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ORIGINAL PAPER

Anti-sense morpholino oligonucleotide assay shows critical involvement for NF-κB activation in the production of *Wnt-1* protein by HepG2 cells: oncology implications

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Abstract The link of proto-oncogenic protein *Wnt-1* production with NF-κB activation has been functionally demonstrated in PC12 cells, a rat pheochromocytoma cell line of neural crest lineage, while it is not yet verified in human cells. The link can be indirectly supported in our previous report that functional proteomics identifies enhanced expression of NF-κB-associated *Wnt-1* production in human hepatocellular carcinoma tissues. This study aimed to further validate this link in human cells using anti-sense strategy. The effects of sequence-specific anti-sense morpholino oligonucleotides (ONs) targeting against pre-mRNA sequences of human p50 and p65 subunits of NF-κB as well as *Wnt-1* genes were investigated. It revealed that all the three morpholino ONs inhibited NF-κB activation in human hepatoblastoma cell line HepG2 cells along with decreased *Wnt-1* production. Chromatin immunoprecipitation assay ascertained the direct binding of NF-κB-p50 to the *Wnt-1* promoter. Additionally, anti-P50 and anti-P65 morpholino ONs also repressed the phosphorylation of Iκ Bα which temporarily correlated with the inhibition of NF-κB activation accompanied by decreased *Wnt-1* production by HepG2 cells. In summary, NF-κB activation is critically involved in the production of *Wnt-1* by HepG2 cells. These results may have important oncology implications in treating patients with NF-κB-associated *Wnt-1*-producing cancers.

Keywords Anti-sense strategy · Chromatin immunoprecipitation assay · HepG2 cells · Iκ B · Morpholino oligonucleotide · NF-κB · *Wnt-1* protein

Abbreviations

ChIP	Chromatin immunoprecipitation
EMSA	Electrophoretic mobility shift assay
Iκ Bs	Inhibitors of NF-κB
IKK	IκB kinase
NF-κB	Nuclear transcription factor-κB
ONs	Oligonucleotides
RNAi	RNA interference
Wnt-1	Wingless-type MMTV integration site gene family, member 1
Wnts	Wnt proteins

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Nuclear transcription factors of the NF-κB/Rel family contains five mammalian NF-κB/Rel proteins: NF-κB1 (also known as p50/p105), NF-κB2 (also known as p52/p100), RelA (also known as p65/NF-κB3), RelB, and c-Rel. RelA, RelB, and c- Rel are synthesized as matured proteins, while p50 and p52 are first synthesized as large precursors p105 and p100, respectively, which are processed by the proteosome [1–3]. Alternatively, NF-κB is the generic name for a family of dimers formed by several proteins. The activity of NF-κB is controlled by shuttling from the cytoplasm to the nucleus responding to cell stimulation. NF-κB dimers containing RelA or c- Rel are retained in the cytoplasm through interaction with the inhibitors of NF-κB (IκBs). In response to a variety of stimuli, IκBs are phosphorylated (Ser 32 and Ser 36 for IκBα and Ser 19 and Ser 21 for IκBβ) by the activated IκB kinase (IKK) complex, followed by rapid ubiquitin-dependent degradation by the 26S proteosome [4, 5]. This allows NF-κB dimers to translocate into the nucleus, where different heterodimers bind to specific promoters to initiate transcription of a wide range of genes that influence the inflammatory response as well as cell death and survival and tissue repair [1–3, 6].

In humans, 19 WNT genes encoding for 19 WNT proteins (Wnts) have been identified [7, 8]. The members of WNT gene family are increasingly discovered in other species [9]. The Wnt signaling pathway, initially discovered by genetic analysis in the wing development of *Drosophila melanogaster*, has been implicated in quite a few cancers [10–12]. The first member of the 19 known human Wnt genes, *Wnt-1*, named by wingless type mouse mammary tumor virus (MMTV) integration site gene family member 1, was first discovered because of its oncogenic properties [13]. The 19 human Wnts are similar in size, ranging from 39 (WNT7a) to 46 kDa (WNT10a) and share 27–83% aminoacid sequence homology as well as a conserved pattern of 23 or 24 cysteine residues [7, 8]. Wnts have diverse roles in regulating many processes during embryonic development, including axis specification, organogenesis, angiogenesis, and stem cell proliferation [7, 10–12, 14–16]. They have also been implicated in bone density maintenance, neurological conditions during adulthood and carcinogenesis of many cancers [10–12, 14–17]. While many Wnts, their cognate receptors of the Frizzled(*Fz*)/planar cell polarity (PCP) and Arrow/low-density lipoprotein (LDL) receptor-related protein 5/6 (LRP5/6) families as well as downstream pathway components have been identified [8, 10, 11, 18], much less is known about the regulation of the production of Wnts, specifically *Wnt-1* production in relation to NF-κB activity.

The link of proto-oncogenic protein *Wnt-1* with NF-κB activity has been functionally demonstrated in PC12 cells, a rat pheochromocytoma cell line of neural crest lineage. It shows that the *Wnt-1*-mediated survival of PC12 cells is dependent on NF-κB activation, and that stable expression of *Wnt-1* increases NF-κB activity [19]. This link can be indirectly supported in our recent report that functional proteomics identifies enhanced expression of NF-κB-associated *Wnt-1* protein in human hepatocellular carcinoma (HCC) tissues [20]. However, a direct evidence of NF-κB activation relating to the production of *Wnt-1* protein in human cells remains elusive. To further validate this link in human cells, one of the three types of anti-mRNA strategies, i.e., anti-sense oligonucleotide, ribozymes, and small interfering RNA (siRNA) [21–23] was used in the present study. The first one, sequence-specific anti-sense morpholino oligonucleotides (ONs) against p50 and p65 subunits of NF-κB as well as *Wnt-1* genes was used to investigate their effects on *Wnt-1* production by human hepatoblastoma cell line HepG2 cells. Morpholino ONs are nonionic DNA analogs, in which the ribose is replaced by a morpholino moiety and phosphoroamidate intersubunit linkages are used instead of phosphodiester bonds and have been designed to bind to specific *cis*-elements within target precursor mRNA to correct aberrant splicing brought about by disease mutations [22, 23]. In contrast, the potential also exists to use this method to selectively delete individual exon of specific pre-mRNA species and thus increase production of variant mRNAs, which are translated into proteins devoid of functional domains [22–26]. In light of this, a similar strategy could be adopted to knockdown specific exon within pre-mRNA of genes, resulting in decreased expression of its downstream protein species [22–26]. By the anti-sense oligonucleotide approach, our current study revealed that NF-κB activation was critically involved in the production of *Wnt-1* protein in HepG2 cells.

Materials and methods

Delivery of morpholino ONs

All anti-sense morpholino ONs used in this study were synthesized by Gene Tools LLC (Corvallis, OR, USA). The Endo-Porter system (Corvallis) that delivers "bare" ONs by an endocytosis-mediated process was applied. This system delivers ONs into the nucleus to affect pre-mRNA splicing. The human hepatoblastoma cell line HepG2 (BCRC No. 60177) was used in the present study because it shows constitutive NF-κB activation associated with *Wnt-1* protein production. Adherent HepG2 cells were obtained by incubating onto 75T flasks in RPMI-1640 medium (Invitrogen Taiwan, Ltd. Taipei, Taiwan) with 10% fetal calf serum and antibiotics in a humidified atmosphere at 37°C, 5% CO_2 incubator. Cultured adherent HepG2 cells at about 80–100% confluent were used for the best delivery of morpholino ONs with the Endo-Porter system. The

procedures for delivery of ONs were carried out according to the manufacturer's instructions. All the anti-sense morpholino ONs used in this study were designed to target against the splicing site of the first intron and the first exon of mRNA sequences of human *Wnt-1* gene as well as p50 and p65 subunits of NF-κB genes with 3'-carboxyfluorescein and green-emitting fluorescent tag. Their nucleotide (nt) sequences are listed as follows:

anti-*Wnt-1* morpholino ON (targeting to nt 198–222, according to NCBI sequence no. NM-005430): 5′-CCAT GGGGCTCTGGGCGCTGTTGCCT-3′;
anti-NF-κB-p50 morpholino ON (targeting to nt 464–488, according to NCBI sequence no. BC051765): 5′-CA GAATGGCAGAAGATGATCCATAT-3′;
anti-NF-κB-p65 morpholino ON (targeting to nt 34–54, according to NCBI sequence no. L19067): 5′-GCGGCA TGGACGAACTGTTCC-3′.

Delivery efficiencies were determined by calculating the number of positive cells with a fluorescence signal within defined fields on an inverted fluorescence microscope. As a control, invert anti-sense morpholino ONs with the same sequences but in a reverse orientation were used.

Definition of successful delivery of morpholino ONs

Unfixed live cells observed under an inverted fluorescence microscope (Nikon TE 2000-S, Tokyo, Japan), the successful delivery of morpholino ONs is indicated by dim and diffuse fluorescence throughout the cytosol/nuclear compartment. Visible diffuse fluorescence requires a fluoresceinated ON concentration up to 10 times higher than the concentration needed for anti-sense action against most targets. Once a successful delivery is confirmed by observing diffuse fluorescence, then there is more labeled morpholino ONs in the cytosol than is needed to inhibit translation of most mRNA targets. Consequently, the morpholino ONs can be used at lower concentrations for the knockdown experiments.

Electrophoretic mobility shift assay

Nuclear and cytoplasmic extracts were prepared from HepG2 cells according to standard protocols [27] by extraction reagents (PIERCE, Rockford, IL, USA) according to the manufacturer's instructions. The protein content of nuclear extracts was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). Electrophoretic mobility shift assay (EMSA) of NF-κB was performed with 10 μg nuclear protein extract using LightShift Chemiluminescent EMSA kit (PIERCE) instead of using ^{32}P isotope probe [28]. Moreover, to show a differential NF-κB activation and *Wnt-1* expression among different hepatoma cells, Hep3B cells (ATCC No. HB-8064), and Alexander cell line PLC/PRF/5 (BCRC No. 60223) which produces HBsAg were also included in the assay. The cell line PC12 (BCRC No. 60048) with constitutive NF-κB activation along with *Wnt-1* protein production was used as the positive control cell line [19]. Briefly, the protein content of nuclear extracts was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). 10 μg of each nuclear extract was incubated with a biotin end-labeled NF-κB-binding consensus oligoduplex probe (Promega, Madison, WI) or a mutant NF-κB probe. The ∼25 bp biotin end-labeled oligoduplex probe contains the NF-κB-binding consensus sequences as follows.

consensus NF-κB probe (oligoduplex): 5′biotin-...AGT TGA GGG CAC TTT CCC AGG C...-3′ and 3′-...TCA ACT CCC GTG AAA GGG TCC G....-biotin 5′.
mutant NF-κB probe (oligoduplex): 5′biotin-...GTG ACG CGG GAA TTT CCG GTG C...-3′, and 3′-...CAC TGC GGC CTT AAA GGC CAC G. ....-biotin 5′.
As a positive control for the assay, biotin end-labeled 60 bp oligoduplex Epstein–Barr virus nuclear antigen (EBNA)-binding probe containing following sequences was used:
5′biotin-...TAGCATATGCTA...-3′and 3′-...ATCGTA-TACGAT...-biotin 5′ .

The EMSA procedures were carried out according to the manufacturer's instructions, and a 50-fold excess of the appropriate unlabeled oligoduplex was used in competition assays.

Assessment of anti-sense morpholino ONs-induced apoptosis

To exclude the possibility that the action of anti-sense morpholino ONs may be via their toxicity on HepG2 cells instead of knockdowning target sequences, cell apoptosis assayed by DNA fragmentation was carried out in parallel as described previously [28]. As a positive control for the apoptosis assay, tumor necrosis factor-alpha (TNF-α)-induced apoptosis was conducted using the strategy of cell sensitization to pretreatment with transient hyperthermia, interferon-γ(IFN-γ) challenge in combination with pyrrolidine dithiocarbamate (PDTC) inhibition [29–31].

Immunoblot analysis of *Wnt-1*, NF-κB p50 and p65 proteins

Western blotting of total protein extracts from HepG2 cells with biotin-conjugated rabbit anti-human WNT1 polyclonal antibody (ZYMED Lab. Inc., South San Francisco, CA , USA) was performed by the procedures described previously [20]. The quantity of *Wnt-1* protein production was

semi-quantitatively estimated on the immunoblot films with ImageMaster TotalLab, Version 2.01 (Amersham Parmacia Biotech, NJ, USA), and data were estimated and expressed as relative density to HepG2 cell control. Likewise rabbit anti-human NF-κB-p50 (**upstate**; Millipore Corporation, Temecula, CA, USA) and p65 (**abcam**, Cambridge, UK) polyclonal antibodies were used to detect p50 and p65 proteins, respectively.

NF-κB p50 chromatin immunoprecipitation assay

Chromatin immunoprecipitation (Ch1P) assays were preformed using the ChIP assay kit (**upstate**) in conjunction with antibodies to NF-κB-p50 (**upstate**) or with an isotype control antibody. The assay procedures were carried out according to the manufacturer's instructions. In brief, log-phase HepG2 cells with *Wnt-1* expression (2×10^6 cells for each ChIP experiment) were cultured and fixed by the addition of formaldehyde (1% (w/v) final concentration) for 10 min. After addition of 125 mM glycine, cells were washed three times with cold phosphate-buffered saline, re-suspended in 900 μl of lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40) containing protease inhibitors and left for 10 min at 4°C. DNA was sheared by sonication to yield an average length of 500–700 bp and cleared by centrifugation at 12,000*g* for 5 min at 4°C. Lysates were diluted with the dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl). 100 μl of the sample was used as the "**Input**," i.e., to be incubated with anti-NF-κB-p50 antibodies; and the rest of sample was incubated with 1 μg of the isotype control (**Control**) antibody beads at 4°C overnight. NF-κB-p50-DNA complexes were isolated by centrifugation at 5,000 rpm in an Eppendorf microcentrifuge for 1 min, and pellets were washed three times with dialysis buffer (50 mM Tris–Cl, pH 8.0, 2 mM EDTA) and three times with immunoprecipitation wash buffer (100 mM Tris–Cl, pH 8.0, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid) for 5 min with rotation. After wash, 200 μl of digestion buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% SDS, 100 μg/ml proteinase K) was added to each sample, incubated at 55°C for 3 h, followed by 6 h at 65°C. Subsequently, phenol–chloroform extraction of DNA was done with standard procedures. PCR was performed with 35 cycles of 96°C for 30 s, 55°C for 45 s, and 72°C for 45 s using primers flanking the putative *Wnt-1* gene promoter [32] as followings: forward primer: 5′-CCC TAA CCG GTG CGC CCT GGT GCC-3′ (nt 181–204); and reverse primer: 5′-AGC GCC CAG AGC CCC ATG GCC TGC-3′ (nt 458–481).

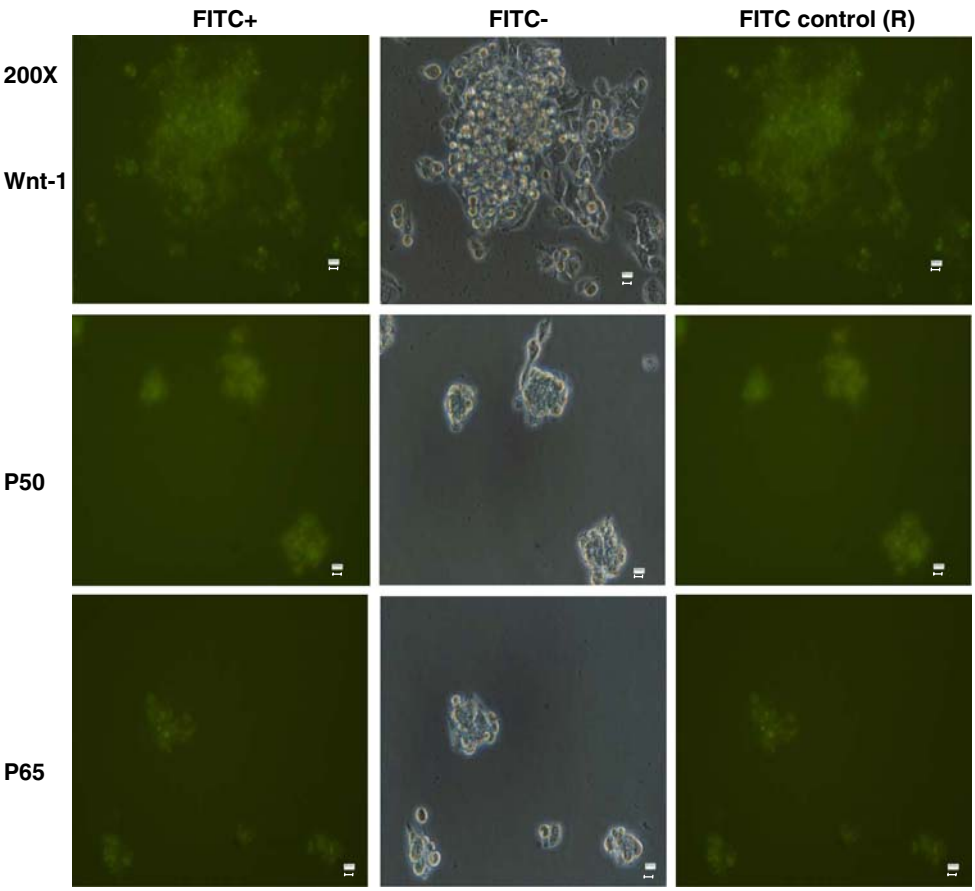

Fig. 1 Delivery of anti-sense morpholino ONs. Under inverted fluorescence microscope, it shows that all the three anti-sense mopholino ONs (a. *Wnt-1*; b. NF-κB p50; c. NF-κB p65) are successfully delivered into HepG2 cells by the Endo-Porter system as evidenced by dim and diffuse fluorescence throughout the cytoplasm/nuclear compartments visualized by inverted fluorescence microscope. FITC+ indicates the cells were visualized on the fluorescence filter and FITC−, on the same cells without the fluorescence filter. Size bar on figures indicates 500 μm. Invert anti-sense morpholino ONs with the same sequences but in a reverse orientation (R) were used as controls

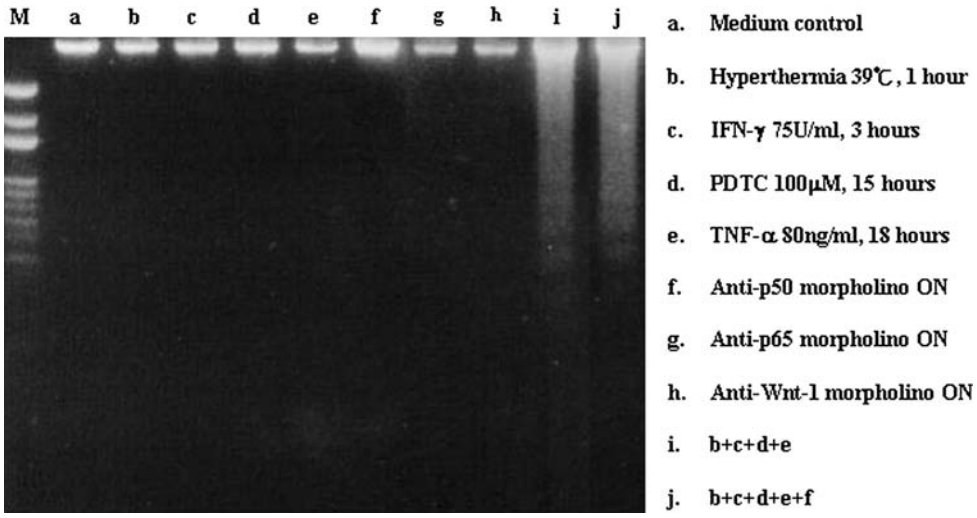

Fig. 2 Apoptosis assay of HepG2 cells after delivery of anti-sense morpholino ONs. HepG2 cells with different treatments shown in right side of the figure were assayed for cell apoptosis by fragmentation of chromosomal DNA. Lane i and j were used as positive control of cell apoptosis

IκBα phosphorylation assay

It is shown that inflammation may enhance tumor promotion through NF-κB-dependent mechanisms and NF-κB is also proposed to promote metastatogenesis [1, 6]. Moreover, activation of IκB kinase α (IKKα) which phosphorylates Ser 32 and Ser 36 on IκB [4, 5] is reported to promote metastatic phenotype in mouse model of prostate cancer [1, 33].

To reflect constitutive activation of IKKα in HepG2 cells, IκBα phosphorylation assay was performed in parallel. Briefly, cytoplasmic protein extract from HepG2 cells was separated on an analytical electrophoresis gel and transferred onto polyvinylidene difluoride (PVDF) membrane (Hybond P, Amersham Biosciences) for Western blot analysis of IκBα (phospho S32 + S36) protein by the standard procedures using biotin-conjugated rabbit anti-rat IκBα (phospho S32 + S36) polyclonal antibodies (abcam). Reaction with the primary antibody was visualized using an enhanced chemiluminescence detection system (ECL-plus, Amersham Biosciences) and exposed to autoradiography film for an appropriate duration. The quantity of phosphorylated IκBα protein was semi-quantitatively determined by the software of ImageMaster TotalLab, Version 2.01.

Results

Fluorescein-tagged morpholino ONs are efficiently delivered into HepG2 cells by the Endo-Porter system

As shown in Fig. 1, fluorescein-tagged mopholino ONs are efficiently delivered into HepG2 cells by the Endo-Porter system. Dominant nuclear staining associated with faint cytoplasmic staining is noted in cells with delivery of all the three anti-sense mopholino ONs including *Wnt-1* and NF-κB p50 and p65 subunits. Similar results could also be obtained in Hep3B cells and PLC/PRF/5 cells, as well as with anti-sense mopholino ONs of rat sequences in PC12 cells (data not shown), indicating the functional consistency of the Endo-Porter system used in the present study.

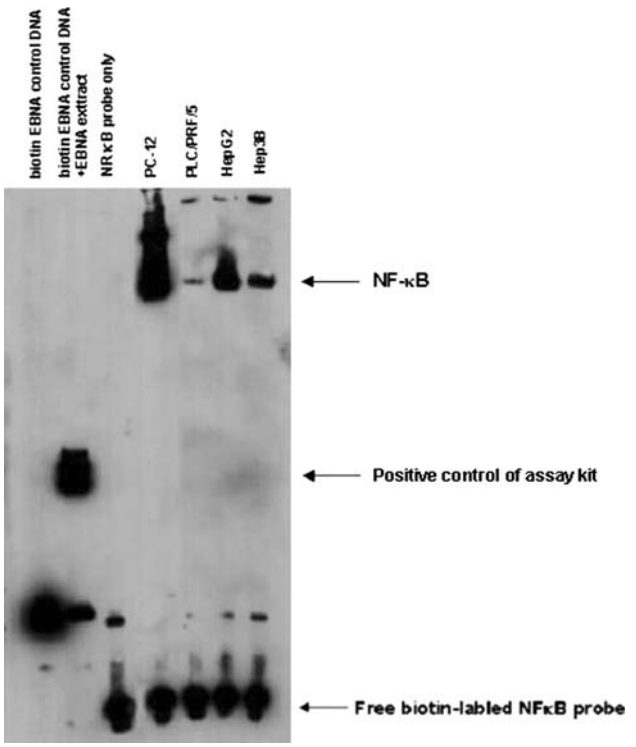

Fig. 3 EMSA on different hepatoma cell lines. Differential NF-κB activation was detected in different hepatoma cell lines by EMSA. PC12 cells were used as positive control

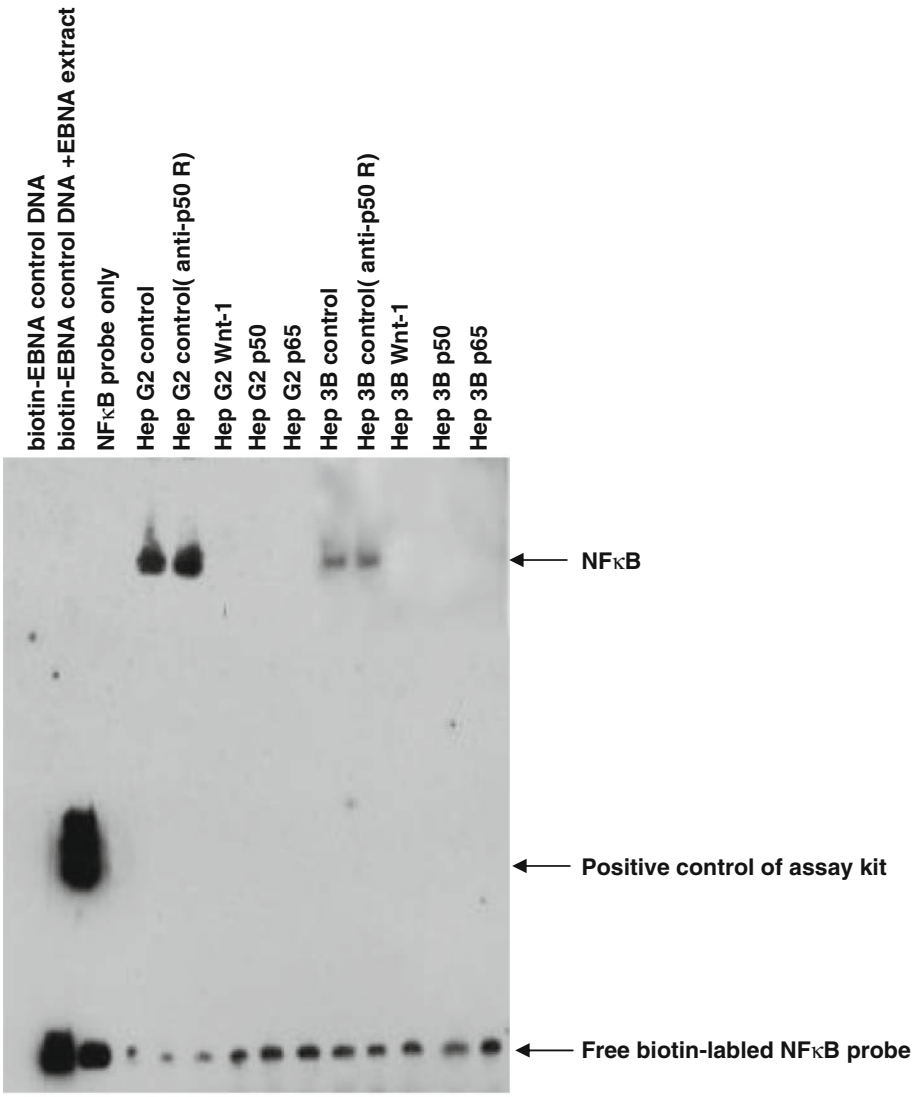

Fig. 4 Inhibition of NF-κB activation by anti-sense mopholino ONs in two hepatoma cell lines. All the three anti-sense mopholino ONs inhibited NF-κB activation. Anti-p50 mopholino ON with reverse orientation (R) which did not inhibit NF-κB activation was used as control of the assay

Anti-sense morpholino ONs do not induce cell apoptosis

No cell apoptosis is detected in HpG2 cells at 48 h after delivery of all the three anti-sense morpholino ONs (Fig. 2), ascertaining that the action of anti-sense morpholino ONs is through the knockdown of target genes instead of cellular toxicity.

All the three anti-sense morpholino ONs inhibit NF-κB activation and block *Wnt-1* protein production by hepatoma cell lines

Differential NF-κB activation (Fig. 3) with varying *Wnt-1* expression (data not shown, Supplementary Fig. 1) is detected in different hepatoma cell lines. By EMSA, activation of NF-κB can be confirmed by the competition assay (data not shown, Supplementary Fig. 2) and is virtually inhibited by all the three anti-sense morpholino ONs after delivery for 48 h as shown in Fig. 4, which demonstrates data for assays on HepG2 and Hep3B cells, for PLC/PRF/5 cells (data not shown). The inhibition runs in parallel with blockade of *Wnt-1* production (Fig. 5). Of note, the expression of NF-κB-p50 protein by HepG2 cells is suppressed by the delivery of both anti-p50 and anti-*Wnt-1* morpholino ONs, but not by anti-p65 morpholino ON (Fig. 6). Likewise, similar results could be obtained in NF-κB-p65 protein expression which is blocked by both anti-p65 and anti-*Wnt-1* morpholino ONs but not by anti-p50 morpholino ON (data not shown).

NF-κB p50 ChIP assay shows direct binding of NF-κB-p50 to the putative Wnt-1 promoter

As shown in Fig. 7, a DNA fragment of 300 bp could be amplified by the NF-κB p50 ChIP assay using primers

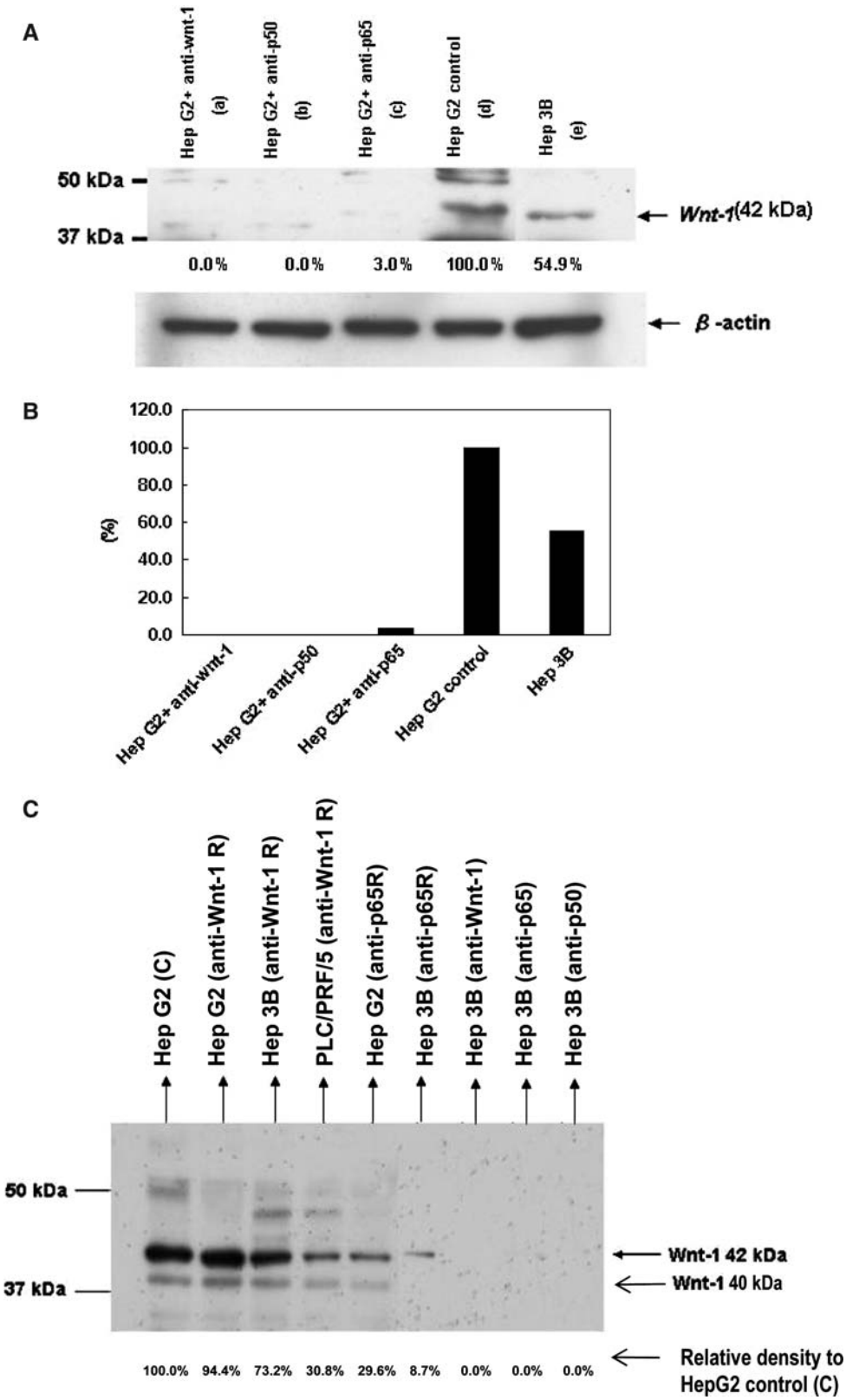

Fig. 5 Inhibition of *Wnt-1* production by anti-sense mopholino ONs. (**A**) *Wnt-1* production by HepG2 cells was virtually inhibited by all the three anti-sense mopholino ONs including *Wnt-1*(lane a), NF-κB p50 (lane b) and p65 (lane c) subunits inhibited. Lane d was used as the control of the assay and for a comparison to Hep3B cells (lane e), showing again the differential *Wnt-1* production in different human hepatoma cell lines. Below each lane, β-actin was used as the protein loading control. (**B**). Box-plot chart shows percent inhibition of *Wnt-1* production by the three anti-sense mopholino ONs and relative *Wnt-1* production by Hep3B cells. (**C**). Control assays for *Wnt-1* expression with anti-sense mopholino ONs in a reverse orientation (R). Lanes 7–9, show the inhibition of *Wnt-1* production by the three anti-sense mopholino ONs in Hep3B cells

flanking the putative *Wnt-1* promoter, in which region DNA sequence is full of CpG islands, a hallmark of gene promoter sequences, could be found. This may support a direct binding of NF-κB-p50 to the putative promoter region of *Wnt-1* gene. Functional assays to assess the binding are being investigated in our laboratory.

Anti-sense morpholino ONs of NF-κB p50 and p65 subunits repress the phosphorylation of IκBα

We investigated simultaneously IκBα phosphorylation and NF-κB activation by anti-sense morpholino ONs in HepG2 cells. Constitutive IκBα phosphorylation associated with NF-κB activation was detected. Notably, anti-sense morpholino ONs of NF-κB p50 (Fig. 8) and p65 (data not shown) subunits also repressed the phosphorylation of IκBα (Fig. 8a) which temporarily correlated with the inhibition of NF-κB activation (Fig. 8b) along with decreased production of *Wnt-1* protein by HepG2 cells (Fig. 8c). These data implicated constitutive activation of IKKα in HepG2 cells.

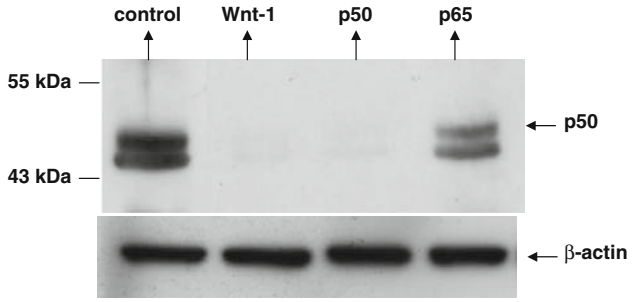

Fig. 6 Effects of anti-sense mopholino ONs on expression of NF-κB p50 and p65 proteins. The expression of NF-κB-p50 protein by HepG2 cells was suppressed by the delivery of both anti-p50 and anti-*Wnt-1* morpholino ONs, but not by anti-p65 morpholino ON

Discussion

The proto-oncogene *Wnt-1*(*int-1*) had been identified in several independently arising mammary tumors in which proviral insertion of MMTV resulted in activation and ectopic expression of *Wnt-1* protein [34]. The *Wnt-1* gene is localized to chromosome 12q13 in human [35], and to chromosome 15 in mouse [36]. Its *cis*-acting regulatory sequences regulating *Wnt-1* expression is being unveiled [7, 8, 10, 11, 37], while much less is known in its transcriptional regulation, specifically regarding NF-κB activity [19].

No cell apoptosis assayed by DNA fragmentation was detected at 48 h after delivery of all the three anti-sense

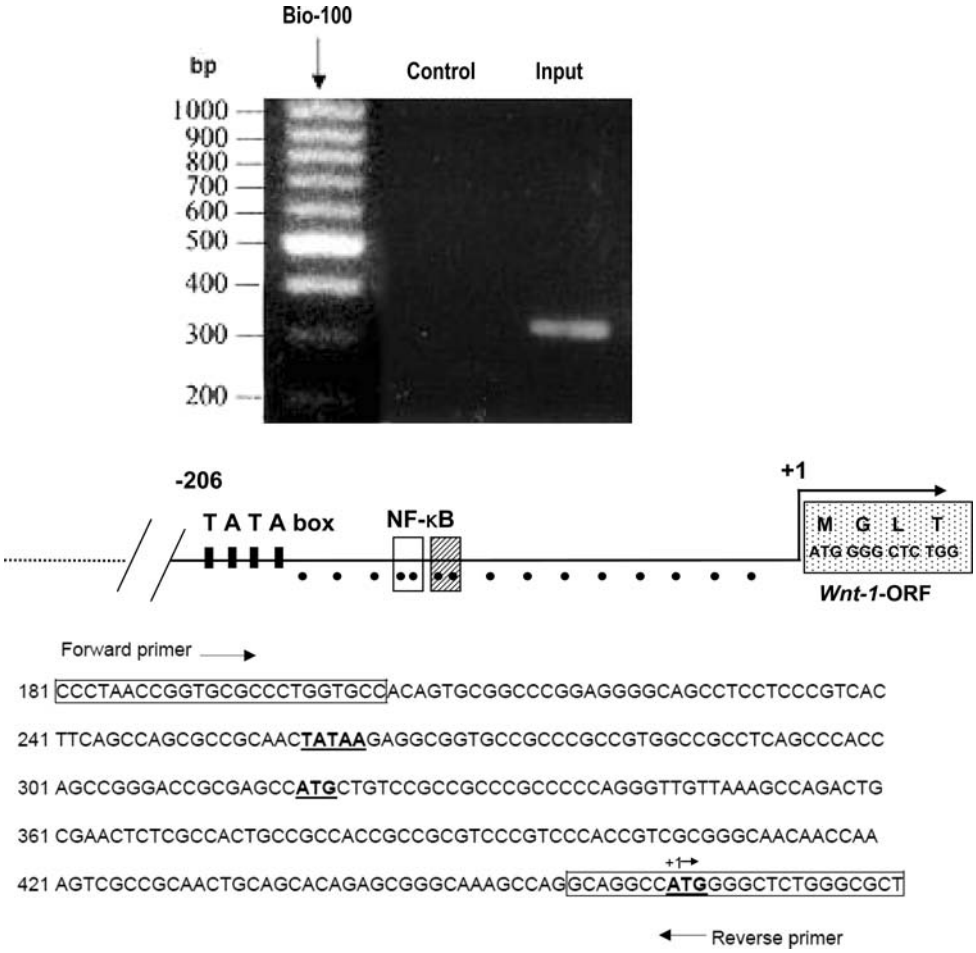

Fig. 7 Results of NF-κB p50 ChIP assay. A scheme shows that a 300 bp DNA fragment could be amplified using primers flanking the putative *Wnt-1* promoter by ChIP assay with NF-κB p50 antibodies (Input), in which region DNA sequences full of CpG islands shown as dot spots below the line after TATA box.This DNA fragment could not be amplified by ChIP assay with isotype control (Control). Boxes indicate the putative NF-κB-binding site. *Wnt-1* open reading frame (ORF), ATG codon and its first four amino acid sequence are in gray square. Primers used for PCR and the putative *Wnt-1* promoter sequence are listed below.

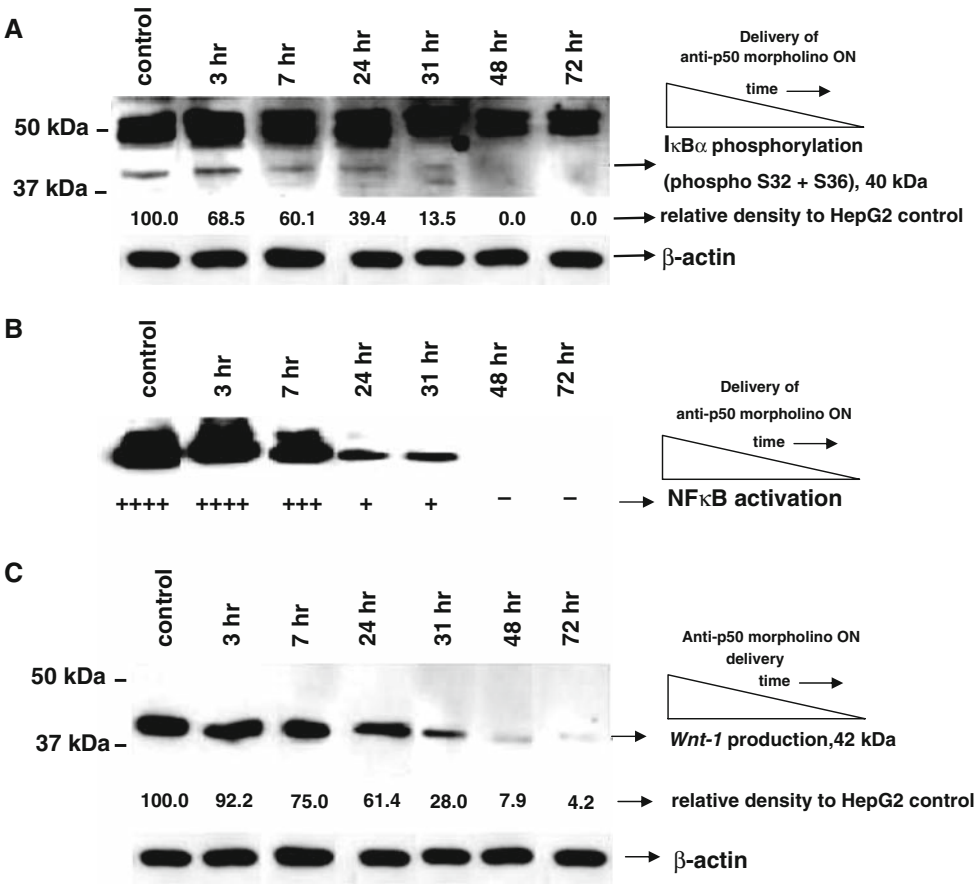

Fig. 8 IκBα phosphorylation assay. Constitutive IκBα phosphorylation and NF-κB activation along with *Wnt-1* production in HepG2 cells was detected (**A, B** and **C**, control lanes). Anti-NF-κB p50 morpholino ON repressed the phosphorylation of IκBα (**A**) which temporarily correlated with the inhibition of NF-κB activation (**B**) accompanied by decreased production of *Wnt-1* protein by HepG2 cells (**C**). *β*-actin was used as the protein loading control

morpholino ONs into HepG2 cells including p50 and p65 subunits of NF-κB as well as *Wnt-1*genes. This indicated that the inhibition of NF-κB activation was via the anti-sense mechanism instead of cellular toxicity. The anti-sense assay with morpholino ONs in human hepatoma cell lines HepG2, Hep3B and PLC/PRF/5 has demonstrated that all the three anti-sense mopholino ONs exhibit inhibition on NF-κB activation associated with blockade of *Wnt-1* protein production, thus ascertaining the critical involvement for NF-κB activation in the production *Wnt-1* protein in human hepatoma cells. The mechanism why anti-*Wnt-1* morpholino ON also inhibits NF-κB activation is not immediately clear. It might attribute to the mutual activation of *Wnt-1* protein and NF-κB [19], as evidenced by reciprocal inhibition on the production of NF-κB-p50 protein by anti-p50 morpholino and anti-*Wnt-1*morpholino ONs, and on p65 protein, by anti-p65 morpholino and anti-*Wnt-1* morpholino ONs.

The finding that over-expression of NF-κB-associated *Wnt-1* protein identified in vast majority of human hepatocellular carcinoma (HCC) samples infected with hepatitis B virus and/or hepatitis C virus (HBV and/or HCV) by functional proteomics [20] and cDNA microarray studies [38] provided indirect evidence supporting the association of NF-κB activation with *Wnt-1* protein production. Furthermore, up-regulation of *Wnt-1* expression is shown to correlate with hepatocyte growth stimulated by HCV core protein [39]. Both HBV and HCV may activate NF-κB [28, 40–45]. A link of HBV with Wnt signaling has also been reported in hepatoma cell line Huh7 showing that X-protein of HBV (HBx) may enhance the stabilization of *β*-catenin, and is essential for the activation of Wnt/*β*-catenin signaling [46]. A hallmark of Wnt signaling is the stabilization of cytoplasmic *β*-catenin, followed by its nuclear translocation and association with T-cell factor/lymphoid enhancer factor (TCF/LEF) protein family, which lead to the transcription of Wnt target genes such as c-myc, cyclin D1, and c-jun [8, 14, 47–49]. HBx/c-myc transgenic mice may induce the development of HCC [50]. In *Wnt-1*-mediated mice model of breast tumorigenesis, *Wnt-1* is shown to be a fundamental signaling event in cancer metastasis progression [51]. Given the overwhelming evidence shows that both NF-κB and Wnt signaling regulate oncogenesis and tumor progression [1, 6, 11, 12, 15, 52–54], the result of direct association of NF-κB activation with *Wnt-1* production is of oncological significance because the over-expression, and sometimes under-expression of Wnt genes appear in many human cancers and dysregulated Wnt signaling frequently occurs in human malignancies [11, 12, 15, 16, 52–54].

Our data also identified a role for IKKα pathway in the production of *Wnt-1* protein. This might be of clinical interest in cancer patients with production of *Wnt-1* protein and/or other Wnts as it is reported that the amount of active nuclear IKKα in mouse model of prostate cancer correlates with cancer metastasis progression [1, 33]. Wnt signaling pathway has been proposed as a potential target for novel treatment of children with medulloblastoma [55, 56] and patients with head and neck squamous cell carcinomas including immunotherapy [57]. Collectively, Wnt signaling together with NF-κB signaling pathways might be potentially used as targets for the novel treatment of patients with NF-κB-associated Wnts-producing cancers [1, 6, 11, 12, 15, 16, 58–60].

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