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1 **Parallel microarray profiling identifies ErbB4 as a determinant of cyst growth in**
2 **ADPKD and a prognostic biomarker for disease progression**

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9 **Running Head**

10 ErbB4 expression and ADPKD disease progression

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16

17 **Abstract**

18 Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the fourth most common cause
19 of end-stage renal disease. The disease course can be highly variable and treatment options
20 are limited. To identify new therapeutic targets and prognostic biomarkers of disease, we
21 conducted parallel discovery microarray profiling in normal and diseased human *PKDI*
22 cystic kidney cells. A total of 1515 genes and 5 miRNA were differentially expressed by
23 more than two-fold in *PKDI* cells. Functional enrichment analysis identified 30 dysregulated
24 signalling pathways including the epidermal growth factor (EGF) receptor pathway. In this
25 paper, we report that the EGF/ErbB family receptor, ErbB4, is a major factor driving cyst
26 growth in ADPKD. Expression of ErbB4 *in vivo* was increased in human ADPKD and *Pkd1*
27 cystic kidneys, both transcriptionally and post-transcriptionally by mir-193b-3p. Ligand-
28 induced activation of ErbB4 drives cystic proliferation and expansion suggesting a
29 pathogenic role in cystogenesis. Our results implicate ErbB4 activation as functionally
30 relevant in ADPKD, both as a marker of disease activity and as a new therapeutic target in
31 this major kidney disease.

32

33 **Keywords**

34 ADPKD, ErbB4, microRNA, Polycystin

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36

37

38

39 **Introduction**

40 Autosomal dominant polycystic kidney disease (ADPKD) is the most common genetic
41 disease affecting the kidney (incidence 1 in 1,000) and is caused by mutations in two genes,
42 *PKD1* (85-90%) or *PKD2* (10-15%) (21). Around 10% of patients requiring renal
43 replacement therapy have ADPKD making it a major economic, medical and psychosocial
44 burden worldwide (21). Recent studies have confirmed strong genic and allelic influences on
45 the age of end-stage renal disease (ESRD) (4). However, the course of disease can be highly
46 variable with evidence of intra-familial variability indicating the likely influence of other
47 genetic factors (modifying genes, microRNAs), epigenetic factors and environmental
48 triggers.

49 The recent approval of the vasopressin type 2 receptor antagonist, tolvaptan, for ADPKD
50 patients with mild to moderate chronic kidney disease (eGFR $>30\text{ml}/\text{min}/1.73\text{m}^2$) and
51 evidence of rapid disease progression represents a step-change in the management of this
52 condition (8, 16). However, the drug is only moderately effective and has significant side-
53 effects including a risk of liver toxicity, limiting its use and requiring frequent monitoring.
54 There is therefore a need to develop more effective and safer drugs, which could also be used
55 in combination. In addition, the identification of new prognostic biomarkers would be a
56 major advance, especially in the early stages of disease, before significant increases in cystic
57 burden occur (as measured by total kidney volume).

58 To identify new therapeutic targets and prognostic biomarkers in ADPKD, we conducted
59 parallel microarray discovery profiling in normal and diseased human *PKD1* cystic kidney
60 cells, generated from nephrectomy tissue. Our results identify 30 dysregulated signalling
61 pathways when genes altered by at least 2-fold in abundance are analysed. Among these was

62 the EGF receptor signalling pathway, several members of which have been previously
63 implicated in PKD pathogenesis. In this paper, we report that expression and activation of the
64 EGF-related receptor, ErbB4, is increased in human and mouse models and that this drives
65 cyst proliferation and expansion in vitro. We also demonstrate that ErbB4 is a target for mir-
66 193b-3p, a novel finding. Finally, we show that urine exosome ErbB4 correlates significantly
67 with the rate of renal disease progression (eGFR slope), providing additional prognostic value
68 to mean kidney length (measured by ultrasound) in ROC analysis. These results indicate that
69 activation of the ErbB4 pathway is functionally relevant in ADPKD pathogenesis, reflects
70 disease activity and represents a new potential therapeutic target in this disease.

71

72 **Materials and Methods**

73 **Materials**

74 All chemicals were purchased from Sigma Chemical (Poole, Dorset, United Kingdom),
75 unless otherwise stated.

76

77 **Cell Lines**

78 Non-cystic (UCL93, RFH) and cystic (OX161, OX938, SKI-001, SKI-002) epithelial cells
79 were immortalised from primary cultures of tubular cells isolated from normal and ADPKD
80 human kidneys removed for clinical indications as previously described (24). SKI-002 cells
81 were found to carry germline (IVS25-3C>G) and somatic (2312_2324del) *PKDI* mutations
82 both of which are predicted to result in premature protein truncation.

83 Cells were grown in Dulbecco's Modified Eagles Medium-Ham's 12 (DMEM-F12,
84 Invitrogen) supplemented with 1% L-Glutamine (Invitrogen), 5% NuSerum (Becton
85 Dickinson) and 1% antibiotic/antimycotic solution (Invitrogen) at 33°C/5% CO₂. HEK-293
86 cells and control CL8 and CL11 kidney epithelial cells were cultured in Dulbecco's Modified
87 Eagles Medium-Ham's 12 (DMEM-F12, Invitrogen) supplemented with 1% L-Glutamine
88 (Invitrogen), 10% FCS and 1% antibiotic/antimycotic solution (Invitrogen) at 37°C/5% CO₂.

89

90 **Transfections**

91 Cells were transfected with a plasmid expressing the JM-A CYT-1 isoform of ErbB4
92 (Addgene plasmid #29527, pcDNA3.1-ErbB4) using Lipofectamine 2000 for 48h prior to the

93 cell proliferation assays. In some experiments, cells were transfected with negative control or
94 mir-193b-3p miRNA mimic (Life Technologies) using RNAiMax (Life Technologies).

95

96 **RNA extraction and microarray**

97 Total RNA was extracted from cells using Trizol according to the manufacturer's protocol
98 (Life Technologies). All human cells were maintained and RNA extracted between passages
99 10-20. Prior to RNA extraction, cells were synchronised by serum starvation for 24h
100 followed by reintroduction of serum for 24h in order to standardise the growth conditions
101 between the different cell lines. RNA quality was determined using an Agilent 2100
102 BioAnalyser. Parallel mRNA and miRNA microarrays were carried out on Agilent human
103 mRNA microarray (SurePrint G3 Human Gene Expression 8x60K v2 Microarray, one glass
104 slide formatted with eight high-definition 60K arrays) or human miRNA microarray (Release
105 19.0, 8x60K, one glass slide formatted with eight high-definition 60K arrays based on
106 miRBase Release 19.0) respectively.

107 A guided workflow based on Agilent single colour expression data within Agilent
108 GeneSpring GX software was used to identify differentially expressed mRNA and miRNA.
109 Briefly the guided workflow carries out a thresholding of the signal values to 5 followed by
110 log transformation. It then normalizes the data to the 75th percentile and performs baseline
111 transformation to the median of all samples. Samples are then divided into experimental
112 groups and entities filtered based on their flag values: P(present), M(marginal) and A(absent).
113 Only entities having the present and marginal flags in at least 1 sample are displayed.
114 Differential expression significance analysis is performed by unpaired T-test and p-values
115 >0.05 were defined as significant. Fold change analysis is then used to identify genes with
116 expression ratios or differences between ADPKD and control cells that are outside of a given

117 cutoff or threshold. Fold change gives the absolute ratio of normalized intensities (no log
118 scale) between the average intensities of the samples grouped. The entities satisfying the
119 significance analysis are passed on for the fold change analysis. All array files are available
120 from the ABI ArrayExpress database (accession number(s) E-MTAB-4188, E-MTAB-4189)
121 Pathway analysis was carried out using The PANTHER (Protein ANalysis THrough
122 Evolutionary Relationships) classification system which was designed to classify proteins
123 (and their genes) in order to facilitate high-throughput analysis. All genes which showed a >2
124 fold change in expression in ADPKD cell lines were mapped to PANTHER pathways which
125 consists of over 177 primarily signalling pathways. Pathways which were statistically
126 overrepresented ($p > 0.05$) were then identified.

127

128 **Quantitative PCR**

129 Relative ErbB4 and mir-193b-3p expression between control (n=4) and ADPKD (n=4) cells
130 as well as in isolated exosomes purified from human urine samples was determined by
131 Taqman qPCR according to the manufacturer's protocol (Life Technologies). Following
132 RNA extraction, cDNA was synthesised from ADPKD, normal cells and exosomes using
133 specific mir-193b-3p RT primers or a total RNA to cDNA kit (Life Technologies). Real time
134 PCR was carried out on an ABI7900 qPCR machine. Normalisation of expression was carried
135 out using specific primers to *GAPDH* or *RNU44*. Results presented are representative of 3
136 separate experiments. *Cre;Pkd1^{del2-11,lox}* mice were generated as previously described (15).
137 RNA was extracted from 4-5 mouse kidneys at 3 months and 4 months after tamoxifen
138 treatment at post-natal day 40, retrotranscribed into cDNA and changes in ErbB4 and mir-
139 193b-3p expression determined. Analysis of gene and miRNA expression were carried out
140 using DataAssist v3.01 (Applied Biosystems) to calculate $1/\Delta Ct$ normalised expression

141 values for each cell line. Data was plotted in Graphpad Prism and p-values >0.05 (adjusted
142 for Benjamini-Hochberg False Discovery Rate) were defined as significant. Relative
143 quantification was also carried out using the comparative Ct ($\Delta\Delta Ct$) method to calculate fold
144 changes in expression between control and ADPKD samples.

145

146 **Small Interfering (siRNA) knockdown**

147 Isoform-specific siRNA to human ErbB4 was chemically synthesized by Cell Signalling
148 Technology. A scrambled negative control siRNA (Silencer) was purchased from Ambion.
149 Transfection of siRNA into cells was achieved using RNAiMax reagent (Life Technologies).
150 Knockdown was confirmed by qPCR 48 h post-transfection.

151

152 **Immunoblotting**

153 Total cell lysates were prepared and processed for immunoprecipitation and Western blotting
154 as described previously (20). Cells were solubilized in detergent lysis buffer (50 mM Tris,
155 0.14 M NaCl, 1% Triton X-100, and 0.5% NP40) supplemented with Complete protease
156 inhibitors and PhosStop phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany).
157 Commercial antibodies against the C-terminus of ErbB4 (EP192Y), phosphoErbB4-Y1188
158 (EPR2271Y) (Abcam, UK), calnexin, pAKT, total AKT, pERK and total ERK (Cell
159 Signalling, USA) were used for western blotting. ECL detection and quantification was
160 carried out using a Biorad Chemidoc XRS+ system running Image Lab automated image
161 capture and analysis software. All quantification was carried out on non-saturated bands as
162 determined by the software. Data is presented as the ratio of ErbB4 to calnexin.

163

164

165 Immunohistochemistry

166 Archival nephrectomy sections were obtained from 6 ADPKD patients with known
167 truncating *PKDI* mutations and from 3 control patients (22). Mouse kidney tissue from
168 *Cre;Pkd1^{del2-11,lox}* mice were obtained at 1, 2, 3 and 4 months post tamoxifen treatment (n=3).
169 Formalin-fixed paraffin sections were dewaxed and hydrated through graded ethanol
170 solutions. For antigen retrieval, the sections were placed in 10 mM citric acid buffer (pH 6.0)
171 and incubated in a pre-heated water-bath maintained at 95°C for 15 min. Endogenous
172 peroxidase activity was quenched by treatment with methanol containing 3% hydrogen
173 peroxide for 30 m. Sections were then incubated in diluted blocking serum for 30 m,
174 followed by incubation overnight at 4°C with ErbB4 antibody (1:100 dilution). For human
175 and mouse kidney sections, a peroxidase-conjugated secondary antibody was employed and
176 peroxidase activity was visualised using a substrate solution of diaminobenzidine (DAB)
177 containing 0.03% hydrogen peroxide. Negative control sections were processed with
178 omission of the primary antibody and a non-immune IgG. All sections were counterstained in
179 haematoxylin.

180

181 Luciferase Reporter Assay

182 A 428bp fragment of the 3'UTR of ErbB4 containing a predicted mir-193b-3p seed sequence
183 was amplified from RNA extracted from HEK-293 cells by PCR using the following primers:

184 Forward: GGTCGTGAGCTCCACACCTGCTCCAATTTCCCC

185 Reverse: CTCGTACTCGAGATGCACACATCAGTTCCTGC

186 Following reverse transcription, ErbB4 3'UTR cDNA was subcloned into a pmirGLO
187 reporter plasmid (Promega). The predicted mir-193b-3p seed sequence was mutated using the
188 following primers according to the Stratagene site-directed mutagenesis protocol:

189 Forward: CTTCTTCTACCCCAAGGCGTCTCGTTTTGACACTTCCCAG

190 Reverse: CTGGGAAGTGTCAAAACGAAGACGCCTTGGGGTAGAAGGAAG

191 For the luciferase assay, HEK293 cells were plated into a 96-well plate at 25,000 cells / well
192 the day before transfection. For 5 replicates, 1.5 μ L of Lipofectamine 2000 reagent was mixed
193 to 50 μ L of OptiMEM medium and 375 μ g of pcDNA 3.1 + 125ng pmirGLO vector together
194 with negative control miRNA or a mir-193b-3p mimic at a final concentration of 25 or 50
195 nM. 24 h after transfection, the luciferase/renilla signals ratios were measured using the Dual
196 Luciferase Reporter Assay from Promega. 100 μ L of Passive Lysis Buffer was added directly
197 into the wells to lysate the cells and 50 μ L from each well were used for signal detection with
198 a Perkin Elmer luminometer.

199

200 **ErbB4 functional inhibition assays**

201 Control and ADPKD cells were plated at 5000 cells/well in a 96well plate. Cells were
202 incubated with a pan-ErbB inhibitor including to ErbB4, JNJ 28871063 hydrochloride
203 (Tocris, USA) for 24 h, an inhibitory ErbB4 antibody (clone H.72.8, Millipore, USA) or non-
204 immune control rabbit antibody for 72 h. To test the effect of the ErbB4 ligand NRG-1, cells
205 were serum starved for 24 h prior to incubation with 100 ng/ml NRG-1 (Cell Signalling,
206 USA) for 72 h. Following the indicated treatment, cell proliferation was measured using a
207 commercial BrdU ELISA kit (Roche, Basel, Switzerland) according to the manufacturer's
208 instructions.

209

210 Matrigel 3D cyst assays

211 OX161 cells (1×10^5 /well) were mixed with 70 μ l Matrigel (Becton Dickinson, UK), plated
212 into 96 well plates in triplicate and incubated for 30 m at 37°C to allow the gel to set. Cells
213 were cultured for 12 d in the presence or absence of 100 ng/ml NRG-1 or HB-EGF. Media
214 was replaced every 2 d. The average cyst area was calculated by measuring cyst areas in
215 individual wells on days 4, 7 and 12. At least 65 cysts were measured in triplicate wells at
216 each time-point.

217

218 Patient recruitment

219 All participants gave their signed informed consent at the time of recruitment. ADPKD
220 patients were recruited through the Sheffield Kidney Institute and healthy normal volunteers
221 were recruited from laboratory staff. Ethical approval for this study was obtained from the
222 National Research Ethics Service Committee Yorkshire & The Humber - Bradford
223 (REC12/YH/0297). Following collection, urine samples were centrifuged at 1000g for 10
224 min at 4°C. Cell pellets were discarded and cell free supernatants stored at -80°C until further
225 analysis.

226

227 Isolation of Urinary Extracellular Vesicles

228 Urinary extracellular vesicles (UEVs) or exosomes were isolated by differential
229 centrifugation as previously reported (25). In brief, 10 ml of urine was initially centrifuged at
230 17000g for 15 min. The supernatant was retained and the pellet resuspended in 200 μ l

231 isolation buffer (250 mM Sucrose, 10 mM Triethanolamine pH7.6, 50 μ l of DDT) to reduce
232 the highly abundant Tamm-Horsfall protein. Following incubation at room temperature for 5
233 min, the sample was briefly vortexed and centrifuged again at 17,000g for 15 min. This
234 supernatant was then combined with the initial supernatant and centrifuged once more at
235 170000 g for 150 min. The final pellet was re-suspended in 50 μ l lysis buffer (50 mM Tris
236 pH 7.4, 150 mM sodium chloride, 1% Triton X-100, 1% sodium-deoxycholic acid and
237 protease inhibitors) on ice for 1 h (3). A protein assay was carried out to normalise protein
238 concentration prior to SDS-PAGE.

239

240 **Electron Microscopy**

241 Electron microscopy was carried out to visualize the morphology of the UEVS isolated.
242 Briefly, the pellet isolated was first resuspended in 50 μ l PBS and a small drop of this
243 mixture was deposited on 200-mesh nickel grids. Negative staining of the mesh was carried
244 out using heavy metal salt (0.5 % Uranium). After drying, the nickel grids were visualized
245 using a Philips electron microscope 400 operated at 80 KV.

246

247 **Statistical Analysis**

248 Data are presented as mean values \pm SEM. Student's *t* test or ANOVA were used for
249 statistical analysis with a p value of <0.05 indicating statistical significance. The degree of
250 significance as determined by GraphPad Prism software is denoted by the following asterisks:
251 *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001, ****P \leq 0.0001

252 **Results**

253 **Parallel mRNA/miRNA expression microarray profiling in ADPKD cell lines**

254 A parallel mRNA and miRNA expression microarray was performed to identify differential
255 changes in mRNA/miRNA expression levels in ADPKD cells compared to non-ADPKD
256 controls. We then used this information to identify predicted mRNA/miRNA interactions
257 which could contribute to ADPKD pathogenesis (**Fig 1A**). The expression of 1515 genes was
258 significantly altered between control and ADPKD cell lines by more than 2 fold and 5
259 miRNAs were significantly altered. In total, 447 genes were significantly up-regulated (**Fig**
260 **2A**) and 1068 genes (**Fig 2B**) significantly down-regulated in ADPKD cells. All the
261 significantly altered miRNAs were down-regulated in ADPKD cells (**Fig 2C**). These
262 miRNAs in turn were predicted to target 5721 genes (TargetScan algorithm) of which 390
263 genes were differentially expressed in the parallel mRNA microarray. Functional enrichment
264 analysis of differentially expressed genes (>2 fold) in ADPKD was carried out using Panther
265 pathway analysis software (<http://www.pantherdb.org/>). A total of 22 signalling pathways
266 were significantly enriched from the list of genes upregulated in ADPKD whereas 8
267 pathways were significantly enriched from the list of genes downregulated in ADPKD (**Fig**
268 **1B**). These included pathways reported to be associated with ADPKD including cadherin,
269 EGF receptor (3.38 fold enrichment), Wnt and G protein signalling. Of the ErbB receptors,
270 the only receptor showing significant differential expression was ErbB4 (~40-fold).

271

272 **Validation of an interaction between mir-193b-3p and ErbB4 mRNA**

273 The differential increase in ErbB4 expression was first confirmed by Taqman qPCR, showing
274 a mean 10-fold increase in ADPKD compared to control cells (**Fig 3A**). We noted that of the

275 5 differentially expressed miRNA identified, mir-193b-3p was predicted to regulate ErbB4,
276 this also being the highest ranked gene identified by a TargetScan prediction algorithm
277 (<http://www.targetscan.org/>, Release 6.2) of 30 predicted targets (**Fig 2D**). Consistent with
278 these predictions, multiple independent miRNA target prediction algorithms (DIANAmT,
279 miRanda, miRDB, miRWalk, RNAhybrid, Targetscan and PICTAR5) identified two
280 potential mir-193b-3p binding sites within the 3'UTR of ErbB4.

281 We next validated the reduction of array mir-193b-3p expression in ADPKD cells by Taqman
282 qPCR showing a 0.5 fold decrease (**Fig 3B**). To provide direct evidence of a functional
283 interaction between mir-193b-3p and ErbB4 mRNA, we generated a luciferase reporter
284 plasmid (pmirGLO) containing a fragment of the ErbB4 3'UTR and mutated the predicted
285 mir-193b-3p seed sequence in a second reporter construct (**Fig 3C**). Co-expression of a mir-
286 193b-3p miRNA mimic significantly reduced luciferase activity in the wild-type but had no
287 effect on the mutated sequence (**Fig 3D**). Finally, expression of the mir-193b-3p mimic in the
288 ADPKD cell line (OX161) resulted in a significant reduction in endogenous ErbB4 mRNA
289 (**Fig 3E**) and ErbB4 protein (**Fig 3F**). These results provide the first evidence that ErbB4
290 mRNA is potentially a target of mir-193b-3p.

291

292 **ErbB4 is over-expressed in ADPKD cell lines as two distinct isoforms**

293 By immunoblotting, we confirmed that full-length ErbB4 protein were increased in all four
294 ADPKD cell lines tested, being almost undetectable in control cells (**Fig 4A**). The
295 *ERBB4* gene however is known to undergo alternative splicing generating several isoforms
296 differing in their N-termini or C-termini (**Fig 4B**). For instance, the two CYT isoforms differ
297 in a stretch of 16 amino acids in the C-terminus encoded by a single exon (CYT-1) that is
298 absent in CYT-2. This structural difference contributes to differential subcellular targeting

299 and even antagonistic functional responses mediated by the two CYT isoforms (40). Apart
300 from the CYT isoforms, tissue-specific alternative splicing can generate JM-a and JM-b
301 isoforms differing in their extracellular juxta-membrane (JM) domain and function (6).

302 To investigate the possibility of an isoform switch in ADPKD, RT-PCR was carried out using
303 specific primers flanking the variable regions using cDNA isolated from OX161 cells (**Fig**
304 **4B**). As a positive control, we used a JM-a/CYT-1 plasmid. For the JM region, the expected
305 sizes were 0.375kb (JM-a) and 0.345kb (JM-b) respectively. A single band corresponding to
306 JM-a (0.375kb) was detected with no evidence of JM-b (0.345kb) (**Fig 4C**). Similar analysis
307 using primers flanking the cytoplasmic site revealed that both CYT-1 and CYT-2 isoforms
308 were expressed, with a higher abundance of CYT-1. Primers designed to a conserved ErbB4
309 motif identified the same control band in both samples. Our results reveal the presence of two
310 isoforms (JM-a/CYT-1 and JM-a/CYT-2) in normal and ADPKD cells with no evidence of
311 an isoform switch (**Fig 4D**).

312

313 **Nuclear ErbB4 expression is increased in human ADPKD kidneys and in a *Pkd1* mouse** 314 **model**

315 The expression of ErbB4 was increased in cyst lining epithelial cells of human ADPKD
316 kidney sections compared to control kidney tissue by immunohistochemistry (**Fig 4E**).
317 Significantly elevated nuclear localisation of ErbB4 was observed in ErbB4 positive cysts
318 (black arrows) indicating increased levels of ErbB4 protein and C-terminal cleavage (**Fig 4E,**
319 **F**). We next analysed ErbB4 expression in a previously reported inducible kidney-specific
320 *Pkd1* mutant mouse model (14). Following induction with tamoxifen at PN40 these animals
321 slowly develop a cystic phenotype with large cysts becoming apparent 4 months after
322 tamoxifen induction. In agreement with the human studies, there was a significant increase in

323 ErbB4 mRNA expression in *Cre;Pkd1^{del2-11,lox}* mice induced with Tamoxifen at 4 months
324 (**Fig 4G**). Similarly, ErbB4 staining was increased in cyst lining epithelial cells of kidney
325 sections. The number of ErbB4 positive cysts increased following Pkd1 deletion with the
326 strongest staining at 4 months after tamoxifen induction. (**Fig 4H**) In agreement elevated
327 nuclear localisation in ErbB4 positive cysts was significant from 2 months after induction
328 (**Fig 4I**). In both human and mouse tissue sections a minority of cysts (~20%) showed no
329 detectable ErbB4 staining although it was not possible to distinguish these from positive
330 stained cysts in terms of size or time following induction. At 4 months, a significant decrease
331 in mir-193b-3p mRNA was detectable in *Cre;Pkd1^{del2-11,lox}* kidneys (**Fig 4J**).

332

333 **Increased expression of cleaved ErbB4 and decreased expression of mir-193b-3p in** 334 **human ADPKD urinary extracellular vesicles**

335 To confirm that these changes also occurred *in vivo*, we isolated urinary extracellular vesicles
336 (UEV) from healthy volunteers (n=12) and ADPKD patients with early (eGFR
337 >60ml/min/1.73m², n=16) or late disease (eGFR <60ml/min/1.73m², n=16) using an
338 established protocol (25). Electron microscopy confirmed the presence of multiple (<100nm)
339 vesicles in the pelleted fraction (**Fig 5A**). The exosome-specific protein TSG-101 was
340 restricted to the pellet and there was clear expression of aquaporin-2 (AQP2) in both cell-free
341 urine and the pellet indicating the origin of some vesicles from collecting ducts (**Fig 5B**).

342 An ErbB4 positive band was detected at 80 kDa in UEVs, corresponding to the known C-
343 terminal intracellular domain (ICD) generated by γ -secretase mediated cleavage (**Fig 5C**). A
344 minor band at 50 kDa was also observed which could correspond to proteolytic degradation
345 or represent a non-specific band. Expression of the 80kDa band was significantly increased in
346 the UEVs of ADPKD patients compared to healthy controls particularly in patients with late

347 disease as defined by baseline eGFR (**Fig 5D**). ErbB4 expression also correlated significantly
348 with the rate of decline in eGFR and outperformed mean kidney length (MKL) measured by
349 ultrasound (**Fig 5E**). In combination, ErbB4 and MKL were additive in ROC analysis,
350 accounting for 0.816 of AUC (p=0.002).

351

352 **Increased ligand dependent proliferation signalling and cyst growth in ADPKD cells**

353 A total of 11 ligands have been reported to bind to members of the EGF receptor family. Of
354 these, several have been shown to activate ErbB4 including the neuregulins (NRG1-4) and
355 HB-EGF (27). However, ErbB4 activation can stimulate or inhibit proliferation in different
356 cells, possibly due to differential expression of ErbB4 isoforms (37, 40).

357 To determine the functional consequence of ErbB4 overexpression in ADPKD cells, we first
358 studied their response to NRG-1. A significant increase in NRG-1 mediated proliferation was
359 seen in the *PKDI* cell lines with almost no effect in control cells (**Fig 6A**). This was mirrored
360 by significant increases in phosphorylated ErbB4 (pErbB4) in response to NRG-1 (**Fig 6B**).
361 Since the OX161 line showed the greatest response to NRG-1, this line was used for further
362 experiments.

363 The JM-a isoforms of ErbB4 are known to undergo two step-wise proteolytic cleavage events
364 when activated, generating a shed N-terminal ectodomain (110kDa) by Tumour Necrosis
365 Factor Alpha Converting Enzyme (TACE) cleavage and a soluble intracellular C-terminal
366 domain (80kDa) by the action of γ -secretase (17). Several downstream signalling pathways
367 are known to be activated including the PI3K/AKT pathway (cell survival), STAT 5
368 (mammary development), Ras/ERK1/2 and PLC γ pathways (cell proliferation). Accordingly,

369 a significantly greater increase in pERK and pAKT was observed in ADPKD (OX161) cells
370 in response to NRG-1 compared to control (UCL93) cells (**Fig 6C**).

371 A small single centre study recently reported that urinary HB-EGF excretion was increased in
372 a cohort of ADPKD patients and showed a positive correlation with more advanced disease ie
373 lower GFR and higher total kidney volumes (10). In contrast, urinary EGF and TGF- α levels
374 decreased with increasing disease severity. Incubation with HB-EGF, similar to NRG-1, was
375 associated with an increase in phosphorylated ErbB4 in ADPKD cells (OX161) suggesting a
376 potential role in activating ErbB4 in ADPKD cells (**Fig 6D**). The effect of both ErbB4
377 ligands on cyst growth was therefore studied in 3D cyst assays. OX161 cells spontaneously
378 form cyst-like structures with well-developed lumen when cultured in matrigel. Both NRG-1
379 and HB-EGF significantly increased cyst growth in OX161 cells as measured by total cyst
380 area in 3D cyst assays (**Fig 6E**). These results confirm that ligand-activated ErbB4 signalling
381 is likely to be a pathogenic factor in ADPKD cyst expansion, by promoting cell survival and
382 stimulating cell proliferation.

383

384 **Inhibition of ErbB4 reduces proliferation in ADPKD cells**

385 Over-expression of ErbB4 in control (UCL93) cells significantly increased basal cell
386 proliferation showing that increasing ErbB4 expression alone was sufficient to induce a pro-
387 proliferative phenotype (**Fig 7A**). Conversely, siRNA knockdown of ErbB4 in OX161 cells
388 inhibited cell proliferation compared to control siRNA treated cells (**Fig 7B, C**).

389 A pan-ErbB inhibitor (JNJ 28871063) which inhibits ErbB1, ErbB2 and ErbB4 (IC₅₀ 21-38
390 nM) (7) reduced proliferation of OX161 cells but had no effect on UCL93 cells at the same

391 doses (**Fig 7D**). These results are consistent with the specificity of JNJ 28871063 on ErbB-
392 driven cancer cell lines but its lack of effect in non-ErbB lines (7).

393 Although ErbB1, ErbB2 and ErbB3 were not differentially expressed in the ADPKD cells,
394 we decided to utilise an ErbB4-specific inhibitory antibody to isolate an ErbB4 specific role
395 in these cells (12, 29, 39). ErbB4 selective blockade reduced basal proliferation in OX161
396 cells to that seen in UCL93 control cells (**Fig 7E**) whereas an irrelevant IgG control antibody
397 had no effect (**Fig 7F**). These results demonstrate that ErbB4 activation could contribute to
398 the increased proliferation rate of ADPKD cells, probably through the action of endogenous
399 ligands such as NRG1 and HB-EGF.

400 **Discussion**

401 In this paper, we report the identification of ErbB4 as a potential new biomarker of disease
402 activity and a new therapeutic target in ADPKD. Parallel discovery microarray profiling in a
403 panel of human ADPKD cell lines previously shown to express germline and somatic
404 mutations in *PKD1* but not *PKD2* (24) identified a total of 1513 differentially expressed
405 genes in ADPKD cells mapping to several pathways previously implicated in ADPKD. We
406 also identified 5 miRNAs that were significantly down regulated in ADPKD cells.

407 The EGF/ErbB receptor family has been previously implicated in experimental models of
408 ADPKD (11). The ErbB receptor family has four members: EGFR/ErbB1, ErbB2/HER2/neu,
409 ErbB3/HER3 and ErbB4/HER4 which belong to the receptor tyrosine kinase superfamily. A
410 total of 11 putative ligands with different receptor selectivity to the ErbB receptors have been
411 reported, with the exception of ErbB2 (27). The complexity of EGF/ErbB signalling is further
412 compounded by the formation of different homo- and heterodimers and the existence of
413 multiple splice forms (especially for ErbB4).

414 The role of EGF/ErbB signalling in ADPKD pathogenesis has attracted attention due to the
415 hyperproliferative phenotype that characterises ADPKD (1). However, inhibition of EGF
416 signalling has yielded conflicting results in different experimental models. For instance, an
417 EGFR and ErbB2 antagonist reduced cystic disease in the Han:SPRD rat and *bpk* mouse
418 models but had a detrimental effect in the *Pck* rat (26, 32, 34, 35). Nonetheless, the
419 physiological importance of the EGF receptor during kidney development, repair and tubular
420 physiology could make this a difficult drug target for ADPKD.

421 We decided to focus our study on ErbB4 for a number of reasons. In murine PKD disease
422 models, renal ErbB4 expression has been shown to be increased (*bpk*, *cpk*) (19, 42). ErbB4
423 plays a key role during nephrogenesis being induced in the earliest murine tubular structures

424 by E14.5 and later in the collecting ducts (37). ErbB4 null mice die by E11 prior to
425 nephrogenesis due to defective heart development (9) but ErbB4 knockout mice rescued with
426 a cardiac myosin-promoter driven ErbB4 transgene have defective mammary gland and
427 neuronal abnormalities but no reported kidney phenotype and display a normal lifespan (33).
428 Consistent with a major role during development, conditional deletion (Pax8-Cre) of ErbB4
429 affecting both renal ErbB4 isoforms (JMa-CYT-1, JMa-CYT-2) from E11.5 altered normal
430 tubular diameter, cell number and differentiation (37). Conversely, transgenic expression of
431 JMa-CYT-2 in the kidney from E11.5 resulted in cortical cysts, tubular proliferation and
432 lumen dilatation (37). Unexpectedly, another group reported that deletion of ErbB4
433 significantly worsened the cystic phenotype of *cpk* mice, a recessive model of PKD (42). One
434 possible explanation for this discrepancy is that both major ErbB4 isoforms were deleted in
435 this study. While deletion of JMa-CYT-2 should be protective (see below), deletion of the
436 JMa-CYT-1 isoform could enhance cystogenesis by removing an inhibitory brake on cell
437 proliferation; the differential expression of each ErbB4 isoform in the kidneys of *cpk* mice
438 was not reported. This imbalance created by deleting both ERbB4 isoforms could have
439 resulted in the surprising results observed in *cpk*/ErbB4 null mice. Formal testing of these
440 possibilities in orthologous models by chemical or genetic manipulation will be required to
441 obtain a definitive answer relevant to ADPKD.

442 We observed a consistent upregulation of ErbB4 expression in human and mouse disease
443 models, both *in vitro* and *in vivo*. In addition, we report for the first time that ErbB4 is a
444 direct target for mir-193b-3p. The increase in ErbB4 mRNA occurs early in disease (2
445 months in *Pkd1* conditional mice, Fig 4G) and is likely to involve increased gene
446 transcription in view of the magnitude of ErbB4 elevation (10-fold) observed in cells. At a
447 later stage of disease, it is probable that the reduction in mir-193b-3p expression also
448 contributes to a post-transcriptional increase in ErbB4 mRNA levels (Fig 4G, J).

449 In the kidney, ErbB4 is expressed predominantly as the isoforms JM-a/CYT-1 and JM-
450 a/CYT-2, with possibly more medullary expression of JM-a/CYT-2 (37, 44). Both isoforms
451 undergo sequential proteolytic cleavage to generate a soluble intracellular domain (ICD) that
452 can translocate to the nucleus to regulate gene transcription. The CYT-1 isoform contains a
453 16 aa sequence which is lacking in the CYT-2 isoform, enabling CYT-1 to couple to PI3K.
454 However, levels of kinase activity, protein stability and nuclear accumulation are greater for
455 the CYT-2 ICD than the CYT-1 ICD (30, 43). CYT-1 ICD inhibits cell proliferation,
456 undergoes ubiquitination but promotes cell differentiation whereas the opposite is true for the
457 CYT-2 ICD (18, 31, 38, 40). Both isoforms were similarly expressed in control and ADPKD
458 cells with a slightly greater expression of CYT-1.

459 The increase in ErbB4 expression was also associated with increased ligand-activated
460 signalling. Addition of two ErbB4 ligands, NRG-1 and HB-EGF, was associated with
461 increased ErbB4 phosphorylation, downstream activation of pERK and pAKT pathways,
462 increased proliferation and cyst formation in ADPKD cells. Conversely, blocking
463 endogenous ErbB4 in cystic cells using siRNA, a kinase inhibitor (JNJ288) or an anti-
464 functional antibody (H.72.8) reduced cell proliferation to that of untreated controls. These
465 findings were confirmed by evidence of increased ErbB4 activation *in vivo*. Cystic human
466 and murine kidney sections showed increased nuclear expression of C-terminal ErbB4.
467 Finally, we detected increased levels of cleaved C-terminal ErbB4 in human ADPKD UEVs
468 compared to healthy controls. ErbB4 expression was significantly raised in patients with
469 reduced kidney function ($eGFR > 60 \text{ ml/min/1.73m}^2$) and in ROC analysis, was significantly
470 correlated with a more rapid rate of eGFR decline ($> 3 \text{ ml/min/pa}$), outperforming ultrasound-
471 measured mean kidney length. These findings suggest that ErbB4 signalling closely parallels
472 disease activity and could represent a prognostic biomarker of disease progression.

473 Our results point to the possibility of targeting ErbB4 to retard cyst growth and disease
474 progression in ADPKD. This could be done at several levels or in combination eg blocking
475 the ligands such as NRG-1 or HB-EGF, targeting ErbB4 itself through anti-functional
476 antibodies (12, 29, 39), reducing its expression through mir-193b-3p mimics (5, 28, 36, 41) or
477 through inhibiting downstream kinase activity with selective small molecule inhibitors.
478 Previous studies have detected a significant increase in NRG-1 (2, 23) or HB-EGF (13) in
479 *Pkd1* cystic kidneys and increased urinary HB-EGF has been detected in human ADPKD
480 (10). Of interest, it was reported in the same study that treatment with the vasopressin V2
481 receptor antagonist (V2RA), tolvaptan, was associated with a significant increase in urinary
482 HB-EGF (10). This simultaneous upregulation of HB-EGF could mean that treatment with
483 tolvaptan is less effective than would be predicted from its effect on cAMP production.
484 Therefore, combining a V2RA with a therapy which blocks ErbB4 signalling could be
485 potentially synergistic.

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637

638

639 **Figure Legends**

640 **Fig 1. Parallel mRNA/miRNA expression microarray profiling in ADPKD cell lines**

641 **(A)** Systematic workflow used to analyse parallel mRNA/miRNA changes between control
642 (n=2) and ADPKD derived cystic cell lines (n=4). **(B)** Pathway enrichment analysis was
643 carried out on the 1515 significantly altered ($p < 0.05$ by TTest) genes which were up or down
644 regulated by >2 fold in ADPKD cell lines using the Panther classification system
645 (<http://www.pantherdb.org/>).

646 **Fig 2. Differential expression of mRNAs and miRNAs in ADPKD cystic cell lines**

647 **(A)**. Differential expression of the top 30 genes up-regulated in ADPKD cell lines expressed
648 as a heatmap. The heatmap was produced by clustering the data matrix of the top 30 genes in
649 GenespringX (Agilent, USA). Analysis of differential expression by TTest was carried out
650 using Agilent GeneSpring GX software and p -values >0.05 were defined as significant. **(B)**.
651 Differential expression of the top 30 genes down-regulated in ADPKD cell lines expressed as
652 a heatmap. The heatmap was produced by clustering the data matrix of the top 30 genes in
653 GenespringX (Agilent, USA). Analysis of differential expression by TTest was carried out
654 using Agilent GeneSpring GX software and p -values >0.05 were defined as significant. **(C)**.
655 Differential expression of the 5 significantly altered miRNAs in ADPKD cell lines expressed
656 as a heatmap. The heatmap was produced by clustering the data matrix of the altered
657 miRNAs in GenespringX (Agilent, USA). Analysis of differential expression by TTest was
658 carried out using Agilent GeneSpring GX software and p -values >0.05 were defined as
659 significant. **(D)**. Differential expression of the top 30 genes predicted to be targets of mir-
660 193b-3p by TargetsScan algorithm which were up-regulated in ADPKD cell lines expressed as
661 a heatmap. The heatmap was produced by clustering the data matrix of the top 30 genes in
662 GenespringX (Agilent, USA).

663 **Fig 3. ErbB4 expression is increased in ADPKD cells and is a direct target for mir-193b-**
664 **3p**

665 (A) Taqman qPCR assays for ErbB4 show a significant increase in expression of transcript in
666 ADPKD derived cell lines (n=4) compared to control cell lines (n=4) representing a 10-fold
667 change relative to control. (B) Taqman qPCR assays for mir-193b-3p show a significant
668 reduction in expression of transcript in ADPKD derived cell lines (n=4) compared to control
669 cell lines (n=4) representing a fold change of 0.5066 relative to control. (C) Identification of
670 the sequence within the 3'UTR of ErbB4 between nt91-98 recognised by the mir-193b-3p
671 seed sequence CCGGUCA. The 3 base pairs in ErbB4 predicted to disrupt binding of mir-
672 193b-3p and which were mutated are shown in italics. (D) ErbB4 (pmirGLO ErbB4) or
673 mutated ErbB4 3'UTR (pmirGLO mErbB4) was cloned into a pmirGLO luciferase reporter
674 vector and transfected into HEK-293 cells. HEK-293 cells were co-transfected with control
675 or mir-193b-3p miRNA mimics at 20 and 50nM. A significant reduction in luciferase activity
676 was seen in cells transfected with ErbB4 3'UTR and a mir-193b-3p mimic but not in cells
677 transfected with mutated ErbB4 3'UTR. A negative non-targeting miRNA control mimic had
678 no effect on luciferase activity. (E) Taqman qPCR assays for ErbB4 show a significant
679 decrease in expression of the endogenous mRNA transcript in OX161 cells when transfected
680 with 50nM mir-193b mimic (n=3) relative to cells transfected with 50 nM scrambled negative
681 control mimic (n=3) representing a fold change of 0.6207 relative to control. (F)
682 Transfection of HEK-293 cells with a mir-193b-3p mimic (50 nM) caused a significant
683 decrease in ErbB4 protein expression as detected with a specific ErbB4 antibody. The blot
684 was reprobbed for calnexin to confirm equal loading. A significant decrease in ErbB4/calnexin
685 ratio (40.5% decrease compared to negative control miRNA) was seen in cells transfected
686 with a mir-193b-3p mimic in triplicate wells. Data was from 3 separate experiments.

687

688 **Fig 4. The expression and activation of two ErbB4 isoforms is increased in ADPKD**
689 **cystic cells and kidney tissue**

690 (A) Expression of full-length uncleaved ErbB4 (180 kDa) was increased in ADPKD cells
691 compared to controls. Blots were reprobbed for calnexin to confirm equal loading. The
692 ErbB4/calnexin ratio in ADPKD cells was significantly increased compared to controls. Data
693 is presented as an average of 3 separate repeat experiments. (B). Schematic diagram of ErbB4
694 depicting the position of primers used to amplify domains present in different isoforms. (C)
695 Isoform specific primers were designed to amplify endogenous JM-a, CYT-1/2 or all ErbB4
696 from OX161 mRNA by RT-PCR. A plasmid expressing the JM-a CYT-1 isoform of ErbB4
697 (pcDNA3.1-ErbB4) was used as a positive control template. Two isoforms, JM-a CYT-1 and
698 JM-a CYT-2, were found to be present in OX161. (D) The two ErbB4 isoforms JM-a CYT-1
699 and JM-a CYT-2 were similarly expressed in all normal and ADPKD cell lines tested. (E)
700 Increased expression of ErbB4 was observed in cyst lining epithelial cells of kidney tissue
701 sections from patients with ADPKD (n=7) compared to controls (n=3). Nuclear localisation
702 of ErbB4 was detected in ErbB4 positive cysts (black arrows) indicating ErbB4 cleavage and
703 activation of gene transcription. (F) Nuclear ErbB4 expression was significantly increased in
704 ErbB4 positive cysts in ADPKD kidneys compared to controls.. *** P<0.001. (G) ErbB4
705 mRNA was significantly increased in kidneys derived from *Cre;Pkd1^{del2-11,lox}* mice at 4
706 months after Tamoxifen compared to untreated control animals (n=4-5) as detected by
707 Taqman qPCR assays. (H) Increased expression of ErbB4 was seen in the cyst lining
708 epithelial cells of cystic kidneys from *Cre;Pkd1^{del2-11,lox}* mice 3 and 4 month after tamoxifen
709 induction compared to control un-induced animals (n=6 each). Nuclear localisation of ErbB4
710 was observed in ErbB4 positive cysts (black arrows) indicating ErbB4 cleavage and
711 activation of gene transcription. (I) Nuclear ErbB4 expression was significantly increased in
712 ErbB4 positive cysts in *Cre;Pkd1^{del2-11,lox}* mice compared to control mice 2, 3 and 4 month

713 after treatment with tamoxifen *** $P < 0.001$. **(J)** mir193b-3p miRNA was significantly
714 decreased in kidneys derived from *Cre;Pkd1^{del2-11,lox}* mice at 4 months after Tamoxifen
715 compared to untreated control animals (n=4-5) as detected by Taqman qPCR.

716

717 **Fig 5. Increased ErbB4 in urinary extracellular vesicles from ADPKD patients**
718 **correlates with renal disease progression.**

719 **(A)** Electron microscopy of purified urinary extracellular vesicles. Extracellular vesicles with
720 a diameter $< 100\text{nm}$ can be seen (arrows) indicating successful purification of urinary
721 exosomes (magnification $\times 26000$). An expanded view of a cluster of vesicles (box in A)
722 shows a measured diameter of 66.7nm in a typical vesicle. **(B)** Immunoblotting of cell free
723 urine, exosome preparation supernatant, concentrated cell free urine and exosome pellet.
724 Expression of TSG-101 was observed only in the exosome pellet fraction, indicating
725 purification of urinary exosomes. Tamm-Horsfall protein (THP) and Aquaporin-2 (AQP2)
726 were present in concentrated cell free urine as well as in the exosome pellet. **(C)**
727 Representative blot showing increased ErbB4 expression in ADPKD patient urine exosomes
728 compared to normal controls (n=12). The arrowhead indicates an 80 kDa ErbB4 C-terminal
729 cleavage product; the asterisk indicates a possible non-specific band around 50kDa . The
730 exosome specific protein TSG-101 was used as a loading control. **(D)** A significant increase
731 in ErbB4 expression was seen in ADPKD patient urine exosomes with an $\text{eGFR} < 60$ (n=16)
732 compared to controls (n=12) or ADPKD patients with an $\text{eGFR} > 60$ (n=16). *** $p < 0.001$,
733 **** $p < 0.0001$. **(E)** ROC curves for exosome associated ErbB4 for dichotomized GFR slope
734 (cut-off $>$ or $< -3\text{ ml/min}$). ErbB4 had a higher AUC than mean kidney length reflecting the
735 ability to discriminate between patients with higher risk of progression. The AUC was
736 significantly increased ($p = 0.002$) when ErbB4 was combined with mean kidney length.

737 **Fig 6. Activation of ErbB4 dependent cell proliferation, signalling and cytogenesis is**
738 **increased in ADPKD cells.**

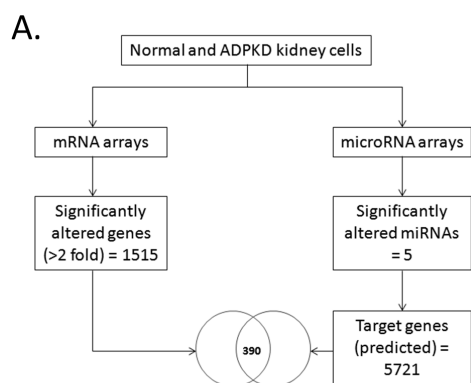
739 **(A)** Addition of the ErbB4 ligand NRG-1 (100 ng/ml) resulted in a significantly greater
740 increase in proliferation from baseline in ADPKD compared to control cells by a BrdU
741 incorporation assay. ** $p < 0.01$, * $p < 0.05$. **(B)** NRG-1 incubation led to a much higher level
742 of phosphorylated ErbB4 (pErbB4) in ADPKD compared to normal controls. pErbB4
743 (Tyr1118) was detected as two bands (arrowheads) representing full-length (180kDa) and C-
744 terminal cleaved (80kDa) proteins. The blot was reprobbed for actin to check for loading. The
745 pErbB4/actin ratio in ADPKD cells was significantly higher compared to controls. Data is
746 presented as an average of 3 separate repeat experiments (graph in lower panel). **(C)** ADPKD
747 (OX161) cells stimulated with NRG-1 (1-100 ng/ml) for 15 min show a greater increase in
748 pERK and pAKT compared to Normal (N, UCL93) cells. Basal pAKT was also noticeably
749 higher in unstimulated ADPKD cells. Equal loading was confirmed by reprobbed for total
750 ERK or AKT. Representative blot of three experiments. The pAKT/total AKT and
751 pERK/total ERK ratio in ADPKD cells were significantly higher following stimulation with
752 10 and 100ng/ml NRG-1 compared to controls. Data is presented as an average of 3 separate
753 repeat experiments (graphs). **(D)** HB-EGF incubation (100ng/ml for 15 min) led to an
754 increase in phosphorylated ErbB4 (pErbB4) in ADPKD cells. pErbB4 (Tyr1118) was
755 detected as two bands representing full-length (180kDa) and C-terminal cleaved (80kDa)
756 proteins. The blot was reprobbed for actin to check for loading. **(E)** OX161 cells form cysts
757 with visible lumen in a time dependent manner when cultured in a 3D matrix (matrigel) over
758 12 days. **(F)** There was a significant increase in average cyst area in cells cultured with 100
759 ng/ml NRG-1 or HB-EGF compared to untreated cells. **** $p < 0.0001$ by 2 way ANOVA.

760

761 **Fig 7. ErbB4 mediates proliferation in ADPKD cell lines.**

762 **(A)** Transfection of control cells (UCL93) with pcDNA3.1-ErbB4 plasmid was associated
763 with a significant increase in cell proliferation as measured by BrDU incorporation,
764 compared to untransfected cells or cells transfected with a pcDNA3.1 empty
765 vector.***p<0.0001. **(B)** Transfection of ADPKD cells (OX161) with specific ErbB4
766 siRNA resulted in a significant reduction in ErbB4 mRNA detected by Taqman qPCR assay
767 representing a fold change of 0.4036 relative to scrambled control siRNA. **(C)** Transfection
768 of OX161 cells with specific ErbB4 siRNA resulted in a significant reduction in proliferation
769 compared to cells transfected with a negative, scrambled control siRNA.*p<0.05. The fold
770 change reduction in ErbB4 mRNA by ErbB4 specific siRNA was detected by Taqman qPCR
771 assay and showed a fold change of 0.4036 relative to scrambled control siRNA. **(D)**
772 Incubation of cells with a pan-ErbB small molecule inhibitor (JNJ 28871063) for 24 h
773 resulted in a dose-dependent decrease in cell proliferation in ADPKD (OX161) cells
774 compared to controls. *p<0.05, **p<0.01. **(E)** Incubation of cells with an ErbB4 specific
775 blocking antibody (H4.72.8) for 24 h led to a significant reduction in cell proliferation in
776 ADPKD (OX161) cells compared to controls. *p<0.05, **p<0.01, ***p<0.001. **(F)** A non-
777 specific control mouse IgG for 24 h had no effect on cell proliferation in ADPKD (OX161)
778 cells compared to controls.

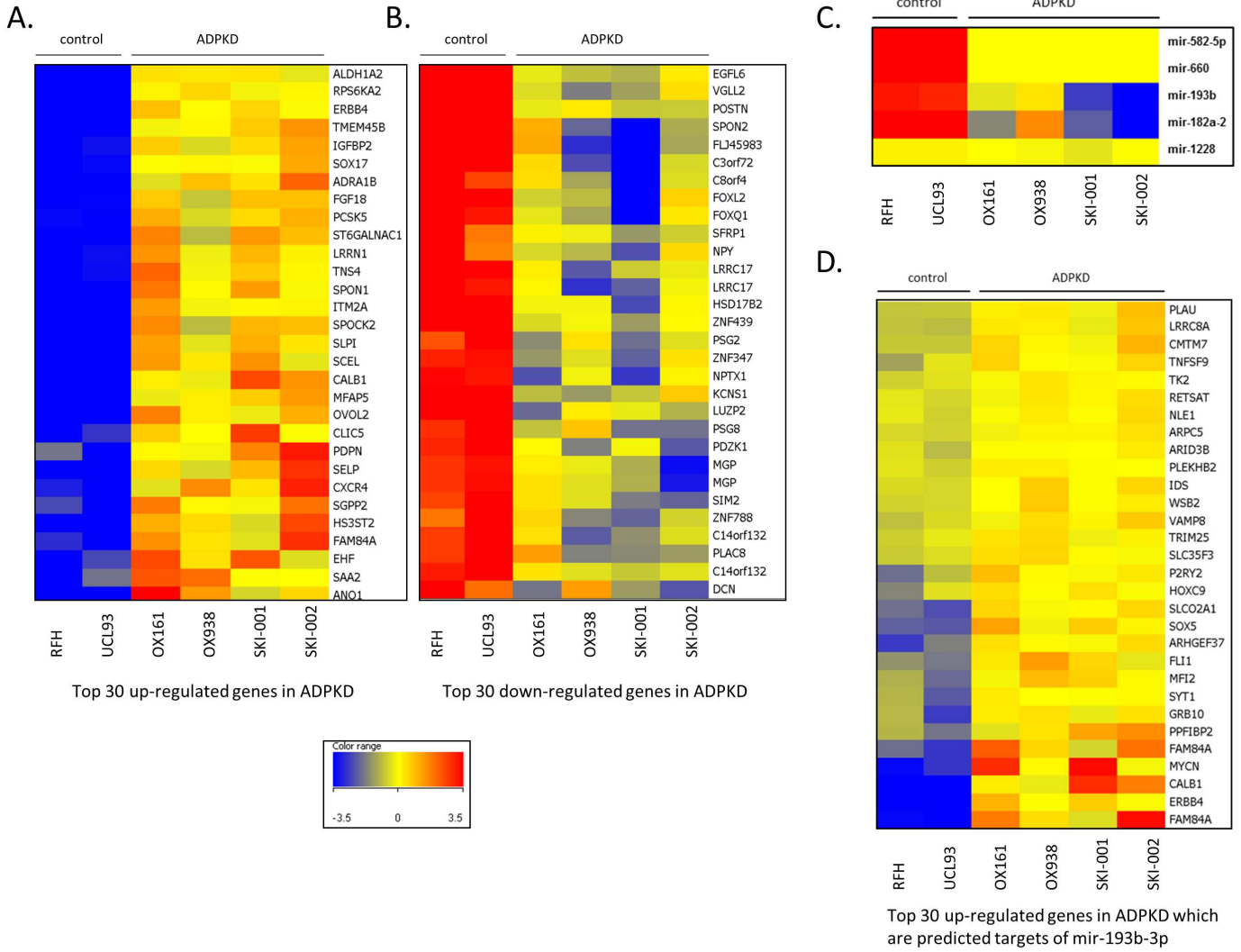
Fig 1



B.

Analysis Type: PANTHER Overrepresentation Test (release 20150430)
 Annotation Version and Release Date: PANTHER version 10.0 Released 2015-05-15
 Reference List: Homo sapiens (all genes in database)

Analyzed List: Up-regulated genes > 2 fold		
PANTHER Pathways	Fold Enrichment	P-value
FGF signaling pathway (P00021)	4.17	1.67E-03
Gonadotropin releasing hormone receptor pathway (P06664)	2.93	4.17E-03
Alpha adrenergic receptor signaling pathway (P00002)	> 5	5.09E-03
Histamine H1 receptor mediated signaling pathway (P04385)	> 5	7.64E-03
EGF receptor signaling pathway (P00018)	3.38	9.46E-03
MYO_signaling_pathway (P06215)	> 5	1.36E-02
GBB_signaling_pathway (P06214)	> 5	1.36E-02
Activinbetasignaling_pathway (P06210)	> 5	1.36E-02
ALP23B_signaling_pathway (P06209)	> 5	1.36E-02
Angiogenesis (P00005)	2.86	2.01E-02
Apoptosis signaling pathway (P00006)	3.19	2.17E-02
Oxytocin receptor mediated signaling pathway (P04391)	5	2.30E-02
Thyrotropin-releasing hormone receptor signaling pathway (P04394)	4.78	2.58E-02
SCW_signaling_pathway (P06216)	> 5	2.69E-02
DPP_signaling_pathway (P06213)	> 5	2.69E-02
DPP-SCW_signaling_pathway (P06212)	> 5	2.69E-02
BMP_signaling_pathway-drosophila (P06211)	> 5	2.69E-02
Wnt signaling pathway (P00057)	2.13	2.75E-02
CCKR signaling map (P06959)	2.6	2.97E-02
5HT2 type receptor mediated signaling pathway (P04374)	4.31	3.34E-02
Thiamine metabolism (P02780)	> 5	4.01E-02
Toll receptor signaling pathway (P00054)	3.93	4.22E-02
Analyzed List: Down-regulated genes > 2 fold		
PANTHER Pathways	Fold Enrichment	P-value
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	2	9.84E-03
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)	2.11	1.07E-02
Ionotropic glutamate receptor pathway (P00037)	2.57	1.44E-02
Enkephalin release (P05913)	3.66	2.51E-02
Cadherin signaling pathway (P00012)	1.72	2.76E-02
Nicotine pharmacodynamics pathway (P06587)	2.77	3.65E-02
Metabotropic glutamate receptor group II pathway (P00040)	2.69	4.06E-02
Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction (P00028)	2.69	4.06E-02



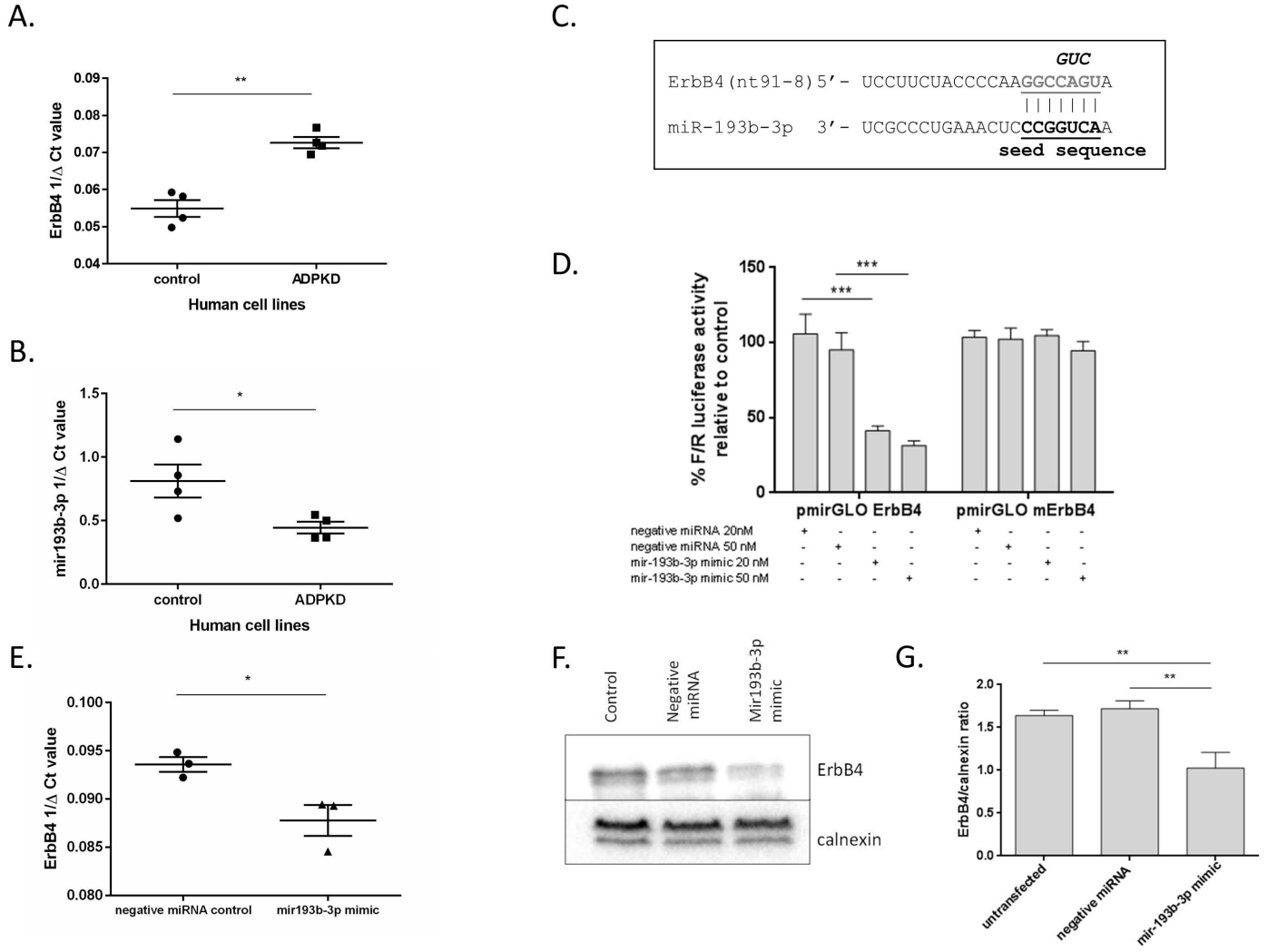
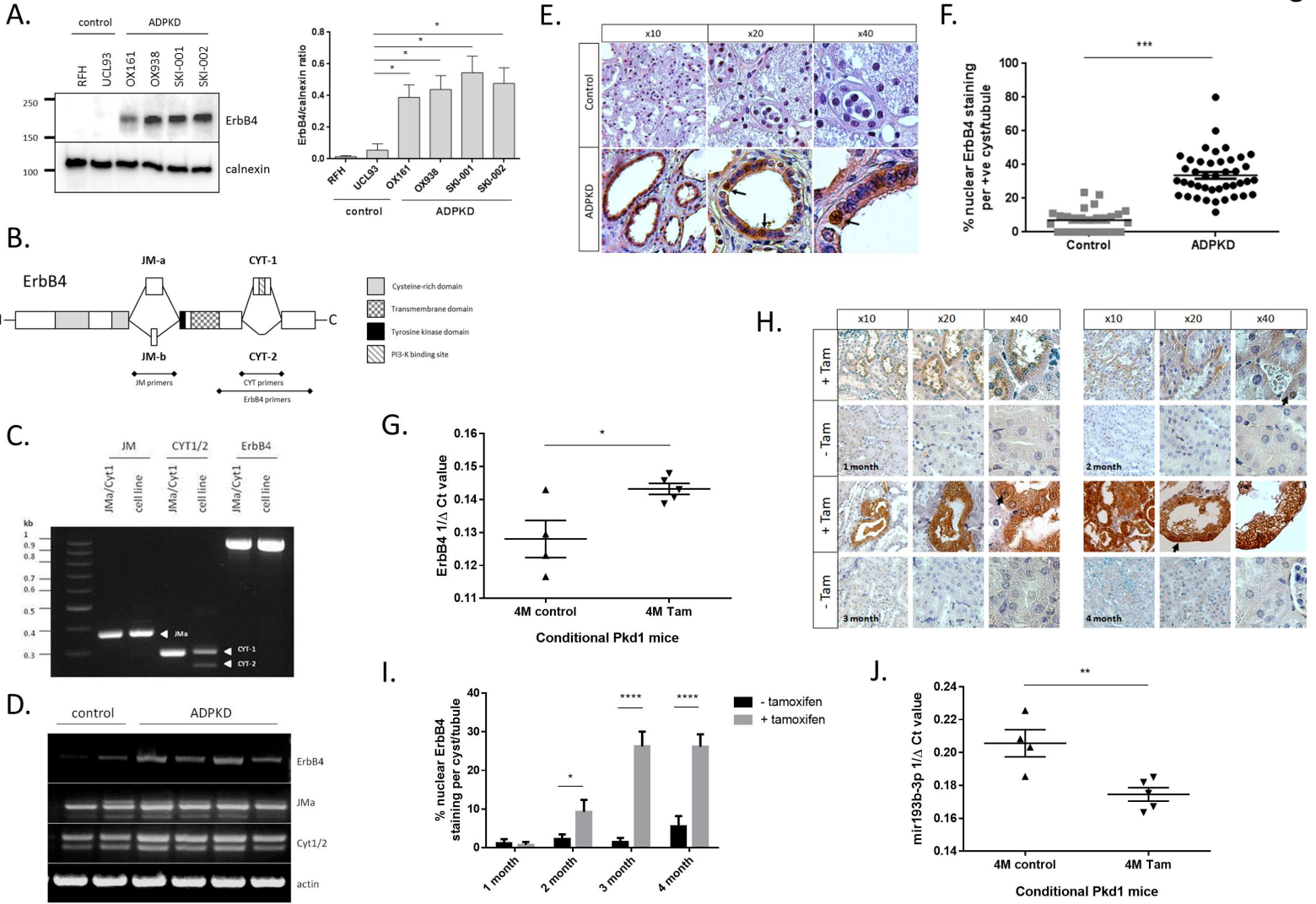
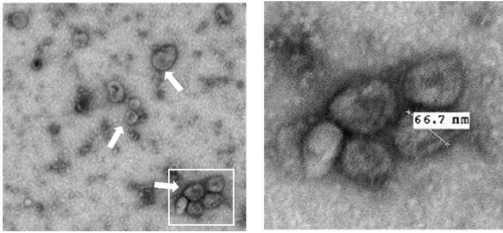


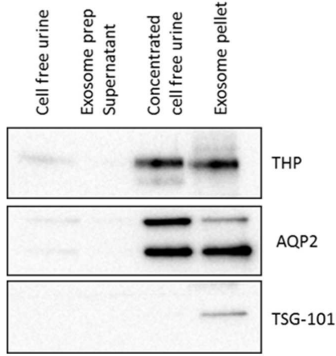
Fig 4



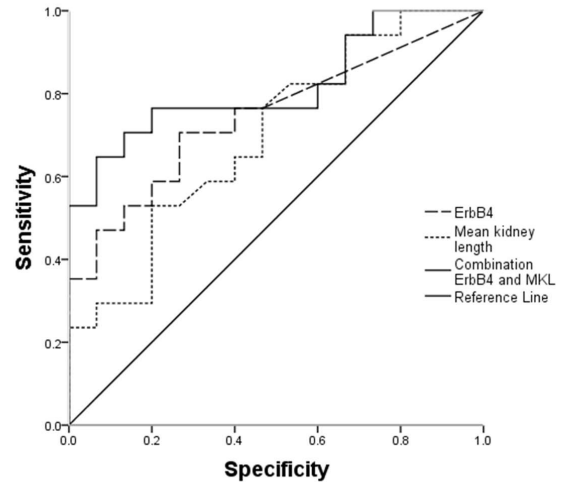
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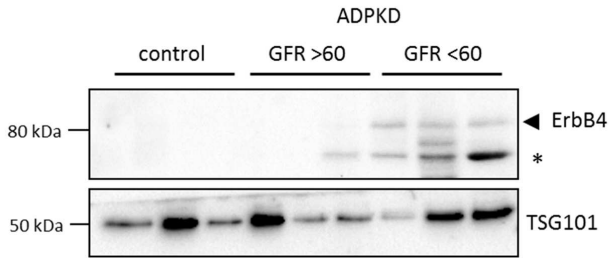
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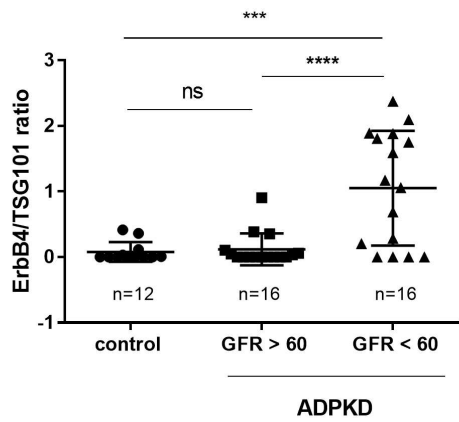
E.



C.



D.



Variables	AUC	95 % CI	P value
ErbB4	0.745	0.573-0.917	0.018
Mean kidney length	0.698	0.516-0.880	0.057
ErbB4 and MKL	0.816	0.665-0.967	0.002

Fig 6

