HOP expression is regulated by p53 and RAS and characteristic of a cancer gene signature

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Received: 20 September 2016 / Revised: 21 November 2016 / Accepted: 7 December 2016 / Published online: 16 December 2016
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Abstract The Hsp70/Hsp90 organising protein (HOP) is a co-chaperone essential for client protein transfer from Hsp70 to Hsp90 within the Hsp90 chaperone machine. Although HOP is upregulated in various cancers, there is limited information from in vitro studies on how HOP expression is regulated in cancer. The main objective of this study was to identify the HOP promoter and investigate its activity in cancerous cells. Bioinformatic analysis of the –2500 to +16 bp region of the HOP gene identified a large CpG island and a range of putative *cis*-elements. Many of the *cis*-elements were potentially bound by transcription factors which are activated by oncogenic pathways. Luciferase reporter assays demonstrated that the upstream region of the HOP gene contains an active promoter in vitro. Truncation of this region suggested that the core HOP promoter region was –855 to +16 bp. HOP promoter activity was highest in Hs578T, HEK293T and SV40-transformed MEF1 cell lines which expressed mutant or inactive p53. In a mutant p53 background, expression of wild-type p53 led to a reduction in promoter activity, while inhibition of wild-type p53 in HeLa cells increased HOP promoter activity. Additionally, in Hs578T and HEK293T cell lines containing inactive p53, expression of HRAS increased HOP promoter

activity. However, HRAS activation of the HOP promoter was inhibited by p53 overexpression. These findings suggest for the first time that HOP expression in cancer may be regulated by both RAS activation and p53 inhibition. Taken together, these data suggest that HOP may be part of the cancer gene signature induced by a combination of mutant p53 and mutated RAS that is associated with cellular transformation.

Keywords HOP · p53 · RAS · RAF · Promoter · Hs578T · HeLa · HEK293T · Reporter assays

Introduction

The HSP90 chaperone machine is composed of various co-chaperones, one of which is the HSP70/HSP90 organising protein (HOP), otherwise known as p60 or the stress-inducible protein 1 (STIP1). HOP is a co-chaperone that is essential for the full functioning of HSP90 with certain client proteins (Hernández et al. 2002; Trepel et al. 2010; Alvira et al. 2014). HOP is specifically responsible for catalysing the client transfer from HSP70 to HSP90, by coordinating the cycle of substrate binding and release through modulating the conformational dynamics of the chaperones. In doing so, HOP facilitates the folding of nascent polypeptides, the productive assembly of multimeric protein complexes and the disassembly of proteins specific to the HSP70 and HSP90 chaperones (Hernández et al. 2002; Caplan 2003; Song and Masison 2005; Trepel et al. 2010; Röhl et al. 2015).

It is now appreciated that co-chaperones of HSP90, including HOP, may contribute to cancer cell proliferation, migration and drug resistance (Erllich et al. 2007; Forafonov et al. 2008; Holmes et al. 2008; Smith et al. 2009; Horibe et al. 2011). HOP has been identified as overexpressed in certain cancers compared to normal cell equivalents (Chao et al.

Electronic supplementary material The online version of this article (doi:10.1007/s12192-016-0755-8) contains supplementary material, which is available to authorized users.

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2013; Carvalho da Fonseca et al. 2014). HOP expression was linked to invasive potential in seven human pancreatic cancers (Walsh et al. 2011), a feature also demonstrated in breast and ovarian cancer cells (Wang et al. 2010b). Kubota et al. (2010) showed that HOP levels were upregulated in colonic carcinoma tissue samples compared to non-tumour tissues obtained from the same patients (Kubota et al. 2010). HOP has been found in a constitutive complex with HSP90 in cancer cells, whereas complex formation is only induced under specific conditions in normal cells (Kamal et al. 2003). Addition of recombinant HOP to glioma cells induced cell proliferation (Erlich et al. 2007), while the disruption of the HOP-HSP90 interaction sensitised cells to inhibitors of HSP90 and reduced proliferation of a range of cancers, including breast, lung and kidney (Horibe et al. 2011; Pimienta et al. 2011). Data from knockdown studies have demonstrated that HOP regulates cancer cell migration by numerous mechanisms, including inhibition of matrix metalloproteinase-2 (MMP2) and regulation of components of the cytoskeleton (actin and tubulin) (Walsh et al. 2011; Li et al. 2012; Willmer et al. 2013). The presence of HOP in the extracellular matrix has been linked to the invasive properties of these cells by Walsh et al. (2011). Collectively, these results suggest a role for HOP in malignancy.

Despite growing evidence for a role for human HOP in cancer, relatively little is known about the mechanisms that control HOP expression. Ruckova et al. (2012) are the only group to have studied the HOP promoter. They identified the presence of HSF-1-binding sites within the HOP promoter, and the binding of HSF-1 to this HOP promoter region was determined by chromatin immunoprecipitation. It was also shown that the protein levels of HSF-1 bound to the putative HOP promoter region increased in cancer cell lines upon the addition of the HSP90 inhibitor 17AAG (Ruckova et al. 2012). However, the region corresponding to the HOP promoter has not yet been cloned or demonstrated in vitro. There is no in vivo information to be found on the DNA sequence encoding the HOP promoter, and there is a paucity of data on the factors that control HOP expression in cancer. In this study, bioinformatic analysis of the putative HOP promoter was performed, in order to predict the regulatory DNA region responsible for controlling HOP expression and allow for an initial investigation into the factors that alter the activity of the HOP promoter in cancer cells.

Methods

Bioinformatic analysis

NCBI accession numbers for sequences are as follows: NT_033903.7 and NM_006819.2 for *Homo sapiens* and NT_082892.2 and NM_016737.2 for *Mus musculus*.

Pairwise global alignments were performed with BioEdit (Hall 1999) using default parameters. Transcription factor binding sites were predicted in silico using Cister (Wingender et al. 2000; Frith et al. 2001) using a hidden Markov statistical model, MATCH™ (BIOBASE—Biological Databases 2001) with a cut-off of 0.7, ALIBABA2.1 (Heinemeyer et al. 1998) with a cut-off of 80% matrix conservation and Promo (Messeguer et al. 2002; Farré et al. 2003) using a dissimilarity <1.0 as a cut-off. CpG islands were identified by MethPrimer (Li and Dahiya 2002), the criteria used being a frequency of observed CG dinucleotides/frequency of expected CG dinucleotides >0.6, GC % >50 and length >100.

Maintenance of cancer cell lines

Hs578T breast cancer cells were maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with GlutaMAX™-I, 10% (v/v) foetal calf serum (FCS), penicillin-streptomycin-amphotericin (PSA; 100 U/ml) and 0.3 U/ml insulin. HeLa cervical cancer cells and MEF-SV40-transformed mouse embryonic fibroblast cells were maintained in DMEM supplemented with GlutaMAX™-I, 10% (v/v) FCS and PSA (100 U/ml). MEF-CF1 mouse embryonic fibroblast cells were maintained in DMEM supplemented with GlutaMAX™-I, 10% (v/v) FCS, PSA (100 U/ml) and MEM non-essential amino acids (MEM NEAAs, 0.1 mM). HEK293T-transformed human embryonic kidney cells were maintained in DMEM supplemented with GlutaMAX™-I, 10% (v/v) FCS, PSA (100 U/ml), MEM NEAA (0.1 mM), 1% (v/v) sodium pyruvate and Geneticin (G418, 500 µg/mL). All mammalian cell lines were maintained in a humidified incubator at 37 °C with 9% CO₂.

Plasmid constructs

The 2-kb, 1.5-kb and 225-bp putative HOP promoter sequences were synthesised by GenScript (USA) and cloned into a pUC57 vector to produce pUC57-HOP-T2054, pUC57-HOP-1500 and pUC57-HOP-225, respectively. The promoterless vector containing the firefly luciferase coding region, pGL4.17 [*luc2/Neo*], was purchased from Promega (USA, cat. no. AE00109). The 2-kb, 1.5-kb and 225-bp putative HOP promoter sequences were cloned out of the pUC57 vector using the *NheI* and *HindIII* restriction sites and into pGL4.17 [*luc2/Neo*] upstream of the firefly luciferase coding region to produce pHOP-T2054, pHOP-1500 and pHOP-225. The −855 to +16 region of the HOP promoter was digested out of pHOP-1500 using *NheI* and *HindIII* restriction enzymes and cloned into pGL4.17 [*luc2/Neo*] to produce pHOP-T855. The −1200 to −1000, −1534 to −1000, −2011 to −1000 and −2500 to −1000 regions of the HOP promoter were PCR amplified using pHOP-T2054 or pHOP-1500 as

templates with primers containing *NheI* and *HindIII* restriction sites on the forward and reverse primers, respectively. The amplicons were purified and digested with *NheI* and *HindIII*, and the digested DNA fragments were gel extracted and ligated into pGL4.17 upstream of the firefly luciferase coding region to produce pHOP-T200, pHOP-T534, pHOP-T1011 and pHOP-T1500. All plasmids were confirmed by sequencing. pLV-eGFP was a gift from Pantelis Tsoulfas (Addgene plasmid no. 36083). The mEGFP-HRas, mEGFP-HRas G12V and mEGFP-HRas S17N plasmids were a gift from Karel Svoboda (Addgene plasmid nos. 18662, 18666 and 18665, respectively). pBabe puro C-Raf 22W was a gift from Channing Der (Addgene plasmid no. 12593). pcDNA3 flag p53 was a gift from Thomas Roberts (Addgene plasmid no. 10838). The expression of proteins from the relevant plasmids was confirmed by Western blot analysis as detailed in the supplementary information (Supplementary Fig. 1).

Promoter activity assays

MEF-SV40T, MEF-CF1, Hs578T, HeLa and HEK293T cell lines were transiently transfected with 500 ng HOP promoter plasmids or pGL4.17 [*luc2/Neo*] and the transfection control reporter plasmid encoding EGFP (pLV-eGFP), using XtremeGENE HP DNA Transfection Reagent (Roche, Switzerland) according to the manufacturer's instructions. The cells were incubated at 37 °C in a CO₂ incubator for 48 h before the promoter activity was determined by measuring the eGFP fluorescence and luciferase activity. The eGFP within the lysate was excited at 488 nm, and the emitted fluorescence was recorded at 509 nm, while luciferase activity was measured after addition of FLAR Buffer (200 mM tricine, 100 µM EDTA, 2.67 mM MgSO₄, 250 µM adenosine triphosphate [ATP], 250 µM luciferin, 17 mM dithiothreitol [DTT]) (Siebring-van Olst et al. 2012). The ratio of luminescence to fluorescence was calculated and defined as the relative luciferase activity. For each experiment, three separate transfections were done, with each biological replicate split into three technical replicates when reading luminescence and fluorescence. The cells were also transiently transfected, as described above, with one of the three RAS plasmids, RAF or p53, along with HOP promoter plasmids. Transfection and luciferase assays were performed as described before. Where relevant, cells were treated with 10 µM cyclic pifithrin-α hydrobromide (CPαH) for 4 or 20 h after a 48-h transfection period (Murphy et al. 2004; Damico et al. 2011).

Statistical analysis

All assays were performed as at least three independent biological replicates each with triplicate technical replicates. Statistical significance was performed using one-way ANOVA with Tukey's multiple comparison tests where a *p*

value below 0.001 was taken as statistically significant unless otherwise stated.

Results

The upstream region of the human HOP gene encodes a functional promoter in vitro

To identify any conserved regions upstream of the HOP gene, the murine and human sequences corresponding to the region of the HOP gene from −500 to +100 bp were aligned and compared using T-Coffee. No specific regional conservation was observed between the human and mouse sequences. A pairwise global alignment of this region by BioEdit (Hall 1999) gave human-mouse identity as 67% (data not shown). The sequences were next searched for specific elements that were known to signify the presence of a promoter (Liu et al. 2008; Anish et al. 2009). A putative TATA box (TATATATA) was identified in the murine sequence at position −102 from the transcriptional start site (TSS), but not in the human sequences (Fig. 1a). This sequence matched the TATA consensus sequence of TATAWAWR, where W represents A or T and R represents A or G. The human sequence had two and the murine sequence a single GC box (GGGCGG) and 6 repeats of CCAAT sequences (also known as stimulatory protein 1 [Sp1]) upstream of the TSS (Fig. 1a).

The putative human HOP promoter sequence identified above, including 2.5 kb upstream of the transcriptional start site, was analysed to identify potential transcription factor binding sites (TFBSs) in order to predict the regulatory DNA region responsible for controlling HOP expression and allow for an initial investigation into the factors that alter the rate of the transcription of HOP in cancer cells. Three prediction programs were utilised to investigate the −2500- to +1-bp region of the human HOP gene. Figure 1b highlights the *cis*-elements for various transcription factors (TFs) that are activated by oncogenic pathways found within the putative HOP promoter. Of interest were the putative *cis*-elements that were downstream effectors of the oncogene RAS, these being ETS-1, Elk-1, ER, NFκB, AP-2α, C/EBPβ and c-Myc. Furthermore, two putative *cis*-elements were observed for the tumour suppressor gene p53 (Fig. 1b). Additionally, 42 putative Sp1 (CCAAT *cis*-elements) as well as 11 TFII-D/I sites were found (data not shown) across the putative promoter region. A CpG island was predicted from −1108 to −287 bp. The lack of a TATA box in the human HOP sequence, as well as the presence of multiple CCAAT *cis*-elements, a GC box and a CpG island, suggested it is a TATA-less promoter. Furthermore, the presence of a TATA box in the murine system suggested the murine HOP may be more of a context- or

Fig. 1 Putative transcription factor binding sites within the human and murine HOP promoter. **a** Schematic diagram of a T-Coffee alignment of human and murine upstream sequences of the HOP genes. Sequences aligned from -500 to +100 with respect to the transcriptional start site (TSS, +1). The putative TATA box, GC boxes (GGGCGG) and CCAAT boxes (Sp1) are shown. **b** Schematic diagram of the 2.5-kb sequences

upstream of the HOP TSS that was identified as a putative promoter region. The distribution of putative transcription factor binding sites (TFBSs) of interest was shown correlating to their position along the promoter. Numbers indicate position relative to the TSS. The CpG island with GC content >50% is shown

tissue-specific promoter, while the human HOP promoter, in part due to the large CpG island, was less so (Sandelin et al. 2007).

To identify the putative HOP promoter in vitro, a series of luciferase reporter constructs spanning the region of -2500 to +16 bp of the human HOP gene was produced (Fig. 2). Luciferase reporter assays were performed 48 h after transfection of the reporter plasmids and transfection efficiency reference plasmid (pLV-eGFP) into two malignant cell lines (HeLa, Hs578T) and one SV40 immortalised cell line (HEK293T) (Fig. 2). Malignant cell lines were chosen because HOP has been found to be upregulated in human cancer cell lines (Kubota et al. 2010; Walsh et al. 2011). In all three cell lines tested, all HOP promoter constructs produced significant levels of promoter activity (except pHOP-T1500 in HEK293T cells). These data suggested that the human HOP gene sequence (from -2500 to +1) contained a functional promoter and illustrated that the HOP promoter activity within the immortalised HEK293T cell line was comparable to that within the malignant Hs578T cell line. However, pHOP-855 produced the highest levels of promoter activity, followed by pHOP-225 and pHOP-1500. The CCAAT regulatory region predicted in silico was conserved within the pHOP-855 truncation, from -750 to -150 bp (Fig. 1b). This suggested that the core promoter for the HOP gene was within 855 bp upstream of the TSS of HOP. This was further supported by the loss of promoter activity

when the first 1000 bp of the HOP promoter were absent from the HOP promoter constructs. The reduction in promoter activity from pHOP-855 to pHOP-1500 suggested that the 645-bp region absent from pHOP-855 contained *cis*-elements responsible for suppressing promoter activity. Interestingly, two putative p53 (a known tumour suppressor) *cis*-elements were observed at -1411 and -1175 bp of the HOP promoter (Fig. 1b). For further studies, pHOP-1500 was utilised as the full-length promoter and is denoted as pHOP-x henceforth. This truncation was chosen as it contained both the core promoter region as well as putative promoter suppressor sites, which were of interest for the purposes of this study.

The HOP promoter activity was subsequently compared in two murine cell lines, MEF-CF1 and MEF-SV40T (Fig. 3). MEF-CF1 cell lines were non-transformed MEFs, while MEF-SV40T cell lines were immortalised by SV40T. The reporter plasmid in the non-transformed murine cells produced no promoter activity, similar to the promoterless pGL4 plasmid, while the SV40-transformed MEFs produced a basal promoter activity 25-fold greater than that of the control plasmid (Fig. 3). These data suggested that the human HOP gene sequence (from -1500 to +1) contained a functional promoter that is active within the murine system but only under specific conditions. The discrepancy in the HOP promoter activity levels in normal and immortalised murine systems suggested that the SV40 transformation was responsible for the activation of the promoter.

a

HeLa

b

Hs578T

c

HEK293T

Fig. 2 The upstream region of the HOP gene encodes a functional promoter. The promoter activity was compared in HeLa (a) and Hs578T (b) cancer cell lines and the HEK293T (c) cell line. Luminescence was measured in RLU. The luciferase activity was determined by normalising the firefly luciferase activity against the fluorescence produced by the control reporter plasmid (pLV-eGFP). The results from the HOP promoter analysis were normalised against the results of the empty vector (pGL4) and the log RFU values plotted. The bars represent the standard error of the mean. One-way ANOVA and Tukey's multiple comparison tests were performed to determine statistical significance (* $P < 0.001$)

p53 overexpression affects the regulation of the HOP promoter

As SV40 transformation is associated with loss of p53 function (Gjoerup et al. 2001) and due to the presence of putative *cis*-elements within the HOP promoter (Fig. 1b), we decided to test the effect of the well-known tumour suppressor p53 on the HOP promoter. The p53 status in HeLa cells is wild type (Bamford et al. 2004), while Hs578T cells contain a missense mutant (V157F) which results in a p53 loss of function (Kovach et al. 1991). The HEK239T and MEF-SV40T cell lines have been immortalised by SV40 transformation and therefore have large amounts of inactive p53 (Gjoerup et al. 2001).

Cells were transfected with pcDNA3 flag p53, and the effect of the overexpression of p53 on the HOP promoter pHOP-x was observed. p53 overexpression had no significant effect on the empty vector control (pGL4) in any cell line. However, within HEK293T and Hs578T cell lines, p53 overexpression caused significant reductions in the activity of the full-length HOP promoter plasmids (Fig. 4). Within Hs578T cells, a 30% decrease was observed (Fig. 4), while in

HEK293T cells, p53 overexpression resulted in a 60% decrease in promoter activity (Fig. 4). In HeLa cells, p53 overexpression caused no significant increase in the activity. These data suggested that reintroducing functional p53 into cell lines containing inactive p53 (HEK293T and Hs578T) caused the suppression of the HOP promoter activity. Whereas overexpressing p53 in a cell line that already contains functional p53 (HeLa) had no significant effect on the promoter.

To confirm the effect of p53 on the HOP promoter, we next inhibited the activity of the wild-type p53 within HeLa cells using cyclic pifithrin- α hydrobromide (CP α H) (Fig. 5) (Murphy et al. 2004; Damico et al. 2011). A significant increase in the HOP promoter activity was observed after a 20-h incubation with CP α H. Thus, the inhibition of wild-type p53 in HeLa cells allowed for the activation of the HOP promoter.

Consequences of overexpression of the RAS oncoprotein on the HOP promoter activity

Having shown that the tumour suppressor p53 could inhibit HOP promoter activity, we next evaluated the effect of a well-known oncogene on the HOP promoter. Since many of the putative TFBSs identified by the *in silico* analysis of the HOP promoter were targets of RAS signalling pathways, it was of interest to determine whether RAS had an effect on the HOP promoter. To test this, cells were transfected with HRAS and c-RAF mutants to determine whether these would increase the activity of the HOP promoter. Cell lines were transfected with RAS plasmids [wild-type HRAS, constitutively active mutated HRAS (G12V) or a dominant negative mutated HRAS (S17N)] or an activated RAF mutant (c-RAF 22W) (McFall et al. 2001; Yasuda et al. 2006), and the effect on the HOP

Fig. 3 HOP promoter activity is minimal in untransformed murine cells. The promoter activity was compared in the murine MEF-CF1 and MEF-SV40T cell lines. Luminescence was measured in RLU. The luciferase activity was determined by normalising the firefly luciferase activity against the fluorescence produced by the control reporter plasmid (pLV-

eGFP). The HOP promoter activities were normalised against the results of the empty vector (pGL4). The bars represent the standard error of the mean. One-way ANOVA and Tukey's multiple comparison tests were performed to determine statistical significance (* $P < 0.0001$)

Fig. 4 Overexpression of p53 decreases the transcriptional activity of the HOP promoter. HeLa, Hs578T and HEK293T cells were co-transfected with p53, pLV-eGFP and pHOP-x. The luciferase activity of the full-length HOP promoter was measured after 48 h. The luciferase activity was determined by normalising the firefly luciferase activity against the

fluorescence produced by eGFP. The results were normalised against the results for the empty vector (pGL4). All bars represent the standard error. Unpaired *t* tests with two-tailed *p* values were performed to determine statistical significance (**P* < 0.001)

promoter activity was analysed (Fig. 6). The Hs578T cell line contains a mutated RAS gene (Q61L HRAS) which renders HRAS in a constitutively active state, whereas the HeLa and HEK293T cell lines have no known RAS mutation (Sepp-Lorenzino and Rosen 1998; Eckert et al. 2004).

Overexpressing HRAS in Hs578T, HEK293T or HeLa cells increased the activity of the HOP promoter significantly (Fig. 6a). HRAS caused a 3-fold increase in the HOP promoter

activity within the Hs578T cells when compared to the negative control (HRAS S17N), while HRAS G12V caused a 3.5-fold increase (Fig. 6a). A similar trend was observed within HeLa cells, whereby the overexpression of both HRAS and HRAS G12V induced a significant 2-fold increase in HOP promoter activity. In HEK293T cells, HRAS did not have a significant effect on the HOP promoter; however, HRAS G12V caused a 1.5-fold increase in the HOP promoter

Fig. 5 Inhibition of wild-type p53 in HeLa cells increases the transcriptional activity of the HOP promoter. HeLa cells were co-transfected with pGL4 or pHOP-x and pLV-EGFP for 48 h. The luciferase activity of pHOP-x was measured after 4 or 20 h post-treatment with 10 μ M cyclic pifithrin- α hydrobromide (CP α H) or the vehicle control (DMSO). The luciferase activity was determined by

normalising the firefly luciferase activity against the fluorescence produced by the eGFP gene. The results were normalised against the results for pHOP-x treated with DMSO. All bars represent the standard error. One-way ANOVA and Tukey's multiple comparison tests were performed to determine statistical significance (**P* < 0.001)

Fig. 6 Constitutively active RAS and RAF increase HOP promoter activity. HeLa, Hs578T and HEK293T cell lines were co-transfected with the HRAS mutants (a) or c-RAF 22W (b), pHOP-x and pLV-EGFP. The relative luciferase activity was determined after 48 h by normalising the firefly luciferase activity against the fluorescence produced by the eGFP gene on each of the RAS plasmids or the pLV-EGFP plasmid. The results were normalised against the results for HRAS S17N or pHOP-x. All bars represent the standard error of the mean. One-way ANOVA and Tukey's multiple comparison tests were performed to determine statistical significance (* $P < 0.001$)

activity. Overexpressing the activated c-RAF 22W mutant caused no significant effect on the HOP promoter activity within Hs578T and HEK293T cells, whereas a 2-fold significant increase in the HOP promoter activity was observed within HeLa cells (Fig. 6b).

Overexpression of p53 inhibits the activation of the HOP promoter by RAS

To determine whether the effect of HRAS on the HOP promoter activity in Hs578T and HEK293T cells was due to the lack of active p53 in these cell lines, both HRAS and p53 were

overexpressed and the effect on the HOP promoter activity analysed (Fig. 7). The combination of HRAS and p53 overexpression reduced the HOP promoter activity compared to HRAS alone within both HEK293T and Hs578T cells, but only significantly within HEK293T cells (Fig. 7). A similar trend was observed with the overexpression of HRAS G12V and p53 in HEK293T cells, whereby p53 significantly decreased the HOP promoter activity compared to expression of HRAS alone. The HOP promoter activity was also reduced 6-fold when HRAS G12V and p53 were overexpressed in Hs578T cells. In both cell lines, a significant increase in HOP promoter activity was observed when both HRAS S17N and p53 were overexpressed (Fig. 7). Thus, in both cell lines, the introduction of active p53 was sufficient to significantly reduce the effect of wild-type HRAS and HRAS G12V on the HOP promoter activity but not the effect of HRAS S17N.

Discussion

The putative HOP promoter region was identified *in silico* and *in vitro*. This is the first report to clone the HOP promoter and evaluate its regulation *in vitro*. We showed that HOP promoter activity was higher in cancer and SV40-transformed cell lines and was negatively regulated by p53. In contrast, the oncogene HRAS was able to increase HOP promoter activity, although overexpression of wild type p53 was able to reduce HOP promoter activity in response to HRAS. These findings suggest for the first time that HOP expression in cancer may be regulated both by RAS activation and p53 inhibition of the HOP promoter (Fig. 8).

The luciferase assays indicated that the predicted promoter region of HOP (−2500 to +16) was able to drive transcription of the luciferase coding region *in vitro*. This suggested that the −2500- to +16-bp region of the human HOP gene could constitute part of or the entire HOP promoter. Since the truncated HOP promoter construct gave significantly higher promoter activity levels than the full-length promoter construct, we suggest that the −2500- to −900-bp region of the HOP promoter contained suppressor elements or a combination of transcription factors that downregulated the transcriptional activity of the HOP promoter.

Cell lines with loss-of-function mutant or inactivated p53 (due to SV40 transformation) had a significantly higher basal HOP promoter activity than cell lines with wild-type p53. The HEK293T and MEF-SV40T cell lines have been immortalised by SV40 transformation, which inhibits the tumour suppressor activity of p53 (as well as tumour suppressor protein pRB and protein phosphatase 2A, PP2A) and thereby disrupts gene regulation by p53 (Jha et al. 1998; Gjoerup et al. 2001; Ahuja et al. 2005). SV40-transformed cells therefore have large amounts of inactive p53. This suggested that the

Fig. 7 Overexpression of p53 inhibits the activation of the HOP promoter by active RAS. Hs578T and HEK293T cells were co-transfected with the HRAS mutants, p53, pHOP-x and pLV-EGFP. The luciferase activity of pHOP-x was measured after 48 h. The luciferase activity was determined by normalising the firefly luciferase activity

against the fluorescence produced by the eGFP gene on each of the RAS plasmids. The results were normalised against the results for HRAS S17N. All bars represent the standard error. One-way ANOVA and Tukey's multiple comparison tests were performed to determine statistical significance (* $P < 0.001$)

deregulation of p53 may have had a downstream effect on the HOP promoter. Introduction of active p53 into cell lines containing inactivated forms of p53 was sufficient to downregulate the HOP promoter. Alternatively, inhibition of wild-type p53 activated the HOP promoter suggesting that p53 has a role in the regulation of HOP transcription. In the case of HeLa cells, overexpressing wild-type p53 had no significant effect on the HOP promoter.

Furthermore, in these cell lines, HRAS caused an upregulation in the activity of the HOP promoter. This effect was undermined when both HRAS and p53 were overexpressed within these cells. This suggested that in a system containing inactive p53, HRAS was able to regulate the HOP promoter.

However, with overexpression of wild-type p53, the HRAS-induced activation of the HOP promoter was inhibited. No significant effect was observed upon the activity of the HOP promoter in Hs578T and HEK293T cells in the presence of overexpressed constitutively active c-RAF, suggesting that in these cells, activation of the HOP promoter via HRAS was not via the MEK/ERK pathway. However, this was not the case in HeLa cells. This might suggest that c-RAF activation was already saturated in Hs578T and HEK293T cells but not within HeLa cells.

One of the most frequent events in cancer is the combined inactivation of p53 and the mutational activation of RAS oncogenes (Bos 1989; Levine 1997). For RAS transformation of

Fig. 8 Proposed model of HOP promoter regulation. p53 putatively inhibits the RAS-mediated activation of the HOP promoter

cells to occur, p53 has to be inactivated so as to prevent p53 from inducing cell arrest or apoptosis. Buganim et al. (2010) have identified a cluster of genes that are downstream targets of RAS, the upregulated expression of which is considered a cancer-related gene signature (CGS) due to the correlation of the CGS with malignancy. These genes are upregulated in conditions whereby wild-type p53 is inactivated and the HRAS G12V mutation is expressed (Buganim et al. 2010; Solomon et al. 2012). Two examples of which are NF κ B and RhoA, both of which are regulated in a CGS-like manner (Xia and Land 2007; Buganim et al. 2010). Since HOP promoter activity is similarly upregulated by the combination of oncogenic H-RAS and inactivated p53, this suggests that HOP expression may be part of the CGS and a contributor to the biology of these cancers. This would correlate with the reports showing that HOP protein levels are elevated in cancer cells (Walsh et al. 2009; Kubota et al. 2010; Wang et al. 2010a; Chao et al. 2013; Willmer et al. 2013; Yuan et al. 2014).

One of the mediators of p53 tumour suppressor signalling is the B-cell translocation gene 2 (BTG2), an early growth response gene. Inactivation of p53 was found to correspond with a reduction in the expression of BTG2; however, the suppression of BTG2 did not cause a consequent reduction in p53 (Boiko et al. 2006). The downregulation of BTG2 was sufficient to allow for the RAS-mediated transformation of murine and human fibroblasts and did not require the inactivation of p53. BTG2 is known to repress the activity of HRAS G12V by binding the protein and reducing its GTP loading state, and the mechanism by which this occurs is yet unknown. This in turn causes a decrease in the expression of CGS (Buganim et al. 2010; Solomon et al. 2012; Mao et al. 2015). This repression of RAS by BTG2 could explain the downregulating effect of wild-type p53 on the HOP promoter in cells containing active RAS, as well as the downregulation effect of overexpression of p53 on the HOP promoter in cells containing inactive p53. Irrespective of the mechanism, taken together, our data demonstrate that HOP overexpression in cancer is likely due to regulation of the promoter by oncogenic factors. As a consequence, elevated levels of HOP may have prognostic importance in a clinical setting or might suggest HOP as a putative drug target for cancer.

Acknowledgements This research was supported by funding from the South African Research Chairs Initiative of the Department of Science and Technology (DST) and the National Research Foundation of South Africa (NRF) (Grant No. 98566), the Cancer Association of South Africa (CANSA), Medical Research Council South Africa (MRC-SA) with funds from the National Treasury under its Economic Competitiveness and Support Package and Rhodes University. SAM was the recipient of a postgraduate bursary from the NRF and German Academic Exchange Service (DAAD). The views expressed are those of the authors and should not be attributed to the DST, NRF, CANSA, MRC-SA or Rhodes University.

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