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SHORT REPORTS

The dioxin/aryl hydrocarbon receptor mediates downregulation of osteopontin gene expression in a mouse model of gastric tumourigenesis

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The dioxin/aryl hydrocarbon receptor functions as a ligand-activated transcription factor regulating transcription of a battery of genes encoding primarily drug-metabolizing enzymes. Expression of a constitutively active mutant of the aryl hydrocarbon receptor (CA-AhR) in transgenic mice results in development of stomach tumours, correlating with increased mortality. We have used suppression subtractive hybridization techniques followed by macroarray analysis to elucidate which genes are differentially expressed during this process. In the glandular stomach of CA-AhR mice, we observed decreased mRNA expression of osteopontin (OPN), a noncollagenous protein of bone matrix that is also involved in several important functions including regulation of cytokine production, macrophage accumulation, cell motility and adhesion. Downregulated expression of OPN during tumour development was confirmed by RT–PCR and RNA blot analysis. Immunohistochemical analysis showed that this decrease was confined to the corpus region, correlating with the restricted localization of the tumours. Decreased OPN mRNA expression was also observed in other organs of CA-AhR mice. Taken together, these results show that OPN is negatively regulated by the dioxin receptor, and that downregulation of its expression correlates with development of stomach tumours in mice expressing a constitutively active mutant of dioxin receptor.
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The dioxin receptor belongs to the basic helix–loop–helix/Per-arylhydrocarbon receptor nuclear translocator(ARNT)-Sim domain (bHLH/PAS) class of transcription factors. This family of proteins is emerging as a group of regulatory factors that respond to various environmental cues (Gu *et al*., 2000). The ligand-activated dioxin receptor interacts with the structurally related partner protein ARNT to mediate transcriptional activation of a network of genes encoding drug-metabolizing enzymes, particularly cytochrome *P*450 1A1 (CYP1A1), which function in the oxidative metabolism of xenobiotics (Pohjanvirta and Tuomisto, 1994). A series of independent loss-of-function studies in mice have not yielded conclusive results with regard to a potential physiological function of the dioxin receptor (Fernandez-Salguero *et al*., 1995, 1997; Schmidt *et al*., 1996; Mimura *et al*., 1997; Lahvis *et al*., 2000). In addition, a potential physiological ligand of the dioxin receptor has not been unequivocally identified. Environmental pollutants such as planar dioxins, biphenyls and polyaromatic hydrocarbons represent the best characterized receptor ligands (Pohjanvirta and Tuomisto, 1994; Gu *et al*., 2000). Consistent with the uncertainty regarding the physiological function of the receptor, there is a paucity of information on target genes of the receptor that are not drug-metabolizing enzymes. Although a series of different gene expression profiling studies have recently indicated dysregulation of numerous genes upon exposure of cells to dioxin (Kolluri *et al*., 1999, 2001; Mimura *et al*., 1999; Puga *et al*., 2000; Frueh *et al*., 2001; Ohbayashi *et al*., 2001; Svensson and Lundberg, 2001; Thomas *et al*., 2001; Oikawa *et al*., 2002 Kurachi *et al*., 2002; Rivera *et al*., 2002), the relevance of these genes for dioxin toxicity and carcinogenicity *in vivo* remains largely unclear.

We have developed in a gain-of-function model transgenic mice expressing a constitutively active dioxin receptor, CA-AhR (McGuire *et al*., 2001). These mice develop invasive tumours of the glandular stomach from 3–4 months of age that correlate with increased mortality beginning at 6–9 months of age (Andersson *et al*., 2002). To identify genes that may be involved in mediating this oncogenic effect, we have performed a gene expression profiling analysis using a modified version of a suppression subtractive hybridization (SSH) procedure (von Stein, 2001).

Stomach tumours in CA-AhR mice are readily detectable in mice 3 months of age as few cystic glandular structures that focally begin to penetrate

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through the muscularis mucosa into the submucosa (Andersson *et al*., 2002). In order to detect relatively early changes in gene expression, tumours from 90-days-old mice were analysed. In these experiments, poly-A-enriched RNA was isolated from the glandular stomachs of male CA-AhR and wild-type (wt) mice and subjected to the SSH procedure followed by cloning and PCR analysis. The resulting PCR products (up- and downregulated genes) were spotted onto a nylon membrane. This macroarray screen demonstrated that 2.1% of the spotted genes were downregulated, whereas 14.6% were upregulated in the glandular stomachs of CA-AhR mice. Among the genes that were differentially regulated in the CA-AhR mouse stomach, decreased expression of the gene encoding osteopontin (OPN) was observed (Figure 1a). This observation was especially intriguing given previous reports indicating a positive association of elevated OPN expression and tumor progression (Brown *et al*., 1994; Ue *et al*., 1998; Furger *et al*., 2001; Agrawal *et al*., 2002). We therefore focused our analysis on a possible link between OPN mRNA expression and stomach tumourigenesis in the CA-AhR mice.

Analysis by RT-PCR of gene expression in the glandular stomach of male mice of different ages demonstrated expression of CA-AhR at 7 days of age (Figure 1b). As a positive control, the established Ah receptor target gene CYP1A1 was found to be upregulated on the resulting filter (Figure 1a) and shown to be induced in CA-AhR mice from 22 days of age (Figure 1b), confirming the transcriptional activity of the CA-AhR. An obvious decrease in OPN expression was observed in the glandular stomach of CA-AhR mice of 58 days of age. Interestingly, despite the CA-AhR being expressed in the glandular stomach expression of CA-AhR throughout postnatal development, a significant decrease in OPN mRNA expression levels was not seen before the mice reached sexual maturity (Figure 1b).

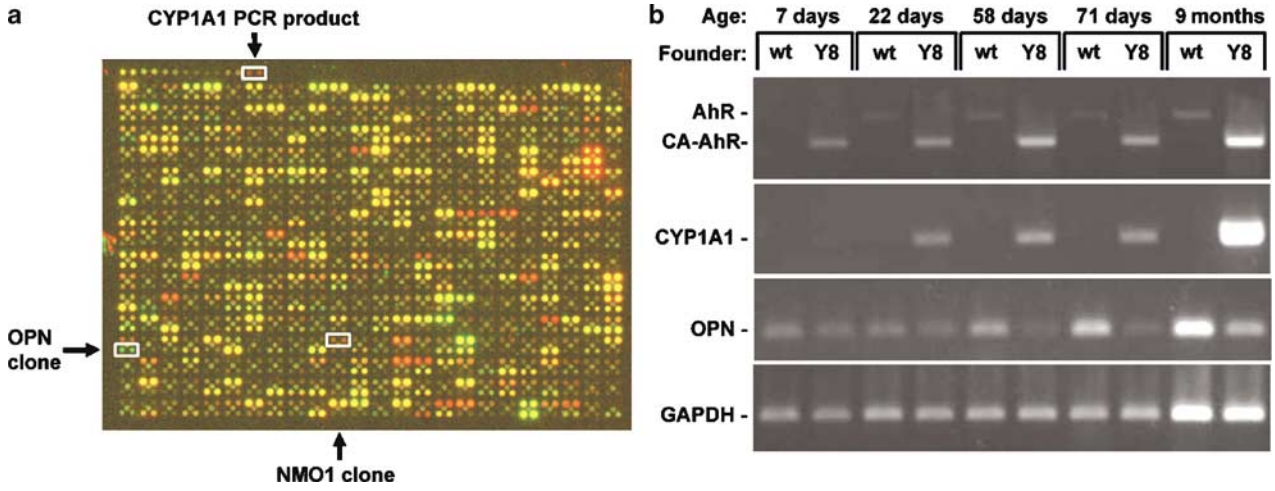

Figure 1 Differentially expressed genes in glandular stomach of CA-AhR mice. (**a**) CA-AhR mice (Andersson *et al*., 2002) bred to homozygosity and wt control animals were of the same mixed genetic background. The glandular part of mouse stomachs was removed and snap-frozen in liquid nitrogen. Total RNA was prepared by tissue homogenization in a guanidinium thiocyanate buffer, followed by $CsCl_2$-gradient centrifugation (Sambrook *et al*., 1989). Poly-(A) RNA was isolated from total RNA using oligo-(dT)-coupled magnetic beads (Dynal Biotech). cDNA synthesis and SSH were performed as previously described (von Stein, 2001). Subtraction efficiency was evaluated by monitoring the expression of a number of housekeeping genes and genes that are known to show differential expression in the glandular stomach of CA-AhR mice (e.g. CYP1A1). Amplified subtracted cDNA was ligated into pSPORT1 vector (Gibco BRL), and the resulting library was transformed into MAX Efficiency DH5α competent *Escherichia coli* cells (Invitrogen). Approximately 4000 bacterial clones from each subtracted library were plated out on LB plates containing 1.5% agar and 100 μg/ml ampicillin. In all, 384 clones were picked from each of the up- and down-subtracted libraries and inoculated in 384 well plates into LB medium containing 100 μg/ml ampicillin using the BioPick machine (BioRobotics, Cambridge, UK). These cultures were used for PCR performed with pSPORT1 polylinker-matched primers in 384-well PCR plates in a volume of 20 μl per sample. The resulting PCR products of up- and downregulated genes were spotted in duplicates onto on Hybond N+ membrane (Amersham) using the MicroGrid II machine (BioRobotics, Cambridge, UK). From such a filter, several replicas were made and used for hybridization with different ^{33}P-labeled cDNA probes as described (Church and Gilbert, 1984). Finally, filters were subjected to phosphoimager analysis and signals were analysed by the ArrayVision software version 6.0 (Imaging Research Inc.). Candidate clones were sequenced (MWG Biotech) and analysed by NCBI BLAST. In all, 14.6% and 2.1% of spotted genes were found up- or downregulated on the processed filter, respectively. One of the downregulated sequenced clones (double spots enclosed in white rectangle frame) contained mouse gene encoding OPN. Upregulated CYP1A1 and NMO1 clones are also marked by white rectangle frames. (**b**) RT-PCR analysis of OPN, CA-AhR and CYP1A1 mRNA expression in the glandular stomach of 7, 22, 58, 71 days and 9-months-old male wt and CA-AhR (Y8) mice. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as input control. First-strand cDNA was synthesized as described (von Stein, 2001) using 2 μg total RNA as a template. The sequences of the gene-specific PCR primers with primer annealing temperature and the size of the amplified fragment, were as follows: OPN, 5′-CCACAGATGAGGACCTCACCTC-3′, 5′-CCTTGTGGCTGTGAAACTTGTG-3′ (54.5°C, 282 bp); CA-AhR, 5′-GCAAT GAATTTGGAAGGGAG-3′, 5′-GGCAAGCCGTGTTCAGCAAA-3′ (55°C, 688 bp), CYP1A1, 5′-CAGGATGTGTCTGGT TACTTTGAC-3′, 5′-CTGGGCTACACAAGACTCTGTCTC-3′ (55°C, 331 bp), GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTA-3′ (53°C, 451 bp). PCR products were analysed on ethidium bromide-stained agarose gels and visualized by UV light

Extended studies of target gene expression by RNA blot analysis confirmed the distinct upregulation of the Ah receptor target genes CYP1A1 and NAD[P]H:menadione oxidoreductase 1 (NMO1) in two independent founder lines of male CA-AhR mice (Figure 2a). OPN mRNA expression levels were decreased to 21.5 and 30.8% relative to the wt levels in the Y8 and A3 founder lines, respectively (Figure 2a, b). A decrease in OPN mRNA expression levels down to approximately 10% of wt levels was also observed in 9- months-old Y8 CA-AhR male mice compared to age-matched control animals (Figure 2a, b).

Other organs previously shown to express the CA-AhR (Andersson *et al*., 2002) were analysed for OPN expression. As reported previously (Brown *et al*., 1992), OPN was expressed at high levels in kidney and was also detected in the liver, lung and brain (Figure 3a). Consistent with the decrease observed in the glandular stomach, OPN expression levels seemed to be reduced also in these organs of CA-AhR mice (Figure 3b).

The present experiments demonstrate that the activated dioxin receptor/ARNT complex induces downregulation of OPN mRNA levels, thus establishing OPN as a potential target gene of the receptor. A bioinformatic analysis (MatInspector, v. 7.3.1 Genomatix Software GmbH, 2004) revealed that the mouse OPN gene contains two putative xenobiotic response element (XRE) motifs that are recognized by the dioxin receptor/ARNT complex. As illustrated in Figure 2c, one of these XREs is located at position −2342 upstream from the reported (Craig and Denhardt, 1991) 5′-distal transcriptional start site (TSS). The other putative XRE was detected in the second intron of the OPN gene at position +316 relative to the described (Miyazaki *et al*., 1990) proximal TSS (Figure 2c). XRE motifs are also found at similar positions in the human OPN gene (Figure 2c). Promoter analysis experiments are now required to investigate whether OPN represents a primary target gene of the activated dioxin receptor. Alternatively, dysregulation of OPN may represent a further downstream event following activation of the dioxin receptor, for example, by receptor-mediated negative regulation of the activity of the c-*jun*/c-*fos* complex (AP-1; Gillesby *et al*., 1997; Suh *et al*., 2002). AP-1 has previously been shown to activate the OPN promoter (Bidder *et al*., 2002; Denhardt *et al*., 2003; Renault *et al*., 2003). As discussed below, in either scenario it will be important to establish a causal link between dysregulation of OPN expression and stomach tumorigenesis.

In the human gastric mucosa, OPN is expressed in mucous and chief cells and has been suggested to have a barrier function and/or participate in host defense against pathogens (Qu *et al*., 1997). Double-staining using antibodies against OPN and intrinsic factor (IF) as a marker for chief cells showed co-localization of these proteins (Figure 4a–e). In contrast to the expression of OPN in the antrum region, which appeared to be unaffected in CA-AhR mice, reduced OPN mRNA expression levels seemed to be reflected by a decrease in OPN protein levels in the corpus region of the stomach

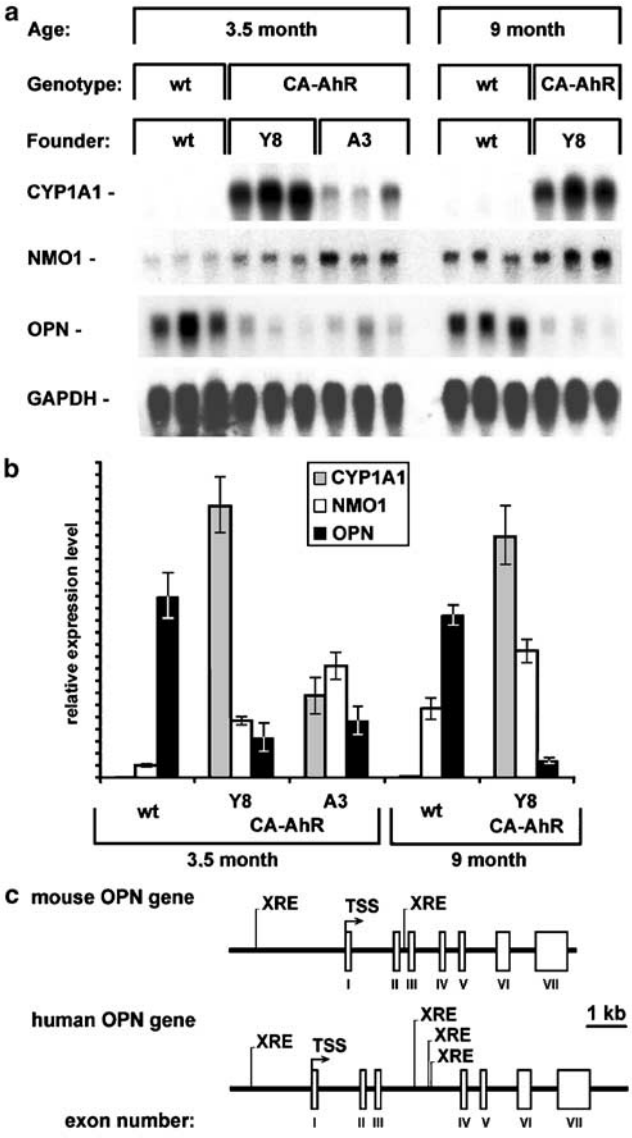

Figure 2 Differentially expressed genes in glandular stomach of CA-AhR mice. (**a**) RNA blot analysis showing expression of following genes: CYP1A1, NMO1, OPN, and GAPDH as loading control in male mice (3.5 and 9 months old) from wt and CA-AhR mice from two independent founder lines (Y8 and A3). Expression level in three individual mice from each group is shown. In all, 30 μg of total RNA per sample was used for RNA blot analysis performed by standard methods as described previously (Andersson *et al*., 2002). (**b**) Quantification of the OPN mRNA expression shown in (**a**), normalized against expression of the house-keeping gene GAPDH. The error bars represent standard deviation. (**c**) Scheme of human and mouse OPN genes. Indicated are dioxin/ARNT complex binding sites which could function as XREs. TSS is designated by an arrow. A bioinformatic analysis for the search of potential transcription factor-binding sites was performed by using MatInspector Release professional v. 7.3.1, October 2004 (Genomatix Software GmbH 1998–2004). The data were confirmed by using the ConSite program with threshold of 0.9 for binding site probability (transcription factor site prediction using phylogenetic footprinting, public service at the Center for Genomics and Bioinformatics, Karolinska Institute)

(data not shown). Interestingly, the stomach tumours observed in the CA-AhR mice always originated in the basal regions of the corpus mucosa, while the antrum

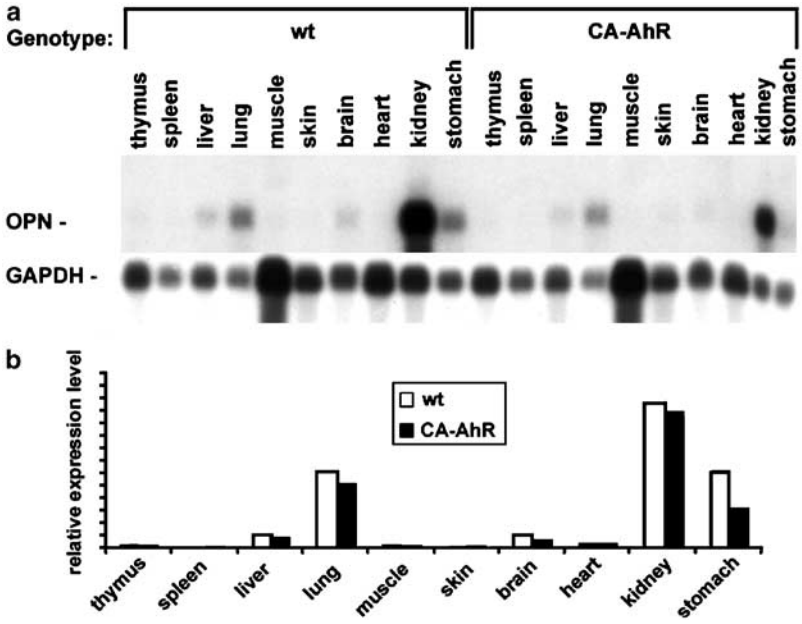

Figure 3 Decreased OPN expression in several tissues of the CA-AhR mice. (**a**) RNA blot analysis (2 μg poly-A RNA) of OPN expression in organs from 8-months-old female wt and CA-AhR mice. Expression of GAPDH is shown as a loading control. (**b**) Bar graph showing OPN expression in wt and CA-AhR tissues normalized against GAPDH

region was unaffected (Andersson *et al*., unpublished data), demonstrating a striking spatial correlation between diminished OPN expression and tumour development. Although the overall OPN mRNA expression levels in glandular stomachs of CA-AhR mice were decreased, some tumour structures showed very strong OPN protein expression (Figure 4f–d). In some instances, this increase in OPN mRNA expression appeared to be associated with a beginning invasion through the muscularis mucosa (Figure 4d).

OPN is also called Eta-1 (for Early T lymphocyte activation-1) factor and is expressed in activated T cells and macrophages, playing an important role in cell-mediated (type-1) immunity (Ashkar *et al*., 2000; Denhardt *et al*., 2001b). Mice lacking OPN show decreased resistance to viral (*Herpes simplex*) and bacterial infections (*Listeria monocytogenes*) (Ashkar *et al*., 2000). Moreover, mice overexpressing OPN have an enhanced population of peritoneal B1 cells (Iizuka *et al*., 1998), cells that are important members of the innate immune system against infectious agents (Baumgarth *et al*., 1999, 2000; Boes *et al*., 1998; Ochsenbein *et al*., 1999; Paciorkowski *et al*., 2000). Consistent with these observations, the peritoneal B1 cell population is reduced in CA-AhR mice (Andersson *et al*., 2003), and it is tempting to speculate that decreased expression of OPN in the CA-AhR mice may be linked to the observed decrease in B1 cells.

OPN is involved in several different physiological and pathological processes, such as bone remodeling, tissue injury (Denhardt and Noda, 1998), arthritis (Yumoto *et al*., 2002) as well as cell attachment and migration via binding to cell surface receptors such as CD44 and integrins (e.g. $\alpha_v\beta_3$) (Denhardt *et al*., 2001a, b). Several studies have reported an association between increased expression of OPN and malignant and metastatic progression of several different human tumours, including cancers of the breast, lung, bladder, kidney (Brown *et al*., 1994; Furger *et al*., 2001), as well as colon (Agrawal *et al*., 2002) and stomach (Ue *et al*., 1998). Considering an important promoting role in immunological responses and that OPN recently has been identified as a target gene for p53 (Morimoto *et al*., 2002), it is possible that an increase in OPN expression is associated with elevated tumorigenicity, probably reflecting an adaptive response to a cellular insult.

The decreased OPN expression observed in stomach tumours of CA-AhR mice apparently contradicts the positive association between OPN expression and tumour progression observed by others. However, the use of OPN-deficient mice in a chemically induced skin squamous cell carcinoma assay showed that these mice demonstrated accelerated tumour growth and progression, with a greater number of metastases (Crawford *et al*., 1998). Moreover, targeted inhibition of OPN expression in mammary epithelial cells results in abnormal development of mammary glands and increased invasiveness both *in vivo* and *in vitro* (Nemir *et al*., 2000). As summarized in a recent review (Rittling and Chambers, 2004), various OPN results suggest a complex role of OPN in tumour development that may be affected by multiple parameters, including, for instance, tumour type and experimental model system, that in turn may be mediated by differential post-translational modifications and/or protein processing of OPN from different sources and by other factors within the tumour microenvironment.

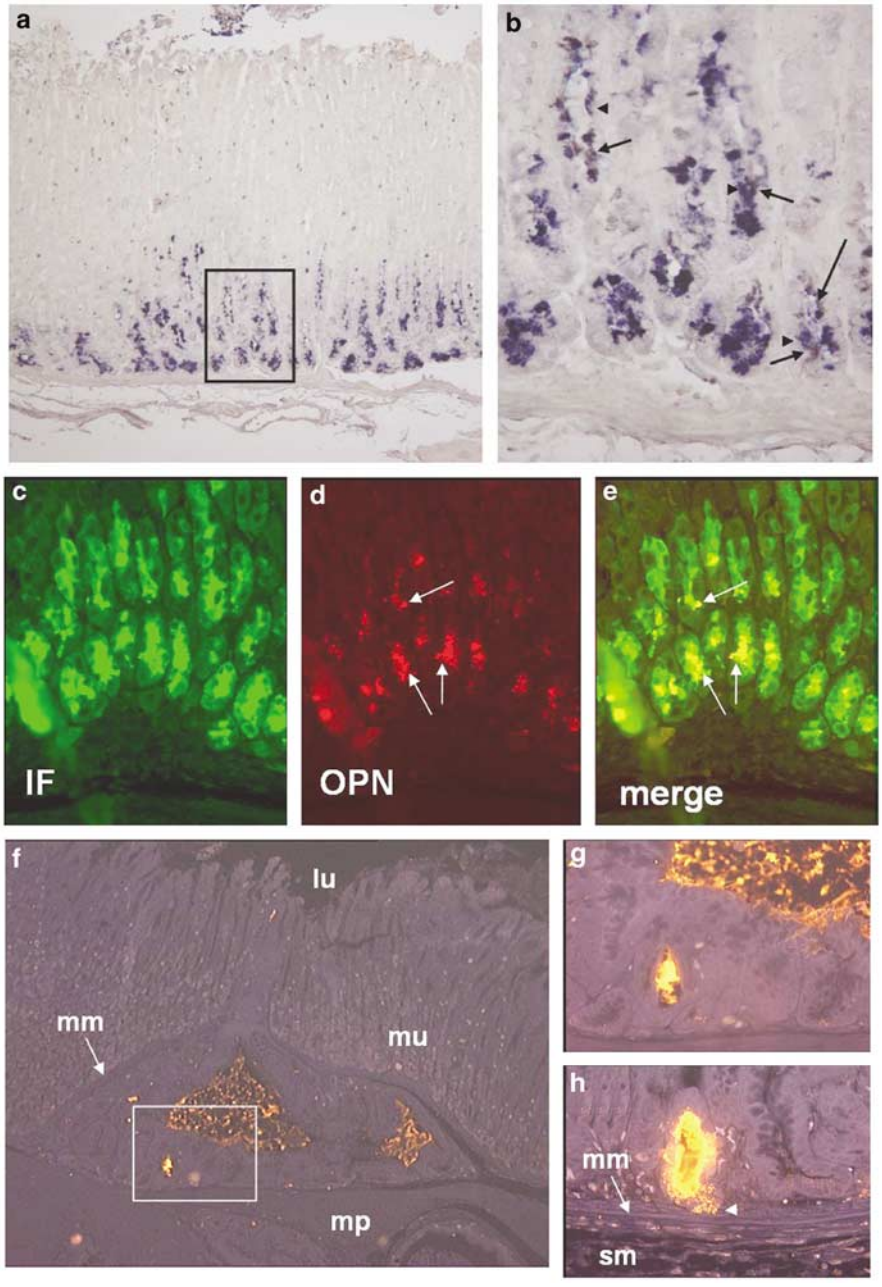

Figure 4 OPN expression in gastric chief cells and in CA-AhR stomach tumors. (**a, b**) Section of mouse stomach stained with antibodies against the chief cell marker IF (dark blue, arrowheads) and OPN (brown; arrows) demonstrates its expression in the basal, chief cell region of the gastric mucosa. (**b**) A magnification of the rectangle area in (**a**). (**c**–**e**). The magnified stomach chief cell region stained with antibodies against IF (green) and OPN (red, arrows) demonstrates co-expression of these proteins (**e**; yellow and arrows). (**f**, **g**) Section of a stomach tumor from a 6-months-old female CA-AhR mouse (Y8 founder line) demonstrates distinct OPN staining (yellow) in both discrete chief cell glands and in excretions into the cavity formed by the tumor (rectangle area in F is magnified in G; lu, stomach lumen; mu, mucosa; mm, muscularis mucosa; mp, muscularis propria). (**h**) Section of a stomach from a 3-months-old female CA-AhR mouse (A3 founder line) demonstrates distinct, focal OPN staining in a gland that appears to adhere closely to the muscularis mucosa (mm, arrow), possibly preceding invasion into the submucosa (sm). Formalin-fixed tissue samples were dehydrated, embedded in paraffin and cut into 4-μm-thick sections. Double-staining of OPN and IF was performed as follows: sections were de-paraffined, rehydrated, and heat-treated for 15 min in Tris–EDTA (pH 9.0). Endogenous peroxidase was blocked in 3.0% H_2O_2 solution in methanol for 15 min. After washing in 1 × PBS, 0.3% Tween-20, sections were incubated in 1 × PBS, 1.0% BSA, 0.5% Tween-20 and then with goat-anti-OPN (Santa Cruz Biotech) and rabbit-anti-human IF (a kind gift from Dr D Alpers, Washington Univ., St Louis, USA) and secondary antibodies (HRP-conjugated anti-goat, Santa Cruz; alkaline phosphatase-conjugated anti-rabbit or Cy2-conjugated anti-rabbit from DAKO) in 1 × PBS, 0.5% BSA, 0.25% Tween-20. All washing steps were carried out in 1 × PBS, 0.3% Tween-20. Diaminobenzidine and NBT/BCIP (Sigma) were used as chromogen substrates. The TCS-rhodamine amplification system (Perkin-Elmer) was used for HRP-mediated fluorescent visualization of OPN

The present data demonstrate that the OPN gene represents a previously unknown gene that is negatively regulated by the activated dioxin receptor/ARNT complex. The dioxin receptor is not only activated by environmental pollutants such as dioxins, but also by compounds derived from the diet, most notably indolo[3,2-b]carbazole, which is formed from dietary indole precursors in the acidic environment of the stomach (Kleman *et al*., 1994). Under physiological conditions, the dioxin receptor may therefore regulate OPN expression in response to cues other than environmental pollutants, for example, dietary receptor ligands, whereas dysregulation of OPN gene in the stomach may contribute to the development of gastric tumours. The CA-AhR model now allows us to further investigate the potential role of OPN in gastric tumour development.

Abbreviations

CYP1A1, cytochrome *P*450 1A1; CA-AhR, constitutively active aryl hydrocarbon receptor; ARNT, arylhydrocarbon receptor nuclear translocator; NMO1, NAD[P]H:menadione oxidoreductase 1; OPN, osteopontin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; XRE, xenobiotic response element; SSH, suppression subtractive hybridization.

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