dueren, Germany). Full-length aHIF was cloned in pcDNA3.1+Hygro plasmid at NotI and NheI sites. Plasmid was amplified in colon bacillus DH-5α (Life Technologies, Carlsbad, CA, USA) and extracted with NucleoBond Xtra Midi EF (Macherey-Nagel). Since, the natural aHIF antisense is not polyadenylated, we did not insert polyadenylation site in our fragment. To avoid the possibility of concatamers formation, full lenght-aHIF pcDNA3.1+ plasmid was linearized with XhoI (Life Technologies) prior to transfection. 2,5 μg of DNA were transfected into cells using Lipofectamine LTX reagent (Life Technologies).

aHIF silencing

For silencing of aHIF by siRNA, A549 cells were transfected using Lipofectamine RNAi MAX reagent (Life Technologies) with either a combination of two siRNAs (Sigma-Aldrich) or selected siRNA (Life Technologies) designed to target aHIF; the AllStar Negative Control (Qiagen, Venlo, Netherlands) wa used as control siRNA. Each siRNAs were used at 100 nM final concentration. The sequences of aHIF targeted by the siRNAs were: 5'-UACAAAGGCUAGAGCUUCUC-3' and 5'-AAUCUGUUCCCAUUAACAGAU-3'. For silencing of aHIF by phosphorodiester oligodeoxyribonucleotides or chimeric oligodeoxyribonucleotides, A549 cells were transfected using Lipofectamine LTX reagent (Life Technologies) with either an ODN specific for aHIF or a control ODN (Sigma-Aldrich) (10 μM final concentration). The sequences of anti-aHIF ODNs was 5'-CCAGGCCCCTTTGATCAGCTT-3', the sequence of control ODNs (Degenerated) 5'-NNNNNNNNNNNNNNNNNNNN-3'.

Protein extraction and western blotting

Cells were pelleted and lysed with buffer containing urea 8 M, β-mercaptoethanol 15 mM The cytoplasmic proteins (35 μg) were separated by NuPAGE 4-12% Bis-Tris Gel (Life Technologies) and electroblotted using an XCell II Blot Module (Life Technologies) for 2 hours onto Protran nitrocellulose transfer membranes (Schleicher & Schuell, Dassel, Germany). The following antibodies were used: mouse monoclonal HIF-1α (BD Laboratory, rabbit polyclonal p53 (SC6243, Santa Cruz Technology, Inc., Santa Cruz, CA, USA), rabbit polyclonal H3 and rabbit monoclonal GAPDH (2118 and 9715, respectively; Cell Signaling, and β-actin and α-tubulin (Sigma-Aldrich). The secondary antibodies included goat anti-mouse IRDye 800CW and goat anti-rabbit IRDye 800CW (Li-Cor Biosciences, Lincoln, NE, USA). The protein bands

were analyzed by the Odyssey Infrared Imaging System (Li-Cor) using the software for protein quantification.
Nucleus/ cytoplasm fractionation Nuclear and cytoplasmic protein and RNA were separately extracted using the Protein and RNA Isolation System PARIS (Life Technologies)

RNA extraction and RT-PCR

RNA extraction, PCR and Real Time PCR Total RNA was isolated from cells with an NucleoSpin II Extraction Kit (Macherey-Nagel), analyzed using Q-bit spectrophotomer (Qiagen) and its quality was assessed by running 500 ng of sample on a 2% polyacrylamide gel. 500 ng of RNA were retrotranscribed using ImProm-II reverse transcriptase (Promega, Madison, Wisconsin, USA) and random examers. cDNA was amplified with specific primers: for *aHIF,* forward 5'-TTTGTGTTTGAGCATTTTAATAGGC-3' reverse 5'-CCAGGCCCCTTTGATCAGCTT-3' (purchased from Sigma-Aldrich); for *hif-1a,* Hs_HIF1A_1_SG QuantiTect Primer Assay (purchased from Qiagen); for *18S,* forward 5'- CGGCTACCACATCCAAGGAA-3' and reverse 5'-GCTGGAATTACCGCGGCT-3' (purchased from Eurofins MWG Operon (Ebersberg, Germany)); for *gapdh*, forward 5'-AACAGCCTCAAGATCATCAGCAA -3' and reverse 5'-CAGTCTGGGTGGCAGTGAT-3'. RT-PCR assays were performed using Go Taq Hot Start Polymerase (Promega). Real-time PCR assays were performed using the SsoFast EvaGreen Supermix (Bio-Rad, Berkeley, CA, USA) and the Rotor-Gene 3000 cycler system (Corbett Research, Sydney, NSW, Australia) or the Applied Biosystem 7500 Fast Real-Time PCR System (Applied Biosystem, Foster City, CA, USA).

RESULTS

aHIF transcript in expressed in several human tumor cell lines

The natural HIF-1α antisense (aHIF) was originally discovered in samples of nonpapillary clear-cell renal carcinomas (Thrash-Bingham C. and Tartof KD., 1999). In addition aHIF was found in several adult (muscle, liver, kidney, thymus, brain, leukocyte, and bone marrow) and fetal tissues (muscle, liver, thymus and lung but not in kidney) as well as in three different types of tumour (mammary carcinoma, bone marrow presenting a chronical myeloid leukaemia and neuroblastoma) (Rossignol F., 2002). In order to know if aHIF could be espressed in tumor cell lines we first examined aHIF transcript expression in three haematological tumor (Raji, Jurkat and HL-60) and in two solid tumors (HeLa and A549) cell lines cultured either in normoxia or in hypoxia-mimetic conditions (cobalt chloride, $CoCl_2$) and we found that aHIF was expressed in all the five cell lines tested, both in normoxia and in hypoxia (Fig. 6).

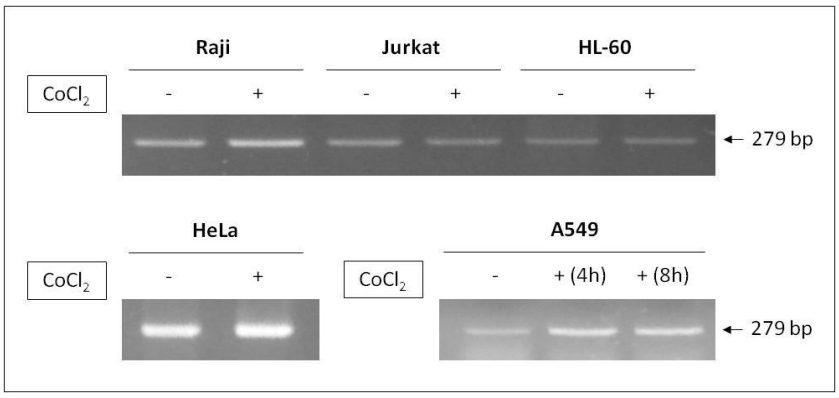

Fig. 6. aHIF transcript detection using RT-PCR in human tumor cell lines. Total RNA extracted from various tumor cell lines was retrotranscribed using random examers: aHIF was amplified using specific primers (see Material and Methods). The amplification products were loaded onto a 2% agarose gel containing ethidium bromide. The figure shows amplification of aHIF in Raji, Jurkat, HL-60, HeLa and A549 human tumor cells.

While expression of aHIF seemed to be independent from treatment with hypoxia in Raji, Jurkat, HL-60 and HeLa cells, in A549 cells aHIF appeared to be inducible after $CoCl_2$ treatment, consistently with the literature.

We then focused on two HeLa and A549 cell lines. HeLa were a very interesting model because in this cell line the research group of Myriam Gorospe demonstrated that HIF-1α protein was up-regulated also at post-transcriptional level by the RNA-binding proteins HuR and PTB in hypoxia: briefly, after 2,5 hours of treatment with $CoCl_2$, HuR and PTB bind to *hif-1a* mRNA with more affinity and promote its translation (Galbán S., 2008). A549 cells was an intriguing model as well because aHIF expression is upregulated following treatment with $CoCl_2$ (Uchida T., 2004).

The hypoxia mimetic $CoCl_2$ elevates of HIF-1α protein expression but does not affect *hif-1a* and aHIF transcripts level in HeLa cells

We first examined the kinetics of HIF-1α protein and mRNA expression in HeLa cells cultured either in normoxic or hypoxic conditions by time-courses experiments. In normoxia HIF-1α protein was practically undetectable but its expression was rapidly upregulated after 2 hour of treatment with $CoCl_2$ (Fig. 7) and reached the maximal expression after 8 hours. HIF-1α protein level, though, decreased in prolonged hypoxia (24 hours) and this could be consistent with a post-transcriptional negative feedback of HIF-1α expression.

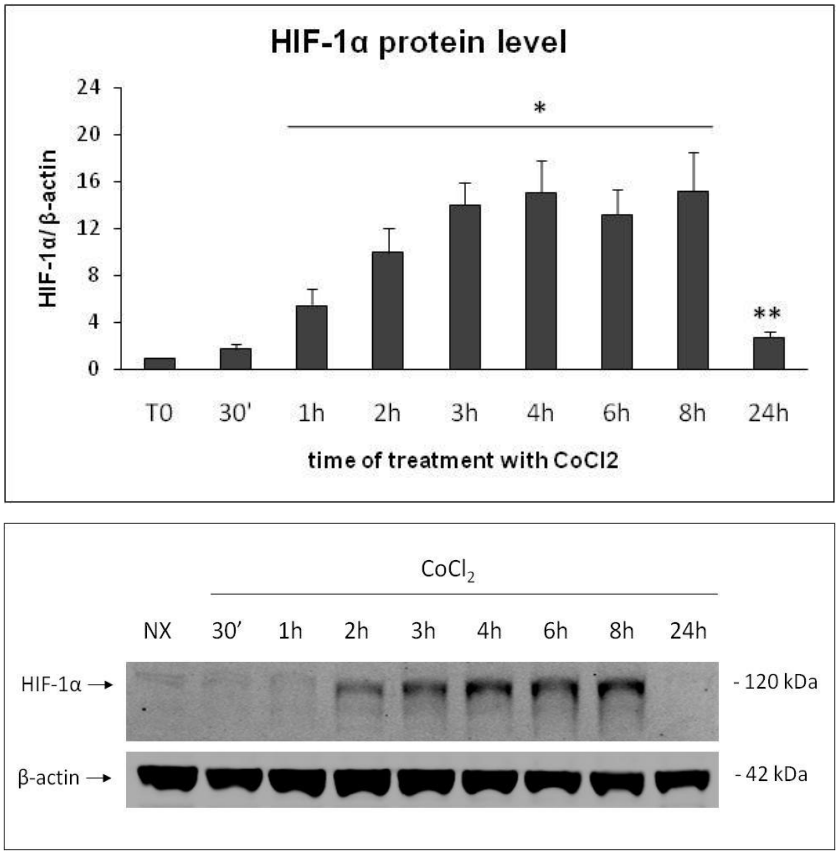

Fig. 7. $CoCl_2$ treatment determines a strong increase of HIF-1α protein level in HeLa cells. HeLa cells were treated with 200 μM $CoCl_2$ for various times prior to whole cell extraction and immunoblot analysis. Time-course experiments were repeated four times with similar results. A representative blot is shown.

Thus, we examined the levels of *hif-1a* mRNA and aHIF transcript and found that expression was unchanged even in prolonged hypoxia (Fig. 8), thus suggesting that in HeLa cells, the downregulation of HIF-1α protein does not depend on mRNA stability nor on aHIF transcript level.

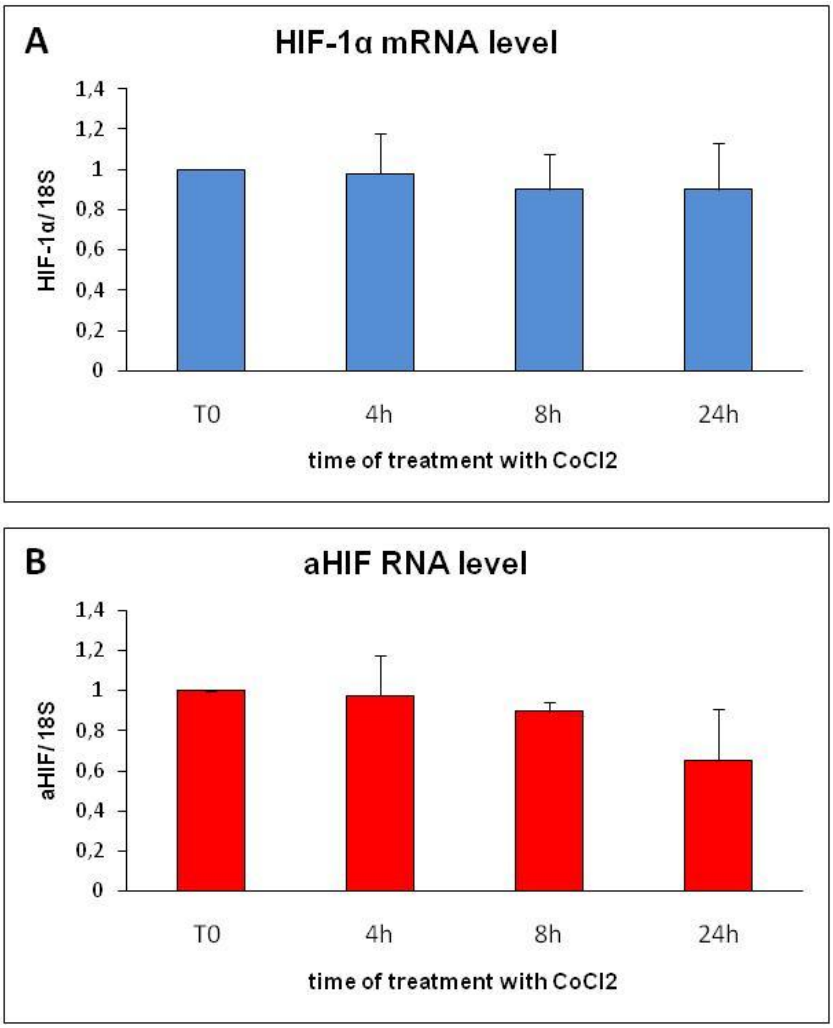

Fig. 8 $CoCl_2$ treatment does not affect *hif-1a* mRNA nor aHIF transcript level in HeLa cells. HeLa cells were treated with 200 µM $CoCl_2$ for various times prior to whole cell extraction and real-time PCR analysis of HIF-1α (A) and aHIF (B) transcripts. 18S was used as housekeeping gene. Each experiment was repeated at least three times with similar results.

$CoCl_2$ elevates HIF-1α protein and aHIF transcript levels and dowregulates *hif-1a* mRNA level in A549 cells

We monitored HIF-1α protein expression in A549 cells and found that its level is rapidly upregulated after treatment with $CoCl_2$, reaching a maximum after 4 hours of treatment. Similarly to what observed in HeLa cells, HIF-1α underwent a significant decrease after 8 hours of treatment (Fig. 9).

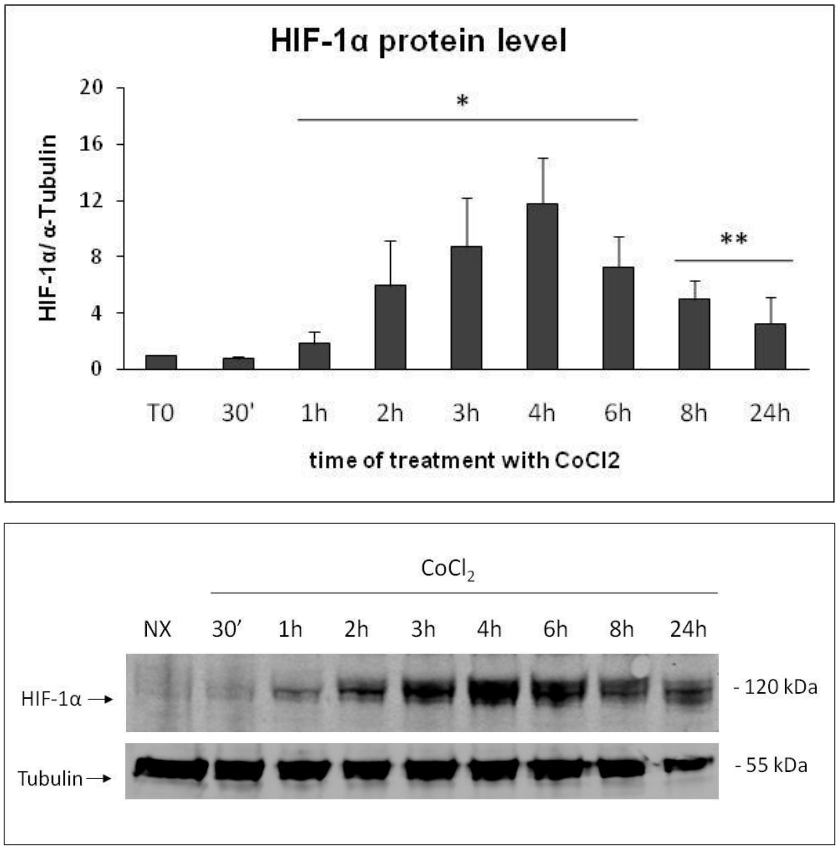

Fig. 9. $CoCl_2$ treatment determines a strong increase of HIF-1α protein level in A549 cells. A549 cells were treated with 250 μM $CoCl_2$ for various times prior to whole cell extraction and immunoblot analysis. Time-course experiments were repeated four times with similar results. A representative blot is shown.

We then evaluated the kinetics of aHIF expression by time-course experiments and found that it was very similar to that of HIF-1α protein in hypoxia-mimetic conditions (Fig. 10A): aHIF level was rapidly upregulated after 2 hours of treatment with $CoCl_2$ and reaches its maximal level after 4 hours. Conversely to what observed by Uchida and collaborators, aHIF level underwent a significant decrease after 6 hours and further decrease in prolonged hypoxia, and this could be consistent with the fact that aHIF is under the transcriptional control of HIF-1. In order to detect aHIF transcript localization we performed a RT-PCR analysis on nuclear and cytoplasmic RNA extract and found that aHIF transcript is localized both in cytoplasm and in the nucleus both in

normoxic and hypoxic conditions (Fig. 10B). GAPDH and H3 hystone were used as control of differential extraction by western blotting analysis (Fig. 10C).

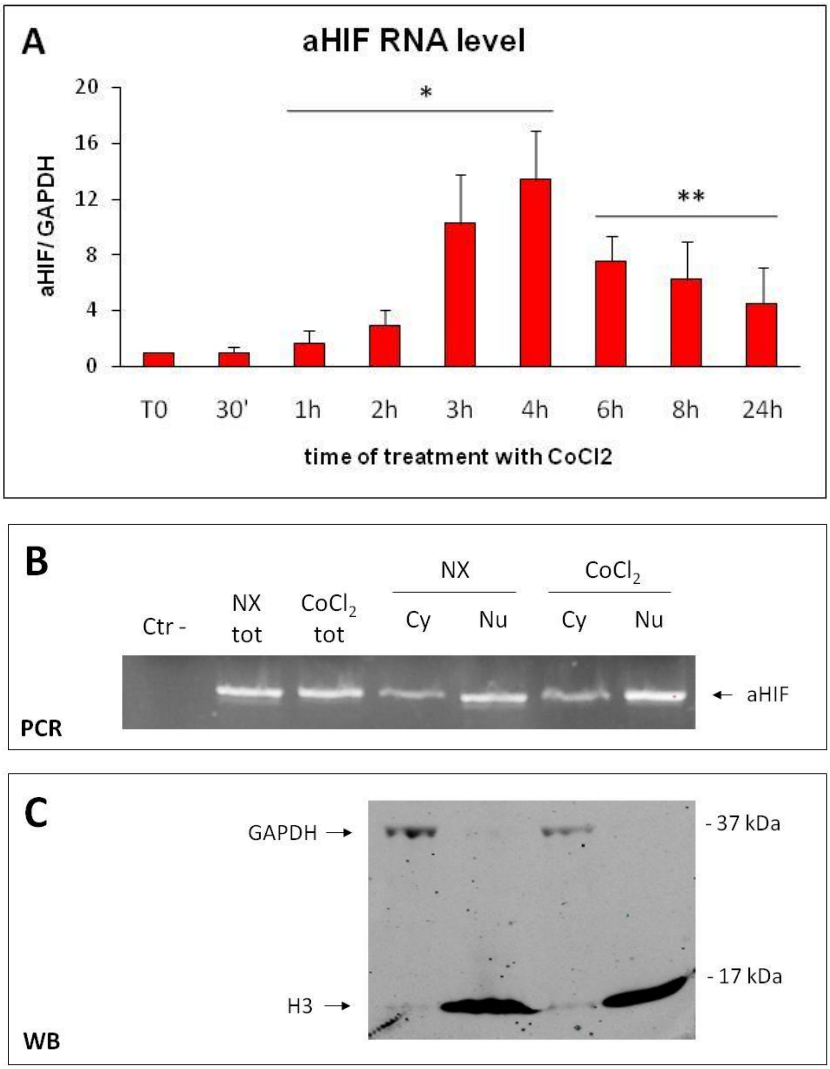

Fig. 10. Treatment with $CoCl_2$ determines a strong increase of aHIF transcript expression in A549 cells. A549 cells were treated with 250 µM $CoCl_2$ for various times prior to whole cell extraction (A) or nuclear/cytoplasmic (B) extraction and RT-PCR analysis. Time-course experiments were repeated seven times with similar results. GAPDH and H3 histone were used as control of extraction (C).

Because hypoxia can modulate the expression and the levels of some important AUBPs (Kim T.W., 2010), we evaluated the levels of two of the AUBPs involved in HIF-1α post-transcriptional regulation which are TTP and HuR: TTP exert a negative regulation on *hif-1a* mRNA promoting its

degradation (KIM T.W., 2010; Chamboredon S., 2011) while HuR promotes *hif-1a* translation in hypoxic condition (Galbán S., 2008). As shown in Fig. 11, immunoblotting analysis revealed that neither protein is affected by treatment with $CoCl_2$.

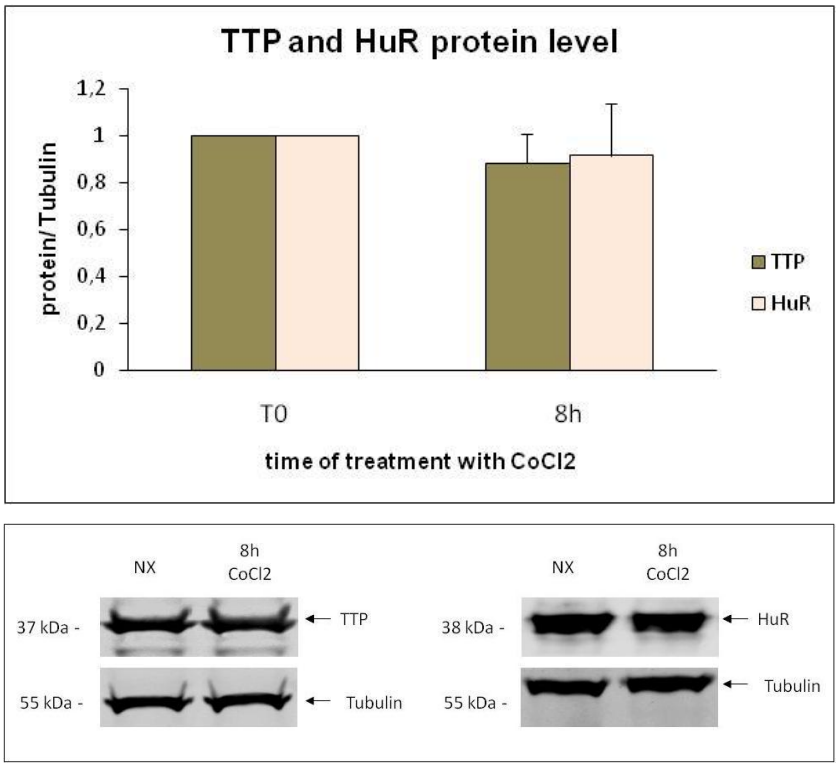

Fig. 11. $CoCl_2$ treatment does not affect TTP and HuR protein levels in A549 cells. A549 cells were treated with 250 µM $CoCl_2$ for 8 hours prior to whole cell extraction and immunoblot analysis. Time-course experiments were repeated four times with similar results. A representative blot is shown.

We then evaluated by real-time PCR analysis the level of HIF-1α mRNA and found that while *hif-1a* mRNA amount remained relatively constant, after six hours of hypoxia its level underwent a dramatic downregulation (Fig. 12).

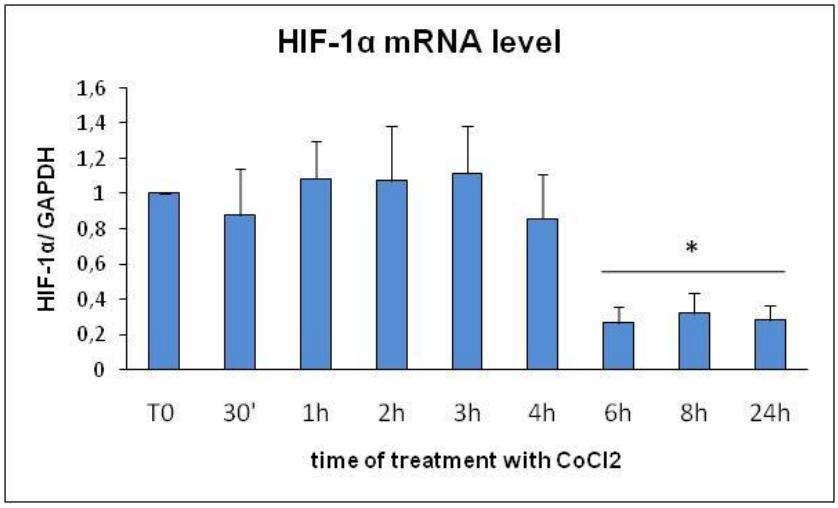

Fig. 12. Treatment with $CoCl_2$ potently downregulates aincrease of *hif-1a* mRNA expression in A549 cells. A549 cells were treated with 250 μM $CoCl_2$ for various times prior to whole cell extraction and real-time PCR analysis. *Gapdh* was used as housekeeping gene. Time-course experiments were repeated seven times with similar results.

Treatment with $CoCl_2$ rapidly upregulated HIF-1α protein in both HeLa and A549 cells but, in prolonged hypoxia, HIF-1α protein underwent a significant decrease. However, while in HeLa cells *hif-1a* mRNA and aHIF transcript levels remained unchanged even in prolonged hypoxia, in A549 cells, $CoCl_2$ increased aHIF transcript level which is paralleled by a decrease of *hif-1a* mRNA level, thus suggesting that aHIF transcript could destabilize *hif-1a* mRNA.

Effect of siRNAs and oligonucleotides on aHIF transcript level

To demonstrate that aHIF is involved in *hif-1a* regulation, we wanted to see the effects of its silencing by using siRNAs targeting aHIF transcript. Unfortunately, neither of three different siRNAs affected aHIF level neither in normoxic nor in hypoxic conditions (Fig. 13).

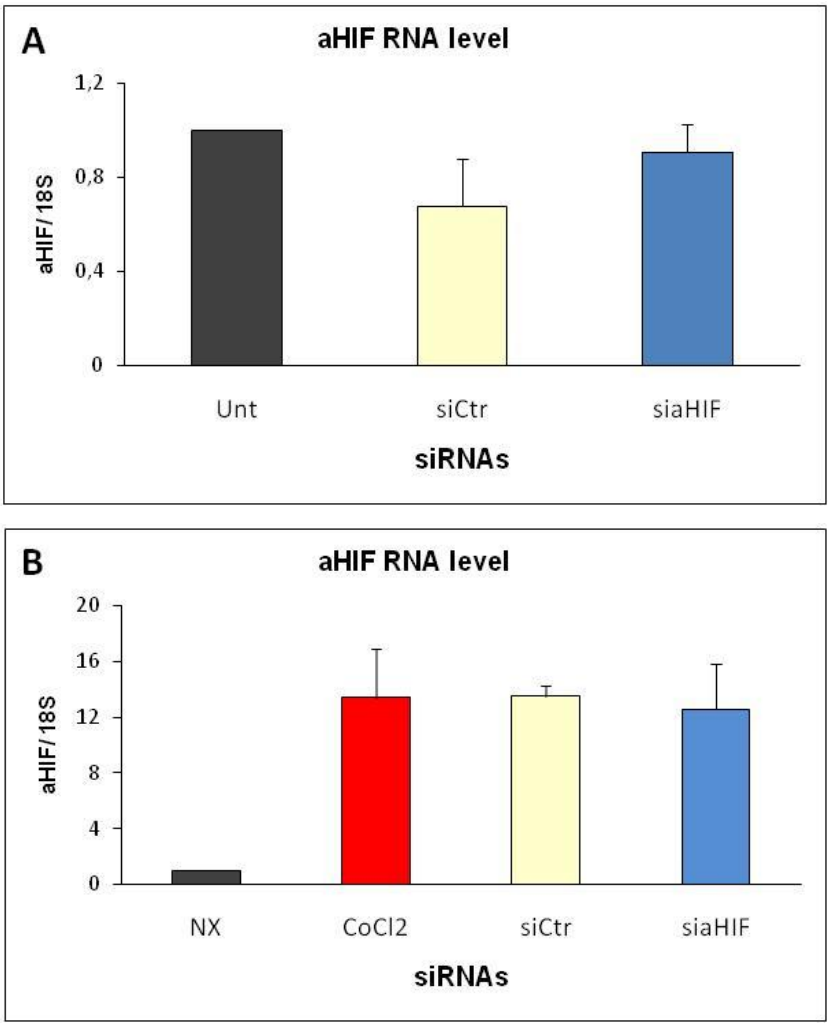

Fig. 13. Silencing of aHIF using siRNAs. A549 cells were lipotransfected three different with targeting (siaHIF) or control (siCtr) siRNAs. 48 hours later, cells were either left untreated (A) or treated with 250 μM $CoCl_2$ for 4 hours (B) prior to whole cell extraction and real-time PCR analysis. 18S was used as housekeeping gene. Experiments were repeated three times with similar results.

We then tried to downregulate the aHIF transcript by means of antisense strategy. For this purpose we designed a sequence-specific antisense oligodeoxyribonucleotide (anti-aHIF-ODN) targeting the aHIF transcript using an aspecific ODN of degenerated sequence (Deg-ODN) as control. Surprisingly, lipotransfection with both the specific ODN and the Deg-ODN induced *per se* a toxic effect in A549 cells: since ODNs have 5' and 3' DNA free ends we hypothesized that transfection with ODNs elicits a cellular response similar to that induced by DNA strand

breaks, a phenomenon similar to that reported in Rat-1luc fibroblasts cells by Papucci and Capaccioli (Papucci L., 2002). Interestingly, transfection with both the specific ODN and the Deg-ODN upregulated the aHIF transcript expression (Fig. 14A). However, while the Deg-ODN provoked a 10-fold increase of aHIF transcript level, the anti-aHIF-ODN provoked only a modest increase of aHIF which suggested that the aHIF transcript was effectively targeted by the sequence of the designed anti-aHIF-ODN and accessible to the RNA-degradation machinery.

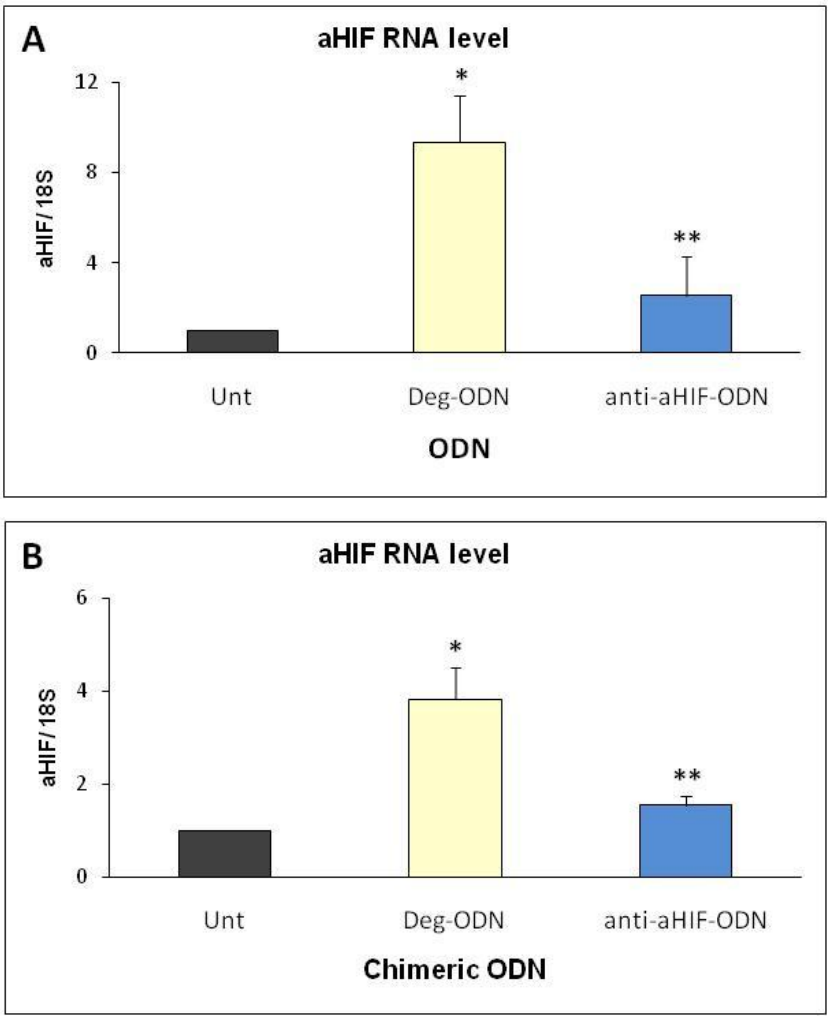

Fig. 14. Lipotrasfection of A549 cells with ODNs and chimeric ODNs provokes aHIF upregulation. A549 cells were lipotransfected with either targeting (anti-aHIF-ODN) or control (Deg-ODN) phosphorotioate-modified (A) or chimeric (B) ODN. 48 hours later, cells were lysed for whole cell extraction and real-time PCR analysis. 18S was used as housekeeping gene. Experiments were repeated three times with similar results.

In order to avoid the hypothesized cellular response to 5' and 3' free ends DNA molecules, we designed a chimeric ODN with the same sequence of the anti-aHIF-ODN and which possessed a DNA core of 17 nt and two ribonucleotides at the 5' and 3' ends. However, although transfection with chimeric control-ODNs determined a much lower induction of aHIF compared to that observed with all-DNA control-ODNs, transfection with chimeric anti-aHIF-ODN had approximately the same effect of all-DNA anti-aHIF-ODN, i.e. it failed in reducing aHIF transcript basal level (Fig. 14B).

Overexpression of the aHIF natural transcript does not affect HIF-1α mRNA levels in both HeLa and A549 cells

Due to the difficulties that we had in silencing aHIF transcript, we tried to demonstrate aHIF functional role by overexpressing it. The longest aHIF sequence reported in GeneBank is 2051 nt long: however, this sequence has not been fully cloned, but derive from an *in silico* prediction of the full-length aHIF based on shorter sequences previously cloned. On this basis our aim was to clone the 2051 full-length sequence of aHIF into an expression vector. In order to obtain an unidirectional cloning, the full-length aHIF cDNA was amplified with specific FW and RV primers bearing a restriction site for NheI and NotI, respectively, and cloned in pcDNA3.1+Hygro plasmid at NotI and NheI sites. Since, the natural aHIF antisense is not polyadenylated, we did not insert polyadenylation site in our fragment: thus, to avoid the possibility of concatamers formation Prior to transfection, both full-length aHIF pcDNA3.1+ and control plasmids were linearized with XhoI. (Fig. 15; for details see Material and Methods).

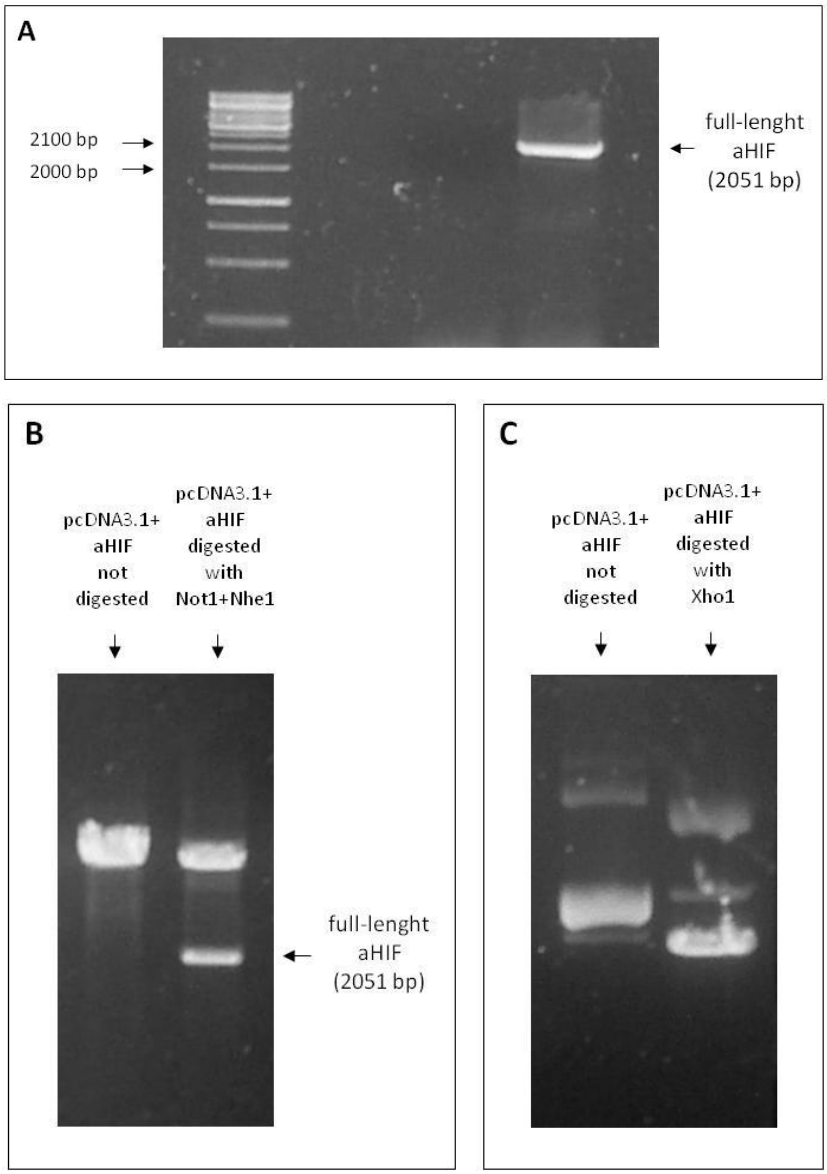

Fig. 15. Construction of the full-length aHIF plasmid. The full-length aHIF cDNA was amplified with specific primers (A) and cloned in a pcDNA3.1+Hygro plasmid at NotI and NheI restriction sites (B). Prior to transfection, full-length aHIF pcDNA3.1+ plasmid was linearized with XhoI (C).

Both HeLa and A549 cells were transfected with either a pcDNA3.1+ Hygro vector containing a 2051 bp aHIF cDNA (full-length aHIF) or an empty pcDNA3.1+ Hygro vector: as shown in Fig. 16, transfection with aHIF-pcDNA3.1+ plasmid resulted in a marked overexpression of aHIF transcript both in HeLa and in A549 cells.

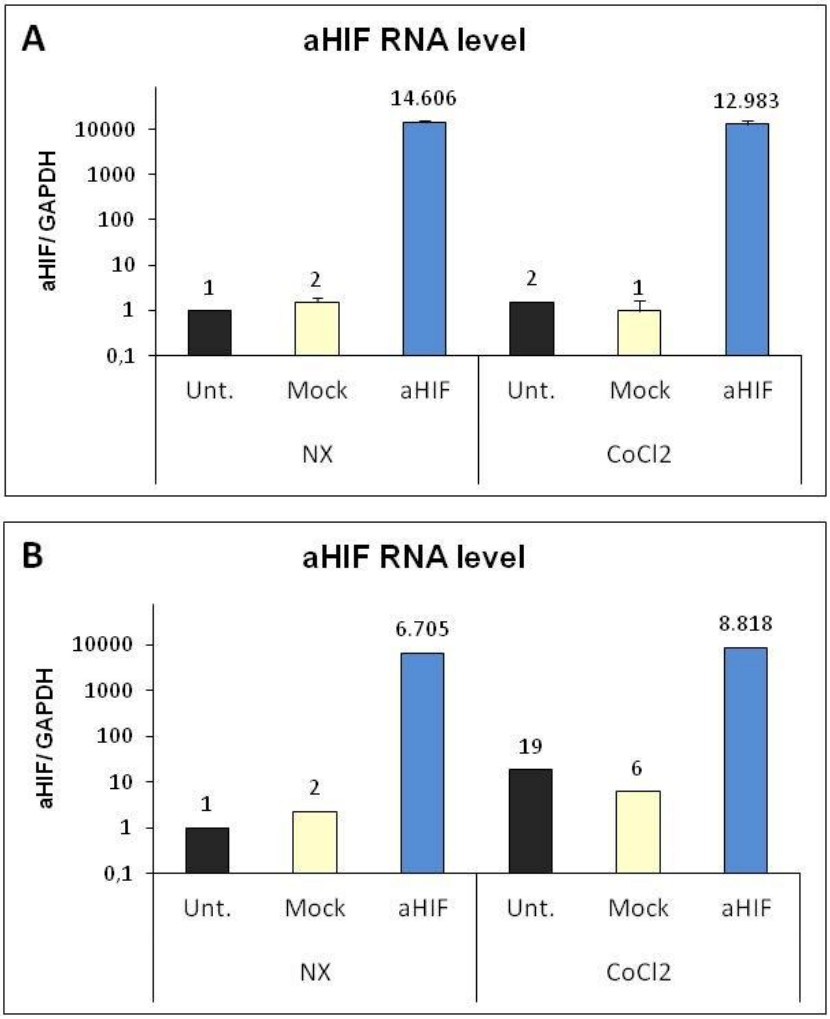

Fig. 16. Transfection of full-length aHIF pcDNA3.1+ vector results in overexpression in both HeLa and A549 cells. HeLa (A) and A549 (B) cells were either left untreated (Unt.) or transfected with an empty vector (Mock) or with the full-length aHIF pcDNA3.1+ vector (aHIF). *Gapdh* was used as housekeeping gene.

We next evaluated the effect of aHIF overexpression on *hif-1a* mRNA level, but, surprisingly, we did not observe any effect: in fact, both in HeLa and A549 cells, transfection with aHIF-pcDNA3.1+ vector did not change *hif-1a* mRNA level compared with those of cells transfected with an empty vector, neither in normoxic nor in hypoxic conditions (Fig. 17).

Overexpression of aHIF had no effect on *hif-1a* mRNA level, neither in normoxia nor in hypoxia. It has been proposed that non-coding RNAs can modulate gene expression via transcriptional interference basing on

the assumption that RNA polymerases bind to the promoters on the anrisense strand could interfere with transcriptional complexes on the sense strand determining inhibiting transcription of the sense RNA. Thus, it is possible that overexpression of aHIF *per se* may not result in a downstream effect on *hif-1a* mRNA and protein expression because HIF-1α gene regulation is related to the transcription of aHIF rather than aHIF itself.

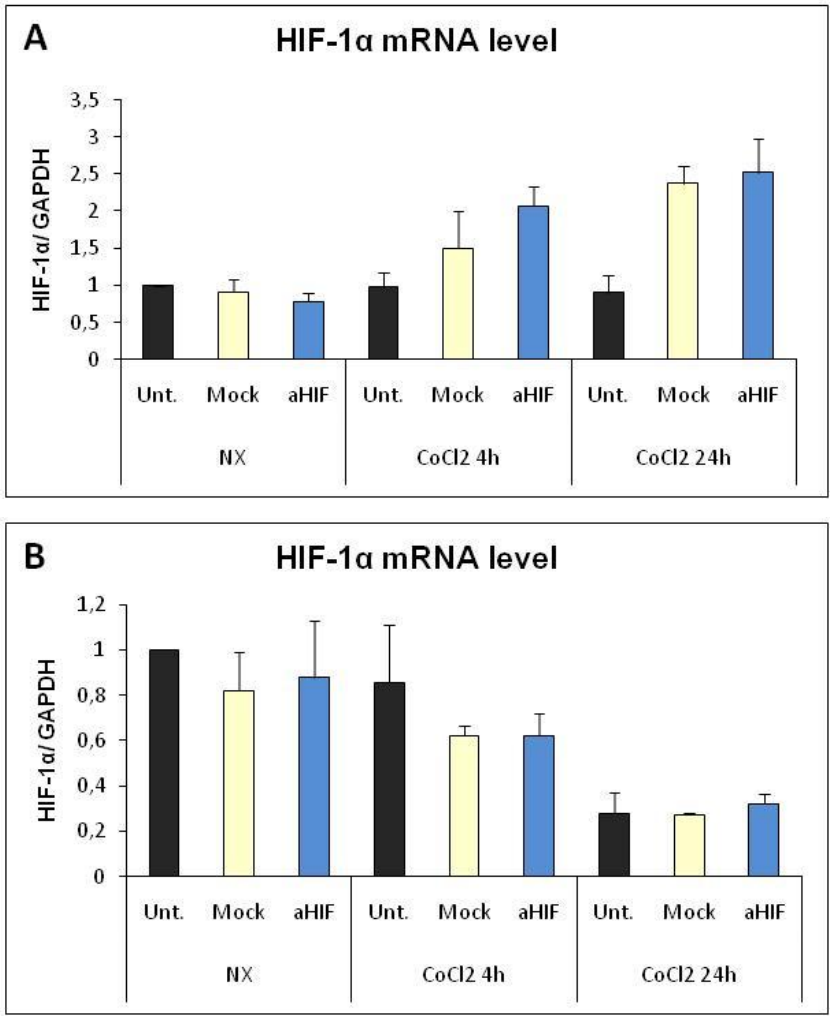

Fig. 17. Impact of aHIF overexpression on *hif-1a* mRNA level in HeLa and A549 cells. HeLa (A) and A549 (B) cells were either left untreated (Unt.) or transfected with an empty vector (Mock) or with the full-length aHIF pcDNA3.1+ vector (aHIF). Both cells were grown in either normoxia or hypoxia (250 μM $CoCl_2$) for 4 or 24 hours. Experiments were repeated three times with similar results. *Gapdh* was used as housekeeping gene.

Treatment with DNA-damaging chemotherapeutic drug Topotecan increased aHIF transcript level

Surprisingly, transfection with phosphorotioate-modified or chimeric ODNs determined upregulation of aHIF expression in A549 cells. It was previously demonstrated that aHIF is under the transcriptional control of HIF-1. Transfection of A549 cells with a truncated form of HIF-2α that form heterodimers with HIF-1β and acts as a dominant-negative for both HIF-2α and HIF-1α subunits (Maemura K., 1999) results in a lower hypoxia-dependent aHIF transcriptional induction but not in its complete abolition (Uchida T., 2004). We therefore hypothesized that DNA-damaging agents could increase aHIF transcript level independently of HIF-1α activity and, to test this hypothesis, A549 cells were treated with Topotecan, an inhibitor of DNA Topoisomerase I which induce DNA double-strand breaks. We used Topotecan for two reasons: the first is that Topotecan is a chemotherapeutic drug which is already used for treatment of lung cancer in patients whose disease has not gotten better with other chemotherapy (*Note: On June 14, 2006, the U.S. Food and Drug Administration approved topotecan hydrochloride (Hycamtin®, made by GlaxoSmithKline) in combination with cisplatin for the treatment of stage IVB recurrent or persistent carcinoma of the cervix (cervical cancer) that is not amenable to curative treatment with surgery and/or radiation therapy. This summary summary is provided by Richard Pazdur, M.D., director of the FDA's Division of Oncology Drug Products.*); secondly, Rapisarda and coworkers demonstrated that Topotecan inhibits HIF-1 transcriptional activity and HIF-1α protein accumulation in either normoxic or hypoxia-treated U251 human glioma cells at concentration of 30 nM or more (Rapisarda A., Cancer Res. 2002) and a similar effect was observed in A549 cells (Choi Y.J., 2009). We first performed dose-dependency experiments in order to evaluate whether treatment with different doses of Topotecan for 4 hours could induce aHIF transcript expression: as shown in Fig. 18, aHIF expression was unchanged when A549 were treated with of 100

nM Topotecan or lower, but its level increased in a dose-dependent fashion when cells were treated with 500 nM Topotecan or higher doses. These results demonstrate that Topotecan exerts a dose-dependent induction of aHIF transcript.

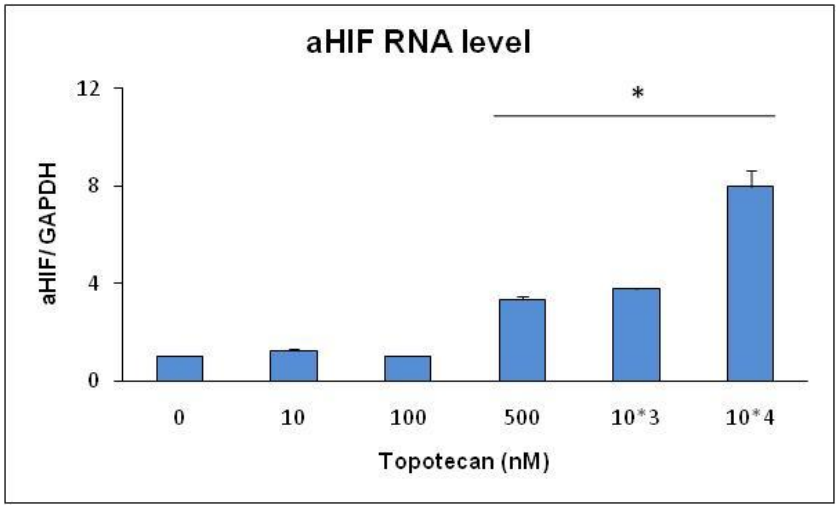

Fig. 18. Dose-dependency of effect of Topotecan on aHIF transcript expression in A549 cells. A549 cells were treated with different doses of Topotecan for 4 hours prior to whole cell extraction and real-time PCR analysis. *Gapdh* was used as housekeeping gene. Time-course experiments were repeated four times with similar results.

Treatment with Topotecan induces p53 but not HIF-1α protein expression

It is known that Topotecan induce DNA double-strand breaks activating p53. We therefore hypothesized that DNA-damage induction of aHIF expression could be related to p53 activation: for this purpose, we examined the level of p53 by immunoblotting. As shown in Fig. 19, p53 level was slightly but significantly upregulated when cell are treated with Topotecan 10 nM for 4 hours and its level increased in a dose-dependent fashion.

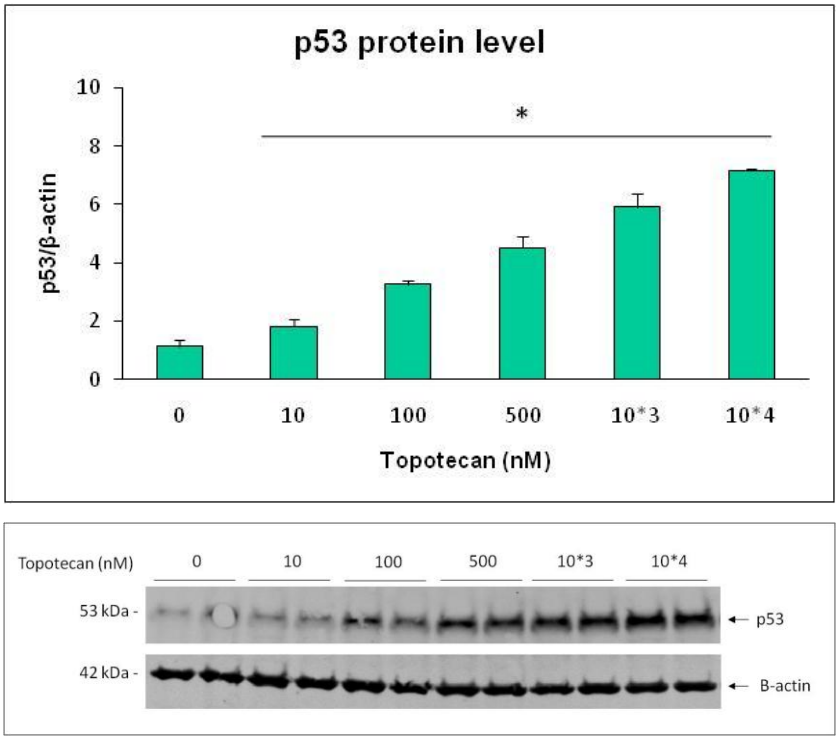

Fig. 19. Treatment with Topotecan determines a consistent increase of p53 protein level in A549 cells. A549 cells were treated with different doses of Topotecan for 4 hours prior to whole cell extraction and immunoblot analysis. Time-course experiments were repeated three times with similar results. A representative blots is shown.

We then performed time-courses experiments in order to evaluate aHIF transcript level as well as p53 and HIF-1α protein level. aHIF expression was rapidly upregulated and reached a plateau after 4 hours of treatment with Topotecan (Fig. 20A). While HIF-1α was not induced by Topotecan even after 24 hours of treatment, p53 levels underwent further increase (Fig. 20B and C). Then, aHIF upregulation in A549 cells following treatment with Topotecan is clearly to be considered an hypoxia-independent and HIF-1-independent phenomenon and could be related to p53 levels and to DNA damage.

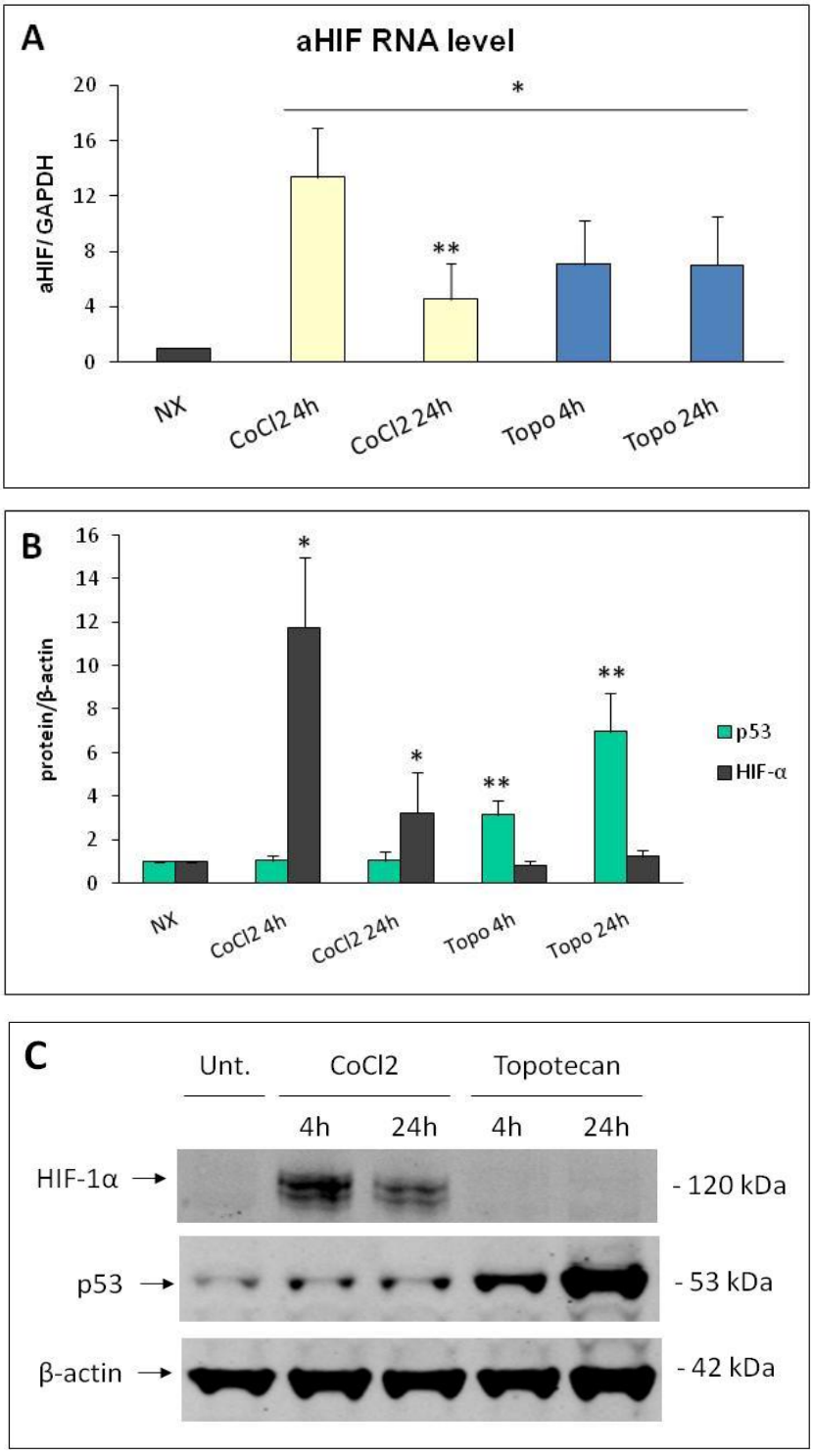

Fig. 20. Time-course of effect of $CoCl_2$ and Topotecan on aHIF transcript and p53 and HIF-1α protein level in A549 cells. A549 cells were treated with 250 μM $CoCl_2$ or 10 μM Topotecan for various times prior to whole cell extraction and real-time PCR analysis to detect aHIF transcript (A) or immunoblot analysis to detect p53 and HIF-1α (B). Time-course experiments were repeated three times with similar results. A representative blot is shown (C).

Treatment with Topotecan decreased *hif-1a* mRNA level

It has been reported that *hif-1a* mRNA level was unchanged in U251 human glioma cells after treatment with Topotecan (Rapisarda A., 2002) and, using a semi-quantitative RT-PCR, Choi and collaborators shown similar result in A549 cells (Choi Y.J., 2009). However, since semi-quantitative RT-PCR could not be enough sensitive to detect slight alteration in RNA levels, we performed real-time PCR analysis to assess whether Topotecan could affect *hif-1a* mRNA level. Interestingly, and similarly to what observed in hypoxia-mimetic conditions, Topotecan determined a slight but significant *hif-1a* decrease (Fig. 21). Once again, these data suggested that aHIF transcript could be involved in HIF-1α expression regulation and, more interestingly, play a role not only in hypoxia but also in a DNA-damage condition.

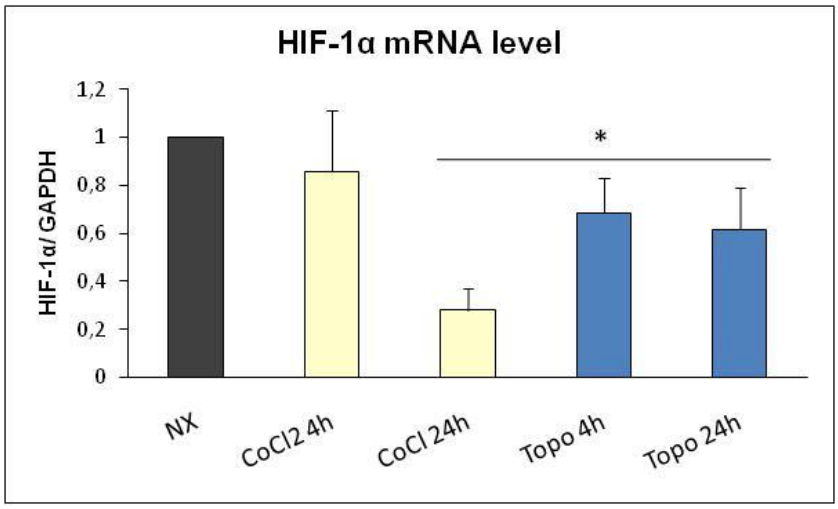

Fig. 21. Time-course of effect of Topotecan on *hif-1a* mRNA expression in A549 cells. A549 cells were treated with 250 μM $CoCl_2$ or 10 μM Topotecan for various times prior to whole cell extraction and real-time PCR analysis to detect HIF-1α mRNA level. *Gapdh* was used as housekeeping gene. Time-course experiments were repeated four times with similar results.

DISCUSSION

The natural HIF-1α antisense (aHIF) was first discovered in nonpapillary clear-cell renal carcinomas (Thrash-Bingham C. and Tartof K.D., 1999). Later, aHIF transcript was detected in several adult and fetal tissues as well as in three different types of tumour (Rossignol F., 2002). Moreover, aHIF was found to be conserved in rodents (Rossignol F., 2004), suggesting that it plays an important role in regulating the HIF-1 system in different physiological and physiopathological situations. Interestingly, hypoxia dramatically decreased the half-life of HIF-1α mRNA in A549 cells (Uchida T., 2004). In addition, aHIF is strongly upregulated after 4 hours of hypoxia, and its expression is, at least in part, under the transcriptional control of HIF-1α. aHIF transcript is a strong independent poor prognostic marker in breast cancer (Cayre A., 2003) and high expression of aHIF was found to associate with decreased metastasis-free survival in paragangliomas (Span P.N., 2011).

However, the role of aHIF still remains unclear: in this study we attempted to demonstrate the functional role of aHIF. We examined aHIF expression in three haematological tumor (Raji, Jurkat and HL-60) and in two solid tumors cell lines (HeLa and A549) and we found that aHIF was expressed in all the five cell lines tested, both in normoxia and in hypoxia. We focused on two experimental models, HeLa and A549 cells: HeLa were very interesting because in this model a post-transcriptional control of HIF-1α expression has already been demonstrated by the research group of Myriam Gorospe: briefly, RNA-binding proteins HuR and PTB binds HIF-1α mRNA with more affinity after treatment with $CoCl_2$ for 2,5 hours and promote its translation (Galbán S., 2008). A549 cells were an intriguing model as well because aHIF is upregulated following treatment with $CoCl_2$. In normoxic HeLa cells HIF-1α protein was nearly undetectable while its level was rapidly upregulated following treatment with $CoCl_2$ reaching a maximum after 8 hours, but its levels decreased after 24 hours. Similarly, in A549 cells

basal HIF-1α protein level was very low but was rapidly upregulated after treatment with $CoCl_2$, reaching a maximum after 4 hours. HIF-1α tends to decrease after 6 hours of treatment, and after 24 hours its levels is strongly diminished. In order to understand whether HIF-1α downregulation could be due to post-transcriptional factors, we examined the levels of *hif-1a* mRNA and aHIF transcript but we observed no change in *hif-1a* mRNA and aHIF transcript levels in hypoxia in HeLa cells. Conversely, and intriguingly, aHIF level was rapidly upregulated in A549 cells following with $CoCl_2$ and reaches its maximal after 4 hours of treatment: then, aHIF levels tend to decrease in prolonged hypoxia. aHIF transcript was localized both in cytoplasm and nucleus either in normoxia or hypoxia. We then evaluated the levels of *hif-1a* mRNA and interestingly we found that after 6 hours of treatment there is a dramatic decrease in *hif-1a* transcript.

To understand whether aHIF could play a role in HIF-1α expression regulation, we tried to downregulate its level by means of either RNAi and antisense strategy but neither siRNAs targeting aHIF nor antisense oligodeoxyribonucleotide (anti-aHIF-ODN) affected aHIF transcript level: conversely, we observed a toxic effect of ODNs transfection on cells. It has been previously reported that lipotransfection of cultured cells with ODNs (30-mers) activate p53. Such effects of ODNs are mediated by a non-antisense mechanism that require the wild-type form of the p53 protein. p53 determines inhibition of mitosis inhibition and induction of apoptosis induction which appear to be determined by the 5' and 3' free ends of ODNs, which activated p53 independently from their sequence. Most probably, this mechanism is analogous to that evoked by genotoxic agent-induced DNA damage or by lipotransfecting cells with heterogeneous DNA. In order to avoid the hypothesized cellular response to 5' and 3' free ends DNA molecules, we designed a chimeric ODN with the same sequence of the anti-aHIF-ODN and which possessed a DNA core of 17 nt and two ribonucleotides at the 5' and 3' ends: although transfection with chimeric ODNs determined a less toxic effect compared with all-DNA ODNs, it had no effect on aHIF basal level.

Surprisingly, lipotransfection with both control-ODNs and chimeric control-ODNs provoked an increase in aHIF level.

The difficulties in silencing aHIF might be due to a close secondary structure of the antisense transcript that do not allow the formation of RNA-ODN eteroduplexes thereby preventing the triggering of RNase-H; this structure could also prevent the access of siRNA-loaded RISC complex.

Despite the unsuccessful attempts to downregulating aHIF expression, aHIF level increment provoked by a DNA damage-mimetic scenario due to lipotransfection with oligodeoxyribonucleotides let us suspect that aHIF expression upregulation could be driven not only by hypoxic stimulus but also by DNA damage.

Overexpression of aHIF had no effect on *hif-1a* mRNA stability, neither in normoxia nor in hypoxia. The recent finding of a novel lnc-RNA covering the 5' region of the HIF-1α gene locus (5'aHIF-1α antisense) suggests that many non-coding RNAs may be involved in HIF-1α regulation (Bertozzi D., 2011). It has been proposed that non-coding RNAs can modulate gene expression via transcriptional interference basing on the assumption that RNA polymerases bind to the promoters of convergent genes on opposing strands of DNA and then proceed towards the 3’ end of sense and antisense genes: this would provoke clash of RNA polymerase complexes elongating in convergent direction thereby determining block of transcription of the sense RNA. Thus, it is possible that overexpression of aHIF *per se* may not result in a downstream effect on *hif-1a* mRNA and protein expression because HIF-1α gene regulation is related to the transcription of aHIF rather than aHIF itself.

It has been demonstrated that aHIF transcription is in part under the transcriptional control of HIF-1; interestingly, we observed that transfection with phosphorotioate-modified or chimeric oligonucleotides determines upregulation of aHIF expression in A549 cells and we therefore hypothesize that DNA-damaging agents could determine an increase of aHIF levels independently of HIF-1α activity. We then treated

A549 cells with Topotecan, an inhibitor of DNA Topoisomerase I which induces DNA double-strand breaks and which also inhibits HIF-1α protein accumulation and HIF-1 transcriptional activity: dose-dependency experiments revealed that treatment with Topotecan for 4 hours induced aHIF expression using doses of 500 nM or higher; this is paralleled by a p53 protein increase and a reduction of *hif-1a* mRNA level. p53 responsive elements tend to cluster within noncoding regions of the gene but they can be found practically anywhere within the target gene locus. Although p53 responsive elements are most commonly located in the promoter at varying distances upstream from the transcription start site (TSS), sometimes they map very close (within approximately 300 bp) to the TSS, or within early intronic sequences and can even be found within exons. (Beckerman R. and Prives C., 2010). Then, aHIF upregulation in A549 cells following treatment with Topotecan is clearly to be considered an hypoxia-independent and HIF-1-independent phenomenon and could be related to p53 levels and to DNA damage.

The interconnections between the hypoxic and the p53 pathways have widely been studied and there are reciprocal influences between these two pathways (Sermeus A. and Michiels C., 2011), many of which appear to be cell type-specific. It has been reported that HIF-1α induces stabilization of p53 and cells exposed to hypoxia accumulate p53 protein (Graeber T.G., 1994), but this was not the case of A549 cells. Interestingly, many studies report that p53 exert a negative regulation on HIF-1α protein amount and activity: thus, the concomitant upregulation of p53 protein and aHIF transcript expression and the subsequent HIF-1α mRNA dowregulation in response to treatment with DNA-damaging drug Topotecan could represent a novel mechanism of p53-mediated HIF-1α negative regulation.

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