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2

3 **The prognostic significance of specific *HOX* gene**  
4 **expression patterns in ovarian cancer**

5

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24

## 25 **Novelty and impact statement**

26 This is the first comprehensive analysis comparing *HOX* gene expression in ovarian cancer  
27 to normal ovarian and fallopian tube tissue. *HOX* expression has been analysed along with  
28 the clinico-pathological features of each patient, identifying the significance of specific *HOX*  
29 genes relating to poor overall survival. This is the first study to analyse changes in *HOX*  
30 expression with the development of platinum resistance, finding increased expression of  
31 specific genes after resistance occurs, identifying them as potential therapeutic targets.

## 32 **Abstract**

33 *HOX* genes are vital for all aspects of mammalian growth and differentiation, and their  
34 dysregulated expression is related to ovarian carcinogenesis. The aim of the current study  
35 was to establish the prognostic value of *HOX* dysregulation as well as its role in platinum  
36 resistance. The potential to target *HOX* proteins through the *HOX/PBX* interaction was also  
37 explored in the context of platinum resistance. *HOX* gene expression was determined in  
38 ovarian cancer cell lines and primary EOCs by QPCR, and compared to expression in  
39 normal ovarian epithelium and fallopian tube tissue samples. Statistical analysis included  
40 one-way ANOVA and t-tests, using statistical software R and GraphPad. The analysis  
41 identified 36 of the 39 *HOX* genes as being overexpressed in high grade serous EOC  
42 compared to normal tissue. We detected a molecular *HOX* gene-signature that predicted

43 poor outcome. Overexpression of *HOXB4* and *HOXB9* was identified in high grade serous  
44 cell lines after platinum resistance developed. Targeting the HOX/PBX dimer with the HXR9  
45 peptide enhanced the cytotoxicity of cisplatin in platinum-resistant ovarian cancer. In  
46 conclusion, this study has shown the *HOX* genes are highly dysregulated in ovarian cancer  
47 with high expression of *HOXA13*, *B6*, *C13*, *D1* and *D13* being predictive of poor clinical  
48 outcome. Targeting the HOX/PBX dimer in platinum-resistant cancer represents a  
49 potentially new therapeutic option that should be further developed and tested in clinical  
50 trials.

51 **Keywords: Ovarian cancer, *HOX* genes, survival, prognosis, targeted treatment**

52

### 53 **Introduction**

54 Ovarian cancer is the 5<sup>th</sup> leading cause of cancer death in women in the western world and it  
55 is estimated there were 22,280 new cases and 15,500 deaths due to the disease in the US  
56 in 2012 (1). It is the most lethal of the gynaecological malignancies largely due to late  
57 diagnosis. Standard treatment involves debulking surgery followed by a combination of  
58 taxane and platinum-based therapy. Initially most women respond to platinum-based  
59 therapy, but the majority suffer disease recurrence due to drug resistance. It is therefore  
60 essential to introduce new therapeutic approaches to improve treatment at diagnosis and/or  
61 provide an effective second line treatment.

62 There are different types of ovarian cancer classified by the cell type they originate from. The  
63 most common form, accounting for more than 90% of ovarian cancers, is epithelial ovarian  
64 cancer (EOC), and the high grade serous (HGS) subtype accounts for approximately 80% of  
65 EOC cases.

66 The epithelial ovarian tumours undergo Müllerian differentiation, which suggests that  
67 differentiation-regulatory factors may contribute to their progression. This mechanism has

68 been shown to involve homeobox (*HOX*) genes (2, 3) which play important roles in tissue  
69 differentiation during embryonic development. The *HOX* genes constitute a family of  
70 transcription factors, and in mammals 39 *HOX* genes have been identified. They are  
71 organised into 4 paralogous clusters (A, B, C and D) located on 4 different chromosomes.  
72 During development of the female reproductive system four *HOX* genes, *HOXA9*, *HOXA10*,  
73 *HOXA11*, and *HOXA13* are expressed uniformly along the Müllerian duct axis. *HOXA9*  
74 becomes expressed in the fallopian tubes, *HOXA10* is expressed in the developing uterus,  
75 *HOXA11* in the lower uterine segment and cervix and *HOXA13* in the upper vagina (4). It is  
76 thought that inappropriate expression of these genes is an early step in epithelial ovarian  
77 neoplasia as they induce aberrant epithelial differentiation. Studies which have analysed  
78 *HOX* gene expression in ovarian cancer cell lines and a small number of tumours have  
79 found dysregulated expression of certain *HOX* genes compared to normal tissue (5).  
80 Numerous studies have also demonstrated dysregulated *HOX* gene expression in other  
81 cancers such as lung, prostate, breast, colon and bladder cancer (6-9). The recent genomic  
82 analysis of HGS ovarian cancer (HGS-OvCa) by the Cancer Genome Atlas (TCGA)  
83 researchers found a number of somatic copy number alterations with three members of the  
84 *HOXB* family, *HOXB2*, *B5* and *B8* among the focally amplified regions. The group divided  
85 HGS ovarian cancer into four expression subtypes 'immunoreactive', 'differentiated',  
86 'proliferative' and 'mesenchymal' on the basis of gene expression, and high expression of  
87 *HOX* genes was a characteristic of the mesenchymal subtype (10). High expression of *HOX*  
88 genes makes them a potential target for therapeutic intervention. One possible method is the  
89 use of a peptide that disrupts the interaction between *HOX* proteins and co-factor PBX.  
90 HXR9 is a small peptide designed to mimic the hexapeptide sequence found in *HOX*  
91 proteins of paralogue groups 1- 9 (11), therefore acting as a specific competitive inhibitor of  
92 the *HOX*/PBX interaction preventing the subsequent binding of the *HOX*/PBX dimer to target  
93 DNA sequences. This in effect inhibits the transcription of target genes. Previous studies  
94 have shown that HXR9 is capable of blocking this interaction *in vitro* and *in vivo* (11-13) and  
95 antagonising the *HOX*/PBX interaction induces apoptosis (11-15).

96 The role of aberrant *HOX* dysregulation in EOC is not yet understood. The aim of the current  
97 study was to establish the prognostic value of *HOX* dysregulation as well as its role in  
98 developing platinum resistance. The potential to target *HOX* function through the HOX/PBX  
99 interaction was also explored in the context of platinum resistance (13).

100

## 101 **Material and methods**

### 102 **Cell lines and reagents**

103 The human ovarian adenocarcinoma-derived HGS cell line SKOV-3, clear cell carcinoma  
104 derived cell line TOV-21G and the endometrioid carcinoma derived cell line TOV-112D were  
105 obtained from the American Type Culture Collection (LGC Promochem, Teddington, UK).  
106 The SKOV-3 cell line has since been reclassified as an endometrioid subtype due to the lack  
107 of a p-53 mutation and the presence of the endometrioid associated ARID1A mutation (16).  
108 Therefore, the SKOV-3 cell line will be considered as an endometrioid cell line in this paper.  
109 SKOV-3 cells were cultured in McCoys's 5A modified medium (Sigma, Poole, UK)  
110 supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Invitrogen Ltd,  
111 Paisley, UK). TOV-112D and TOV-21G cells were cultured in 1:1 mixture of MCDB 105  
112 medium (Sigma) supplemented with 1.5 g/L sodium bicarbonate and Medium 199  
113 (Invitrogen), with 15% heat-inactivated FBS (Invitrogen). The epithelial HGS carcinoma cell  
114 line derived from peritoneal ascites COV-318 and the paired HGS ovarian carcinoma cell  
115 lines PEO1, PEO4, PEO14 and PEO23 were obtained from the HPA Cell Culture Collection  
116 (HPA, Salisbury, UK) (17). These cell lines were authenticated by either STR profiling (DDC  
117 Medical, OH, USA) or LCG Standards (Middlesex, UK). COV-318 cells were cultured in  
118 DMEM medium (Sigma) with 10% heat-inactivated FBS and 2mM glutamine (Sigma). PEO  
119 cell lines were maintained in RPMI1640 media with 10% heat-inactivated FBS. All media  
120 was supplemented with 1% penicillin (10,000 U/ml) / streptomycin (10 µg/ml) (Sigma). Cell

121 cultures were maintained at 37°C in a humidified, 5% CO<sub>2</sub> incubator. Cisplatin sensitivity of  
122 cell lines was verified by MTS assay after 72 hour cisplatin treatment.

### 123 **RNA isolation, cDNA production and quantitative Real Time PCR (qRT-PCR)**

124 Two total RNA samples from normal human ovarian tissue were purchased from OriGene  
125 (Cambridge, UK). All cell lines were grown in normal growth medium in 6-well plates at a  
126 density range to ensure overnight growth resulted in until 80% confluency before RNA  
127 extraction took place. RNA was isolated from cell lines using the RNeasy<sup>®</sup> Plus Mini Kit  
128 (Qiagen Ltd, Crawley, UK) following the manufacturer's instructions. This included the use of  
129 gDNA eliminator columns to remove genomic DNA contamination. Total RNA extracted from  
130 20–30mg ovarian tumour or ovarian normal tissue stored in RNA<sup>later</sup><sup>®</sup> (Sigma) was isolated  
131 using the gentleMACS dissociator followed by RNA extraction using the RNeasy<sup>®</sup> Plus Mini  
132 Kit (Qiagen). RNA purity was verified by the 260nm/280nm absorbance ratio, measured  
133 using the Nanodrop (Thermo Fisher, MA). Ratios of 1.9-2.0 were considered 'pure' RNA as  
134 described by manufacture. cDNA was synthesised from RNA using the Cloned AMV First  
135 Strand Synthesis Kit (Invitrogen) following the manufacturer's protocol. qRT-PCR was  
136 performed using the Stratagene MX3005P Real Time PCR machine (Agilent Technologies  
137 UK Ltd, Stockport, UK) and SYBR<sup>®</sup> Green JumpStart<sup>™</sup> Taq ReadyMix<sup>™</sup> (Sigma).  
138 Oligonucleotide primers were designed to facilitate the unique amplification of  $\beta$ -actin and  
139 each *HOX* gene. Melt curves and gels were run originally to validate the primers and check  
140 for single bands of the correct product size. Relative expression was calculated using the  
141 Livak comparative Ct method (18).

### 142 **Synthesis of HXR9 and CXR9 peptides**

143 HXR9 is an 18 amino acid peptide consisting of the previously identified hexapeptide  
144 sequence that can bind to PBX and nine C-terminal arginine residues (R9) that  
145 facilitate cell entry (11). The N-terminal and C-terminal amino bonds are in the D-  
146 isomer conformation, which has previously been shown to extend the half-life of the  
147 peptide to 12 h in human serum (11). CXR9 is a control peptide that lacks a

148 functional hexapeptide sequence but still includes the R9 sequence. All peptides  
149 were synthesized using conventional column based chemistry and purified to at least  
150 80 % (Biosynthesis Inc., USA).

151

## 152 **Analysis of cell death and apoptosis**

153 Cells were plated in flat bottomed 96-well plates and incubated for 24 hours until  
154 70% confluent. Cells were treated with HXR9 or CXR9 at a range of dilutions for 2  
155 hours. Cell viability was measured via the MTS assay (Promega, Southampton, UK)  
156 according to the manufacturer's instructions. To detect morphological changes  
157 consistent with apoptosis, cells were plated in 24-well plates and incubated overnight  
158 to reach 70% confluency. Cells were then treated for 2 hours with 2% FBS media,  
159 the control peptide CXR9 or the active peptide HXR9 at the IC<sub>50</sub> (Concentration of  
160 drug needed to induce 50% cell death, as determined by the MTS assay) and double  
161 the IC<sub>50</sub>. Cells were then harvested by incubating in trypsin-EDTA (Sigma) at 37°C  
162 until detached and dissociated. Apoptotic cells were identified using a Beckman  
163 Coulter Epics XL flow cytometer (argon laser, excitation wavelength 488 nm, FL-2  
164 and FL-4 detectors) and the Annexin V-PE apoptosis detection kit (BD Pharmingen)  
165 as described by the manufacturer's protocol. Caspase-3 activity was measured using  
166 the EnzCheck Caspase-3 Assay Kit (Molecular Probes), using the protocol defined  
167 by the manufacturer.

168

## 169 **Calculating synergy**

170 To measure synergistic interaction between HXR9 and cisplatin, cells were plated in  
171 a 96-well plate and treated with either HXR9 or cisplatin alone or in combination at  
172 concentrations of the drugs IC<sub>50</sub> and  $\pm$  2-, 4- and 8- fold this concentration. Cell  
173 viability was then measured by the MTS assay (as described above) and the  
174 presence of synergy was analysed based on the Chou-Talalay method using  
175 CalcuSyn version 2.0 software (Biosoft, Stapleford, UK) (19). The interaction  
176 between HXR9 and cisplatin was quantified by determining the combination index  
177 (CI). Using this method, CI < 1 indicates synergism, CI = 1 indicates an additive  
178 effect antagonism (CI > 1) between drugs.

179

180 **Clinical data**

181 A cohort of 99 patients with corresponding age, stage, time to progression (TTP), overall  
182 survival (OS), histology and chemotherapy information was used in the analysis of primary  
183 ovarian tumours (Supplementary Table 1). Fresh biopsy tissue specimens were obtained  
184 during surgery from human subjects with ovarian cancer or other gynaecological conditions  
185 from the Royal Surrey County Hospital, Guildford following informed consent and ethical  
186 approval. Samples were immediately stored in RNA<sup>later</sup>® and stored at -20°C for later use.  
187 Each biopsy was confirmed by a pathologist to be either cancerous of ovarian origin or  
188 normal ovarian tissue. OS and TTP were measured from the date of diagnosis. The duration  
189 of OS was measured up to the date of death or, for patients still alive the 1st October 2012,  
190 when statistical analysis was performed. The duration of TTP was the minimum amount of  
191 time until clinical progression, or death. Only cases where causes of death were due to  
192 disease were used to calculate OS. *HOX* gene expression was obtained by qRT-PCR and  
193 values were normalised to housekeeping gene  $\beta$ -actin. All sample and data collection  
194 received an ethical approval by the institutional ethics committee (MREC-09/H1103/50).

195 **Mouse *in vivo* study**

196 All experiments were conducted in accordance with the United Kingdom Co-ordinating  
197 Committee on Cancer Research (UKCCCR) guidelines for the Welfare of Animals in  
198 Experimental Neoplasia (20) and were approved by the University of Surrey Ethics  
199 Committee. The mice were kept in positive pressure isolators in 12 hour light/dark cycles  
200 and food and water were available ad libitum. Six-8 week old female balb/C NUDE mice  
201 (Charles River, Kent, UK) were inoculated subcutaneously with a suspension 100 $\mu$ L Hanks  
202 media (Sigma) containing 10<sup>6</sup> SKOV-3 cells in 50% matrigel (BD Bioscience). Once  
203 tumours reached a volume of approximately 100mm<sup>3</sup>, mice were randomised into 4  
204 treatment groups, each containing 10 mice: PBS alone, Cisplatin alone, HXR9 alone,  
205 Cisplatin and HXR9 in combination. Mice in the HXR9 group received an initial dose of

206 100mg/kg HXR9 intratumorally (IT), with subsequent dosing of 10 mg/kg twice weekly. The  
207 cisplatin treatment group received a weekly dose of 3mg/kg via intraperitoneal injection (IP).  
208 PBS was used as a control. Drug concentrations were used based on previous experiments  
209 (13). The mice were monitored carefully for signs of distress, including behavioural changes  
210 and weight loss.

## 211 **Statistical analysis**

212 All data analysis and manipulation of primary ovarian tumours were performed using R (an  
213 integrated set of software tools for data manipulation, calculation and graphical display).

214 Four test statistics were used to evaluate the change of gene expression. For variables with  
215 two groups (i.e. Age, OS and chemotherapy) the t-test was used for parametric analysis and  
216 the Mann-Whitney test was used as a non-parametric analysis. For variables with three or  
217 more groups (i.e. TTP and Stage) the one-way ANOVA was used for parametric analysis  
218 and the Kruskal-Wallis was used as a non-parametric analysis. Differential expression and  
219 interactions based on ANOVA. The Benjamini and Hochberg and the Bonferroni correction  
220 was applied to cell line data and ovarian tumour data, respectively, to account for multiple  
221 testing. Principle component analysis (PCA) was performed and the first two principle  
222 components are plotted. The heatmaps include row Z-score transformation (genes), and are  
223 plotted in red–blue colour scale with red indicating high expression and blue indicating low  
224 expression. Analysis of OS was calculated using the Kaplan-Meier method using GraphPad  
225 PRISM Version 5.0 (GraphPad Software). Hazard ratio (HR) and confidence intervals (CI)  
226 were calculated using the Log rank model.

227

228

229

230

231 **Results**

232 ***HOX* gene expression in ovarian cancer cell lines and normal ovarian and fallopian**  
233 **tube tissue**

234 In order to evaluate the changes in *HOX* gene expression in EOC we compared the relative  
235 expression of all 39 *HOX* genes in normal ovarian and fallopian tube tissue to a number of  
236 ovarian cancer cell lines.

237 The *HOX* expression profile was analysed in a panel of 5 HGS ovarian cancer cell lines, 2  
238 endometrioid cell lines and 1 clear cell carcinoma cell line and compared with 10 normal  
239 ovarian and 3 fallopian tube tissue samples. A highly dysregulated pattern of *HOX* gene  
240 expression was found in the EOC cell lines whereas normal tissue showed very little or no  
241 *HOX* gene expression (Figure 1).

242 The HGS cell lines showed marked dysregulation but this varied significantly across the  
243 panel. The COV-318 (HGS) cell line showed two *HOX* genes, with *HOXA9*, being  
244 significantly upregulated, whilst the PEO14 (HGS) cell line had 23 *HOX* genes that were  
245 significantly upregulated when compared to normal tissue.

246

247 ***HOX* expression in platinum sensitive and resistant ovarian cancer cell lines**

248 To evaluate differences in *HOX* expression between platinum sensitive and resistant EOC, 2  
249 paired HGS cancer cell lines derived from patients with platinum sensitive and resistant  
250 disease were analysed. Each pair was acquired from separate patients at the time when the  
251 tumour was deemed clinically sensitive to platinum and at a later time-point after developing  
252 platinum resistance. PEO1 and PEO14 - platinum sensitive cell lines, were compared with  
253 PEO4 and PEO23, platinum resistant cell lines, respectively (21). We found significant  
254 differences in the *HOX* expression profile in platinum resistant and platinum sensitive cell  
255 lines. The PEO4 (platinum resistant cell line) showed a significant increase of *HOXB3* and  
256 *HOXB4* gene expression compared to its paired sensitive cell line, PEO1. PEO23 (platinum-

257 resistant) also has a relatively higher expression of *HOXB4* when compared to its platinum-  
258 sensitive counterpart-PEO14, and in addition, elevated expression of *HOXB9*. Cell line gene  
259 expression data was pooled according to platinum sensitivity status and the resistant cell  
260 lines showed an overall higher *HOX* expression compared to normal and sensitive cell lines  
261 (Figure 2).

### 262 ***HOX* expression in primary EOC**

263 To comprehensively evaluate *HOX* gene expression profiles in clinically relevant HGS EOC  
264 we analysed tumours from a cohort of 73 HGS ovarian cancer patients and compared it to  
265 10 normal ovarian and 3 fallopian tube tissue samples (patients' characteristics are  
266 summarized in Table 1). HGS ovarian tumours exhibited a significant upregulation in the  
267 expression of 36 of the 39 *HOX* genes when compared to their expression in normal tissue  
268 samples (Figure 3). The strongly overexpressed genes included *HOXA9* ( $p = 1.86 \times 10^{-8}$ ),  
269 previously reported to be related to the HGS histotype (3), however, *HOXA3* was expressed  
270 to a far higher level, ( $p = 9.55 \times 10^{-10}$ ).

271 There were significant differences in *HOX* expression profiles between the HGS and  
272 endometrioid histological subtypes with up-regulation of *HOXA7*, *A9*, *A10*, *A13*, *B1*, *B4*, *B5*,  
273 *B13*, *C9*, *C13*, *D9* and *D10* in the endometrioid samples. *HOXB2* was the only gene to show  
274 a significant difference between HGS and clear cell carcinomas, although this might reflect  
275 the small sample size.

### 276 **A 5-*HOX* gene signature predicts poor OS**

277 The *HOX* expression profile in HGS EOC was subsequently correlated with clinical  
278 characteristics such as age, stage, TTP and OS.

279 The *HOX* expression profile in EOC also correlated with OS. We found that 5 *HOX* genes:  
280 *HOXA13*, *B6*, *C13*, *D1* and *D13* were expressed significantly more strongly in the tumours of  
281 patients with poor survival with higher expression of these genes found in all deceased

282 patients. Each of these genes were individually analysed by the Kaplan-Meier method and  
283 the result from the analysis are summarised in Table 1.

#### 284 **Targeting the HOX/PBX dimer in platinum-resistant EOC**

285 The aberrant *HOX* expression found in EOC makes them a potential therapeutic target. As  
286 the function of *HOX* genes is partly based on the binding of HOX proteins to the PBX and  
287 MEIS co-factors, targeting these co-factors could impair the oncogenic potential of HOX.  
288 PBX and MEIS proteins are present in both the nucleus and cytoplasm in ovarian  
289 carcinomas, however only MEIS is expressed in normal ovarian epithelia (22). These co-  
290 factors are important for ovarian carcinogenesis, most likely through potentiating the function  
291 of HOX proteins. A peptide called HXR9 has been designed to target the interaction between  
292 HOX proteins (members of paralogue groups 1-9) and PBX (11). This drug has been shown  
293 previously to induce apoptosis in cancer cells with highly dysregulated *HOX* expression  
294 profiles (11, 12, 14, 15), including the ovarian cancer cell line SKOV-3 (13). SKOV-3 is  
295 platinum-resistant, although its origin has recently been questioned (16).

296 In view of the gross *HOX* dysregulation pattern seen in platinum-resistant tumours we have  
297 used HXR9 alone and in combination with cisplatin to evaluate its efficacy in this setting.  
298 HXR9 and its control peptide –CXR9 (which has an identical polyarginine cell penetrating  
299 sequence to HXR9 but has a single alanine substitution in its hexapeptide sequence that  
300 renders inactive) have been described previously (11). All cell lines treated with HXR9  
301 demonstrated an increase in *cFOS* expression, which is thought to be at least partly  
302 responsible for HXR9-induced cell death (data not shown) (11). When analysed with flow  
303 cytometry for Annexin-V-PE there was a significant increase in the number of cells in late  
304 apoptosis after HXR9 treatment compared to untreated cells (Figure 4a, b). Previous  
305 publications have also demonstrated the apoptosis inducing capacity of HXR9 in ovarian  
306 cancer cell lines showing PARP cleavage and caspase-3 activity in treated cancer cells (11).  
307 The *in vitro* experiments showed that HGS cell lines were all sensitive to HXR9 treatment but

308 not to CXR9 and when combined with cisplatin there was synergy between HXR9 and  
309 cisplatin as shown in Supplementary Table 2. There was also enhanced cell killing *in vivo*  
310 using a combination of HXR9 and cisplatin over each drug used alone when treating mice  
311 bearing SKOV-3 tumours (Figure 5). Despite a good synergy effect seen *in vitro*, the effect *in*  
312 *vivo* was not as powerful and the combination of HXR9 and cisplatin was only marginally  
313 more active than HXR9 alone. This however may be cell line dependent. Combined HXR9  
314 and cisplatin provided a survival advantage, with a hazard ratio of 1.98 (95% CI, -0.88–6.58;  
315  $p = 0.098$ ) determined by the Log-rank model.

## 316 **Discussion**

317 This study confirms that *HOX* genes are highly dysregulated in ovarian cancer and that  
318 targeting the HOX/PBX interaction in platinum resistant tumours is of therapeutic value. Little  
319 to no *HOX* expression was found in normal ovarian tissue, whereas increased expression of  
320 certain groups of *HOX* genes was found in the majority of ovarian cancers.

321 The HGS carcinoma subtype shows the highest degree of heterogeneity in *HOX* expression  
322 for both cell lines and primary tumours, whereas endometrioid subtypes show a very distinct  
323 *HOX* expression profile. The HGS histological subtype is known to have a very  
324 heterogeneous nature, exhibiting a wide range of underlying genetic alterations, which may  
325 explain this variation. However, the functional redundancy between *HOX* genes may mean  
326 the net effect of *HOX* overexpression is similar even in cells expressing different sets of *HOX*  
327 genes (23).

328 Previous studies have shown that the over-expression of specific *HOX* genes determines the  
329 histological subtype, with *HOXA9* being overexpressed in HGS subtypes, *HOXA10* in  
330 endometrioid and *HOXA11* in mucinous (3). In this study we found that *HOXA9* is  
331 overexpressed in only 3 of the 8 HGS cell lines, but is also expressed in the clear cell and  
332 endometrioid cell lines. With regards to the primary tumours, *HOXA9* is significantly  
333 overexpressed in the HGS samples; however *HOXA10* and *HOXA11* are also expressed at

334 high levels in HGS tumours, which has not been previously reported. The endometrioid cell  
335 lines show an overall higher level of *HOX* expression than the HGS cell lines, including  
336 *HOXA9* and *HOXA10*.

337 *HOXA7* has been previously reported to play a role in the differentiation of ovarian surface  
338 epithelia (OSE) into EOC (24). We found that *HOXA7* is overexpressed in the HGS cancers  
339 as well as in the endometrioid carcinomas compared to normal ovarian tissue. In addition,  
340 *HOXA13* is overexpressed in the endometrioid tumours. This suggests that the *HOXA* genes  
341 play a role in the determination of histological subtypes, but the differences in expression are  
342 not as clear as previously suggested by Cheng *et al.* The high expression of *HOXA10* in the  
343 endometrioid cell lines and primary tumours does support a role for this gene in the  
344 differentiation of endometrioid tumours; however the high level of heterogeneity in cancer  
345 calls for caution in the interpretation of the results as the level of gene expression may differ  
346 in individual tumour samples.

347 Although the function of the *HOX* genes in cancer remains unclear, there have been reports  
348 that they act as tumour suppressor genes or oncogenes. In ovarian cancer both *HOXB7* and  
349 *B13* expression has been linked to the invasive characteristics of ovarian cancer cells (25),  
350 and *HOXB7* has been shown to be a regulator of bFGF- a potent mitogenic and angiogenic  
351 factor (26) and involved in double strand break repair (27), whereas *HOXB13* promotes cell  
352 proliferation (28).

353 We found that 9 out of the 10 *HOXB* genes were upregulated, the most significant being  
354 *HOXB4*, *B5*, *B7* and *B13*. *HOXB4* upregulation has been shown to be associated with the  
355 development of platinum resistance in cell lines, and its over-expression in ovarian cancer  
356 has been reported previously in a relatively small study using only 4 cell lines and 7 ovarian  
357 cancer tumour samples (29), but no oncogenic function for this gene has been proven.  
358 *HOXB4* has been implicated as a cancer-related gene in other malignancies, including  
359 breast cancer, leukaemia and lung cancer (30-32). The recent genomic analysis of HGS

360 ovarian cancer by the Cancer Genome Atlas (TCGA) researchers found a number of  
361 somatic copy number alterations and three members of the *HOXB* family, *HOXB2*, *B5* and  
362 *B8* were among the focally amplified regions (33) further supporting a possible oncogenic  
363 role of *HOXB* genes in ovarian cancer and emphasising the overlapping functions which  
364 exist between *HOX* genes (34).

365 Significant differences in *HOX* gene expression were found between platinum sensitive and  
366 resistant cell lines. Platinum-resistant cell lines show upregulation of *HOX* genes from the  
367 *HOXB* cluster. Although there was a difference between the three paired cell lines tested,  
368 *HOXB4* and *HOXB9* overexpression was common in two of the three cell lines (when  
369 compared to the platinum-sensitive counterpart). These results therefore demonstrate that  
370 *HOXB* genes are likely to play a role in developing platinum resistance; although further  
371 work is needed to understand the mechanism of this interaction.

372 Survival analysis revealed a cluster of 5 *HOX* genes, *HOXA13*, *B6*, *C13*, *D1* and *D13*, that  
373 was strongly associated with a poor OS in HGS patients. *HOXA13* is usually expressed in  
374 the upper vagina (4) playing a role in Müllerian duct differentiation during development, but  
375 has been reported to be overexpressed in ovarian cancer cell lines (25). *HOXA13* was  
376 linked to poor OS in oesophageal squamous cell carcinoma patients, and the same study  
377 found its expression in cell lines enhanced tumour growth *in vitro* and *in vivo* (35). High-  
378 throughput microarray analysis of gastric cancer patients revealed *HOXA10* and *A13* over-  
379 expression with *HOXA13* upregulation significantly associated with an aggressive  
380 phenotype, and a prognostic marker for poor OS (36). Highly deregulated expression of the  
381 *HOXA* cluster has also been found in hepatocellular carcinoma (HCC), in particular *HOXA13*  
382 (37).

383 Up-regulation of *HOXB6* has also been reported in ovarian cancer before, in addition to  
384 *HOXB7* (38). Data from this study and previous reports of high *HOXB* expression in ovarian  
385 cancer suggests that the *HOXB* gene products play a role in ovarian tumourgenesis.

386 *HOXC13* has a role in DNA replication (39), supporting an oncogenic function. A role in  
387 human cancer has also been reported with overexpression found in metastatic melanoma  
388 (40) and fusion with NUP98 has been associated with acute myeloid leukaemia (AML) (41).  
389 The *HOXD1* gene appears to be involved in cell differentiation (42), whereas *HOXD13* is  
390 deregulated in breast and cervical cancer and melanoma (43-45). A large *HOXD13*  
391 expression analysis by Cantile and colleagues in 79 different tumour types also supports its  
392 role in neoplastic transformation (46).

393 Determination of *HOX* gene dysregulation may be undertaken routinely in the clinical setting  
394 using fresh or archived patient tissue and such information could be used for stratifying  
395 patients in terms of prognosis. Furthermore, we have shown that our novel agent HXR9, a  
396 peptide capable of disrupting *HOX* gene function by inhibiting HOX binding to its co-factor,  
397 PBX, has significant anti-tumour efficacy (11-15), which is increased when used in  
398 combination with cisplatin. This synergy could be explained due to the role of *HOX* genes in  
399 DNA repair pathways (27) but further work investigating this is needed. *HOX* gene  
400 dysregulation therefore represents a potential ovarian cancer target with a low likelihood of  
401 cross resistance to conventional chemotherapeutic agents. Both HXR9 and small molecule  
402 inhibitors of the HOX/PBX dimer are currently being evaluated as novel cancer agents in  
403 preclinical models.

## 404 **Conclusion**

405 This comprehensive analysis of *HOX* gene expression in ovarian cancer cell lines and  
406 primary ovarian tumours demonstrates that these genes are profoundly dysregulated  
407 compared to normal ovary. Increased expression of *HOXA13*, *B6*, *C13*, *D1* and *D13* in EOC  
408 patients is associated with a poor prognosis and a more aggressive malignancy. It is  
409 possible to target HOX function by disrupting its binding to PBX, and further development of  
410 therapeutic compounds to achieve this is warranted.

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414 **Acknowledgements**

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416 **Conflicts of interest statement**

417 The authors report no conflicts of interest.

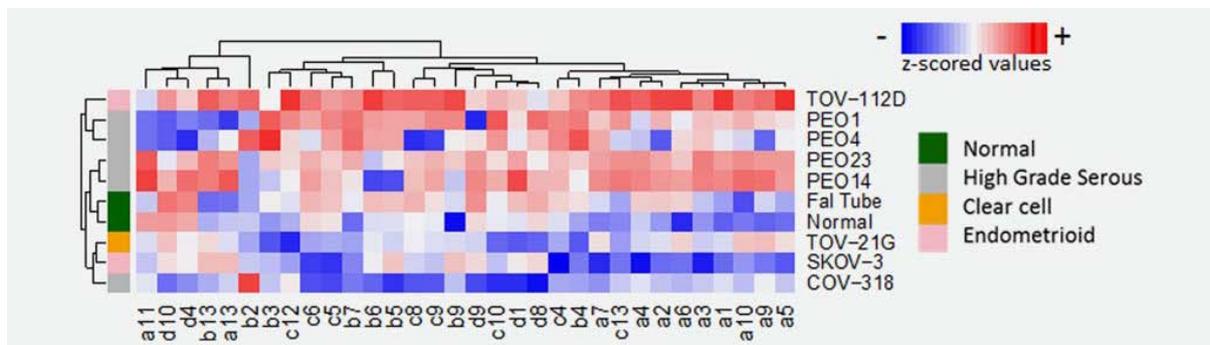
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419 **Figure legends**

420 **Figure 1**

421 ***HOX* expression in ovarian cancer cell lines of high grade serous (HGS), endometrioid**  
422 **and clear cell carcinoma subtypes and normal ovarian and fallopian tube tissue.** Heat  
423 map showing differential *HOX* expression between 5 HGS, 2 endometrioid and 1 clear cell  
424 ovarian carcinoma cell lines and 10 normal ovarian and 3 fallopian tube tissue samples.  
425 Expression of each gene was determined by quantitative PCR (qRT-PCR) from at least  
426 three independent experiments and expression is relative to the house keeping gene  $\beta$ -actin.

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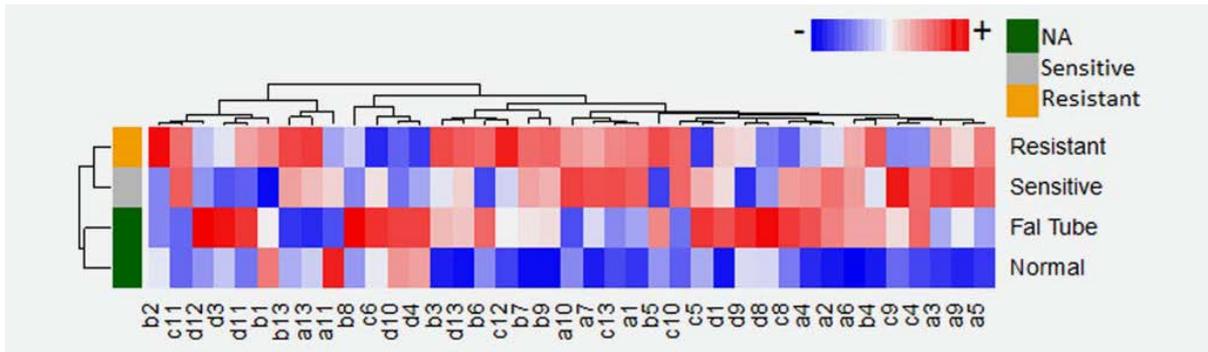
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431 **Figure 2**

432 ***HOX* gene expression of paired platinum sensitive and platinum resistant ovarian cancer**  
433 **cell lines.** The comparison of *HOX* gene expression profiles between patient derived cell  
434 lines before (PEO1 and PEO14) and after (PEO4 and PEO23) developing platinum resistance.  
435 Heat map shows differential *HOX* expression between platinum sensitivity statuses of cell  
436 lines.



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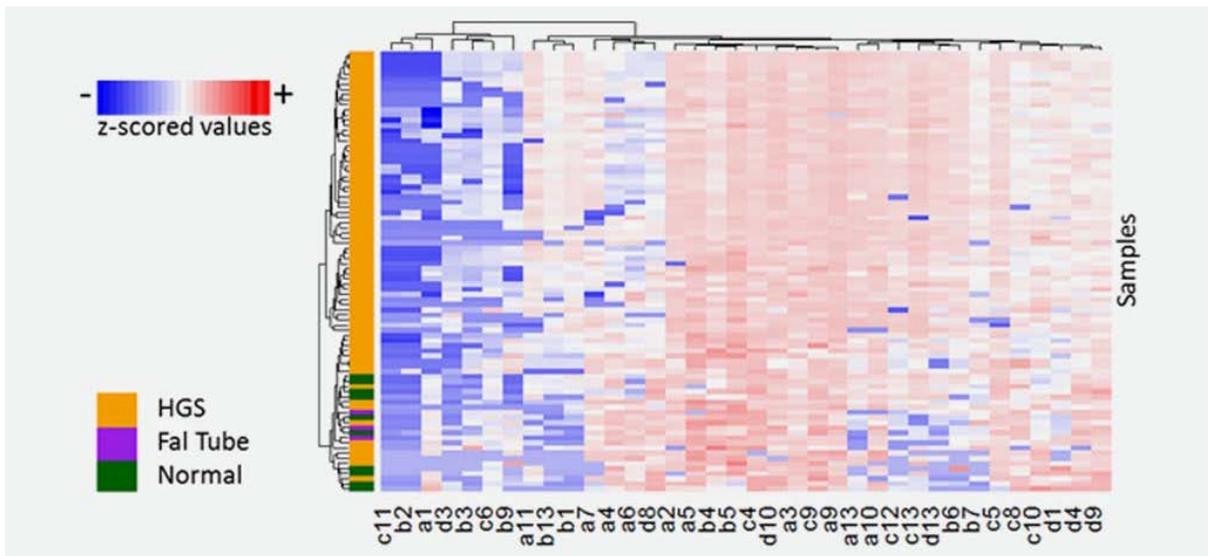
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449 **Figure 3**

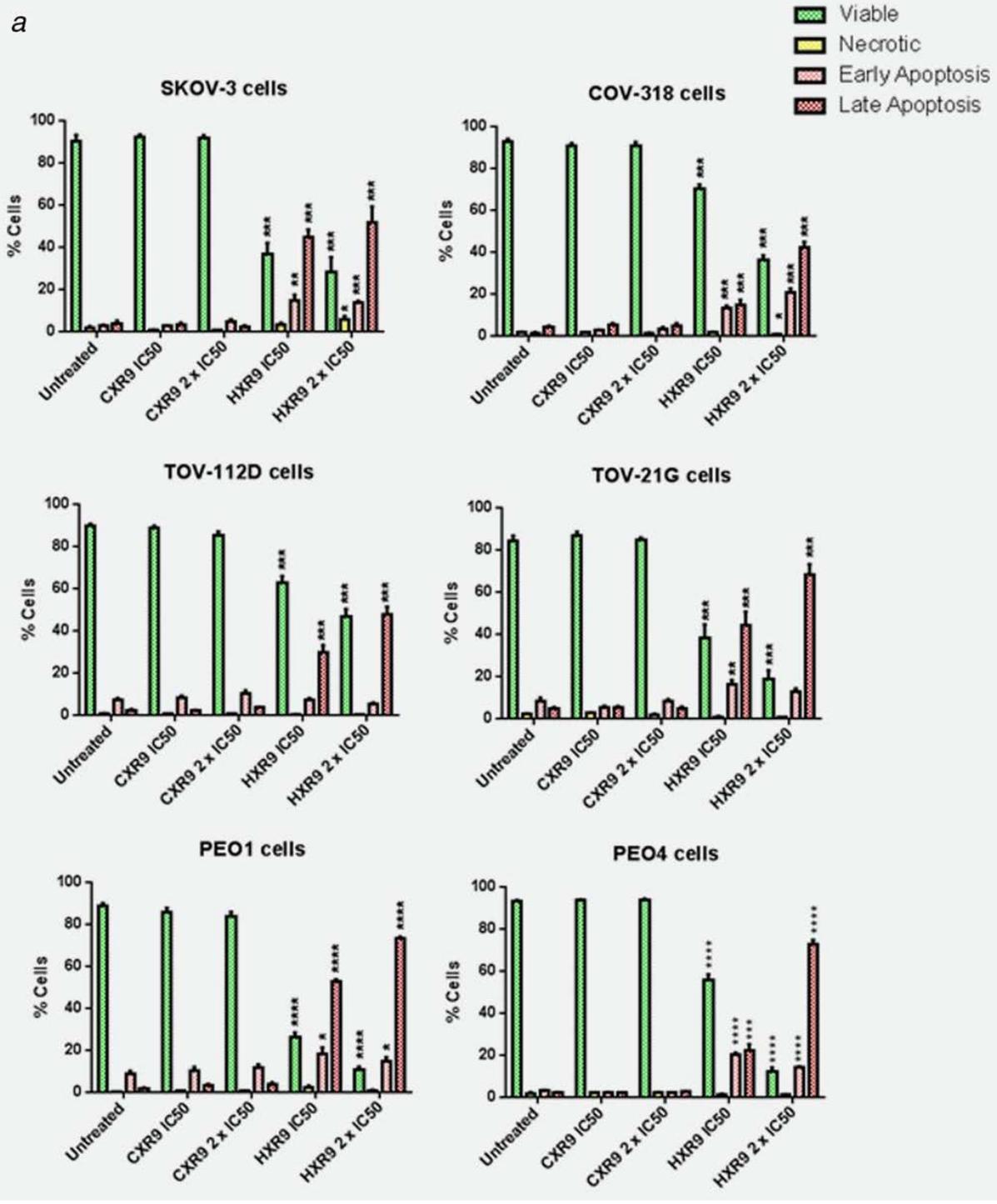
450 ***HOX* gene expression in high grade serous ovarian tumours.** a) Heat map showing  
451 differentially expressed *HOX* genes between high grade serous (HGS) ovarian tumours and  
452 normal ovarian and fallopian tube tissue. *HOX* gene expression data for HGS tumours  
453 (yellow) were compared to 10 normal ovarian tissues (green) and 3 fallopian tube sample  
454 (purple) to find upregulation of 36 genes in the HGS tumours. *HOX* expression profiles were  
455 determined by quantitative PCR (qRT-PCR) and normalised to housekeeping gene  $\beta$ -actin.  
456 Each column represents a gene and each row represents a sample. Column-wise z-score  
457 transformation (genes) was used. Red colour for a gene indicates expression above the  
458 median and blue indicates expression below the median.



466 **Figure 4**

467 **HXR9 induces apoptosis in ovarian cancer cell lines.** Ovarian cancer cell were assessed  
468 for apoptosis or necrosis through annexin/propidium iodine staining after HXR9 treatment. a)  
469 The bargraphs show the percentage of cells in early apoptosis, late apoptosis, and necrosis,  
470 as well as viable cells, when untreated, treated at the HXR9 IC<sub>50</sub> dose or double the IC<sub>50</sub>  
471 dose for each cell line or equivalent CXR9 dose. Error bars show the SEM. *P*-values <0.05  
472 are denoted as \*, <0.005 \*\* and < 0.001 as \*\*\* with respect to untreated cells. Example flow  
473 cytometry plots for untreated; CXR9 25µM; CXR9 50µM; HXR9 25µM and HXR9 50µM  
474 treated SKOV-3 cells

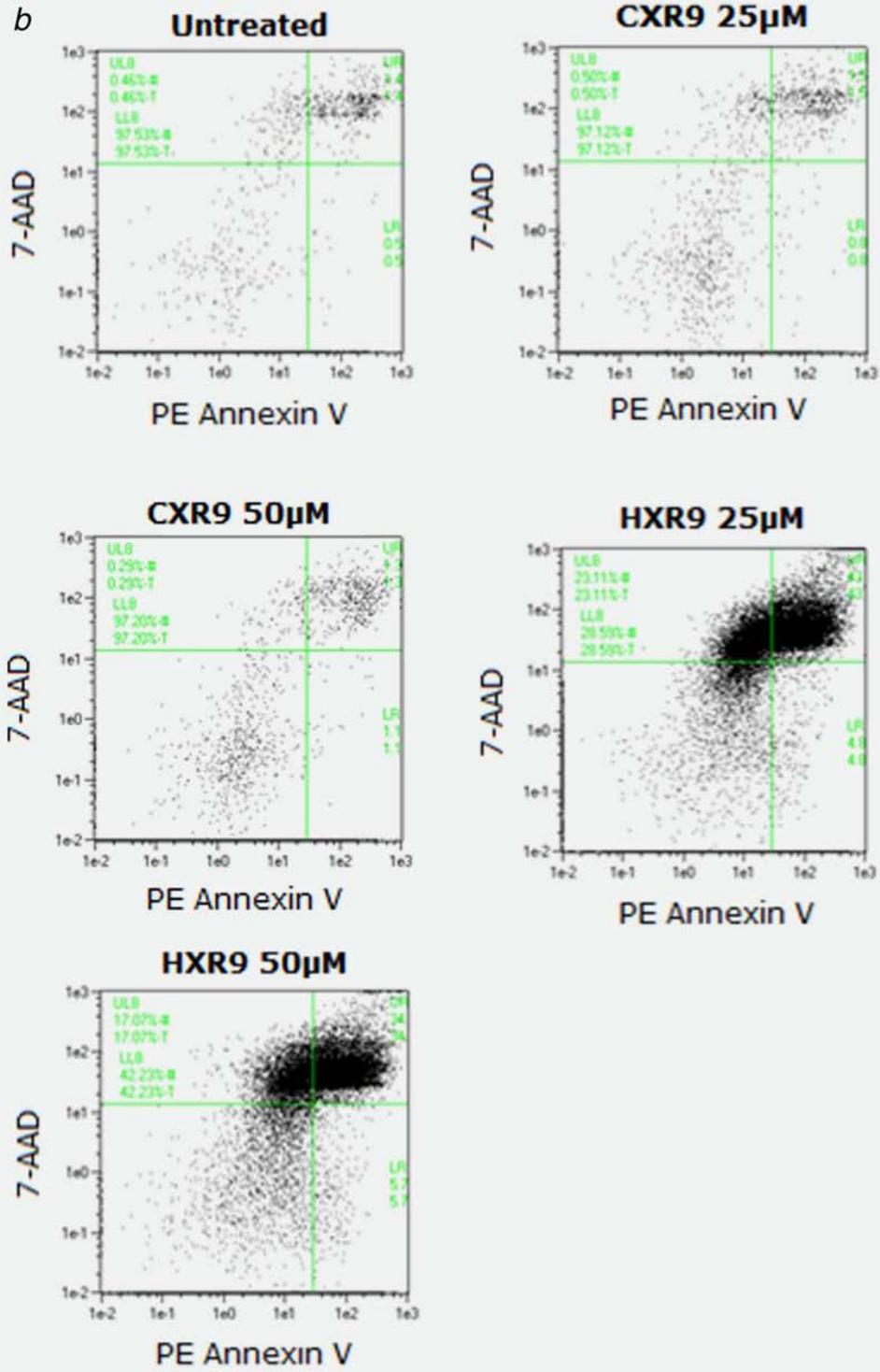
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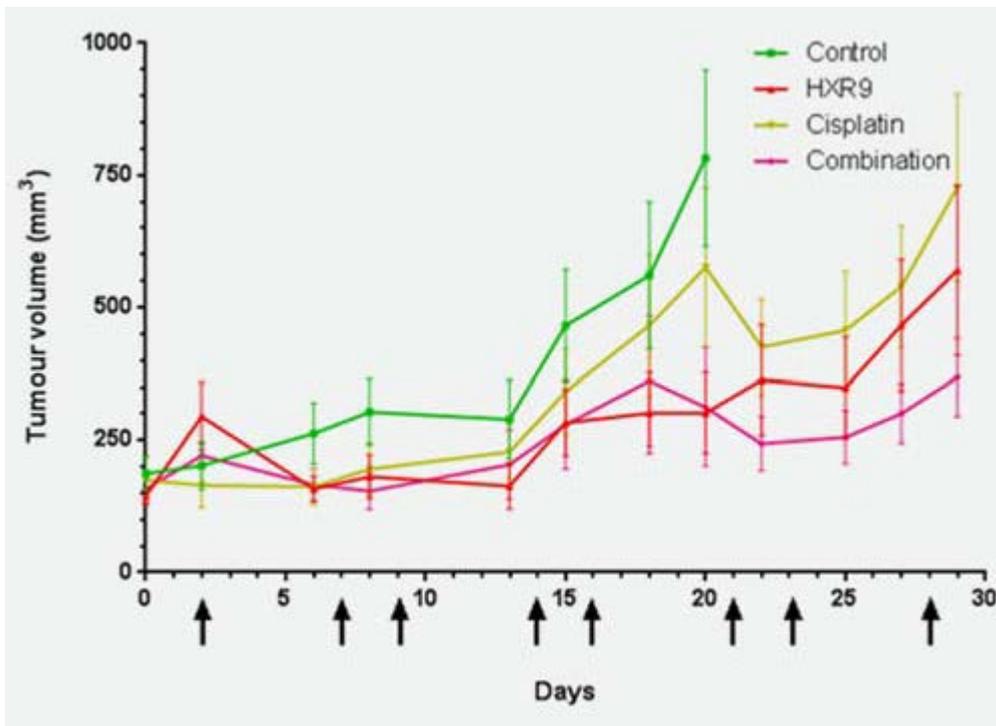
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482 **Figure 5**

483 ***In vivo* combination study of HXR9 and cisplatin.** Antitumor activity of HXR9 and cisplatin  
484 alone or in combination against ovarian cancer (SKOV-3) xenografts. Nude female mice  
485 were inoculated SC with  $1 \times 10^6$  SKOV-3 cells (Day 0). Treatment was initiated when  
486 tumours reached an approximate volume of  $100\text{mm}^3$ . An initial dose of HXR9 was given IT at  
487  $100\text{mg/kg}$ , followed by twice weekly doses at  $10\text{mg/kg}$ . Cisplatin was administered IP at  $3$   
488  $\text{mg/kg}$  weekly. Combinational studies consisted of both treatments; PBS was used as a  
489 control. Arrows indicates drug administration. A minimum of 6 mice in each group was set as  
490 the cut-off point for each curve.



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497 **Table 1**

498 Kaplan-Meier analysis of the 5-*HOX* gene prognostic signature showing the median overall  
 499 survival for patients who do not express the gene as compared to patients whose tumours  
 500 show expression of the genes listed below. Hazard ratios and Confidence intervals were  
 501 calculated using the Log rank model

Gene	Median overall survival (months)	No. of patients	p values	Hazard ratio	95% Confidence interval
HOXC13	36	37	0.0128	8.264	1.396–12.75
HOXB6	36	36	0.0145	8.286	1.365–14.67
HOXA13	44	39	0.0317	4.508	1.145–12.17
HOXD13	36	37	0.0308	6.834	1.153–12.79
HOXD1	36	37	0.025	4.692	1.206–11.61

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504 **Supplementary Table 1**

505 **Patient characteristics.** Tumour samples and patient information was obtained from the  
 506 Royal Surrey Hospital, Guildford.

507 **Supplementary Table 2**

508 **Synergy between HXR9 and cisplatin in ovarian cancer cell lines.** Cells plated in 96-well  
 509 plates were exposed to graded concentrations of HXR9 or cisplatin either alone or in  
 510 combination. Cells were treated with cisplatin for a total of 72 hours and with HXR9 for 2  
 511 hours. Cell viability was then measured by the MTS assay and the presence of synergy was  
 512 analysed based on the Chou-Talalay method using CalcuSyn version 2.0 software (Biosoft,  
 513 Stapleford, UK) (19). The interaction between HXR9 and cisplatin was quantified by  
 514 determining the combination index (CI) at ED50, ED75 and ED90 (doses which produce the  
 515 cell kill in 50, 75 and 90 per cent of a population, respectively), where  $CI < 1$  indicates  
 516 synergism,  $CI = 1$  indicates an additive effect antagonism ( $CI > 1$ ) between drugs.

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