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Neuropeptide Y functions as a facilitator of GDNF-induced budding of the Wolffian duct

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Ureteric bud (UB) emergence from the Wolffian duct (WD), the initiating step in metanephric kidney morphogenesis, is dependent on GDNF; however, GDNF by itself is generally insufficient to induce robust budding of the isolated WD in culture. Thus, additional factors, presumably peptides or polypeptide growth factors, might be involved. Microarray data from in vivo budding and non-budding conditions were analyzed using non-negative matrix factorization followed by gene ontology filtering and network analysis to identify sets of genes that are highly regulated during budding. These included the GDNF co-receptors GFRα1 and RET, as well as neuropeptide Y (NPY). By using ANOVA with pattern matching, NPY was also found to correlate most significantly to the budded condition with a high degree of connectedness to genes with developmental roles. Exogenous NPY [as well as its homolog, peptide YY (PYY)] augmented GDNF-dependent budding in the isolated WD culture; conversely, inhibition of NPY signaling or perturbation of NPY expression inhibited budding, confirming that NPY facilitates this process. NPY was also found to reverse the decreased budding, the downregulation of RET expression, the mislocalization of GFRα1, and the inhibition of AKT phosphorylation that resulted from the addition of BMP4 to the isolated WD cultures, suggesting that NPY acts through the budding pathway and is reciprocally regulated by GDNF and BMP4. Thus, the outgrowth of the UB from the WD might result from a combination of the upregulation of the GDNF receptors together with genes that support GDNF signaling in a feed-forward loop and/or counteraction of the inhibitory pathway regulated by BMP4.

KEY WORDS: Kidney development, Neuropeptide Y, Wolffian duct budding

INTRODUCTION

The initiating step in kidney development is the formation of the ureteric bud (UB) from the Wolffian duct (WD). Glial cell line-derived neurotrophic factor (GDNF), produced in the metanephric mesenchyme (MM), interacts with its receptors on the WD where it binds to the GPI-linked co-receptor GFRα1, which then signals through the receptor tyrosine kinase RET (Sariola and Saarma, 2003). GDNF is expressed in the MM adjacent to the caudal portion of the WD, whereas RET and GFRα1 are expressed throughout the WD prior to the formation of the UB. After the UB emerges from the WD, the expression of RET and GFRα1 becomes limited to the UB (Costantini and Shakya, 2006). GDNF signaling appears to be the central modulator of UB formation; mice lacking GDNF or its receptors GFRα1 or RET are characterized by kidney agenesis (Schuchardt et al., 1994; Schuchardt et al., 1996). Similar phenotypes are found in mice in which upstream activators of GDNF expression, such as EYA1, SIX1, PAX2 and GDF11 are knocked out (reviewed by Brodbeck and Englert, 2004; Li et al., 2003; Sampogna and Nigam, 2004; Shah et al., 2004). The proper expression of GDNF is also important in limiting the formation of the UB to a single site; transgenic misexpression of GDNF throughout the WD in vivo (Shakya et al., 2005) or the application of GDNF-soaked beads next to the WD in organ culture (Sainio et al., 1997) caused multiple, ectopic UBs to emerge. BMP4, one of the endogenous inhibitors of budding, regulates the budding process downstream of GDNF expression (Costantini and Shakya, 2006); however, the mechanism of this inhibition has not yet been clarified. In some cases, GDNF signaling might be bypassed through the activation of signaling pathways by stimulation from FGF-family growth factors and/or through the inhibition of activin signaling; this might explain why some RET and GFRα1 knockout animals manage to form rudimentary kidneys (Maeshima et al., 2006; Maeshima et al., 2007).

Microarray analysis of gene expression during kidney organogenesis has revealed broad patterns of expression changes (Stuart et al., 2001; Stuart et al., 2003; Tsigelny et al., 2008). Further analysis of the in vitro cultured kidney components (UB and MM) demonstrated differences in gene expression within the various compartments of the kidney, suggesting there are distinct gene networks responsible for UB branching and MM induction (McMahon et al., 2008; Stuart et al., 2003). Similar analyses have aided in the identification of potential novel regulators of kidney development (Schmidt-Ott et al., 2007; Schmidt-Ott et al., 2005). These and other studies demonstrate the utility of microarray analysis to investigate developmental systems. Various methods of unsupervised data clustering exist, such as hierarchical clustering (HC), self-organizing maps (SOM) and non-negative matrix factorization (NMF) (Brunet et al., 2004; Tsigelny et al., 2008). NMF clusters many thousands of genes together into a metagene to simplify the expression pattern and to extract biological correlations in microarray data. This patterning is less dependent on initial conditions than are HC and SOM clustering. Here, we performed microarray analysis on several in vivo conditions with budded and unbudded

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morphologies to determine which genes are important for the initial formation of the UB. Using this approach, we have identified a novel modulator of in vitro WD budding, neuropeptide Y (NPY).

NPY is a linear 36 amino acid neuropeptide expressed throughout the central and peripheral nervous systems (Tatemoto, 1982) that has been shown to play a role in the development of enteric neurons in response to GDNF (Anitha et al., 2006). NPY belongs to a family of neuropeptides that include the homologous peptide YY (PYY) and pancreatic polypeptide (PP), which share 69% and 50% identity in their amino acid sequences, respectively. NPY and its homologs signal through five G protein-coupled receptors belonging to the $G_{i/o}$ class: Y1, Y2, Y4, Y5 and y6. The existence of the Y3 receptor is unclear and the y6 receptor is not present in rats (Burkhoff et al., 1998). The Y1, Y2, Y4 and Y5 receptors are known to increase mitogen-activated protein kinase (MAPK) levels in transfected cells (Mannon and Mele, 2000; Mannon and Raymond, 1998; Mullins et al., 2002; Nie and Selbie, 1998). NPY has also been shown to increase the phosphorylation of AKT in enteric neurons (Anitha et al., 2006). Both of these pathways appear to be involved in GDNF signaling. NPY expression has been shown to be modulated by various growth factors: brain-derived neurotrophic factor in cortical neurons, nerve growth factor in avian sympathoadrenal cells, and GDNF in enteric neurons (Anitha et al., 2006; Barnea et al., 1995; Barreto-Estrada et al., 2003). As NPY was the transcript that was most significantly altered in the budded and nonbudded conditions among nearly 30,000 transcripts, we sought to define the effect of NPY on the budding of the WD. In order to study the effect of NPY on UB formation, we used a recently devised in vitro culture system in which the WD without the attached mesonephros was induced to undergo budding without relying upon artificial matrices (Maeshima et al., 2006; Rosines et al., 2007). Our results support a key role for NPY in facilitating, in concert with GDNF, the formation of the UB from the WD.

MATERIALS AND METHODS

Reagents

Recombinant human BMP4, FGF7, follistatin and rat GDNF were from R&D Systems (Minneapolis, MN, USA). Recombinant FGF1 was from Calbiochem (EMD, San Diego, CA, USA). NPY, PYY, NPY 3-36 and NPY 13-36 were from GenScript (Piscataway, NJ, USA). PYX-1 and BIBP3226 were purchased from Bachem Bioscience (King of Prussia, PA, USA). DMEM/F12 was purchased from Gibco (Invitrogen, Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Biowhittaker (Walkersville, MD, USA). Donor donkey serum was from Gemini Bio-Products (West Sacramento, CA, USA). Fluorescein-labeled Dolichos biflorus (horse gram) agglutinin was from Vector Laboratories (Burlingame, CA, USA). Goat anti-GRFα1 was from R&D Systems. Anti-ZO-1 and anti-E-Cadherin were from Zymed (Invitrogen). Rabbit anti-phospho-AKT (Ser473), anti-total-AKT, anti-phospho-p44/42 MAPK (Thr202/Tyr204), and anti-p44/42 MAPK were from Cell Signaling Technology (Beverly, MA, USA). Alexa Fluor 488 or 594 secondary antibodies were from Molecular Probes (Invitrogen). All signaling inhibitors were from Calbiochem. All other reagents were from Sigma (St Louis, MO, USA).

Isolation and culture of the Wolffian duct

Wolffian duct (WD) cultures were prepared as previously described (Maeshima et al., 2006). Briefly, embryos from timed-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) at day 13 of gestation were used for all cultures. The WDs along with a thin layer of attached mesodermal mesenchymal cells were dissected using a stereomicroscope and fine forceps (FST, Foster City, CA, USA). The WDs were placed on 0.4 μm pore size Transwell filters (Costar, Cambridge, MA, USA) in 12- or 24-well tissue culture dishes. Culture medium consisting of DMEM/F12, 10% FBS and growth factors was added below the Transwell. The ‘standard’ GDNF-dependent budding control consisted of 125 ng/ml GDNF with 125 ng/ml FGF1.

NPY knockouts

$NPY^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA), strain name 129S-Npy^{tm1Rpa}/J, stock number 004545. The use and care of animals reported in this study conformed to the procedures of the laboratory’s animal protocol approved by the Animal Subjects Program of the University of California, San Diego.

siRNA

ON-TARGETplus SMARTpool Rat NPY siRNA was purchased from Dharmacon (Thermo Fisher Scientific, Chicago, IL, USA) with target sequences of: GAUGCUAGGUAACAAACGA, CCUUGUUGUCGUUGUAUAU, GCAUUCUGGCUGAGGGGUA and UCAUCACCAGACAGAGAUA. Control siRNA-targeted cyclophilin B, also known as peptidylprolyl isomerase B, had the sequence 5′-GGAAAGACUGUUCCAAAAA-3′ (siGENOME D-001136-01-20, Dharmacon). siGLO Green was used as a fluorescent oligonucleotide control transfection indicator (D-001630-01-02, Dharmacon). A non-targeting mismatch control oligonucleotide was also used: On-TargetPlus non-targeting siRNA #1 (D-001810-01-05, Dharmacon).

Four to 6 hours before transfection, WDs were placed on Transwell filters above culture medium (DMEM/F12 + 10% FBS). DharmaFECT IV (Dharmacon) was diluted to 3% in Opti-mem (Gibco). siRNA was diluted to 1 μM and siGLO was diluted to 100 nM in Opti-mem reduced serum media (Gibco). The mixtures were allowed to incubate separately for 5 minutes at room temperature. Afterwards, either the siRNA or the siGLO mixture was combined with the DharmaFECT mixture and the mixtures gently mixed together at room temperature for 20 minutes. The final concentration of siRNA oligomers was 500 nM or 50 nM for siGLO transfection indicator. This mixture was then applied on top of the Transwell filter, in direct contact with the WD cultures. GDNF and FGF1 (125 ng/ml) were added to the media underneath the Transwell and the culture was allowed to grow for 24 to 48 hours.

Immunohistochemistry

Cultured WDs were fixed with 4% paraformaldehyde (PFA) for 1-2 hours at room temperature, followed by incubation with the primary antibody in blocking solution overnight at 4°C. Three rinses in PBS with 0.1% Tween (PTW) was followed by incubation with the secondary antibody in blocking solution with 10% donkey serum overnight at 4°C. The samples were then thoroughly rinsed with PTW and viewed with a confocal microscope (Nikon D-Eclipse C1).

Western blot

Samples were lysed in buffer containing 100 mM NaCl, 1% Triton X-100, 0.5% sodium dodecyl sulfate, 20 mM EDTA, 10 mM HEPES and 0.5% sodium deoxycholate with Sigma protease inhibitor cocktail (1:10), 20 mM DTT and 10 mM Na_3VO_4. Protein concentration was determined by BCA analysis (PIERCE, Rockford, IL, USA). Protein from each sample (20 μg) was run on SDS-PAGE using NuPAGE Novex Bis-Tris Gels (4-12%) (Invitrogen) and transferred to a nitrocellulose membrane. The membrane was blocked with 2% milk (w/v), 1% Triton X-100, 0.01 M EDTA in 0.04 M Tris-HCl pH 7.5, incubated with primary antibody for 1 hour, washed with Tris-buffered saline with 0.1% Tween-20 (TBST), and incubated with a peroxidase-labeled secondary antibody for 1 hour. After rinsing with TBST, the membrane was exposed to HyBlot CL autoradiography film (DENVILLE, Metuchen, NJ, USA) using Supersignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL, USA).

RT-PCR

RNA was isolated from WDs or kidneys using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and converted to cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Amplification of cDNA by PCR was performed using the HotStartTaq Master Mix Kit (Qiagen). The primers used were:

NPY (NM_012614), 5′-GGCCAGATACTACTCCGCTCTGCG-3′ (forward) and 5′-TTCACAGGATGAGATGAGATGTG-3′ (reverse) (Chottova Dvorakova et al., 2008);

PYY (NM_001034080), 5′-CTCTGTTCTCCAAACTGCTC-3′ (forward) and 5′-ACCAAACATGCAAGTGAAGTC-3′ (reverse);

PP (NM_012626), 5′-CATACTACTGCCTCTCCCTG-3′ (forward) and 5′-GTTTCGTATTGAGCCCTCTG-3′ (reverse);

NPY Y1 receptor (X95507), 5′-AAATGTATCACTTGCGGCGTTCA-3′ (forward) and 5′-GCGACCACGATGGAGAGCAG-3′ (reverse) (Jackerott and Larsson, 1997),

Y2 receptor (NM_023968), 5′-CCCGGATCTGGAGTAAGCTAAA-3′ (forward) and 5′-GTGGAGCACATCGCAATAATGT-3′ (reverse) (Chottova Dvorakova et al., 2008);

Y4 receptor (NM_031581), 5′-TTGCAGTTCTCTGGCTGCCCCTG-3′ (forward) and 5′-CTTGCTACCCATCCTCATAGAT-3′ (reverse);

Y5 receptor (NM_012869), 5′-CCAGGCAAAAACCCCCAGCAC-3′ (forward) and 5′-GGCAGTGGATAAGGGCTCTCA-3′ (reverse); and

β-actin (NM_031144), 5′-TCATCACTATCGGCAATGAGC-3′ (forward) and 5′-CTCCTTCTGCATCCTGTCAGC-3′ (reverse).

The PCR conditions were 15 minutes at 95°C, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 1 minute at 72°C, and concluding with 10 minutes at 72°C.

Real-time quantitative PCR

All primers were designed with PerlPrimer (Marshall, 2004). The primers used on rat-derived samples were:

GAPDH, 5′-ATGATTCTACCCACGGCAAG-3′ (forward) and 5′-CTGGAAGATGGTGATGGGTT-3′ (reverse);

NPY, 5′-GACATGGCCAGATACTACTC-3′ (forward) and 5′-ATCTCTTGCCATATCTCTGTC-3′ (reverse);

RET, 5′-CCCTATATGTAAATGACACGGA-3′ (forward) and 5′-CTTCTTCTGCAATGTATGTCCC-3′ (reverse); and

cyclophilin B, 5′-CAATATGAAGGTGCTCTTCG-3′ (forward) and 5′-CAAAGTATACCTTGACTGTGAC-3′ (reverse).

The primers used on mouse-derived samples were:

GAPDH, 5′-TGGCCTCCAAGGAGTAAGAAAC-3′ (forward) and 5′-GGGATAGGGCCTCTCTTGCT-3′ (reverse);

PYY, 5′-GTTCTCCAAACTGCTCTTCACAGA-3′ (forward) and 5′-TTCACCACTGGTCCAAACCTT-3′ (reverse); and

cyclophilin B, 5′-CTGTCTGTGTGGGTCCTGTCA-3′ (forward) and 5′-GGGTGGCACAGAACCTTGTG-3′ (reverse).

PowerSybr Green master mix (5 µl, Applied Biosystems), 1 µM primers, cDNA and water to a total of 10 µl were run on an Applied Biosystems 7500 Fast Real-Time PCR machine. The program was set to 50°C for 2 minutes and 95°C for 10 minutes, with 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, and concluded with a dissociation step.

Microarray analysis

RNA from the various tissues was isolated with the Abion RNAqueous Micro Kit (Applied Biosystems) following the manufacturer's protocol. The purified RNA was processed by the UCSD Microarray Core facility and hybridized to the Rat Genome 230 2.0 Array (Affymetrix). Data normalization and fold-change analysis were performed with Genespring GX (Agilent). Briefly, the data were normalized to unity per chip and per gene.

NMF was performed using the GenePattern (Broad Institute, MIT) server and client software (Brunet et al., 2004; Reich et al., 2006). The data were pre-processed to remove genes that did not vary by 8-fold or 800, which reduced the data set from 31,000 to 2007 genes. The number of metagenes was set to four and the rest of NMF variables were kept at the default values. We selected four metagenes based on the maximum cophenetic coefficient score after performing NMF-consensus clustering with our data (i.e. it was the largest value that also had a cophenetic coefficient of 1.0).

ANOVA and pattern matching (Pavlidis, 2003; Pavlidis and Noble, 2001) were performed with data normalized with Genespring GX. The pattern was set as budding or non-budding. The budding sample was the isolated ureteric bud. The two non-budding samples were the two uncultured, unbudded WDs [iWD and WD(+IM)]. The test type was set to parametric test, without assuming equal variances. The false discovery rate was varied between 0.001 and 0.00001. No multiple testing correction or post-hoc tests were performed. The software reported the *P*-value for each gene. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEP series accession number GSE18260.

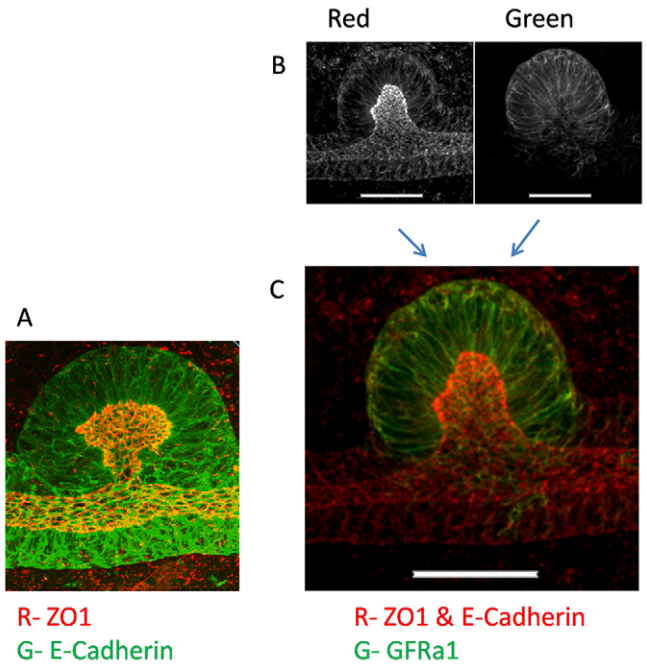

Fig. 1. WD budding. (**A**) Budded WD stained for the epithelial marker E-cadherin (green) and the tight junction protein ZO-1 (red), which delimits the apical surface. (**B**,**C**) Budded WD stained for GFRα1 (green) and both ZO-1 and E-cadherin (red). Scale bars: 50 µm.

RESULTS

A microarray-based approach identifies a subset of genes potentially involved in UB formation from the WD

In order to identify novel factors that modulate the budding process (Fig. 1), we obtained genome-wide transcriptional profiles of rat tissue with either a budded or a nonbudded morphology by using the Affymetrix Rat Genome Array 230 2.0. We selected one budded condition [isolated ureteric bud (iUB)] and two unbudded conditions: uncultured isolated E13 WDs with and without attached mesenchymal cells [WD with intermediate mesoderm (WD+IM) and isolated WD (iWD), respectively]. Three biological replicates were performed for each condition.

Non-negative matrix factorization (NMF), which is used for computer pattern recognition in image and natural language processing (Devarajan, 2008), was used to separate out the 'budding genes' enriched in the iUB from those enriched in either of the two nonbudding conditions. NMF sorts the data into the specified number of metagenes consisting of hundreds or thousands of genes without input from the user. The original matrix, M, comprising the number of samples multiplied by the number of genes on the microarray, is factored into the matrices W and H. The W matrix has the dimensions of the number of genes on the microarray multiplied by the number of metagenes. The H matrix represents the data in metagene format and has the dimensions of the number of samples multiplied by the number of metagenes (Brunet et al., 2004). The data for four metagenes are shown (Fig. 2A). Because we were interested in the growth factors and signaling events that regulate WD budding, the genes that were enriched in the iUB were filtered by gene ontology to select genes that were involved in signal transduction (Fig. 2C). This reduced the number of genes to approximately 64. This reduced set of genes was then fed into Ingenuity Pathway Analysis, a

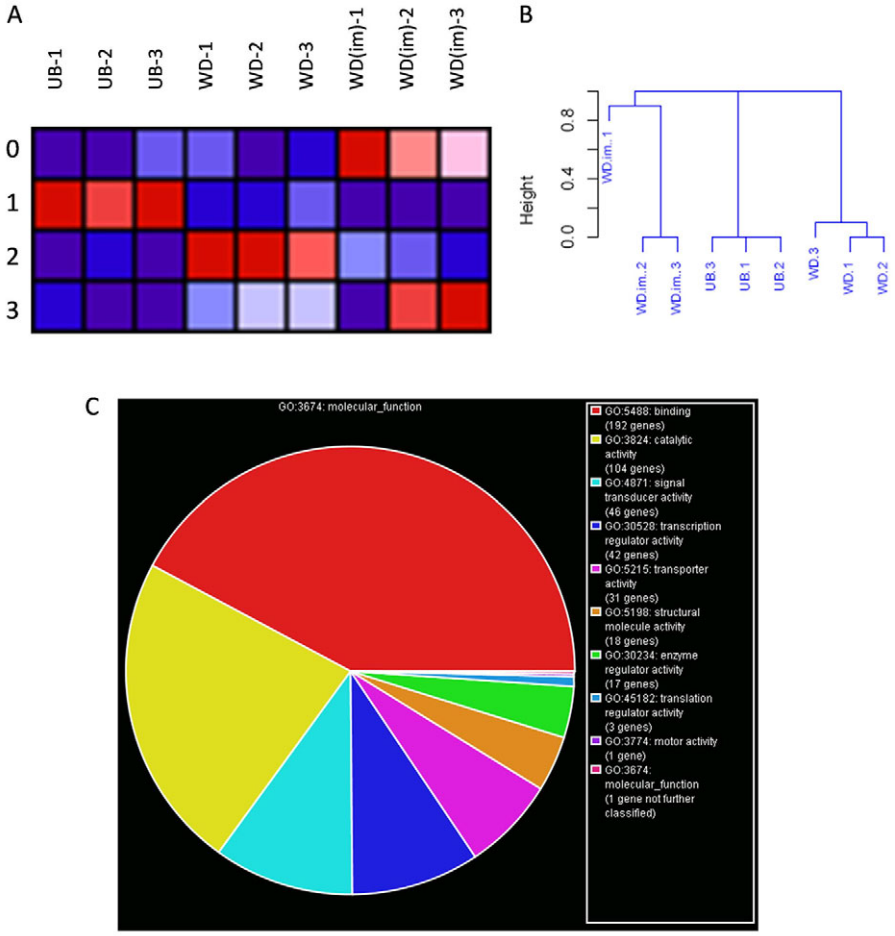

Fig. 2. Microarray analysis. (**A**) Non-negative Matrix Factorization (NMF) was applied to the microarray data that were pre-processed to include genes that changed at least eightfold and by 800 units. The data were factored into four metagenes. UB, isolated ureteric bud; WD, isolated Wolffian duct; WD(IM), Wolffian duct with intermediate mesoderm. (**B**) Ordered linkage tree of samples. (**C**) Gene ontology classification of genes enriched in the ureteric bud.

hand-curated interaction dataset, which resulted in two networks containing a number of genes from the list of 64 (Fig. 3). The first network included the GDNF receptors RET and GFRα1, and was categorized as 'Cellular Development' and 'Nervous System Development and Function'. The second network was categorized as 'Behavior, Digestive System Development and Function' and 'Cell Signaling' and had nine genes, which included three secreted ligands: neurexophilin 1 (NXPH1), a neuropeptide-like ligand that binds to receptor-like proteins expressed on many neuronal cell surfaces (Missler et al., 1998); neuropeptide VF precursor (NPVF), a member of the neuropeptide FF family that modulates opioid tolerance and gut motility (Hinuma et al., 2000); and neuropeptide Y (NPY; see Table 1).

The data were also analyzed using a method that compares the data using ANOVA and pattern matching without the need for arbitrarily setting a fold-change threshold (Pavlidis, 2003; Pavlidis and Noble, 2001). The pattern was set as budded or non-budded (Fig. 4A), with the output being a *P*-value for each gene. Greater than 1800 genes had a *P*-value of less than 0.001 (Fig. 4C), 481 genes had a *P*-value of less than 0.0001 (Fig. 4D), and 110 genes had a *P*-value of less than 0.00001 (Fig. 4E). The ANOVA method does not distinguish between positive correlation and negative correlation; however, a simple fold-change analysis can determine this to establish which genes positively correlate to budding. Table 2 lists selected genes upregulated in the iUB together with their *P*-value. Using this analysis, NPY had the lowest *P*-value (2.01×10^{-8}) of the genes that correlated with budding.

That these two independent methods of data analysis identified NPY among the several thousand genes present on the array suggested the relevance of NPY in the budding process. We also analyzed the expression of NPY in the developing embryonic kidney and found that it has high levels of expression at the initiation of UB formation (data not shown), which is consistent with its levels being elevated in the isolated UB. Given these results, further studies on NPY in WD budding were performed.

Table 1. IPA networks

Molecules/genes	Score	Top functions
Crk, Cxcr4, Ednrb, Erbb3, Fst, Gfra1, Ghr, Gnaq, Homer1, Kit, Kitlg, Lasp1, Ldlr, Met, Nov, Nr4a1, Ret	43	Cellular development, hair and skin development, nervous system development and function
Fcgr2a, Htr5b, Il13ra1, Lphn2, Npvf, Npy, Nxph1, Pax8, Unc13b	19	Behavior, digestive system development and function, cell signaling

Expression and localization of NPY and its receptors

We first proceeded to verify the aforementioned results by qRT-PCR. Consistent with the microarray, NPY was markedly upregulated in WDs budded in culture compared with either uncultured WDs or WDs cultured with a budding inhibitor (Fig. 5A). In order to further characterize the possible role of NPY during WD budding, we sought to determine the presence of NPY, its homologs PYY and PP, and the cognate receptors Y1-Y5

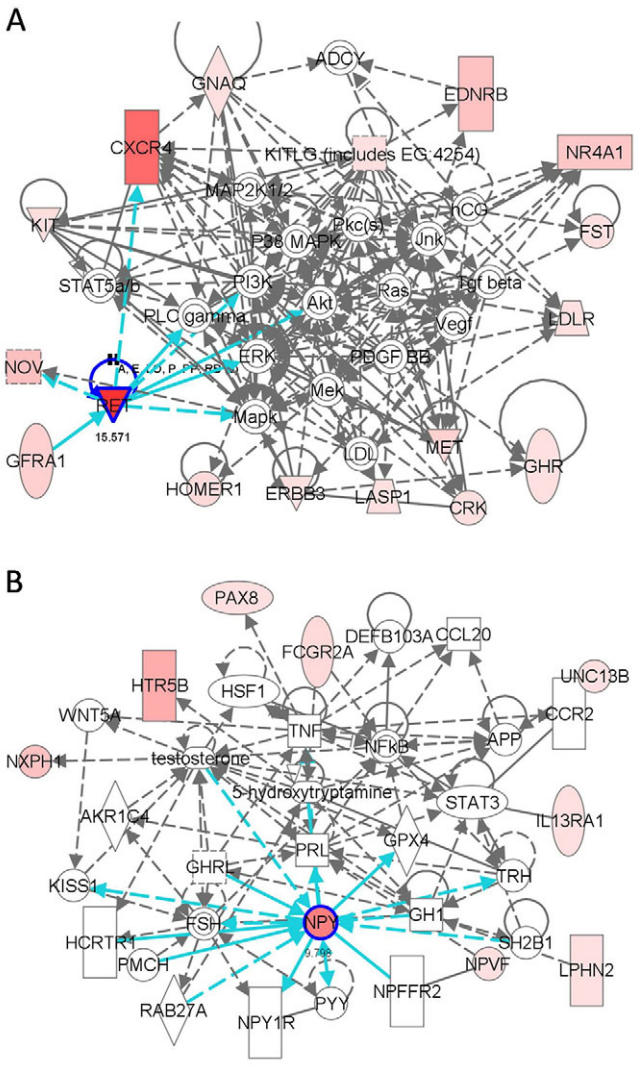

Fig. 3. Ingenuity pathway analysis (IPA) of microarray data generated several genetic networks. (**A**) Network with RET classified as 'Cellular Development' and 'Nervous System Development and Function'. (**B**) Network with NPY as a connection hub classified by IPA as 'Behavior, Digestive System Development and Function' and 'Cell Signaling'. See Table 1.

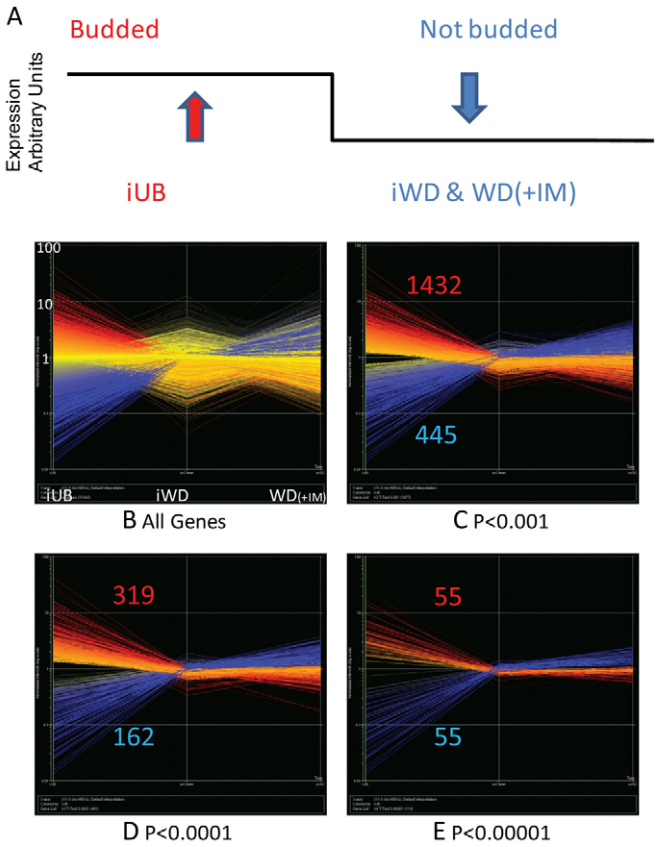

Fig. 4. ANOVA and pattern matching. (**A**) Pattern set for ANOVA analysis. iUB, isolated ureteric bud; iWB, isolated Wolffian duct; WD(+IM), Wolffian duct with mesodermal cells. (**B**) Line graph showing all genes. *y*-axis shows normalized expression and the *x*-axis shows the samples: iUB, iWD and WD(+IM). (**C-E**) Line graphs corresponding to budding (red) or non-budding (blue) for (C) $P<0.001$ 1877 genes (1432 upregulated in budding, 445 downregulated in budding), (D) $P<0.0001$ 481 genes (319 upregulated in budding, 162 downregulated in budding) and (E) $P<0.00001$ 110 genes (55 upregulated in budding, 55 downregulated in budding). Graphs display normalized log intensity on the *y*-axis. The iUB, iWD and WD(+IM) are plotted along the *x*-axis.

within the developing kidney. We found that NPY was expressed in both the WD and the developing kidney, whereas PYY was not present in the WD (Fig. 5B). PP was not detected in the tissues tested. All NPY receptors were present in both the WD and the kidney (Fig. 5C).

In vitro budding with GDNF

We went on to evaluate the role of NPY in WD budding in vitro. It has previously been shown that the WD isolated from mesonephric tubules can undergo robust budding in the presence of GDNF only if another growth factor such as FGF1 is also added (Maeshima et al., 2007; Rosines et al., 2007). However, FGF1 is not highly expressed at this time, suggesting that the physiological growth factor supporting GDNF budding is something other than FGF1. Based on the microarray network and statistical analysis, as well as on qRT-PCR data, NPY seemed like a promising candidate for this role. The WDs have attached intermediate mesodermal cells (WD+IM). Addition of only NPY, without other growth factors, did not result in budding (Fig. 6A), whereas the addition of 50 nM NPY to WDs cultured in the presence of GDNF (without an FGF) resulted in impressive budding of 90% of the WDs (compared to the minimal budding seen in only 10% of WDs cultured in the presence of GDNF alone; Fig. 6B,C). It was also found that inhibition of the Y1 receptor (using either the peptide inhibitor PYX-1 or a chemical inhibitor, BIBP3226) blocked budding in WDs cultured in the presence of FGF1 and GDNF (used to induce budding of the WD+IM cultures). The length and area of the buds that formed in the presence of NPY were also markedly enlarged compared with the buds that formed in the presence of GDNF alone (250% and 384%, respectively; Fig. 6D).

To determine whether PYY was also capable of facilitating budding, this neuropeptide was added to the cultured WDs. PYY, which was found to be expressed in the developing kidney, induced

Table 2. ANOVA and pattern matching selected genes enriched in the isolated ureteric bud

Probe name	*P*-value	Common	Description
1387154_at	2.01×10^{-8}	NPY02; RATNPY	Neuropeptide Y
1370177_at	1.41×10^{-7}	Taa1; Tage4	Poliovirus receptor
1380168_at	1.54×10^{-7}	Etv4; Pea3	ets variant gene 4 (E1A enhancer binding protein, E1AF) (predicted)
1367869_at	4.04×10^{-7}	MGC93253	Oxidation resistance 1
1390141_at	5.76×10^{-7}	Fthfsdc1_predicted	formyltetrahydrofolate synthetase domain containing 1 (predicted)
1392064_at	6.55×10^{-7}	Dlx1_predicted	Distal-less homeobox 1
1380062_at	9.94×10^{-7}	Mpp6_predicted	Membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6) (predicted)
1381545_at	1.22×10^{-6}		UI-R-CU0-bus-a-01-0-UI.s1 UI-R-CU0 Rattus norvegicus cDNA clone
1373625_at	1.24×10^{-6}	Shmt; mShmt	Serine hydroxymethyl transferase 1 (soluble)
1384828_at	1.75×10^{-6}	Kif7_predicted	Kinesin family member 7 (predicted)
1380749_at	2.09×10^{-6}	Sh2d4a	SH2 domain containing 4A
1373653_at	2.58×10^{-6}	Papd1_predicted	PAP associated domain containing 1 (predicted)
1386540_at	2.60×10^{-6}	Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
1384950_at	2.61×10^{-6}	MGC94512	Phosphatidylinositol 4-kinase type-II beta
1388485_at	2.79×10^{-6}	BRAK; chemokine	Chemokine (C-X-C motif) ligand 14
1368032_at	4.14×10^{-6}	Nopp140	Nucleolar and coiled-body phosphoprotein 1
1369473_at	4.63×10^{-6}	Pgm1	Phosphoglucomutase 1
1368674_at	4.68×10^{-6}	Pygl	Liver glycogen phosphorylase
1370162_at	5.02×10^{-6}	Pp4r1	Protein phosphatase 4, regulatory subunit 1
1374947_at	5.41×10^{-6}	Bcar3_predicted	Breast cancer anti-estrogen resistance 3 (predicted)
1373379_at	5.79×10^{-6}	Irak1_predicted	Interleukin-1 receptor-associated kinase 1 (predicted)
1374748_at	6.20×10^{-6}	Shmt; mShmt	Serine hydroxymethyl transferase 1 (soluble)
1368931_at	6.22×10^{-6}	SH3P13; Sh3d2c1	SH3 domain protein 2 C1
1368943_at	6.37×10^{-6}	Rnase4	Ribonuclease, RNase A family 4
1368290_at	6.58×10^{-6}	MGC93040	Cysteine rich protein 61
1376711_at	6.61×10^{-6}	Cldn11	Claudin 11
1373336_at	7.00×10^{-6}	Gprc5b_predicted	G protein-coupled receptor, family C, group 5, member B (predicted)
1395555_at	7.12×10^{-6}	p28	Golgi SNAP receptor complex member 1
1372088_at	7.63×10^{-6}	Rnf25	Ring finger protein 25
1389367_at	7.74×10^{-6}	Schip1_predicted	Schwannomin interacting protein 1
1368174_at	7.79×10^{-6}	LOC497816	Hypothetical gene supported by NM_019371
1374105_at	7.79×10^{-6}	Hig1	Hypoxia induced gene 1
1377631_at	9.49×10^{-6}	Col9a3_predicted	Procollagen, type IX, alpha 3 (predicted)
1368305_at	9.81×10^{-6}	Mch2; MGC93335	Caspase 6
1393101_at	9.88×10^{-6}	Fbxl10_predicted	F-box and leucine-rich repeat protein 10
1388912_a_at	1.16×10^{-5}	Ret	Ret proto-oncogene

ectopic budding similar to that seen with the addition of NPY (data not shown), raising the possibility that a redundant system exists that might compensate for the absence of any one component. Regardless of this, taken together, the microarray, network, statistical and functional data suggest that NPY is a key supporting growth factor for GDNF-dependent budding.

Role of NPY in GDNF-dependent budding

Because NPY is present in the thin layer of intermediate mesoderm (IM) surrounding the epithelial component of the WD, we sought to determine whether this was a potential source of the NPY necessary for GDNF-dependent budding in the WD(+IM) system. We attempted to block expression of NPY via RNA interference. With control fluorescent siRNA, good penetration of the IM surrounding the WD was observed; penetration of the epithelial cells of the WD was variable (Fig. 7A). We used a commercially available pool of four siRNAs targeted to rat NPY and added that to our culture system. An effect was seen in approximately half of the experiments. In the successful experiments, siRNA treatment of WD cultures targeting NPY resulted in decreased budding compared with a negative control treated with a non-targeting siRNA (Fig. 7B-G). The application of non-targeting siRNAs did not significantly alter the target gene expression in the WD when compared with negative controls that had no siRNA delivery components added. The number of buds decreased by 60% and the length of the buds that formed was decreased by 30% (Fig. 7E,F). In experiments in which the total level of NPY in all cultured WDs was decreased to 61% of that of non-targeting siRNA controls, there was no effect on budding (data not shown), whereas experiments in which the total NPY expression of all cultured WDs was decreased to 40% of that of the controls showed decreased budding (Fig. 7G), suggesting that there might be a threshold at which NPY reduction will have an effect. Based on the good penetration of the labeled siRNA into the IM but not the WD, we assume that the effect observed is due to >50% blockade of NPY expression in the IM.

NPY augments GDNF-induced budding but not GDNF-independent budding

The DNA array data described above suggested that GDNF stimulation leads to NPY expression. However, budding can be elicited without the presence of GDNF, providing a possible explanation for how rudimentary kidneys form in the absence of extant GDNF-RET signaling (Maeshima et al., 2007). Conceivably, NPY could be upregulated during budding either in general or primarily as a response to GDNF signaling. Thus, it is important to determine whether NPY supports both GDNF-dependent and GDNF-independent budding, or whether its role is specific to GDNF-dependent budding. To assess this, the expression of NPY in GDNF-dependent versus GDNF-

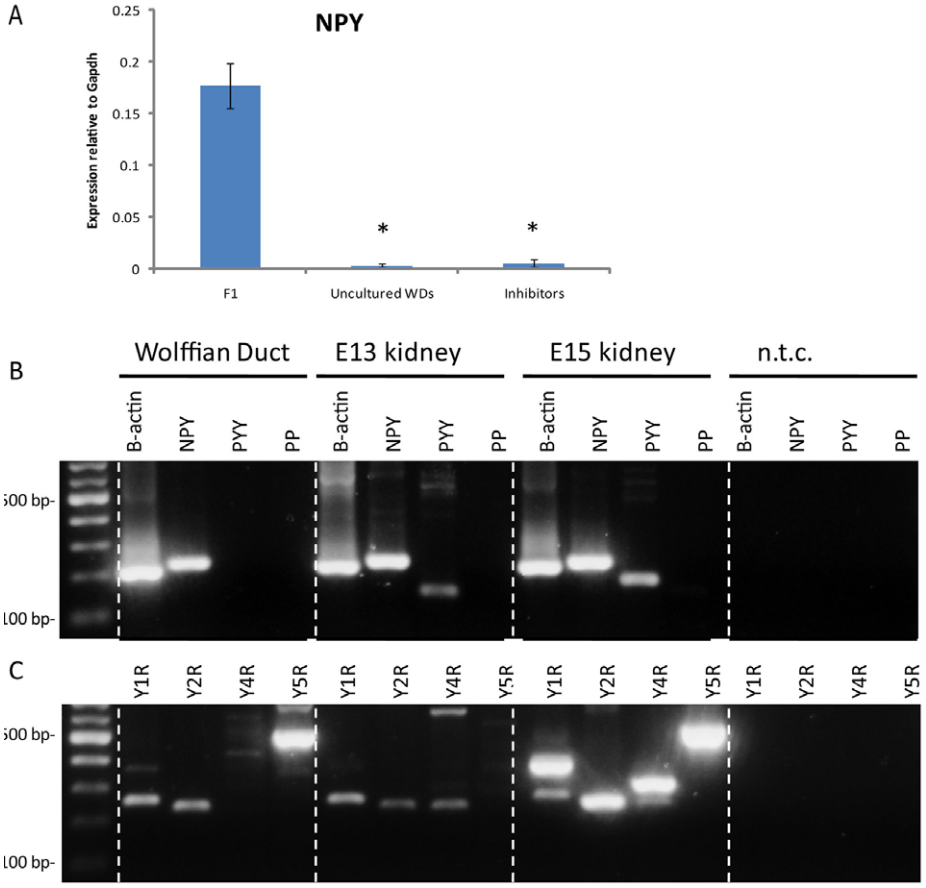

Fig. 5. NPY expression. (**A**) Comparison of NPY expression relative to GAPDH in WDs [WD(+IM)] cultured with GDNF and FGF1 (budded), in uncultured unbudded WDs, and in WDs cultured with GDNF and FGF1 with BMP4, activin A, or AKT inhibitor IV. **P*<0.05. (**B**) RT-PCR of NPY (234 bp), PYY (141 bp) and PP (142 bp) in the WD, E13 and E15 kidneys, and in a no template control (n.t.c.). (**C**) RT-PCR of NPY receptors Y1 (258 bp), Y2 (235 bp), Y4 (292 bp) and Y5 (524 bp).

independent budding was gauged. GDNF-dependent budding was obtained in vitro by adding GDNF plus an additional factor added to the culture medium, which could be an FGF (such as FGF1 or FGF7), or NPY (Fig. 8A). GDNF-independent budding was elicited through the addition of FGF7 plus the inhibition of activin A [via follistatin (FST) or a neutralizing anti-activin antibody; Fig. 8B] (Maeshima et al., 2007). NPY expression was increased in the GDNF-dependent condition, whereas the buds formed via the GDNF-independent budding mechanism did not exhibit increased NPY expression (Fig. 8C). In contrast to the GDNF-

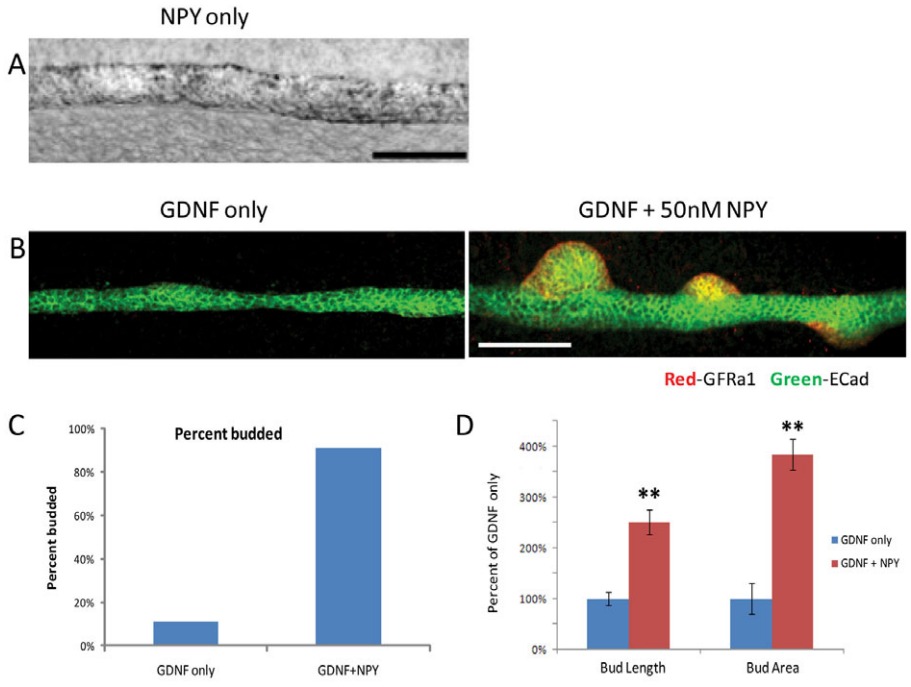

Fig. 6. NPY augments bud formation in vitro. (**A**) Addition of only NPY did not induce budding in the WD. Scale bar: 100 μm. (**B**,**C**) Addition of concentrations ranging from 50 nM to 2 μM of NPY to WDs cultured with 125 ng/ml GDNF resulted in 90% budding (right) compared with 10% budding in WDs cultured without added NPY (left). Confocal images in B show GFRα1 (red) and E-cadherin (green) staining. Scale bar: 100 μm. (**D**) Quantification of length and area increase caused by the addition of NPY (compared with those WDs exposed to just GDNF that did form a bud). ***P*<0.01.

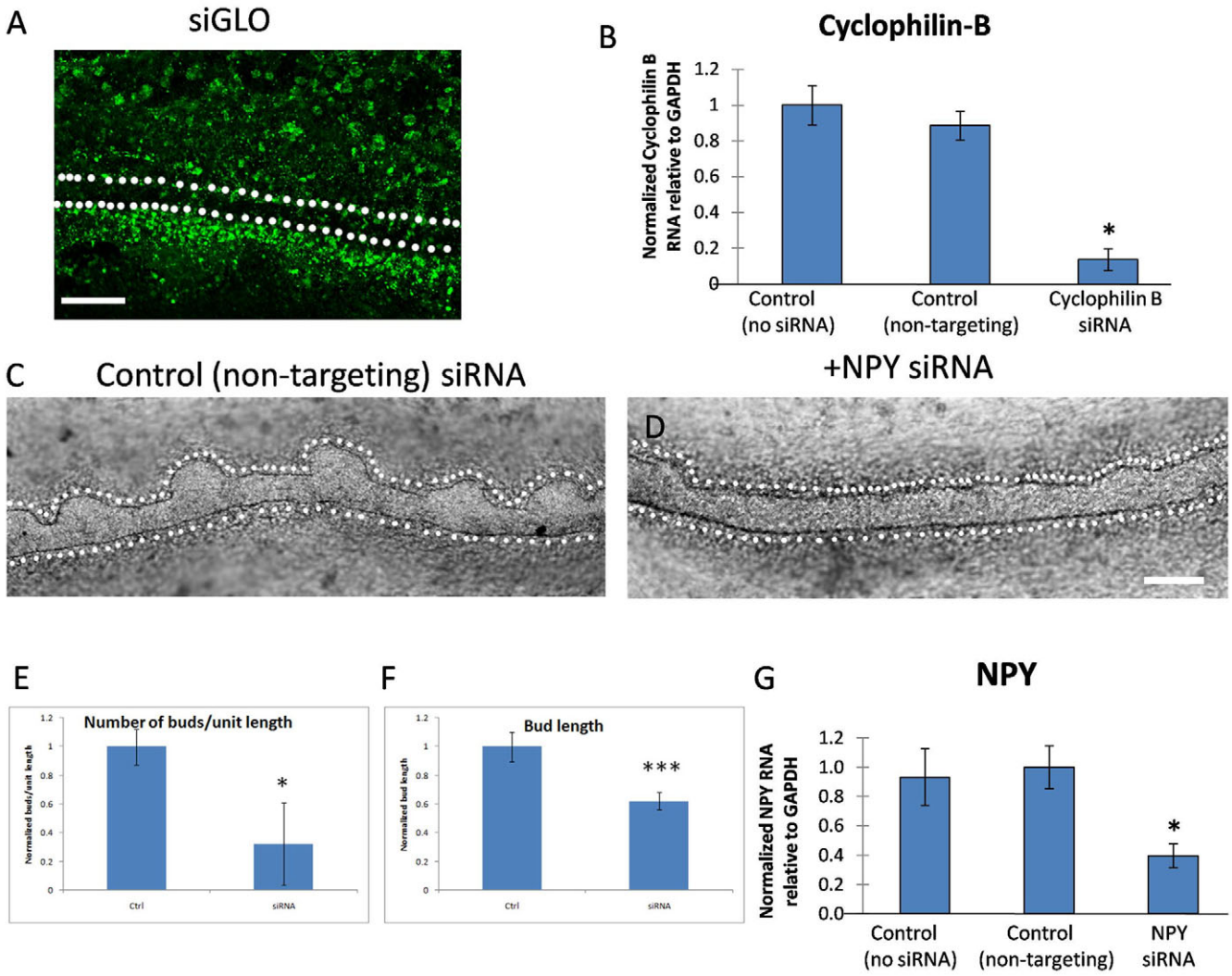

Fig. 7. siRNA silencing of NPY. (**A**) siGLO added for 24 hours showed penetration into the IM and slightly inside the WD epithelium (dotted lines). (**B**) WDs cultured with siRNA directed against cyclophilin B as a positive control reagent showed a decrease to approximately 20% of the expression levels observed in negative controls cultured with non-targeting siRNAs. The application of nontargeting siRNAs did not significantly alter the target gene expression in the WD when compared with negative controls with no siRNA delivery components added (n=10 for each condition). (**C**,**D**) WDs cultured with siRNA directed towards NPY (D) showed a decrease to approximately 40% of the expression levels observed in negative controls (C; n=10 for each condition). (**E**,**F**) The number of buds (E) per unit length decreased after culture with NPY siRNA, as did the length of the bud (F). (**G**) Experiments that showed a decrease in budding had a reduction of NPY mRNA to approximately 40% of the expression levels observed in controls cultured with either no siRNA or a mismatch oligonucleotide, n=10. *P<0.05, ***P<0.001. Scale bars: 100 μm.

dependent budding, addition of the NPY Y1, Y2 and Y5 receptor inhibitors did not inhibit formation of buds in WDs cultured in a GDNF-independent manner (data not shown). Furthermore, when NPY was added to WDs that were induced to bud without GDNF, this did not result in increased budding events (Fig. 6A). These results suggest that the role of NPY is specific to GDNF-dependent budding.

Rescue of BMP4 inhibition of budding by NPY and restoration of AKT phosphorylation

Because GDNF signals through a variety of pathways, we sought to determine which pathway NPY might be assisting. Prior studies suggest that NPY might act through the MAPK, the MEK/ERK or the PI3-kinase/AKT pathways (Anitha et al., 2006; Mannon and Raymond, 1998; Pierce et al., 2001). It has been previously shown that BMP4 inhibits GDNF-mediated budding of the WD both in vivo and in vitro (Tang et al., 2002), and it is believed that this balance is one of the important elements in limiting budding to a single site in vivo. However, the mechanism is not well understood. In our studies, addition of BMP4 decreased budding and also the localization of GFRα1 to budding regions (cf. Fig. 9A,C), suggesting that BMP4 directly affects signaling by GDNF. Significantly, addition of BMP4 decreased expression of RET in the WD (Fig. 9G). This inhibition of both GDNF receptors by BMP4 might be the endogenous means by which budding is suppressed by BMP4.

At 100 ng/ml of BMP4, budding was almost completely inhibited (Fig. 9C); however, addition of NPY resulted in restoration of budding, and of GFRα1 localized zones of budding, to levels similar to those of WDs cultured without BMP4 (Fig. 9D). In order to determine which pathway NPY activated during the rescue of budding, we performed a western blot for phosphorylated and total ERK and AKT of WDs cultured under conditions of BMP4

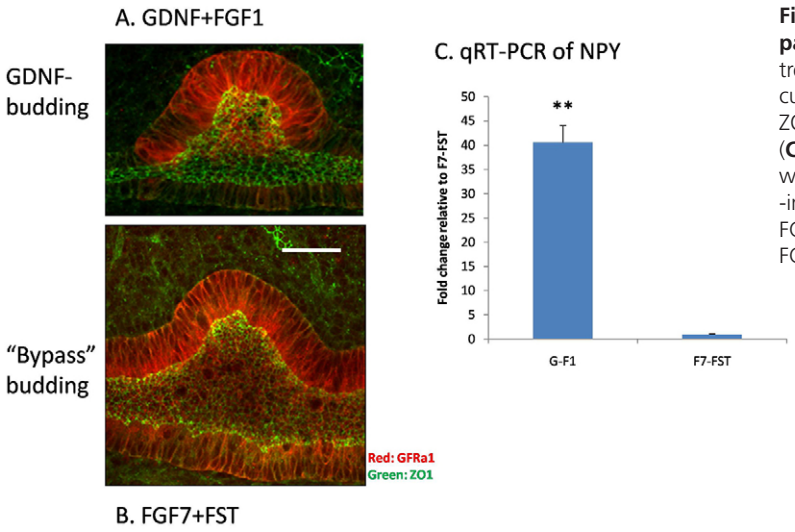

Fig. 8. Budding in GDNF dependent and independent pathways. (**A**) GDNF-dependent budding; cultures were treated with GDNF and FGF1. (**B**) GDNF-independent budding; cultures were treated with FGF7 + follistatin (FST). GFRα1, red; ZO-1 and E-cadherin, green (bright spots). Scale bar: 50 μm. (**C**) Relative expression of NPY in budded WDs cultured with or without GDNF. NPY expression in GDNF-dependent and -independent budding induced to bud with GDNF+FGF1 or FGF7+FST, respectively, normalized to WDs cultured with FGF7+FST using the Delta-Delta Ct method. ***P*<0.01.

suppression of GDNF-dependent budding with or without 200 nM NPY (Fig. 9E,F). WDs rescued with NPY showed elevated levels of phosphorylated AKT compared with WDs not treated with NPY; however, the relative amount of phosphorylated ERK (p44/42 MAPK) was not enhanced. These data suggest BMP4 acts to quench AKT signaling and that NPY rescues budding from the effects BMP4 treatment, possibly via reactivation of the AKT pathway.

Analysis of NPY deficient mice showed no readily apparent kidney defect at E11, E18 or in adult mice (data not shown), a finding that is in agreement with the reported phenotype (Erickson et al., 1996). This might be explained by a redundant network and overlapping functions in the NPY family, such as is the case when the Y2 receptor is upregulated in Y1 deficient mice (Wittmann et al., 2005). In the case of NPY deficiency, PYY or other factors (e.g. FGFs) might compensate for the missing neuropeptide (Maeshima et al., 2007). Although treatment of cultured WDs isolated from NPY-deficient embryos with siRNA targeting PYY inhibited budding (six out of six WDs with 79±6% PYY knockdown by qRT-PCR) compared with a non-targeting control siRNA, budding of the NPY knockout controls was less robust than had been previously observed in wild-type WDs (data not shown).

DISCUSSION

Based on the data presented here, we propose a feed-forward mechanism of budding where GDNF signals through its receptor RET to stimulate AKT (Fig. 10). Activation by GDNF or downstream signaling leads to increased expression of NPY (as well as of other facilitatory factors, such as PYY and FGFs), which in turn upregulates RET expression, thus amplifying the budding signal by increasing the sensitivity to GDNF. A complicated underlying network of interconnected genes supports the budding process. The known

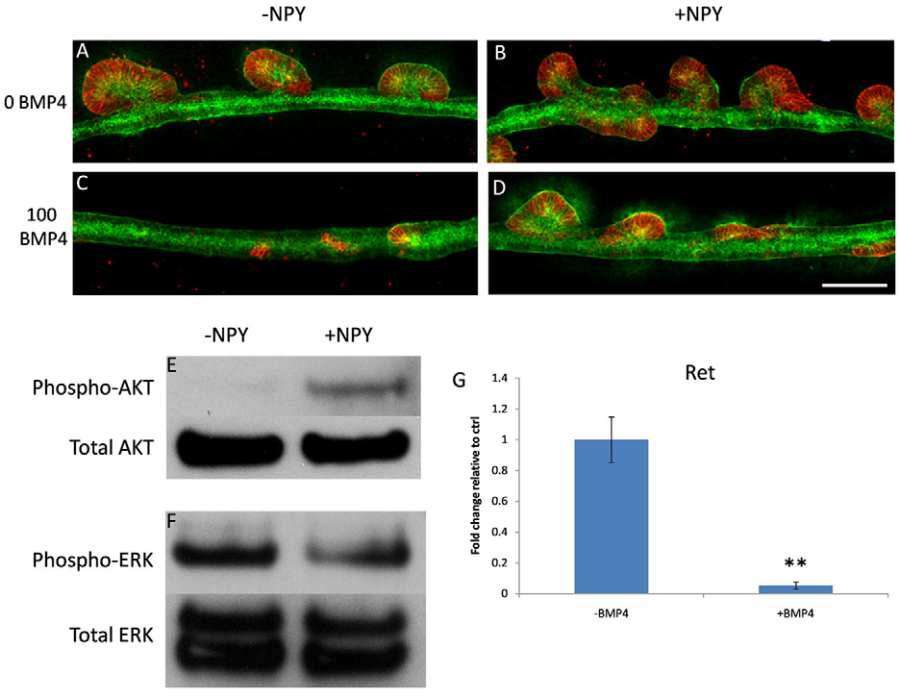

Fig. 9. NPY rescue of BMP4 inhibition. (**A-D**) WDs cultured with 125 ng/ml of GDNF and FGF1 without NPY (A,C) with 0 (A,B) or 100 (C,D) ng/ml BMP4 added. WD cultured with 125 ng/ml of GDNF and FGF1 with 200 nM NPY added (B,D). GFRα1, red; DB, green. Scale bar: 100 μM. (**E**,**F**) Western blot of (E) phospho-AKT (Ser473) and total AKT, and (F) phospho-ERK (p44/42 at Thr202/Tyr204) and total ERK in WDs treated with 100 ng/ml BMP4 without NPY (left, inhibited) or with 200 nM NPY (right, budded). (**G**) Relative expression of RET in WDs without and with BMP4 treatment. ***P*<0.01.

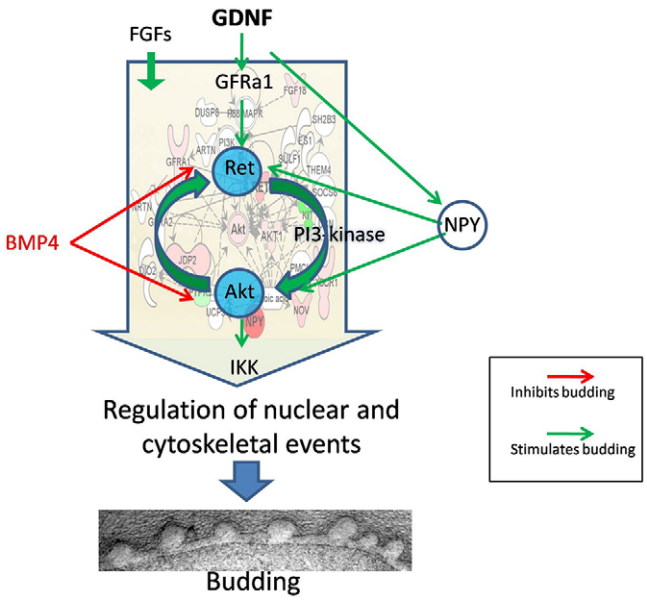

Fig. 10. Budding signaling diagram. Feed-forward budding with GDNF signaling through its receptor RET. GDNF stimulation leads to an increase in NPY expression and an upregulation in RET expression, thereby amplifying the signal. The inhibitor BMP4, conversely, works to suppress RET expression and AKT phosphorylation, thereby preventing signaling and budding. Various FGF proteins support budding, and inhibition of activin signaling might also be important. A complicated underlying network supports this budding process.

budding inhibitor, BMP4, conversely, might suppress budding by two mechanisms: (1) downregulating RET and GFRα1 expression and/or localization, thus preventing amplification of signaling and budding from occurring; and (2) blockade of the PI3-kinase/AKT signaling. Whether these two events are directly related, or whether one causes the other, remains to be determined. The GDNF-independent budding mechanisms, which are presumably responsible for rudimentary kidney formation when GDNF signaling is disrupted, bypass GDNF-RET and stimulate the PI3-kinase/AKT pathways, leading to budding that does not increase NPY, as shown in Fig. 8B (Maeshima et al., 2007).

Formation of the UB via budding from the WD is the key initiating step in kidney development. Failure of this step results in renal agenesis. The regulation of RET by endogenous inhibitors of budding, such as BMP4 and activin A, might be a key mechanism by which ectopic budding is regulated. Although GDNF appears to be the central modulator of UB formation, the downstream pathways and effector molecules that regulate epithelial outpouching remain undefined. By using a recently described in vitro model system of WD budding, we were able to study the effect of NPY and BMP4 on WD budding. In these in vitro experiments, the WD was separated from the mesonephros and gonadal ridge to isolate key morphogenetic processes involved in WD budding. In the data we presented, 10% of the WDs with a layer of IM [WD(+IM)] budded (minimally) with addition of GDNF alone, compared with nearly 100% that budded impressively with GDNF when the mesonephros and gonadal ridge were present, raising the possibility that cells within these tissues might play a role in the modulation of the budding process (Maeshima et al., 2006; Rosines et al., 2007). The PI3-kinase/AKT pathway was reported to be essential for bud formation in a mixed culture system that included the whole mesonephros (Tang et al., 2002). The PI3-kinase pathway appears to be activated in both the GDNF-dependent and GDNF-independent (FGF7) mechanisms, suggesting that common downstream pathways might be activated through separate initial signaling events (Maeshima et al., 2006; Rosines et al., 2007; Tang et al., 2002). Although FGF7 is usually not expressed during early kidney development, we have found it and other FGFs upregulated in a few of the developing kidneys in vivo in the absence of the RET receptor, which suggests that this or other genes not typically present during normal kidney development might be responsible for the significant number of UBs that do form in the absence of GDNF signaling (Maeshima et al., 2007). Blockade of activin signaling might also be important (Maeshima et al., 2006). This redundant budding mechanism might, in part, contribute to the robustness of kidney development.

We used a systems biological approach to analyze budded and unbudded tissue isolated from the developing rat kidney. Diverse tools, such as NMF for pattern recognition, gene ontology filtering and pathway analysis, were combined to narrow down the genes of interest into two primary gene networks. Not surprisingly, the first network included both GDNF receptors. The second grouping suggested that NPY was a worthy candidate for study. An additional method of analyzing the data was to use ANOVA and pattern matching to discern genes that are significant in the budding process. From this analysis, NPY was found to be the gene with the greatest correlation to budding.

Alternative methods of analysis generally resulted in RET, GFRα1 and NPY being selected as significant genes. If GO filtering were skipped and the genes were sent directly to IPA, six networks were generated. The first network (Cell Morphology, Cellular Growth and Proliferation) included the GDNF receptors RET and GFRα1. NPY was in the fifth network (Cellular Development, Nervous System Development and Function, Cancer). Some of the other networks included cell cycle, cell death and cellular growth. If, by contrast, the GO Signal Transduction list were further subdivided into signaling ligands (GO:5102 receptor binding), 17 genes remained and IPA generated only one network that contained NPY (along with several other genes known to be involved in early kidney development such as *Kit*, neurturin and *Wnt11*). These receptor-binding ligands might act to modulate the budding process in concert with GDNF-RET.

The power of this approach to identify novel factors in developmental processes is highlighted here because NPY-knockout animals do not display an overt kidney phenotype (Erickson et al., 1996), a finding that we have confirmed (data not shown). However, as we describe here, NPY/PYY/PP and the Y1-Y5 receptors are likely to form a redundant, though crucial, system. The effect of NPY on UB emergence from the WD seems unlikely to be a pure in vitro phenomenon, as NPY is dynamically expressed in the in vivo developing kidney. Furthermore, our data demonstrate that NPY rescues budding in BMP4-treated WDs. Thus, NPY modulates two of the key pathways known to regulate in vivo budding, and the in vitro model reproduces in vivo-based predictions. It has been suggested that endogenous BMP4 acts to suppress RET expression along the cephalic portion of the WD in vivo, thereby preventing the formation of ectopic buds; thus, NPY might assist GDNF in overcoming BMP4 suppression of budding in the WD. That NPY acts through the PI3-kinase/AKT pathway was suggested by the finding that addition of NPY restored phospho-AKT signaling in WDs exposed to BMP4. Stimulation of the PI3-kinase pathway has been shown to support renal epithelial cell proliferation during tubular development and

regeneration (Cantley et al., 1994; Derman et al., 1995; Zhuang et al., 2007), thus it is plausible that NPY stimulation of the PI3-kinase pathway leads to the proliferation of epithelial cells required to initiate UB formation. This is supported by our data demonstrating that buds stimulated by NPY are quantitatively larger than their counterparts without NPY added. NPY has been shown to exert similar proliferative effects in both the central and enteric nervous systems (Anitha et al., 2006).

Our data suggest that the role of NPY in UB formation is to assist GDNF-mediated budding. GDNF induces expression of NPY in enteric neurons; likewise, GDNF-induced bud formation in the WD leads to increased NPY expression. By contrast, buds formed through the 'bypass' mechanism do not show such upregulation. It seems plausible that GDNF stimulation of NPY leads to a feed-forward effect, whereby epithelial cell proliferation and morphogenesis is sustained by NPY once the process is initiated by GDNF. NPY, and other factors stimulated by GDNF, might directly or indirectly lead to increased RET receptor expression, increasing the local responsiveness of the WD epithelia to GDNF. BMP4 suppression of budding along the caudal portion of the WD downregulates RET expression; however, at the location of UB emergence, GDNF and NPY (and other factors, such as gremlin and FGFs) might act synergistically to overcome BMP4 inhibition (Maeshima et al., 2007; Michos et al., 2004). As many of these same factors regulate UB branching (Bush et al., 2004; Qiao et al., 1999; Shah et al., 2004), it seems plausible that NPY plays a similar role in GDNF-dependent branching. However, this remains to be tested experimentally.

The results of this study suggest there is a core set of genes required for budding to occur. Although GDNF appears to be a central modulator of UB formation, we demonstrate that other factors, such as NPY, contribute to this process. Loss of these factors might not manifest as obvious kidney phenotypes when genetically perturbed, but they are likely to contribute to the robustness of the developmental processes and may play a role in the determination of nephron number and disease (Nigam and Brenner, 1992; Shah et al., 2004). The identification of these types of augmenting factors can only be achieved through an approach such as the one described in this study, as the in vivo single knockout of these factors is unlikely to result in a readily detectable developmental phenotype. It will only be through the elucidation of these pathways that a comprehensive network for kidney development can be proposed (Monte et al., 2007; Nigam, 2003; Nigam et al., 2008; Sampogna and Nigam, 2004; Tsigelny et al., 2008).

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