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**HES5 silencing is an early and recurrent change in prostate tumourigenesis**

Charles E Massie¹, Inmaculada Spiteri¹, Helen Ross-Adams¹, Hayley Luxton¹, Jonathan Kay¹, Hayley C Whitaker¹, Mark J Dunning¹, Alastair D Lamb¹,⁶,⁷, Antonio Ramos-Montoya¹, Daniel S Brewer³, Colin S Cooper²,³, Rosalind Eeles²,⁴, UK Prostate ICGC Group¹, Anne Y Warren⁵, Simon Tavare´¹, David E Neal¹,⁶,⁷ and Andy G Lynch¹

¹Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge, CB2 0RE, UK
²Division of Genetics and Epidemiology, The Institute of Cancer Research, Sutton, UK
³Department of Biological Sciences and School of Medicine, University of East Anglia, Norwich, UK
⁴Royal Marsden NHS Foundation Trust, London and Sutton, UK
Departments of ⁵Pathology, ⁶Urology and ⁷Surgical Oncology, Addenbrooke’s Hospital, Hills Road, Cambridge, UK
Group participants are listed in the supplementary materials

**Abstract**

Prostate cancer is the most common cancer in men, resulting in over 10 000 deaths/year in the UK. Sequencing and copy number analysis of primary tumours has revealed heterogeneity within tumours and an absence of recurrent founder mutations, consistent with non-genetic disease initiating events. Using methylation profiling in a series of multifocal prostate tumours, we identify promoter methylation of the transcription factor HES5 as an early event in prostate tumourigenesis. We confirm that this epigenetic alteration occurs in 86–97% of cases in two independent prostate cancer cohorts (n=49 and n=39 tumour–normal pairs). Treatment of prostate cancer cells with the demethylating agent 5-aza-2'-deoxycytidine increased HES5 expression and downregulated its transcriptional target HES6, consistent with functional silencing of the HES5 gene in prostate cancer. Finally, we identify and test a transcriptional module involving the AR, ERG, HES1 and HES6 and propose a model for the impact of HES5 silencing on tumourigenesis as a starting point for future functional studies.

**Key Words**

- prostate cancer
- epigenetics
- methylation
- HESS
- HES6
- AR
- ERG
- NOTCH

**Introduction**

Current analysis of cancer genome sequencing has revealed disease processes and genomic alterations that may underlie disease initiation or evolution (Nik-Zainal et al. 2012, Baca et al. 2013, Tarpey et al. 2013). These approaches have identified and enumerated recurrently mutated driver genes in several cancer types, such as KRAS which is mutated in 93% of pancreatic cancers (Biankin et al. 2012) and TP53 which is mutated in 96% of high-grade serous ovarian cancers (Cancer Genome Atlas Research Network 2011), 69% of oesophageal cancer (Weaver et al. 2014) and over 50% of colorectal cancers (Cancer Genome Atlas Network 2012). In contrast with these highly recurrent mutations, a recent study of 112 aggressive primary prostate cancers has reported that the...
most significantly mutated gene (SPOP) was altered in only 13% of cases, with the next most commonly affected gene TP53 affected in only 6% of prostate tumours (Barbieri et al. 2012).

Therefore, while genome sequencing approaches have provided important insights into the biology of prostate cancer (Berger et al. 2011, Baca et al. 2013, Lindberg et al. 2013, Weischenfeldt et al. 2013) the high intra- and inter-tumour heterogeneity coupled with the small samples sizes may have limited the identification of genetic driver events in primary tumours. Indeed, previous genome sequencing studies have reported few common mutations between different tumour foci within the same prostate (Lindberg et al. 2013), highlighting marked intra-tumour heterogeneity and the absence of a genetic founder mutation. This complexity has led many groups to focus on late-stage, aggressive disease with the aim of identifying genomic events associated with disease progression (Barbieri et al. 2012, Grasso et al. 2012). However, their remain important unanswered questions over the early stages of prostate tumour evolution where genetic events appear to be for the most part heterogeneous. One notable exception to this is gene fusions involving ETS (E26 transformation-specific) transcription factors that have been found to occur in approximately half of all prostate cancers (Tomlins et al. 2005, Perner et al. 2006). However, these androgen receptor (AR)-driven gene fusions alone are insufficient to initiate prostate tumours in disease models (Carver et al. 2009, Chen et al. 2013) and may not be early ‘founder’ events in disease evolution (Barry et al. 2007, Mertz et al. 2013, Minner et al. 2013).

Therefore current evidence would seem to suggest that if a common initiating driver event exists it is not genetic, implicating other mechanisms in disease aetiology. In addition to somatic mutation several other disease-initiating pathways have been proposed in prostate cancer including germline predisposition (Kote-Jarai et al. 2011, Eeles et al. 2013), telomere shortening (Sommerfeld et al. 1996, Heaphy et al. 2013), chronic inflammation (Elkahwaji et al. 2009, Caini et al. 2014), metabolic stress (Freedland 2005, Kalaany & Sabatini 2009) and epigenetic alterations (Lee et al. 1994, Kanwal et al. 2014). It is likely that non-genetic and genetic alterations interact during tumourigenesis and several studies have identified interactions between somatic mutations and micro-environmental changes (Garcia et al. 2014), inflammation (Kwon et al. 2014) and metabolism (Kalaany & Sabatini 2009). Current technologies allow accurate identification and quantification of epigenetic alterations and are therefore a tractable second line of enquiry to identify driver events in prostate tumourigenesis.

We have recently identified a role for the enhancer of split transcription factor HES6 in prostate cancer and AR signalling (Ramos-Montoya et al. 2014). Herein, we characterise an epigenetic alteration at the promoter of the related HESS gene, which has been recently reported in a panel of genes that showed promise as a prostate cancer marker in biopsy samples (Paziewska et al. 2014). We profile this change in detail and show it to be an early event in prostate cancer development and highly recurrent across three unrelated prostate tumour cohorts. We then characterise an interaction between the epigenetic silencing of HESS and the expression of HES6 and provide evidence for interactions with known oncogenic pathways in prostate cancer (namely AR signalling and ERG gene fusions), highlighting a transcriptional network that is altered in prostate cancer development first by an epigenetic change and then by a genomic rearrangement.

Materials and methods

Sample cohorts

In a series of four radical prostatectomy specimens, we systematically dissected the whole prostates, identified regions containing tumour and harvested 17 tumour-rich samples from 13 spatially separated tumour cores (median 46% tumour, interquartile range (IQR) 36–62%), four adjacent benign samples and three whole-blood samples (Fig. 1a and Supplementary Figure 1a, see section on supplementary data given at the end of this article). Each tumour core was taken from a 5 mm tissue slice and the tumour content of samples used for DNA extraction was assessed by a pathologist using H&E staining of immediately adjacent sections (Warren et al. 2013). From two such cores, we also took three sets of sections for DNA extraction to allow assessment of heterogeneity within cores in addition to the spatial heterogeneity within and between cancerous prostates (Supplementary Figure 1a). These samples were used for global methylation profiling using Infinium HumanMethylation450 arrays (see below for details).

In a separate cohort of 39 matched prostate tumour and adjacent benign samples, we performed targeted bisulphite sequencing of the HESS promoter, to assess the frequency of HESS hypermethylation in prostate cancer. This analysis provides a promoter-wide view of DNA methylation changes at the HESS promoter (in contrast to the limited number of CpGs assessed using methylation profiling arrays).
In an unrelated, larger cohort of prostate cancers with publicly available methylation array data (n = 304 tumours, n = 49 matched normal samples) (Weinstein et al. 2013), we assessed the recurrence of HESS promoter methylation.

DNA methylation profiling in blood, benign prostate and multiple spatially separate tumour foci

Clinical samples for analysis were collected from prostatectomy patients with full research consent at the Addenbrooke’s Hospital, Cambridge, UK. The prostates were sliced and processed as described previously (Warren et al. 2013). A single 5 mm slice of the prostate was selected for research purposes. Tissue cores of 4 mm or 6 mm were taken from the slice and frozen. The frozen cores were mounted vertically and sectioned transversely giving a single 5 μm frozen section for H&E staining followed by 6 x 50 μm sections for DNA preparation using the Qiagen Allprep kit. Using the Infinium HumanMethylation 450 BeadChip kit, DNA was subjected to bisulphite conversion, amplification, fragmentation, hybridisation, extension and labelling, according to the manufacturer’s instructions (Illumina, Little Chesterford, Essex, UK). Bead summary data from Infinium HumanMethylation450 arrays were processed using the Minfi package in the R statistical software (Aryee et al. 2014, R-Core-Team 2014). As previously described, probe types were normalised separately (Marabita et al. 2013) before generating M- and B-values for exploratory analysis. Summary plots were generated in the R statistical software (R-Core-Team 2014). Raw and processed data have been uploaded to the ArrayExpress portal under accession E-MTAB-2964, in addition all code used to generate figures in the paper are included as part of the R-markdown HTML document available on our group webpage.

Targeted bisulphite sequencing

PCR primers were designed to amplify a 441 bp fragment from the HESS promoter containing 60 CpGs (HESS-BSx-F: 5'-GAGGGGTTAGGTTGGTT-3'; HESS-BSx-R:
5′-ACCCACCTACTCTTTAAAAAC-3′). The amplicons were generated separately for 39 matched tumour normal sample pairs and assessed before preparing barcoded sequencing libraries using a Nextera XT kit (Illumina). Barcoded DNAs were quantified and equal amounts of each indexed library were then pooled and sequenced on an Illumina MiSeq (PE300). Fastq data files were split using index sequences and downstream methylation analysis was performed using Bismark (Krueger & Andrews 2011) and summary plots and test statistics were generated using the R statistical software (R-Core-Team 2014). This analysis gave a median sequencing coverage of 786× (Supplementary Figure 3, see section on supplementary data given at the end of this article). All code used to generate figures in the paper are included as part of the R-markdown HTML document available on our group webpage.

Data mining

An R markdown document containing all code required to reproduce our analysis and all figures has been included as a supplementary HTML document (available on our group webpage). Briefly, DNA methylation 450k array data for LNCaP prostate cancer cells and PrEC benign prostate epithelial cells (CC-2555, Lonza, Basel, Switzerland) were obtained from GEO (triplicate data for each cell line from GSE40699) (Statham et al. 2012, Varley et al. 2013) and summary plots were generated using the R statistical software (R-Core-Team 2014). Gene expression data from LNCaP cells treated with the demethylating agent 5-aza-2′-deoxycytidine were retrieved from GEO (GSE25346). Gene expression data from human prostate benign and tumour tissues were obtained from GEO (GSE3325). Gene expression data from control and ERG-knockdown VCaP cells was retrieved from GEO (GSE60771). All GEO data were retrieved using the GEOquery package in the R statistical software and summary plots were generated using the same software (Davis & Meltzer 2007, R-Core-Team 2014). Transcriptional networks were drawn using the BioTapestry application (Longabaugh 2012) constructing models using ChIP-seq binding profiles, expression correlations and published transcriptional links.

**HES5 motif enrichment analysis**

The position weight matrix for HES5 was obtained from Yan et al. (2013) and used to search the genomic sequence of the HES6 gene locus (including 1 kb upstream and 1 kb downstream sequence). Motif searches were carried out using the RSAT matrix-scan (with human ‘upstream-noorf’ background control) (Turatsinze et al. 2008), and motif scores were visualised using BioSAVE (Pollock & Adryan 2008).

**Androgen time-course gene expression profiling in LNCaP and VCaP cells**

Following 72-h steroid depletion in the media containing 10% charcoal-stripped FBS, LNCaP and VCaP cells were subjected to androgen stimulation (1 nM R1881) or vehicle control treatment (0.01% ethanol). The cells were harvested at the indicated timepoints over a 24 h period following treatment and RNA extracted using Trizol (Life Technologies). For the LNCaP treatment time-course, a full analysis has been published (Massie et al. 2011) and raw and normalised data have been deposited at GEO (GSE18684). Data for the VCaP androgen treatment time-course have also been deposited at ArrayExpress (E-MTAB-2968). Expression data were analysed using the beadarray software, with spatial artefacts identified and removed automatically (BASH) and curated manually (Dunning et al. 2007, Cairns et al. 2008). The resulting data set was summarised with outliers removed to obtain mean log-intensity and standard error for each probe/array combination.

**Results**

**HES5 promoter methylation is an early event in prostate tumorigenesis**

In order to investigate the epigenetic landscape within and between prostate tumours, we systematically dissected four radical prostatectomy specimens, harvesting 17 tumour-rich samples from 13 spatially separated tumour cores (median 46% tumour, IQR 36–62%), four adjacent benign samples and three whole-blood samples (Fig. 1a and Supplementary Figure 1a). Consistent with previous reports (Lindberg et al. 2013), these spatially separated tumour cores appeared to be only distantly related by somatic mutations and therefore our aim was to identify early (common ‘trunk’) epigenetic events. Analysis of the methylation distributions for all assayed CpGs revealed that global methylation profiles were similar between tumour and benign prostate samples (Spearman’s rank correlation of tumour vs benign methylation profiles 0.94–1.00; Supplementary Figure 1b,c, d and e). A recent study has highlighted eight genomic loci that showed differential methylation in a series of unmatched tumour and benign prostate samples (i.e. from...
different individuals), a subset of which were proposed as molecular markers to support pathological diagnosis of biopsies (Paziewska et al. 2014). We assessed the reproducibility and clonality of these eight differentially methylated regions in our cohort of cases with multiple spatially separate tumour samples, matched benign tissue and blood DNA samples (Fig. 1b and Supplementary Figure 1f, g, h, i, j, k, l, m).

In our cohort, the promoter region of the HESS gene showed the largest and most consistent increase in methylation in tumour samples compared with matched normal tissue (median 7.6-fold increase, median variance = 0.003), together with consistently low methylation in adjacent normal tissue (median normal methylation = 0.08, median variance = 0.0006; Fig. 1b, c, d and Supplementary Figure 1f, g, h, i, j, k, l, m). The study by Paziewska et al. (2014) showed low HESS promoter methylation in benign prostatic hyperplasia and hypermethylation in prostate tumour biopsies. Among the other regions examined, we found that tumour methylation at the ITGB2 and mir10B loci showed no difference with examined, we found that tumour methylation at the APC locus showed variable

The homogenous hypermethylation of the HESS promoter across genetically heterogeneous tumour cores is consistent with this being an early event in tumourigenesis (Fig. 1c and Supplementary Figure 1m).

Therefore using our cohort of cases with multiple tumour foci and matched benign samples, we found that hypermethylation at the HESS promoter region was observed across tumour samples from all patients and in all spatially separated tumour foci from the same patient. The homogenous hypermethylation of the HESS promoter across genetically heterogeneous tumour cores is consistent with this being an early event in tumourigenesis (Fig. 1c and Supplementary Figure 1m).

Figure 2
Validation of HESS promoter methylation as a common event in two additional independent prostate cancer cohorts. (A) CpG methylation summary of the HESS promoter as determined by bisulphite sequencing from a representative tumour–normal pair. Each column represents one CpG assayed (n = 60), red and blue stacked bars represent the proportion of methylated and unmethylated reads, respectively, at each CpG. Column widths are proportional to sequencing coverage (median = 786×).

(B) Scatter plot summary of HESS promoter methylation for 39 tumour–normal pairs. (C) Histogram summary of significance testing for increased HESS promoter methylation in tumour vs normal sample pairs (n = 39 pairs from panel-C; paired Wilcoxon rank sum test; log2 P values are plotted to visualise distributions). (D) Boxplot summary of HESS promoter methylation for 304 tumour and 49 benign prostate samples on Illumina 450k arrays (TCGA data). (E) Histogram summary of significance testing for increased HESS promoter methylation in TCGA tumour vs normal sample pairs (n = 49 pairs from panel-E; paired Wilcoxon rank sum test; log2 P values are plotted to visualise distributions). (F) ROC curve for HESS promoter methylation using data from bisulphite sequencing of 39 tumour normal pairs (A, B and C) and methylation array profiling of 49 tumour normal pairs (D and E).
**HES5 promoter methylation is a recurrent event in prostate tumours**

To assess the frequency of HES5 hypermethylation in prostate cancer, we performed targeted bisulphite sequencing of the HES5 promoter in a separate cohort of 39 matched tumour and adjacent benign samples. This analysis included 60 CpGs in the HES5 promoter and gave a median sequencing coverage of 786 × (Supplementary Figure 3). This analysis provided a comprehensive view of DNA methylation across the HES5 gene promoter, in
contrast to the four CpGs assessed using methylation arrays and a narrow genomic window in a previous study (Paziewska et al. 2014). Benign samples showed hypomethylation across the entire HES5 promoter, whereas matched tumour samples had consistent hypermethylation across all 60 CpGs assayed (Fig. 2a, b and Supplementary Figure 4, see section on supplementary data given at the end of this article). This pattern of hypomethylation in benign tissue and hypermethylation in tumours was consistent in 38/39 matched tumour normal pairs (97% at P<0.05, Wilcoxon test; Fig. 2c). In the single discordant sample pair, there was increased methylation in the matched benign sample that was maintained in the tumour (median methylation 20.7 and 15.4 respectively; Supplementary Figure 4), consistent with either a pre-transformation change in this single case or tumour contamination of this normal tissue core.

We also assessed HES5 methylation in an additional prostate cancer patient cohort using publicly available methylation array data (n=304 tumours, n=49 matched normal samples) (Weinstein et al. 2013). In this second validation cohort, we again observed hypermethylation in tumours and hypomethylation in benign samples (42/49 pairs, 86% at P<0.05, Wilcoxon test; Fig. 2d and e). Receiver operating characteristic (ROC) curve analysis for these two geographically distinct validation cohorts run on different platforms revealed high sensitivity and specificity (positive predictive value (PPV)=0.92, area under the curve (AUC)>0.9, Fig. 2f). These results clearly demonstrate that in addition to being an early event in prostate tumourigenesis HES5 methylation is a highly recurrent event in prostate cancer, suggesting potential as a specific disease marker and an early acquired (or selected) event in prostate tumourigenesis.

**HES5 is silenced in prostate cancer cells and demethylation restores expression**

Consistent with observations in human tumours, we found that LNCaP prostate cancer cells exhibit hypermethylation of the HES5 promoter, in contrast to HES5 hypomethylation in benign epithelial cells PrEC (Fig. 3a). The expression of HES5 is low or undetectable in cultured prostate cancer cell lines and is also low in human prostate tumours (Supplementary Figure 5a, c, see section on supplementary data given at the end of this article and Fig. 3d, f), consistent with epigenetic silencing of HES5 in prostate cancer (Supplementary Figure 5g and h). Treatment of LNCaP cells with the DNA demethylating agent 5-aza-2’-deoxycytidine caused de-repression of the HES5 gene (Fig. 3b), consistent with active epigenetic silencing of the HES5 gene in prostate cancer cells.

**HES5 epigenetic silencing is associated with HES6 expression**

HES5 is known to play a role similar to that of HES1 in developmental processes (Hatakeyama et al. 2004, 2006, Tateya et al. 2011), and both are involved in negative feedback loops with HES6 (Fior & Henrique 2005, Jacobsen et al. 2008), which antagonises the activity of HES1 and HES5 (Bae et al. 2000, Salama-Cohen et al. 2005). Of note, HES6 has been recently reported to play an important functional role in prostate cancer enhancing oncogenic signalling through the AR (Ramos-Montoya et al. 2014). Although a rare HES6 gene fusion has been reported (Annala et al. 2014), no molecular mechanism has been found for the frequent up-regulation of HES6 in prostate cancer. In prostate cancer cells, de-repression of HES5 with the demethylating agent 5-aza-2’-deoxycytidine resulted in increased expression of HES6, confirmed by qPCR and western blotting (data not shown). Relationships between gene expression, dashed quadrant lines indicates the mid-point of expression values for each gene. Plots on the right show the relationship between the level and difference in expression for each pair of genes (using median centred values for each gene). Divergence from the dashed zero line indicates an inverse relationship, red trend lines depict loss regression. (I) Simple models of the putative expression networks in benign prostate, prostate cancer and ERG-positive prostate cancer involving the AR, HES5, HES6, ERG and HES1. Genes are depicted by thick horizontal lines, connecting lines depict transcriptional targets of each encoded transcription factor. Connectors with arrowheads depict positively regulated targets, while connectors with flat ends depict repressed targets. Genes shown in grey depict low/no expression in a given condition. On the HES5 gene open circles depict hypomethylation and filled circles depict hypermethylation. ARGs denotes AR-regulated genes. Model drawn using BioTapestry.
in a delayed downregulation of \textit{HES6} (Fig. 3c), consistent with \textit{HES5} repression of \textit{HES6}. We also observed an inverse relationship between \textit{HES5} and \textit{HES6} expression in a series of primary tumours compared with benign prostate samples, where \textit{HES5} expression decreased and \textit{HES6} expression increased in tumour vs benign prostate samples (Fig. 3d and e). In our cohort of multiple spatially separated tumour samples, we found that \textit{HES5} expression
was decreased in tumour cores compared with matched benign tissue and that HES6 was also increased in some of those tumour cores, consistent with HESS silencing in tumourigenesis and additional mechanisms regulating HES6 expression (Supplementary Figure 5e and f). However, we found no evidence of a correlation between HESS methylation and expression in a larger series of tumours (n=39), nor between HESS and HES6 expression in this tumour cohort (Fig. 3f). This lack of correlation may at least in part be explained by the low or absent expression of HESS in prostate tumour samples (Figs 2 and 3d, f) confounding such correlative analysis. Indeed, we found that HESS expression appeared to be low and showed little variation in this series of 39 prostate tumours (Fig. 3f). The few samples that had slightly higher HESS expression also had low HES6 expression (Fig. 3f), which although not compelling alone is consistent with our other data supporting an inverse relationship between HESS and HES6 in addition to highlighting the recurrent silencing of HESS in tumourigenesis. There are no successful HESS genomic binding data nor chromatin immunoprecipitation grade antibodies for HESS; therefore; we could not assess direct binding of HESS at the HES6 gene locus (Yan et al. 2013). However, the preferred consensus DNA-binding sequence of HESS has been determined experimentally (Yan et al. 2013) and we found strong HESS consensus sites in and around the HES6 gene (Supplementary Figure 5i, j and k). Taken together our observations of i) the inverse correlation between HESS and HES6 in cancer cells treated with 5-aza-2’-deoxycytidine, ii) their inverse correlation in tumour-normal comparisons and iii) strong consensus HESS binding sites at the HES6 gene locus suggests that HESS may repress HES6 in prostate epithelial cells. The ubiquitous HESS silencing in tumours may therefore potentiate (or de-repress) HES6 expression in prostate tumours.

**ERG and HES6 expression show an inverse relationship**

Despite the early and frequent silencing of HESS in prostate cancer, we observed variable expression of the HESS transcriptional target HES6 in prostate tumour samples (Fior & Henrique 2005) (Fig. 3f and Supplementary Figure 5f), prompting us to investigate other factors that may regulate HES6 expression in prostate tumour cells. We found that variations in HES6 expression showed an inverse relationship with expression of the frequently rearranged ERG gene in prostate tumours, highlighted by an inverse correlation (r=−0.28) and mutual exclusivity of HES6 and ERG expression (i.e. no samples have both high ERG and HES6 expression, Fig. 3g left panel). This inverse relationship is illustrated clearly by the increasing difference between ERG and HES6 at higher levels of expression (i.e. divergence from zero with increasing expression, Fig. 3g right panel).

**ERG and HES1 expression show a positive correlation**

In contrast the other major HES6 antagonist HES1 (Bae et al. 2000, Hatakeyama et al. 2004, 2006, Jacobsen et al. 2008) showed a strong positive correlation with ERG expression (r=0.65; Fig. 3h), suggesting an ERG–HES1–HES6 transcriptional network in ERG-fusion positive prostate cancer cells (Fig. 3g, h and i). In support of this prediction, we found evidence for extensive ERG binding at the HES1 gene locus (Fig. 4f) and also confirmed the previously reported AR binding sites upstream of the HES6 gene (Ramos-Montoya et al. 2014) by using multiple data sets (Fig. 4g).
A transcriptional network involving HESS, AR, ERG and HES6

Combining our observations of HESS silencing in prostate cancer with expression correlations in prostate tissue, DNA binding profiles for ERG and the AR and published transcriptional links (i.e. between HESS and HES6 (Fior & Henrique 2005), HES1 and HES6 (Jacobsen et al. 2008), reciprocal HES6 and HES1/S negative-feedback (Bae et al. 2000, Salama-Cohen et al. 2005, Hatakeyama et al. 2006) and AR and HES6 (Ramos-Montoya et al. 2014)), we constructed models of putative gene expression networks in benign prostate, prostate cancer and prostate cancer harboring ERG-rearrangements (Fig. 3). In this model, we predict that i) HESS expression in benign epithelial cells contributes to HES6 repression and ii) HESS promoter methylation and silencing in prostate tumours potentiates AR activation of HES6 to start an oncogenic feed-forward transcriptional signalling network (Fig. 3). Finally, our model suggests that in tumour cells harbouring an ERG gene fusion iii) AR activation of the ERG fusion gene creates a dynamic negative feedback loop impacting on both the AR and HES6, creating a more complex transcriptional network (Fig. 3). Negative feedback loops are common motifs in biological networks and have been shown to increase robustness and speed-up response times of transcriptional circuits (Rosenfeld et al. 2002, Shen-Orr et al. 2002, Austin et al. 2006, Nevozhay et al. 2009). Therefore, our model may highlight a previously unknown signalling node in ERG-positive tumours that may increase the robustness and response-rates of key pathways in prostate cancer.

ERG-fusion status affects HES1 and HES6 regulation by the AR

We tested the putative AR–HES6 and AR–ERG–HES1–HES6 transcriptional networks in AR-positive prostate cancer cells with and without TMPRSS2–ERG gene fusions (VCaP and LNCaP, respectively; Fig. 4). Using an androgen stimulation time-course, we were able to both track changes in gene expression and map their dynamics in prostate cancer cells with and without AR-regulated ERG-fusion expression following AR stimulation (Tomlins et al. 2005, Massie et al. 2011). We observed early up-regulation of the known AR-target gene TMPRSS2 in both ERG-fusion positive and ERG-fusion negative cells in response to androgen stimulation (Fig. 4a), while ERG induction only occurred in TMPRSS2–ERG fusion positive cells (Supplementary Figure 7a, see section on supplementary data given at the end of this article). Consistent with its epigenetic silencing, we found low expression and no change in HESS expression in either cell type (Supplementary Figure 7b). HES1 expression was not significantly changed in ERG-fusion negative cells, but showed strong androgen induction in ERG-fusion positive cells (Fig. 4b). HES6 expression was increased in ERG-fusion negative cells but was downregulated in ERG-fusion positive cells (Fig. 4c). Defining the timing of gene expression changes (‘change-points’) for these genes in ERG-fusion positive cells shows the sequence of events: i) TMPRSS2–ERG upregulation; ii) HES1 upregulation; iii) HES6 downregulation (Fig. 4e). These data show that HES1 is only induced by androgen signalling in ERG-fusion positive cells and that induction precedes HES6 repression. This transcriptional data are supported by genome-wide binding profiles showing that the AR is recruited to the HES6 gene locus (Fig. 4g) but not to the HES1 gene locus in ERG-fusion negative cells (Fig. 4f). However, in ERG-fusion positive cells, ERG binding is widespread at the HES1 locus (Fig. 4f), consistent with direct ERG regulation of the HES1 gene.

HES1 expression is dependent on ERG

To test this further, we looked at the expression of HES1 following ERG knockdown in VCaP cells (Mounir et al. 2014) (Fig. 4h and Supplementary Figure 6b, c, see section on supplementary data given at the end of this article) and found that HES1 expression was dependent on the expression of ERG (Fig. 4i and Supplementary Figure 6d, e), further supporting our model. In addition to the timing of expression changes in response to androgen stimulation, these data support an AR–ERG–HES1–HES6 transcriptional network in ERG-fusion positive prostate cancer cells. While in ERG-fusion negative cells, a simpler AR–HES6 network seems to occur. In each case, these transcriptional networks may have been preceded (and potentiated) by HESS epigenetic silencing in early tumourigenesis.

Discussion

Our data are consistent with an early role in prostate tumourigenesis for promoter-wide hypermethylation of HESS, supported by the very high frequency of this epigenetic change and our observation that this was a common alteration in a series of multi-focal tumours. While the functional role of HESS methylation in prostate tumourigenesis is yet to be determined, we found that demethylation resulted in downregulation of the HESS-target gene HES6, which has recently been shown to drive...
progression in prostate cancer via the androgen receptor (Ramos-Montoya et al. 2014). Therefore, we speculate that one potential effector mechanism of HESS silencing could be de-repression of HES6 that in turn enhances AR regulation of key oncogenic targets, contributing to transformation and/or priming cells for subsequent acquisition of aggressive phenotypes. In addition, HESS has established roles in tissue patterning during development (Sancho et al. 2013). Intriguingly defective NOTCH signalling has recently been shown to drive clonal expansions of PS3 mutant cells (Alcolea et al. 2014), raising the possibility that HESS silencing early in prostate tumourogenesis might drive clonal expansions and contribute to the ‘field effect’ observed in prostate tumours (Bostwick et al. 1998, Hanson et al. 2006, Mehrrotra et al. 2008). However, these and other downstream consequences of the early and common epigenetic silencing of HESS will require careful dissection in future studies.

It is intriguing that this HESS–HES6/AR–HES6 transcriptional network is affected by TMPRSS2–ERG gene fusion status. While the functional consequences of this remain to be explored, the implication of both AR and ERG oncogenic signalling axes provides further weight for the importance of the HES transcriptional network in prostate cancer. Future studies will need to include overexpression of HESS in prostate cancer cells to establish the direct consequences on HES6 and AR signalling, as well as the phenotypic consequences of bypassing HESS silencing. In addition, depletion of HESS in 5-aza-2′-deoxycytidine-treated prostate cancer cells (both ERG-positive and ERG-negative) will allow an assessment of de-repression of the endogenous HESS locus on gene expression and cellular phenotypes. Finally, future studies should also address the mechanisms upstream of HESS silencing, the high frequency of which would be consistent with either a strong-selective pressure or a targeted silencing of HESS, for example via loss of GCM as described in neural stem cells (Hitoshi et al. 2011).

This report highlights HESS silencing as an early and frequent event in prostate tumourigenesis that may serve as a useful biomarker or as a starting point for preventive medicine or targeted intervention strategies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
C E Massie carried out analysis, directed the project and wrote the manuscript; I Spiteri designed and carried out bisulphite sequencing experiments; H Ross-Adams prepared samples for bisulphite sequencing and tumour gene expression data; H Luxton, J Kay and H C Whitaker co-designed and provided all samples for the initial methylation profiling, expression and validation experiments; M J Dunning, A D Lamb and A Ramos-Montoya provided tumour gene expression data; A Y Warren carried out systematic pathology of prostatectomy samples and identified tumour and benign cores for sampling; S Tavare designed the study and drafted the manuscript; D S Brewer, C S Cooper, R Eeles and the ICGC Working Group provided 3D prostate reconstructions, contributed to study design and drafted the manuscript; D E Neal co-designed the study and drafted the manuscript; A G Lynch co-designed the study, carried out analysis, directed the project and co-authored the manuscript.

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