Cross-talk of anosmin-1, the protein implicated in X-linked Kallmann's syndrome, with heparan sulphate and urokinase-type plasminogen activator

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Defective function of anosmin-1, the protein encoded by *KAL-1*, underlies X-linked Kallmann's syndrome (X-KS), a human hereditary developmental disorder. Anosmin-1 appears to play a role in neurite outgrowth and axon branching, although molecular mechanisms of its action are still unknown. Anosmin-1 contains a WAP (whey acidic protein-like) domain and four contiguous FnIII (fibronectin-like type III) repeats; its WAP domain shows similarity to known serine protease inhibitors, whereas the FnIII domains contain HS (heparan sulphate)-binding sequences. To investigate the functional role of these domains, we have generated both wild-type and mutant recombinant anosmin-1 proteins using a *Drosophila* S2 cell expression system. Here we present the first biochemical evidence demonstrating the high-binding affinity between HS and anosmin-1, as measured by SPR (surface plasmon resonance) ($K_d = 2$ nM). The FnIII domains, particularly the first, are essential for dose-dependent HS binding and HS-mediated cell surface association. Furthermore, we have identified uPA (uro-

kinase-type plasminogen activator) as an anosmin-1 interactant. Anosmin-1 significantly enhances the amidolytic activity of uPA *in vitro*; and anosmin-1–HS–uPA co-operation induces cell proliferation in the PC-3 prostate carcinoma cell line. Both the HS interaction and an intact WAP domain are required for the mitogenic activity of anosmin-1. These effects appear to be mediated by a direct protein interaction between anosmin-1 and uPA, since anosmin-1–uPA could be co-immunoprecipitated from PC-3 cell lysates, and their direct binding with high affinity ($K_d = 6.91$ nM) was demonstrated by SPR. We thus propose that anosmin-1 may modulate the catalytic activity of uPA and its signalling pathway, whereas HS determines cell surface localization of the anosmin-1–uPA complex.

Key words: anosmin-1, fibronectin-like type III domain, heparan sulphate, Kallmann's syndrome, serine protease inhibitor, whey acidic protein-like domain (WAP domain).

INTRODUCTION

Anosmin-1, the modular protein encoded by the *KAL-1* gene, was the first identified molecule implicated in the pathogenesis of X-linked Kallmann's syndrome (X-KS). X-KS is characterized by anosmia (loss of smell) and hypogonadotrophic hypogonadism, due to a rostral telencephalic developmental defect resulting in olfactory bulb (OB) dysgenesis and failed migration of GnRH (gonadotrophin-releasing hormone) neurons from the olfactory placode to the septo-preoptic hypothalamus. Additional phenotypic features include mirror movements (bimanual synkinesis) and unilateral renal agenesis [1,2]. Studies have revealed the biological functions of anosmin-1 during neurogenesis. Anosmin-1 is able to induce neurite outgrowth and axon branching and act as an extracellular adhesion molecule for neuronal and kidney epithelial cells [3–5]. However, the molecular mechanisms underlying the link between anosmin-1 and olfactory system development and kidney morphogenesis are largely unknown.

Structurally, anosmin-1 comprises an N-terminal signal peptide, a CR (cysteine-rich) region, a WAP (whey acidic protein-like) four-disulphide core motif and four contiguous FnIII (fibronectin-like type III) domains, followed by a histidine-rich C-terminus. Using protein sequence alignment from invertebrates (*Drosophila melanogaster*, *Caenorhabditis elegans*) to vertebrates (*Gallus gallus*, *Brachydanio rerio*, *Homo sapiens*), the most highly conserved region of anosmin-1 was found to be the N-terminal WAP and the first FnIII domain, suggesting a fundamental functional role of these two regions [6]. Transgenic studies using *Kal-1* over-expression in *C. elegans* have provided evidence that these two

domains individually or collectively direct axonal branching and the mis-routing activity of sensory neurons [3]. The characterization and identification of the domain-specific interactants of anosmin-1 may thus help unravel the molecular mechanisms of anosmin-1 function.

Based on amino acid sequence comparison and molecular modelling studies, FnIII domains have been predicted to interact with HS (heparan sulphate) via their large positively charged basic surface. HS comprises disaccharide repeat units of alternating glucuronic acid or iduronic acid and glucosamine residues, with variable sulphation patterns responsible for the many binding and regulatory properties of HS towards proteins [7]. There are five predicted HS-binding sequence motifs within or among the individual FnIII domains of anosmin-1 [8]; three loss-of-function missense mutations [N267K (Asn²⁶⁷ → Lys), E514K, F517L] in the first and third FnIII domains have been found in X-KS patients. All these three residue substitutions are in close proximity to the large basic surface, potentially interfering with binding to HS [8]. Furthermore, cell adhesion assays using a 32-amino-acid peptide have implicated the first FnIII domain as the dominant HS-binding site [5]. The importance of the FnIII-domain–HS interaction is further supported by the observations that the absence of either 6-O-sulphotransferase or C5 epimerase (required for HS 6-O-sulphation or conversion of glucuronic acid unit to iduronic acid) virtually abolishes *Kal-1*-induced phenotypes in *C. elegans* [9]. However, the binding characteristics and kinetics between the C-terminal FnIII domain of anosmin-1 and HS have not hitherto been systematically investigated.

Abbreviations used: CR, cysteine-rich; ECM, extracellular matrix; FGF2, fibroblast growth factor 2; FGFR1, fibroblast growth factor receptor 1; FnIII, fibronectin-like type III; GFP, green fluorescent protein; GnRH, gonadotrophin-releasing hormone; HS, heparan sulphate; OB, olfactory bulb; SLPI, secretory leucocyte proteinase inhibitor; SPR, surface plasmon resonance; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; WAP domain, whey acidic protein-like domain; X-KS, X-linked Kallmann's syndrome; N267K etc., Asn²⁶⁷ → Lys etc.

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Figure 1 Structure of recombinant anosmin-1

The functional domains represented are: CR region, WAP domain, FnIII domains, H (histidine-rich region), V5 (the V5 epitope tag derived from simian virus 5) and 6His (His₆) tag. The missense mutation (C172R) within the WAP domain present in mPIWF4¹⁷² and mPIWF1¹⁷² is indicated by the star.

On the other hand, the N-terminal WAP domain contains eight conserved cysteine residues forming four intramolecular disulphide bonds. This four-disulphide core motif is common to the serine protease inhibitors of the WAP protein family, including elafin, SLPI (secretory leucocyte proteinase inhibitor), PS20 and WDNM1 [10,11]. Elafin and SLPI are well-defined serine protease inhibitors acting on elastase, chymotrypsin and trypsin. The three-dimensional structures of elafin and SLPI have been revealed by X-ray crystallography in complexes with serine proteases. The active centre of these proteins contain a highly variable region, V, and a conserved methionine residue in the P₁' position [12,13]. Members of WAP protein family play important roles in cell growth control, differentiation and tissue remodelling through inhibition of protease activity [14]. For example, elafin inhibits pulmonary artery smooth muscle cell proliferation by inhibiting elastase-mediated proteolytic release of the mitogen FGF2 (fibroblast growth factor 2) from ECM (extracellular matrix) [15]. PS20, a WAP protein with a 31.2% amino acid sequence similarity to anosmin-1, has been shown to inhibit proliferation of prostate carcinoma PC-3 cells [14]. The functional importance of the WAP domain of anosmin-1 is supported by an X-KS pedigree study where three affected brothers shared a C172R missense mutation, which is predicted to disrupt a highly conserved Cys¹⁵⁷–Cys¹⁷² intramolecular disulphide bond [1].

We hypothesized that anosmin-1 may interact with and regulate a cellular serine protease to exert its biological function, and sought to identify a WAP-domain-interacting molecule. Firstly, we reviewed the spatio-temporal expression patterns of several known serine proteases in the developing OB where anosmin-1 may be functionally active. Several serine proteases are expressed, including uPA (urokinase-type plasminogen activator), tPA (tissue-type plasminogen activator) and thrombin [16–18]. Of these, early expression of uPA has also been observed in spinal cord (by embryonic day 12.5) and kidney epithelial cells [19,20]. uPA has been reported to influence cell proliferation, tissue remodelling, synaptogenesis and neurite outgrowth through the pericellular conversion of the zymogen plasminogen to plasmin, which in turn catalyses the activation of the pro-matrix metalloproteinases. It can also act via a proteolysis-independent mechanism [21,22]. uPA was thus investigated as a putative serine protease candidate ligand for the N-terminus of anosmin-1.

In the present study, we generated a series of recombinant anosmin-1 proteins containing various numbers of FnIII domains, namely PIW, PIWF1, PIWF2, PIWF3 and PIWF4, together with two corresponding C172R mutants (mPIWF4¹⁷² and mPIWF1¹⁷²) (see Figure 1). We showed that the FnIII domains, notably the

first, are endowed with high HS-binding affinity ($K_d = 2$ nM), and that anosmin-1 unexpectedly and specifically enhances uPA amidolytic activity in a dose-dependent manner, but had no effect on thrombin amidolytic activity. Moreover, in a human PC-3 prostate cancer cell line known to express an elevated level of uPA as well as HS, anosmin-1 showed a mitogenic effect dependent on HS, uPA and the intact WAP domain, and directly formed a protein complex with uPA. The direct binding between anosmin-1 and uPA was demonstrated by SPR (surface plasmon resonance). Taken together, our data demonstrated a novel growth-promoting function of anosmin-1 through its interaction with uPA and regulation of uPA's enzymic activity.

EXPERIMENTAL

Generation of recombinant anosmin-1

KAL-1 cDNA encoding anosmin-1 without its signal peptide sequence was amplified by PCR, using the 5'-CCGGATCCGCGCCCGGCCCGGCGCGGCTGCT-3' forward oligonucleotide primer with a 5' *Bam*HI restriction site. The reverse primers were complementary to the 3' end of either the full coding sequence (PIWF4), or were designed to amplify truncated fragments of anosmin-1 comprising the N-terminal CR and WAP domain (PIW), followed by variable numbers of FnIII repeats (PIWF1, PIWF2 and PIWF3) with a 3' *Xba*I site (Figure 1). The reverse primer sequences were as follows: PIW, 5'-CCTCTAGAACCTTTGTACAGAGTCTTGGGTACTTG-3'; PIWF1, 5'-CCTCTAGAGGCA-GATGGATCTTTGGAAGAACGGAA-3'; PIWF2, 5'-CCTC-TAGAGGTTGCATGTGTCGATGTGAAGTGAAG-3'; PIWF3, 5'-CCTCTAGACTTAAGAGCAGAGCATGGTGGAGTAGT-3'; PIWF4, 5'-CCTCTAGAGTATCTTTCTGGAGAAGGCTTGTA-ATG-3'.

PCR was performed with an annealing temperature at 55 °C and the amplified cDNA fragments were subcloned into a *Drosophila* expression vector PMT/BiP/V5/6His (Invitrogen), which incorporated *Drosophila* secretory signal at 5' end and the V5 plus His₆ epitopes at 3' end. The QuikChange™ site-directed mutagenesis kit (Stratagene) was used to introduce the C172R substitution in mPIWF1¹⁷² and mPIWF4¹⁷² mutant constructs. For expression of these constructs, *Drosophila* S2 cells seeded at 1 × 10⁶ cells/ml in DES™ expression medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum, and antibiotics were transfected using a calcium phosphate kit (Invitrogen). At 24 h post-transfection, transient expression of the desired protein was induced by addition of 500 μM CuSO₄, and the conditioned medium was collected after 48 h. For protein

expression in the absence of serum, transfected cells were weaned into serum-free medium before induction. Cell membrane-associated proteins were extracted using 350 mM NaCl in serum-free medium. To generate stable cell lines, empty vector or vectors expressing PIWF4, PIWF1, their corresponding C172R mutants and GFP (green fluorescent protein) were co-transfected into S2 cells with pCoHygro plasmid (Invitrogen). Hygromycin resistant transfectants were selected with 700 $\mu\text{g/ml}$ hygromycin-B (Invitrogen) and subclones showing the highest expression efficiencies were weaned into serum-free medium. After induction, salt extracts were collected, mixed with 50% Ni-NTA (Ni²⁺-nitrilotriacetate) agarose (Qiagen) and subjected to Ni-NTA affinity chromatography. Proteins were eluted from the beads with 250 mM imidazole and dialysed against PBS. Protein concentration was estimated by the Bradford assay (Bio-Rad).

For Western blot analysis, proteins generated in conditioned medium, salt extract and cell lysate in Laemmli sample buffer were separated by SDS/PAGE and probed with anti-His monoclonal antibody as described previously [8].

Generation of polyclonal antibody against anosmin-1

Purified PIWF1 protein was used to generate specific polyclonal anti-anosmin-1 antibody (AIWF1) in rabbits (CN Bioscience). The sensitivity and specificity of generated antibody was confirmed by ELISA and Western blotting using purified PIWF1 and PIWF4. Antibody was then purified with Protein G–Sepharose affinity chromatography using a MAB Trap Kit (Amersham Pharmacia).

HS competition assay using *Drosophila* S2 cells

Purified HS of bovine kidney origin (Sigma, H7640) was added to serum-free medium at 100 $\mu\text{g/ml}$ simultaneously with the CuSO₄ induction.

Solid-phase ELISA

HS dissolved in 0.1 M Mes (Sigma), pH 5.6, at 2.5 mg/ml, was thoroughly mixed with 2.5 mM EZ-linked biotin-hydrazine (Pierce) and 0.5 mM EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride] (Sigma) for 24 h. The mixture was dialysed against distilled water to remove untreated biotin, freeze-dried and re-dissolved in 0.3 M NaCl. Immunlon 4HBX plates with 96 microtitre wells (Thermo Labsystems) were coated with anosmin-1 and BSA at the appropriate concentrations before biotin-conjugated HS (12.5 $\mu\text{g/ml}$) was added and incubated for 18 h at 4 °C. Wells were rinsed with TBS (Tris-buffered saline) and alkaline phosphatase-conjugated avidin (Sigma) was added prior to incubation at 37 °C. Wells were then rinsed thoroughly, followed by incubation with alkaline phosphatase substrate. Absorbance was measured at 405 nm.

Kinetic measurement using SPR

A Biocore 3000 surface plasmon response-based biosensor (BIAcore AB) was used to quantify binding parameters for the interaction between anosmin-1 and immobilized biotin conjugated heparin (Calbiochem). A streptavidin sensor chip (Biosensor AB) was coupled with biotinylated heparin (1 $\mu\text{g/ml}$) in HEPES-buffered saline [10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, pH 7.4, containing 0.005% (v/v) P-20] at a flow rate of 5 $\mu\text{l/min}$, giving low levels of immobilized ligand (200 resonance units) to minimize mass transfer effects. Three flow cells were coupled and one flow cell contained a blank sensor chip to serve as a reference surface. The bulk shift due to changes in refractive index

was measured using a reference surface and was subtracted from the binding signal to correct for non-specific signals. Purified proteins at six different concentrations were injected at a 5 $\mu\text{l/min}$ flow rate to ensure recovery of data prior to curve fitting. Between binding cycles, the coated chip was regenerated by a 2 M NaCl injection. For the measurement of anosmin-1–uPA binding, human high-molecular-mass uPA (American Diagnostic) at 20 $\mu\text{g/ml}$ in 10 mM sodium acetate buffer, pH 5.0, was covalently immobilized by amine coupling to a CM5-certified grade sensor chip (Biosensor AB) pre-activated with NHS/EDC [*N*-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride]. Coupling was achieved by injection of uPA at a flow rate of 5 $\mu\text{l/min}$ for 6 min, resulting in a coupling yield of 5000 resonance units. Unreacted groups were blocked with 1 M ethanolamine, pH 8.5. A flow cell without protein coupling, but with the same treatment of activation and blocking, served as a control. Binding was measured at a flow rate of 5 $\mu\text{l/min}$ using different concentrations of PIWF1 and PIWF4 dissolved in HEPES-buffered saline, followed by dissociation for 10 min. Sensor chips were regenerated at the end of each run by injection of 0.1 M acetic acid containing 0.5 M NaCl. For the analysis of anosmin-1 and uPA binding specificity, 250 nM PIWF1 was pre-incubated with 0, 60, 120 or 300 nM uPA prior to injection. The set of sensorgrams and kinetic parameters were analysed using BIA Evaluation software version 3.0.

uPA and thrombin amidolytic activity assay

uPA and thrombin activities were quantified in PBS containing 20 mM Tris/HCl, pH 7.5, using the specific chromogenic substrate S2366 for thrombin and S2444 (Chromogenix) for uPA. Triplicates of anosmin-1 were pre-incubated with 0.8 nM thrombin and 1 nM uPA in the absence and presence of 10 $\mu\text{g/ml}$ HS at 25 °C for 15 min. Specific substrates were then added for a further incubation at 37 °C and substrate hydrolysis was measured at A_{405} at different time points. Residual amidolytic activity of proteases was calculated as the ratio between the initial reaction velocity with anosmin-1 treatment and that of a control group.

PC-3 cell proliferation assay

To assess cell proliferation, Cell Titer 96 AQ_{ueous} non-radioactive cell proliferation kit (Promega) was used according to the manufacturer's instructions. Briefly, 5×10^3 PC-3 cells were seeded in 96-well flat bottom microtitre plates (Nunc) in Coon's modified Ham's F12 medium (Sigma) containing 7% fetal bovine serum and antibiotics. The cells were serum starved for 24 h before treatment with recombinant anosmin-1 in the presence or absence of heparinase III, HS, polyclonal antibodies against PIWF1 and uPA or non-specific rabbit anti-sera (Santa Cruz Biotechnology). After 48 h, the D_{492} was measured. Each experiment was performed in five replicates and repeated more than twice. The culture without any treatment was used as a negative control. Effects of different treatments were measured as the percentage absorbance compared with that of the control group set to 100%. To prove that this assay directly correlates with the cell proliferation, actual cell counting was also performed after treatment with 10 nM PIWF1, PIWF4 and GFP proteins. Trypsinized cells were resuspended and intact cells, determined by Trypan Blue exclusion, were counted using a haemocytometer.

Immunocytochemistry

To visualize cell surface association of the recombinant anosmin-1, immunocytochemistry using anti-PIWF1 antibody was performed under non-permeabilizing conditions. PC-3 cells were cultured in serum-free medium for 48 h in a chamber slide with

Figure 2 Western blot analysis of recombinant anosmin-1 in *Drosophila* S2 cells

(a) Transient expression of recombinant anosmin-1 was induced by 500 μM CuSO₄ in 10% serum-containing medium. Recombinant anosmin-1 proteins present in cell lysate (L), salt extract (S) and conditioned medium (M) were identified using an anti-His monoclonal antibody. The molecular-mass-markers are shown. Transiently transfected *Drosophila* S2 cells in serum-free medium culture were induced to express proteins in the absence (b) and presence (c) of 100 μg/ml HS. (d) PIWF1 and PIWF4 proteins generated from stable *Drosophila* S2 cell line in serum-free medium were analysed in presence (+) or absence (-) of HS in comparison with vector (control), GFP, mPIWF1¹⁷² and mPIWF4¹⁷².

10 or 100 nM PIWF1 in the presence and absence of 100 μg/ml exogenous HS. Cells were then rinsed with PBS and blocked in BSA for 30 min before overnight incubation with anti-PIWF1 antibody, followed by anti-rabbit Texas Red fluorescent antibody (Jackson). Cells were fixed in 2% paraformaldehyde/2% glutaraldehyde, rinsed and put on to coverslips in Vectashield (DBA), and the slides were viewed using a Zeiss Axioplan microscope.

Immunoprecipitation

After 48 h incubation in serum-free medium, 6 × 10⁶ PC-3 cells were scraped and lysed in 0.5 ml lysis buffer [50 mM Tris/HCl, 350 mM NaCl, 1% Triton X-100, 1% Nonidet P40, 1 mM EDTA, 1 mM EGTA, pH 7.4, protease inhibitor cocktail (Boehringer Mannheim) and 0.02% NaN₃] at 4 °C for 30 min. The cell lysate was then incubated with 1 μg of PIWF1 at 4 °C for 2 h before immunoprecipitation with 5 μg of polyclonal or monoclonal anti-uPA antibody (American Diagnostics) and anti-anosmin-1 antibody (AIWF1). Precipitated protein complexes captured on Protein A beads were separated by SDS/PAGE and probed with anti-uPA and anti-His antibodies.

Statistical analysis

The results are expressed as the means ± S.D. from more than two independent experiments performed. The statistical significance compared with the control group was calculated using the Student's *t* test (*P* < 0.01).

RESULTS

Generation of recombinant anosmin-1 and characterization of its HS-binding features in *Drosophila* S2 cells

Independent research groups have previously reported the production of recombinant anosmin-1 protein in different cell lines, but only with low efficiency [23] and not in a purified form [3]. For the first time, we have generated purified recombinant anosmin-1 protein on a relatively large scale using a *Drosophila* S2 cell expression system. Five different anosmin-1 cDNA constructs were generated, as shown in Figure 1. PIWF4 represents the full-length wild-type protein. Four truncated anosmin-1 recombinants, PIW, PIWF1, PIWF2 and PIWF3, each containing 0, 1, 2 or 3 FnIII domains respectively, were also constructed to investigate FnIII domain function. After transient transfection of S2 cells with each construct, cells were grown in 10% serum-containing medium for 48 h. For serum-free cultures, the transfected cells were weaned into serum-free medium before harvesting. Samples of total cell lysate, salt extracts (cell surface-associated proteins) and conditioned medium (secreted proteins) were prepared from serum-containing (Figure 2a) and serum-free (Figure 2b) cultures, and the samples were subjected to Western blot analysis using an anti-His monoclonal antibody, which confirmed the expression of corresponding recombinant proteins of expected molecular masses under both conditions (Figures 2a and 2b). As compared with the serum-free culture, the secreted recombinant anosmin-1 is much more abundant in the presence of serum, particularly

in the PIWF protein devoid of FnIII domains. While the majority of the PIWF protein was secreted into the serum-containing medium, other anosmin-1 recombinants containing increasing number of FnIII domains (PIWF1–PIWF4) showed a higher level of presence in salt extract, indicating that FnIII domains are necessary for cell surface association of anosmin-1. PIWF1-expressing cells showed the highest yield of recombinant protein production. In serum-containing culture, PIWF3 and PIWF4 samples also contained a band of approx. 20 kDa and 45 kDa respectively (Figure 2a). On the basis that they are detected by the anti-His antibody, are similar to the size of the third FnIII domain alone or the third plus the fourth FnIII domains, and are not present in serum-free medium cultures, we speculated that these were serum-induced artifacts generated by cleavage of recombinant anosmin-1 proteins near the end of the second FnIII domain. A similar proteolytic cleavage phenomenon has been reported in COS7 cells where anosmin-1 was generated in serum-containing medium [24]. To determine whether the cell surface association of anosmin-1 is the consequence of an interaction with HS, a competition assay was performed in serum-free medium by adding exogenous HS. In transiently transfected *Drosophila* S2 cells, co-incubation with 100 $\mu\text{g/ml}$ HS resulted in a significant dissociation of recombinant anosmin-1 proteins (PIWF1–PIWF4) from the cell surface into the medium (Figure 2c), indicating that the endogenous HS proteoglycans mediated the attachment of anosmin-1 to the cell surface. To further investigate these observations and systemically characterize the biochemical properties of anosmin-1, we established a stable cell line expressing PIWF1 or PIWF4. Western blot analysis confirmed the expression of expected proteins in these established lines. The detected band intensity of PIWF1 and PIWF4 was greater in medium when exogenous HS was added, consistent with the results using transient transfection method (Figure 2d). In order to rule out the possible involvement of the V5 and His₆ tag in cell-surface binding, the stable cell lines transfected with either the empty vector or the GFP-expressing vector were established. Western blot analysis using the anti-His antibody showed that the epitope containing bands were only present in conditioned medium, rather than in salt extract (Figure 2d), indicating that these epitopes alone are not able to bind to the cell surface.

To further understand the role of the evolutionarily conserved WAP domain and to study the effects of C172R mutation, mPIWF1¹⁷² and mPIWF4¹⁷² expression constructs were also generated. Expression of mPIWF1¹⁷² and mPIWF4¹⁷² proteins was also confirmed by Western blotting (Figure 2d). The C172R substitution, however, did not disrupt the anosmin-1–HS interaction, since these mutants were able to associate to the cell surface in a manner comparable with their wild-type counterparts (Figure 2d). This result is consistent with the conclusion that it is the FnIII, not WAP, that is essential for HS interaction.

Measurement of HS and anosmin-1 interaction

To further characterize the different anosmin-1 mutants in terms of their HS binding, a solid-phase ELISA was used. Increasing concentrations of purified anosmin-1 was immobilized under a constant concentration of biotinylated HS as the soluble component. As shown in Figure 3, all anosmin-1 analogues bind effectively to HS in a dose-dependent manner. Both wild-type (PIWF4) and WAP-mutated (mPIWF4¹⁷²) full-length anosmin-1 exhibited higher HS-binding affinities than their truncated counterpart. The presence of increasing numbers of FnIII domain repeats therefore leads to stronger HS binding. Next we used SPR, which detects real-time ligand–analyte interactions, for a

Figure 3 ELISA analysis of anosmin-1–HS binding

Immobilized proteins at the indicated concentrations were coated on to microtitre wells and incubated with 12.5 $\mu\text{g/ml}$ soluble biotinylated HS. Alkaline phosphate-conjugated avidin and an alkaline phosphatase substrate were added and the A_{405} was measured. The results are expressed as the means $\pm$ S.D. ($n = 5$).

Figure 4 BIAcore analysis of anosmin-1 interaction with heparin

Representative sensorgrams are shown for the interaction of immobilized heparin with soluble PIWF4 (a), mPIWF4¹⁷² (b), PIWF1 (c) and mPIWF1¹⁷² (d). Six different concentrations of analyte were used for these analyses: 25, 37.5, 50, 75, 100 or 200 nM for PIWF1 and mPIWF1¹⁷², 12.5, 25, 37.5, 50, 75 or 100 nM for PIWF4 and mPIWF4¹⁷². The top sensorgram line in each graph represents the highest concentration of proteins, and the bottom line the lowest concentration. RU, response unit.

quantitative estimate of anosmin-1–heparin binding affinity. Representative sensorgrams for the binding of the truncated and full-length anosmin-1 proteins to immobilized heparin are shown in Figure 4. The initial rising portion of the binding curve represents association of injected proteins with immobilized heparin, the final portion of the curve corresponding to protein dissociation from the sensor chip surface. The relatively steep association phase with shallow dissociation phase in all four recombinant anosmin-1 indicated high-binding affinity with heparin. The resulting curves were analysed by curve fitting and conformed to a 1:1 Langmuir model. The dissociation constant (K_d) was calculated as the ratio of dissociation (k_{off}) to association constants (k_{on})

Table 1 BIAcore kinetic data for binding of anosmin-1 to heparin

Association rate constants (k_{on}), dissociation rate constants (k_{off}) and apparent dissociation constants (K_d) for soluble anosmin-1 binding to immobilized heparin are shown. The values are shown as the means $\pm$ S.D. derived from triplicate measurements.

Soluble analyte	k_{on} ($\times 10^{-5}$, $M^{-1} \cdot s^{-1}$)	k_{off} ($\times 10^3$, s^{-1})	K_d (nM)
PIWF4	4.36 ± 0.7	1.04 ± 0.12	2.51 ± 0.60
mPIWF4 ¹⁷²	5.72 ± 1.92	1.14 ± 0.47	1.97 ± 0.42
PIWF1	7.71 ± 3.57	3.09 ± 0.97	4.31 ± 0.99
mPIWF1 ¹⁷²	1.82 ± 0.80	1.37 ± 0.47	7.89 ± 1.49

(Table 1). All anosmin-1 proteins tested showed K_d values less than 10 nM, confirming their high heparin-binding affinity. The truncated anosmin-1 protein PIWF1, containing a single first FnIII domain, had a lower binding affinity ($K_d > 4$ nM) than the full-length PIWF4 protein ($K_d = 2$ nM). Although the disruption of WAP domain in mPIWF1¹⁷² increased K_d value slightly as compared with its wild-type counterpart PIWF1, similar binding affinities were obtained in PIWF4 and its C172R substitution mutant mPIWF4¹⁷². These results further confirm that it is the

FnIII domains, rather than WAP domain, that contribute to the high HS-binding affinity of anosmin-1, consistent with the ELISA data (Figure 3).

Anosmin-1 increased uPA amidolytic activity, but did not alter thrombin activity

To investigate whether the N-terminal WAP domain of anosmin-1 has a serine protease inhibitor function and whether the FnIII domain co-operates in this process, different anosmin-1 recombinants were tested on two serine proteases, uPA and thrombin, which are known to be expressed during OB development. As shown in Figure 5(a), the *in vitro* thrombin activity was not altered by increasing the concentration of PIWF1 and PIWF4 treatment, regardless of the presence or absence of HS. By contrast, the *in vitro* amidolytic activity of uPA was unexpectedly and significantly enhanced by addition of all anosmin-1 analogues at concentrations of 10 nM and above. Up to a 300 % enhancement of activity was induced by both wild-type and WAP-mutated full-length anosmin-1, PIWF4 and mPIWF4¹⁷² (Figure 5b). The truncated PIWF1, however, showed only 100 % maximum

Figure 5 Anosmin-1 increases uPA but not thrombin amidolytic activity

(a) Triplicates of anosmin-1 and 0.8 nM thrombin were pre-incubated with or without 10 μ g/ml HS. Full-length (b) and truncated (c) anosmin-1 were incubated with 1 nM uPA in the absence and presence of 10 μ g/ml HS. Serine protease-mediated hydrolysis of the chromogenic substrates was monitored spectrophotometrically and the percentage of the initial velocity in comparison with that of the control was calculated. The results are expressed as the means $\pm$ S.D. ($n = 3$).

enhancement, approx. 60% less than the full-length proteins (Figure 5c). In the absence of uPA, no discernible stimulation of amidolytic activity was detected, and purified recombinant GFP caused a negligible effect on uPA activity, confirming the specificity of anosmin-1 action (results not shown). These results demonstrated that the wild-type and WAP-mutated full-length anosmin-1 variants behave similarly in induction of uPA activity; thus the C172R substitution itself does not cause a complete impairment of this particular function of anosmin-1. Alternatively, it may also suggest that disruption of a solitary disulphide bond is insufficient to significantly block the interaction of anosmin-1 with uPA. However, loss of three FnIII domains, as in PIWF1, seems to cause a decrease in the effect of anosmin-1 in this assay. Curiously, the truncated WAP mutant mPIWF1¹⁷² exhibited higher magnitude of activation compared with the full-length proteins (Figure 5c). The reason why the WAP mutation in the truncated PIWF1 protein further enhances uPA activation is not clear.

HS is known to modulate the proteolytic activity of serine protease through interaction with its inhibitors. For instance, binding of HS to antithrombin III increased its inhibitory activity on thrombin 1000-fold in the coagulation cascade [25]. The effects of HS on anosmin-1-induced uPA amidolytic activity were therefore investigated. When anosmin-1 was co-incubated with 10 µg/ml HS, the catalytic activity was unchanged, except that a slight increase was observed with mPIWF1¹⁷², indicating that anosmin-1-induced enhancement of uPA activity is independent of HS in this assay (Figures 5b and 5c). Taken together, although anosmin-1 unexpectedly enhances uPA activity rather than inhibiting it, which is contradictory to our initial expectation and previous suggestions of the role of the conserved WAP domain often associated with serine protease inhibitor function, our data provide the first evidence identifying uPA as an anosmin-1 interactant whose activity is regulated by anosmin-1.

Anosmin-1 induces PC-3 cell proliferation in a HS dependent manner

Since anosmin-1 binds to HS with high affinity and increases uPA catalytic activity *in vitro*, we sought to examine possible co-operation between these molecules at a cellular level and, in particular, the contribution of FnIII and WAP domains. Previous studies had shown that uPA not only initiates metastasis via ECM degradation, but also promotes cell proliferation during tumorigenesis [26]; moreover, these processes are known to be modulated by HS [27]. To establish whether a functional interaction between anosmin-1, uPA and HS exists in living cells and whether these interactions have any effect on cell proliferation, PC-3 cells, known to express high levels of endogenous uPA and cell surface-associated HS, were employed as a model system. PC-3 cells were starved of serum for 24 h and then incubated with the PIWF1 or PIWF4 proteins for a further 48 h. To assess the effects of anosmin-1 on cell growth, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assays were performed and absorbance values from the control group were designated as being 100%. Both PIWF4 and PIWF1 at concentrations of 1–10 nM caused a 20–30% increase in cell growth compared with control (Figures 6a and 6b). These observations were confirmed by direct cell counting, in which total viable cell number was significantly increased by 10 nM PIWF4 and PIWF1 treatments, whereas purified GFP had no effect (Figure 6c). The mitogenic activity of anosmin-1 proteins on PC-3 cells was blocked by pre-incubation with AIWF1 (Figure 6b), a specific antibody raised against PIWF1, confirming the specificity of these effects.

To determine whether HS is functionally required for anosmin-1 the ability of anosmin-1 to induce cell proliferation,

Figure 6 Effect of anosmin-1 on PC-3 cell proliferation

PC-3 cell proliferation was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay with PIWF4 and mPIWF4¹⁷² (a), PIWF1 and mPIWF1¹⁷² (b) respectively in the absence or presence of heparinase III (hep III), exogenous HS or anti-PIWF1 antibody. Culture without addition of anosmin-1 was used as control. The histogram shows the percentage change in *D* (OD) compared with the control cultures. (c) Following 10 nM anosmin-1 or GFP treatment, cell viability was determined by Trypan Blue exclusion and counted using a haemocytometer. The Student's *t* test (two-tailed) was used to analyse the difference between treatment and control; ***P* < 0.01. Each value represents the means ± S.D. (*n* = 5).

the effect of heparinase III, an enzyme that specifically degrades the under-sulphated regions of HS, was examined. PIWF4 induced cell proliferation was completely blocked by 2.5 units/ml heparinase III treatment (Figure 6a), whereas PIWF1 required up to 5 units/ml heparinase III for complete attenuation of its mitogenic effect (Figure 6b). These results demonstrate that the growth-promoting activity of anosmin-1 is HS dependent. A greater sensitivity to heparinase III digestion observed with PIWF4 may imply that the full-length anosmin-1 with more FnIII domains requires longer HS chains for its biological function. If cell surface-associated HS mediates anosmin-1 function, addition of excess exogenous HS should be able to neutralize the activity of anosmin-1 by competitively dissociating anosmin-1 from the cell surface. Immunofluorescence staining using anti-PIWF1 antibody under non-permeabilizing condition clearly demonstrated that

Figure 7 Immunofluorescence analysis of PIWF1 dissociation from PC-3 cell following addition of exogenous HS

Following incubation with 10 nM and 100 nM PIWF1, with or without addition of exogenous 100 µg/ml HS, PC-3 cell surface protein was detected using anti-PIWF1 antibody and Texas Red-conjugated secondary antibody. The bar scale represents 5 µm.

PC-3 cells incubated with 10–100 nM PIWF1 exhibit association of PIWF1 on the cell surface, which is significantly attenuated following addition of 100 µg/ml HS (Figure 7). Incubation with HS completely attenuated PIWF4- and PIWF1-induced PC-3 cell proliferation and a high concentration of HS (up to 500 µg/ml for PIWF4, 100 µg/ml for PIWF1) resulted in a 20–40 % decrease in cell growth (Figures 6a and 6b).

We next asked whether the WAP domain is functionally required for this mitogenic activity of anosmin-1. Figures 6(a) and 6(b) show that both mPIWF4¹⁷² and mPIWF1¹⁷² have lost the cell proliferation effect in the absence or presence of 100 µg/ml HS, suggesting that an intact WAP domain is necessary in this process. The fact that a higher concentration of HS cannot only block the growth promoting effect induced by PIWF4 and PIWF1, but actively causes growth suppression (Figures 6a and 6b) raised the question of whether this growth inhibitory activity of anosmin-1 is also mediated by the WAP domain. Figure 8 shows proliferation of PC-3 cells incubated with 10nM of different anosmin-1 proteins under increasing concentrations of HS. The growth inhibitory effect of PIWF1 and PIWF4 was observable in the presence of relatively high concentrations of HS. However, the WAP mutated counterparts showed no differences. Taken together, these data provide direct and clear evidence that anosmin-1 associates with the HS present on the cell surface through its FnIII domain, allowing the WAP domain to exert its growth promoting/inhibiting activity on PC-3 cells.

Anosmin-1 induces PC-3 cell proliferation through interaction with uPA

We next questioned whether uPA is directly involved in anosmin-1-induced PC-3 cell proliferation. As our results showed that proliferative activity induced by PIWF4 was retained by its truncated version, we opted to use PIWF1 in our subsequent studies of uPA–anosmin-1 interaction, since it had a higher yield of recombinant protein production. Addition of anti-(human uPA) antibody with 10 nM PIWF1 resulted in total abolition of PIWF1-induced PC-3 cell proliferation, whereas non-specific rabbit anti-sera did not show this effect; thus uPA seems to be required for anosmin-1-induced mitogenesis (Figure 9a). To determine if a

Figure 8 Growth suppression effect of anosmin-1 in PC-3 cells

PC-3 cells were treated with 10 nM PIWF4 and mPIWF4¹⁷² (a) or PIWF1 and mPIWF1¹⁷² (b) in the presence of increasing concentrations of HS. Cell proliferation was measured by percentage change in *D* (O.D.) compared with the control. The results are expressed as the means ± S.D. (*n* = 5).

physical association between anosmin-1 and uPA exists, a co-immunoprecipitation assay was performed using total cell lysates in the presence and absence of PIWF1. As shown in Figure 9(b), both monoclonal and polyclonal anti-uPA antibody co-precipitated PIWF1 and uPA, as detected by Western blotting with anti-His (for PIWF1) or anti-uPA antibody. Anti-PIWF1 immunoprecipitation also generated the corresponding uPA and PIWF1 bands. These reciprocal immunoprecipitations clearly demonstrated complex formation between these two proteins, an effect absent in negative controls. As uPA had also been previously reported to have high HS-binding affinity [28,29], we sought to investigate whether anosmin-1 can directly interact with uPA independent of HS. To assess the direct binding properties between these two proteins, SPR was performed using uPA-coupled sensor chips. The sensorgrams shown in Figure 10 give values of $k_{on} = 2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{off} = 1.38 \times 10^{-3} \text{ s}^{-1}$ for PIWF4, and $k_{on} = 1.33 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{off} = 4.36 \times 10^{-3} \text{ s}^{-1}$ for PIWF1. The calculated dissociation constant K_d was 6.91 nM for PIWF4 and 328 nM for PIWF1, demonstrating a relatively high direct binding affinity between anosmin-1 and uPA. The specificity of their interaction was confirmed by two approaches: pre-incubation of PIWF1 with increasing concentrations of uPA significantly reduced the binding of anosmin-1 with the uPA-coupled sensor chip surface in a dose-dependent manner (Figure 10c), and purified GFP did not show any binding ability (results not shown).

DISCUSSION

The molecular pathogenesis of X-KS has not been fully elucidated and the systematic analysis and identification of the interactants

Figure 9 Involvement of uPA in anosmin-1-induced PC-3 cell proliferation

(a) Proliferation of PC-3 cells treated with 10 nM PIWF1 in presence of anti-uPA antibody (Ab) or non-specific rabbit anti-sera. The results are expressed as the means $\pm$ S.D. ($n = 5$). **(b)** PC-3 cell lysate were incubated with PIWF1 and immunoprecipitated (IP) with the corresponding antibodies. The immunoprecipitated complex was analysed by SDS/PAGE and Western blotting using anti-His or anti-uPA antibody. Bands corresponding to PIWF1 and uPA are shown. pAb, polyclonal antibody; mAb, monoclonal antibody.

with anosmin-1 are likely to lead to greater understanding of this developmental disease. Our results have provided the first experimental evidence for a direct interaction between the FnIII domain at the C-terminus of anosmin-1 and HS, as well as detailed analysis of their binding kinetics. Furthermore, we have identified uPA as a novel ligand for anosmin-1.

The affinity and specificity of HS–protein binding is determined by the HS polysaccharide sequence, the monosaccharide sulphated residues and the peptide sequence of the protein ligand, and is mediated by three types of interaction: ionic interaction through the basic residues of the proteins, hydrophobic interaction and hydrogen bond formation [30]. In the present study, all anosmin-1 analogues bound to the *Drosophila* S2 cell surface could be displaced by a high salt-containing medium, and anosmin-1 proteins bound to the immobilized heparin sensor chip surface could also be eluted by 2 M NaCl solution, indicating that charged residues are crucially involved in the anosmin-1–HS interaction. The large positively charged surfaces of the FnIII domains are likely to participate in an ionic interaction with the negatively charged HS, whereas the WAP domain contains more acidic residues, leading to a more negatively charged surface, and is unlikely to be involved. This prediction was confirmed by using FnIII domain truncated or WAP domain mutated anosmin-1 constructs in both cell-based HS competition assay (Figure 2) and the solid-phase analysis using immunosorbant and SPR techniques (Figures 3 and 4). Recombinant anosmin-1 with greater numbers of FnIII repeats exhibits stronger cell surface association and greater

Figure 10 BIAcore analysis of anosmin-1 binding to uPA

Representative sensorgrams are shown for the interaction of immobilized uPA with soluble PIWF4 **(a)** and PIWF1 **(b)**. Five different concentrations of analyte were used for these analyses: 50, 100, 175, 250 or 375 nM for PIWF4, and 250, 375, 500, 750 or 1000 nM for PIWF1. The top sensorgram line in each graph represents the highest concentration of proteins, and the bottom line the lowest concentration. **(c)** Specificity of anosmin-1 binding to the uPA-coupled sensor surface. PIWF1 (250 nM) was pre-incubated with uPA (upper to lower sensogram curves: 0, 60, 120 and 300 nM) prior to injection. RU, response unit.

HS-binding affinity, whereas the C172R mutation of the WAP domain does not alter HS-binding characteristics of PIWF4, as indicated by real-time kinetic measurements. The HS-binding affinity of PIWF1 is only slightly lower than that of the full-length PIWF4 (4.31 ± 0.99 nM and 2.51 ± 0.60 nM respectively), indicating that the first FnIII domain constitutes the main HS-binding module. These results are consistent with previous studies by Saoussi-Yanicostas et al. [5] who described that a synthetic 32-amino-acid peptide derived from the first FnIII domain induced heparin-associated cell adhesion, and by Bulow et al. [3] who observed that the introduction of a missense mutation (corresponding to the human N267K substitution) in the first FnIII of the *C. elegans* *Kal-1* abolished heparin-dependent cell adhesion and axon-branching activity [3]. Our data show that disruption of one of the disulphide linkages within the WAP domain can interfere with the FnIII-domain–HS interaction in PIWF1, but not in PIWF4, suggesting that the structural alteration of the WAP domain may influence the charged residue distribution in the first

FnIII domain, especially when not followed by additional FnIII repeats.

The high binding affinity of anosmin-1 to HS has functional importance, since treatment with heparinase III or exogenous HS can abolish anosmin-1 induced PC-3 cell proliferation (Figures 6a and 6b), with a concomitant dissociation of anosmin-1 from the cell surface, as confirmed by immunofluorescence (Figure 7). Indeed, HS has pleiotropic actions through binding to a wide range of soluble and insoluble molecules, including growth factors and their receptors, cell adhesion molecules, serine proteases and specific inhibitors [31–33]. On the cell surface, HS chains act as receptors or co-receptors to regulate the ligand binding and subsequent signalling in diverse ways: converting ligands from inactive to active forms through conformational changes, promoting ligand and receptor dimerization inducing signal transduction, and stabilization of active ligand–receptor complexes through ternary complex formation [34,35]. With respect to anosmin-1, HS could, through binding with the FnIII domain, serve at least three functions: (i) induce conformational changes of anosmin-1 thereby exposing the CR and WAP domains' active sites, (ii) act as a co-receptor or provide a stabilization platform, as is known to occur with FGFs and FGFRs (FGF receptors), and (iii) to increase local concentration of anosmin-1 on the cell surface, allowing more effective binding of the interacting serine protease ligands.

A crucial role for the WAP domain is supported by the cell-based evidence in which PIWF1, with its solitary FnIII domain, can fully recapitulate the biological actions of the full-length protein, and yet WAP mutants with similar HS binding affinity have completely lost their mitogenic activity in PC-3 cells.

Prior to this study, the identity of serine protease ligand(s) that interacts with anosmin-1 was unknown. Our data show that anosmin-1 augments uPA amidolytic activity *in vitro* and has a direct protein–protein interaction with uPA, suggesting that the serine protease uPA represents a potential molecular target for anosmin-1. Although four-disulphide core motifs are present in the WAP family of proteins, they do not all exhibit the same serine protease inhibitory activities. For example, although elafin and SLPI share the same distribution of disulphide bridges and a methionine residue in the P1' position of the active loop, SLPI inhibits a far wider range of serine proteinases than elafin [11]. Moreover, the Na⁺, K⁺-ATPase inhibitor with identical four disulphide bond organization to elafin and SLPI, shows inhibitory activity only on Na⁺, K⁺-ATPases, but not on any other serine proteases [36]. We have previously conducted a sequence and structural analysis of anosmin-1's WAP domain in comparison with that of elafin and revealed that Cys¹⁴⁷ of anosmin-1 is located to the opposite site of the corresponding Cys⁸³ of elafin with respect to the protein surface loop, resulting in the rearrangement of Cys¹⁴⁷–Cys¹⁶⁸ disulphide bridge in anosmin-1 [8]. These structural differences of anosmin-1 may explain why it increases rather than inhibits uPA activity, as initially thought.

Our data demonstrate the functional co-operation between HS, uPA and anosmin-1 on PC-3 cell proliferation. Anosmin-1, associated with the cell surface through its FnIII-domain–HS interactions, may come in contact with uPA via its N-terminus including the WAP domain, causing uPAR (uPA receptor) activation and/or induction of uPA-mediated proteolysis. Studies have shown a role for uPA and uPAR during cell proliferation *in vitro*, and in human malignant tumours overexpression of uPAR is often observed [21]. Enhancement of uPA proteolytic activity can lead to activation of a number of growth factors such as insulin-like growth factor, FGF2 and transforming growth factor- β [37,38]. It is not known, however, whether this mitogenic activity is dependent on the uPA mediated proteolysis or uPA binding to uPAR.

Figure 11 Hypothesis of molecular mechanism of anosmin-1 action

HS side chains of HSPG anchor anosmin-1 to the cell surface via binding to FnIII domains, thus facilitating N-terminal anosmin-1–uPA interaction. Increased uPA proteolytic activity induces plasmin–matrix-metalloproteinase-mediated ECM degradation, releasing a number of growth factors involved in cell proliferation, migration and tissue remodelling. Different functional domains of uPA are schematically represented: growth-factor-like domain (circle); kringle domain (rectangle); catalytic domain (triangle). HSPG, HS proteoglycans, MMP, matrix metalloproteinase.

The identification of HS and uPA as ‘cross-talk’ partners for anosmin-1 provides novel insights into the molecular basis of the neuronal and non-neuronal manifestations of X-KS. In developing OB, anosmin-1 and uPA expression is detected within the large mitral and tufted cells around embryonic day 14, a time at which a HS proteoglycan, N-syndecan, is also expressed by migrating olfactory and GnRH neurons [16,39]. Furthermore, uPA expression is observed in kidney medullary and tubular epithelial cells [19,20], and dysfunction of glypican-3 results in decreased proliferation of kidney medullary collecting duct cells and medullary degeneration [40]. Thus any interruption of the interaction among these three molecules (anosmin-1, uPA and HS) may lead to the olfactory as well as the renal developmental defects seen in X-KS patients.

In conclusion, we postulate that uPA-induced activation of the plasmin cascade system on the cell surface causes proteolytic degradation of ECM components to relieve cell–cell and cell–ECM adhesion and release activated growth factors, thereby facilitating cell migration, tissue remodelling, angiogenesis and neuronal synaptogenesis. Anosmin-1 may modulate these processes by increasing uPA catalytic activity, whereas the role of HS may be to concentrate and restrict uPA–anosmin-1 distribution on the cell surface (Figure 11). FGFR1 has been recently identified as a locus for autosomal dominant KS, suggesting that anosmin-1 may also be involved in FGFR signalling pathways [41]. Functional relationship between uPA and FGF system has been previously suggested. For instance, FGF2 up-regulated uPA expression through MAPK (mitogen-activated protein kinase) signalling, whereas FGF2-dependent phosphoinositide 3-kinase activity was down-regulated [42]. Moreover, FGF2-induced smooth muscle cell proliferation requires endogenous uPA and its catalytic domain [43]. Therefore, anosmin-1 may function through multiple molecular mechanisms in a tissue and developmental stage-specific manner, leading to different phenotypic outcomes such as cell proliferation during kidney morphogenesis or cell differentiation in neuronal development.

We thank Professor Paul Travers and the Anthony Nolan Research Institute for help with the SPR experiments. This work was supported by grants from Action Research and the Hobson Charity.

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