Porcine familial adenomatous polyposis model enables systematic analysis of early events in adenoma progression

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We compared gene expression in low and high-grade intraepithelial dysplastic polyps from pigs carrying an APC1311 truncating mutation orthologous to human APC1309, analysing whole samples and microdissected dysplastic epithelium. Gene set enrichment analysis revealed differential expression of gene sets similar to human normal mucosa versus T1 stage polyps. Transcriptome analysis of whole samples revealed many differentially-expressed genes reflecting immune infiltration. Analysis of microdissected dysplastic epithelium was markedly different and showed increased expression in high-grade intraepithelial neoplasia of several genes known to be involved in human CRC; and revealed possible new roles for GBP6 and PLXND1. The pig model thus facilitates analysis of CRC pathogenesis.

Functional disruption of the adenomatous polyposis coli (APC) tumour suppressor initiates formation of most adenomas in the human gut, and is responsible for most cases of familial adenomatous polyposis (FAP), an inherited predisposition to colorectal cancer (CRC)1. While the severity of the condition varies considerably, FAP patients typically develop tens to hundreds of adenomatous polyps in the colon and rectum in early life and have greatly increased risk of developing CRC2.

Several genetic and epigenetic alterations have been implicated in this progression, including tumour suppressor genes and proto-oncogenes (TP53, KRAS, BRAF, SMAD2/4, PIK3CA), chromosomal instability, aberrant DNA methylation and histone modification1. However the events that determine whether an early stage polyp proceeds towards cancer have not yet been identified. As Sievers et al.4 recently reported, human precancerous polyps are very heterogeneous, which hinders comparative molecular analyses.

We investigated gene expression in the premalignant progression of dysplastic polyps in pigs carrying an engineered translational stop signal at codon 1311 in the APC gene (APC1311) orthologous to a human APC1309 mutation responsible for FAP5, to determine if molecular changes in the pig model parallel events in small polyps in humans, and whether pigs can be used to reveal new events in early CRC development. The value of pigs in modeling CRC and other cancers has been discussed in several publications e.g. refs 6, 7. The APC1311 pig model has the advantage that samples can be taken from many individuals with the same initiating mutation, avoiding an important source of variation. Changes held in common across such samples are more likely to have a function in premalignant transformation.

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Results and Discussion

Phenotypic characterisation of APC\textsuperscript{1311/+} pigs. To enable systematic investigation of CRC and its precursor lesions, we previously generated pigs carrying an APC\textsuperscript{1311} mutation, orthologous to human APC\textsuperscript{1309} responsible for a severe form of FAP\textsuperscript{5}. Four generations of APC\textsuperscript{1311/+} pigs have now been examined regularly by colonoscopy (Fig. 1a). Findings to date have confirmed that the APC\textsuperscript{1311} FAP model recapitulates key aspects of the human disease, most notably multiple polyps in the colon and rectum\textsuperscript{5}. Polyps have not so far been observed in other parts of the pig gastrointestinal tract. We also observe that, as in human patients\textsuperscript{8}, polyp severity varies between individual pigs carrying the same germ line mutation. Histological evaluation of biopsy samples reveals typical epithelial features of the adenoma-carcinoma sequence including aberrant crypt foci, adenomatous polyps with low (LG-IEN) and high-grade intraepithelial (HG-IEN) dysplasia and carcinoma in situ (Figure S1).

Figure 1. Polyposis and transcriptome patterns specific for low- (LG-IEN) and high-grade intraepithelial (HG-IEN) dysplasia in polyps of APC\textsuperscript{1311/+} pigs. (a) Endoscopic images of an F1 APC\textsuperscript{1311/+} animal (ID 73). Photographs were taken at approximately the same location (\textasciitilde 15 cm depth) at ages 7, 15, 20 and 27 months as indicated. The tumour shown at 20 and 27 months was \textasciitilde 2.5 cm diameter, and at the later date had become hardened and more irregular. (b) Heatmap of the top 60 genes showing transcriptome pattern specific for LG-IEN and HG-IEN whole samples ranked by normalised expression differences between the two groups and P-value. (c) Illustrative quantitative PCR analysis of IL7 in LG-IEN and HG-IEN polyps (n = 20 per group, P = 4.02 \times 10^{-7}). qPCR measurements were normalised to porcine normal colonic mucosa samples (n = 6). (d) Gene set enrichment analysis for human microsatellite stable (MSS) T1 stage polyps versus human normal colonic mucosa from TCGA database and LG-IEN versus HG-IEN colon polyps in APC\textsuperscript{1311/+} pigs.
Comparison of LG-IEN and HG-IEN polyps by gene expression analysis of whole biopsy samples. The process of polyposis in APC<sup>1311/11</sup> pigs shows clear morphological, histological and immunohistological similarities to human CRC. To gain insight into the transition from LG-IEN and HG-IEN, we investigated transcriptional changes occurring in early polyps in the pig model. HG-IEN is the more advanced stage, where the risk of malignancy increases considerably. Whole biopsy samples from 10 polyps, each ~1 cm diameter, taken from six individual pigs at sites from 20 to 40 cm deep, histologically classified as either LG-IEN (n = 4) or HG-IEN (n = 6), were analysed by next generation RNA sequencing, and transcriptome clusters specific for LG-IEN and HG-IEN polyps identified. More than 300 genes were expressed at significantly different levels between the two groups (P < 0.01, Fig. 1b, Table S1), most of which (n = 192) showed lower expression in HG-IEN than LG-IEN polyps. Reported functions of genes in this subset include metabolic processes (MMP20, TEO2, HERC2, MAPK4, S100A8, S100A9), intracellular transport (TMEM8B, SLCL12A6, SLCL46A1), and many were immune related (IL7, IL17D, CD276, IL21, IL20RA, CD40). Differential expression of selected genes was validated by quantitative PCR (qPCR) (e.g. Fig. 1c, Table S2). The gene AHNK showed the greatest reduction in HG-IEN polyps (3.3 fold; P = 4 × 10<sup>−6</sup>). AHNK has been reported as a negative regulator of cell growth, and as a tumour suppressor that potentiates TGFβ signalling<sup>19</sup>. Inactivation of epithelial TGFβ signalling promotes development of intestinal cancer in mouse and human colon cells <i>ex vivo</i><sup>11, 15</sup>. Of those genes expressed at higher level in HG-IEN polyps, the stress-responsive gene HSPA1L (P = 1.7 × 10<sup>−4</sup>) , a member of the HSP70 family, showed the greatest difference (3.6 fold). Others had functions related to Wnt signalling (WISP1), the TNF pathway (SLCL12A6), and cell apoptosis and tumour microenvironment (S100A8, S100A9). Pathway analysis of differentially expressed genes using PANTHER software revealed that the histamine H1 receptor mediated signalling and B cell activation pathways were significantly over-represented (P < 0.05, Bonferroni corrected for multiple testing). Histamine receptors are known to play an important role in cancer development including CRC<sup>18, 19</sup>. It should however be pointed out that most genes studied (n = 238) were unclassified in the pathways, which is a limitation when analysing the pig genome.

To assess the relevance of our animal model to human disease, we carried out gene set enrichment analysis (GSEA)<sup>14</sup>. Equivalent data from human LG-IEN versus HG-IEN polyps was not available, precluding direct side-by-side comparison with the pig. Differential expression data for human T1 stage microsatellite stable (MSS) and microsatellite unstable (MSI) colorectal cancers 15. There was a striking degree of similarity between changes in gene sets in pig, GSEA results are summarised in Fig. 1d. It is known that APC mutations are frequent in non-hypermutated (MSS) colon cancers<sup>14, 15</sup>. There was a striking degree of similarity between changes in gene sets in pig and human MSS polyps and several close parallels in key functional sets, e.g. upregulation of MYC non-hypermutated (MSS) colon cancers 15. There was a striking degree of similarity between changes in gene sets in pig and human MSS polyps and several close parallels in key functional sets, e.g. upregulation of MYC target genes, the E2F group of genes involved in cell cycle regulation and DNA synthesis, and the cell cycle G2/M checkpoint DNA damage gene set, and downregulation of interferon alpha and gamma response targets. This was clearly only a broad comparison and there were some anomalies, such as the apparent lack of Wnt pathway involvement.

Immune cells make a significant contribution to transcriptome data using whole biopsies. As mentioned, a substantial proportion (18%) of genes with the most marked differences in expression between pig LG-IEN and HG-IEN were immune-related or known to be specific to immune cells. Immunohistochemical analysis of polyp samples confirmed infiltration of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells (Figure 5). The role of immune cells in CRC is well documented, several studies have reported that cytokines produced by immune cells influence CRC progression<sup>18</sup> and are linked to poor prognosis at early stages<sup>30</sup>. Our findings indicate a similarly important role in the pig model. However the presence of infiltrating immune cells in whole samples is likely to obscure transcriptome changes in the dysplastic epithelium.

Comparison of microdissected LG-IEN and HG-IEN adenoma by gene expression analysis. To restrict our analysis to events in the epithelium, we microdissected dysplastic epithelial cells from five distinct and separate LG-IEN and similarly five HG-IEN adenomas, each from the same polyps, and again
performed RNA sequencing (Fig. 2a, Table S3). To our knowledge, similar studies have not been performed on human adenomas, although Lechner et al.21 examined a limited number of cancer-related genes in microdissected human LG-IEN polyps. Our analysis of microdissected samples revealed marked differences to results from whole samples. Several genes identified as differentially expressed in the whole samples were not represented, e.g. IL7, IL17D, S100A8, S100A9, AHNAK, indicating that such transcriptional changes occurred in other tissues, such as stroma and infiltrating immune cells. New gene expression differences were also revealed by the more defined analysis. Indeed, none of the top 20 genes identified as differentially expressed in microdissected epithelium were significant in the whole sample analysis. Most notable were higher expression of PLXND1 and GBP6 in HG-IEN (>2-fold, $P < 3 \times 10^{-6}$; Table S4). While the top gene PLXND1 did show as differentially expressed in whole biopsy samples, this fell below statistical significance after correction for multiple testing. Analysis of gene expression data from both Gene Expression Omnibus and TCGA databases revealed that PLXND1 expression decreases in later stages of CRC 22, 23, but GBP6 showed no significant expression changes. PLXND1 encodes a transmembrane signal transduction receptor known to bind semaphorins and other ligands. It is upregulated in a wide variety of human tumour cells 22 and has been reported as mediating epithelial-to-mesenchymal transition in ovarian cancer41, and associated with tumour invasiveness and metastasis in human CRC23. GBP6 belongs to the family of guanylate binding proteins and is best known as a gene induced by interferon-γ signalling. Interferon-γ is produced predominantly by NK and T-cells, so increased GBP6 expression in HG-IEN may reflect interplay between the epithelium and infiltrating immune cells. GBP6 has not previously been implicated in tumorigenesis although a related gene, GBP1, is involved in Interferon-γ dependent CRC progression26. Other genes expressed at higher level in HG-IEN have reported functions in oxidative stress and anti-inflammatory activity (SLC30A1), p53 regulation and cell proliferation (VASH1), Wnt pathway regulation (SMARCD3), and DNA binding (RABGAP1, RBM18). Of those genes expressed at lower level in HG-IEN, the greatest differences (>1.75-fold, $P < 2.08 \times 10^{-6}$) were in RAD23B, a DNA repair enzyme, and CHST12 (Table S4). CHST12 encodes a carbohydrate sulphotransferase involved in extracellular matrix formation and its reduction in later stage dysplasia may reflect disruption of extracellular matrices as tissue organisation is affected. Reduced expression of other members of this family has been associated with invasiveness in liver carcinoma cells27.

GSEA of microdissected epithelium revealed a more restricted set of changes than in whole samples. Of those with q value $< 0.05$, five gene sets were upregulated in HG-IEN, all of which were also highlighted in whole biopsy samples, and eight gene sets showed downregulation in HG-IEN. Eight gene sets identified in whole samples showed no change in the microdissected epithelium, e.g. unfolded protein response, bile acid metabolism, adipogenesis, fatty acid and haeme metabolism (Fig. 2b), suggesting that such transcriptome changes occur in other components. The oxidative phosphorylation gene set was more highly expressed in microdissected than in whole HG-IEN samples. Again this can probably be explained by the exclusion of immune cells. Immune cells such as T-cells are known to downregulate oxidative phosphorylation in favour of glycolysis28.
Analysis of allele specific expression. Changes in cell subpopulations over time are a common feature of many tumours, and can be monitored by measuring the proportional expression of different alleles of the same gene. Allele specific expression (ASE) is reportedly common in CRC, but early polyps have not so far been investigated. Analysis of our RNA sequencing data from whole samples revealed that, as expected, the mutant \( \text{APC}^{1311} \) allele was expressed at higher level than the wild-type \( \text{APC} \) allele \(^5\), \(^30\) (Fig. 3). We then quantified transcriptome-wide ASE in the LG-IEN and HG-IEN polyp whole samples. Variant calling revealed 705,000 heterozygous SNPs, of which 48,000 (6%) showed allelic imbalance in at least one sample either in LG-IEN and HG-IEN polyps (\( P_{\text{value Adj}} < 0.05 \), Table S5). Imbalanced allele expression was detected for SNPs in several cancer-related genes such as \( \text{EpCAM} \), \( \text{MSH2} \), \( \text{MMP7} \), \( \text{MMP12} \), \( \text{PLOR1D} \) and \( \text{CCL5} \). To specifically investigate the dysplastic epithelium, we selected genes with known function in epithelium and CRC progression (\( \text{MMP9} \), \( \text{CEACAM7} \), \( \text{LMAN2} \)) or immune cells (\( \text{SLA2} \)) and analysed allele expression in microdissected samples from distinct LG-IEN and HG-IEN adenoma from the same polyp (Fig. 4). The allele proportion of SNPs analysed in

Figure 3. Proportion of \( \text{APC}^{1311} \) and \( \text{APC} \) allele expression in LG-IEN and HG-IEN whole samples. Mutant and wild type \( \text{APC} \) alleles can be distinguished by the replacement of G by T in codon 1311 (GAA), which introduces a premature stop codon. The allele proportion was based on raw RNA sequencing reads mapped in this region.

Figure 4. Allele specific expression of selected genes in whole and microdissected samples quantified using pyrosequencing. LG1, LG2, HG1, HG2 represent microdissected adenomas from the same polyp. The following SNPs were analysed: \( \text{MMP9} \) - rs341963098A/G; \( \text{SLA2} \) – 1.0 rs337323048A/G; \( \text{LMAN2} \) – rs339673049C/T; \( \text{CEACAM7} \) – 6:45194986C/T. All SNP positions are according to the Scrofa10.2 porcine genome reference sequence.
LG-IEN more closely resembled normal mucosa than did SNPs in HG-IEN. These differences were more evident in microdissected epithelium than in whole samples, consistent with the emergence of cell subpopulations in HG-IEN even at this premalignant stage. Reduced detection of one allele by PCR, as observed for SNPs in the MMEP9 and LMAN2 genes in HG-IEN polyps, suggests overgrowth of cells carrying that allele by other faster growing cells with the other allele. These ASE results for HG-IEG polyps are consistent with the high frequency of chromosomal imbalances reported for MSS colorectal cancers.

Conclusions
In summary, pigs genetically predisposed to colorectal polyposis based on a single, well-defined genetic mutation enable systematic analysis of CRC precursor lesions, offering an important complement to the study of human patients and existing genetically-modified mouse models. Gene expression data from whole samples obtained by biopsy of polyps from APC1311 pigs correlates with alterations in molecular pathways involved in early pathogenesis of human colorectal cancer. Microdissection and specific analysis of the epithelial component revealed that many transcriptome changes apparent in whole samples actually reflect events outside the epithelium, notably immune infiltration. Allele-specific expression was clearly evident, especially in microdissected HG-IEN, consistent with the emergence of cell subpopulations. Analysis of microdissected dysplastic epithelium also revealed possible roles for cancer-related genes not previously associated with CRC (GBP6, PLXND1) in early stage adenoma progression.

Material and Methods

Animals. Animal experiments were approved by the Government of Upper Bavaria (permit number 55.2-1-54-2532-6-13) and performed according to the German Animal Welfare Act and European Union Normative for Care and Use of Experimental Animals. All pigs were fed with normal pig diet. Blood and tissue samples were collected every 6 months.

Stereomicroscopy and endoscopy. Macroscopic images of colonic lesions were taken in vivo with a STORZ colonscopy system and ex vivo with a Zeiss Stemi 11 stereomicroscope. Colonoscopy examination was carried out approximately every 6 months, starting at 3 months old. Polyp and normal mucosa biopsy samples were divided and portions were fixed as described below, frozen in 2-methylbutane (OCT) for cryosection, and snap frozen and stored at −80°C for molecular analyses.

Histochemistry. For histopathology analysis, specimens were fixed in 4% buffered paraformaldehyde, embedded in paraffin, sectioned (3μm) and stained with haematoxin and eosin (H&E). Polyps were classified as LG-IEN or HG-IEN according to the AJCC TNM staging system.

For immunohistochemistry, specimens were fixed using the HOPE technique, embedded in paraffin and 3μm sections cut. Sections were dewaxed, microwaved (10 min, 600 watt), and incubated with mouse anti-porcine primary antibodies: CD3 (1:100, clone BB23-8E6), CD4 (1:100, clone 74-12-4), CD8 (1:100, clone 76-2-11). All primary antibodies were obtained from SouthernBiotech. Tissue sections were incubated with biotinylated anti-mouse IgG (1:200, Vector Laboratories) followed by Elite ABC kit incubation. Antibody binding was detected with a DAB Peroxidase Substrate kit (Vector Laboratories).

Laser microdissection. Laser microdissection (LMD) of cryosectioned samples was performed immediately after H&E staining using a Leica Microsystems Laser Microdissection Systems 6000 and Leica Application Suite software (Leica). In total, ten LMD captured crypts per polyp sample were cut, providing 300–500 cells, and collected into 50μL lysis buffer from the AllPrep® DNA/RNA Micro Kit (Qiagen), and a further 50μL lysis buffer added after dissection. Dissected samples were stored at −80°C. Genomic DNA and total RNA from the same sample were isolated using the AllPrep® DNA/RNA Micro Kit according to the manufacturer’s instructions (Qiagen).

Whole transcriptome amplification of LMD samples. For allele expression assessment by pyrosequencing, 100 pg total RNA isolated from LMD samples was used for whole transcriptome amplification using the QuantiTect Whole Transcriptome Kit according to the manufacturer’s instructions (Qiagen).

Library preparation. Libraries for RNA sequencing of whole biopsies were prepared using the TruSeq Stranded mRNA LT Kit (Illumina). RNA integrity and fragment size were tested using the RNA6000 Nano kit (Agilent) on an Agilent Bioanalyzer 2100 (Agilent). Libraries for RNA sequencing of LMD samples were prepared using SMART-Seq. 2 Ultra-low kit (Clontech) and Nextera XT DNA SMP preparation kit according to the manufacturers protocol (Illumina). RNA and dscDNA integrity and fragment size were tested using RNA6000 Pico and High Sensitivity DNA kits (Agilent).

NGS RNA sequencing. Libraries were sequenced with a HiSeq. 2500 ultra-high-throughput sequencing system (Illumina) to produce 100-base-paired end reads. An average of 45 million reads per sample were generated and mapped to the porcine reference genome (Sscrofa10.2) using the 2-pass method of the STAR aligner with default parameters. 80% of these reads uniquely hit to the reference genome. Duplicate reads were denoted with the MarkDuplicates tool of Picard (http://broadinstitute.github.io/picard). Aligned reads (omitting duplicate reads) were assigned to gene sequences as defined in the 10.2.77 porcine gene set and counted with featureCounts. Normalisation of read counts and estimation of fold change was carried out using DESeq. Hierarchical clusters and heat maps for 60 genes with the most significantly different levels of expression were generated using the python (http://www.python.org) packages spatial and cluster of scipy along with matplotlib.
Variant calling based on STAR alignments was performed according to GATK\textsuperscript{39} best practice recommendations for RNAseq\textsuperscript{38–41}. The GATK tool SplitNCigarReads was used to split reads into exons and remove false variants resulting from overhangs. This step included reassignment of the STAR alignment mapping qualities. GATK recalibration of base scores was based on the Ensembl release 83 variant database. Variant calling was carried out using GATK HaplotypeCaller with the dontUseSoftClippedBases option. GATK VariantFiltering was applied to clusters of at least 3 SNPs within a window of 35 bases between them with the following parameters: Fisher strand value (FS) >30.0 and a quality by depth value (QD) <2.0. The probability of allelic imbalance for each SNP was calculated based on the number of reference and alternate allele reads in heterozygous animals using a two-sided binomial test. P values were adjusted for false discovery rate (q value) to take account of multiple testing.

**Gene set enrichment analysis.** Gene expression data (HTSeq-counts) for stage I human colon cancers (n = 80) and normal samples of colonic mucosa (n = 41) were retrieved from The Cancer Genome Atlas (TCGA) using the R/Biocounter package TCGAbiolinks (version 2.2.3). From the TCGA, we also retrieved gene expression data (HTSeq-counts) for stage I microsatellite stability (MSS) (n = 23) and stage I low level microsatellite instability (MSI-L) (n = 11) human colon cancers. Log2 fold change of gene expression was analysed using the included functions TCGAanalyze_DEA and TCGAanalyze_LevelTab. For porcine colon polyps, the numbers of mapped reads to each gene were counted using the featureCount tool implemented in the subread package (version 1.5.0-p2)\textsuperscript{35}. The gene annotation file was downloaded from Ensembl (Ensembl release 86). Log2 fold change of gene expression (HG-IE\textsuperscript{N} versus LG-IE\textsuperscript{N}) was analysed using the R/Biocounter package DESeq. 2 (version 1.14.0)\textsuperscript{36}. Gene set enrichment analysis\textsuperscript{43} was performed using Human Genome Organization (HUGO) gene symbols together with log2 fold change of gene expression as input. The tool GSEA\textsuperscript{44} ranked implemented in GSEA (version 2.2.3) was used to estimate gene set enrichment with the following parameters: classic enrichment statistics, 10,000 permutations, and hallmark gene sets collection from MapMan Database (version 5.2)\textsuperscript{45}. P values were adjusted for false discovery rate (q value) to take account of multiple testing.

PANTHER Gene List Analysis\textsuperscript{44} was used to perform functional pathway analysis for the top >300 differentially expressed genes with PANTHER pathways as annotation data set and statistical overrepresentation test with default settings.

**Quantitative RT-PCR.** Total RNA was extracted using Direct-zol RNA Mini Prep Kit (Zymo Research) according to the manufacturer’s instructions. 200 ng total RNA was used to synthesise complementary DNA (cDNA) using Superscript IV reverse transcriptase (Invitrogen). cDNA was amplified by RT-PCR, and allele expression for selected SNPs was analysed by pyrosequencing using the Pyromark Q24 system (Qiagen). Pyrosequencing primers were designed using the PyroMark Assay Design Software 2.0 (Qiagen). Allele expression differences at individual SNPs were evaluated using Students t-test.

**Allele quantification using pyrosequencing.** Genomic DNA was extracted using the GenElute Mammalian Genomic DNA Kit (Sigma Aldrich) and used for allele quantification in heterozygous SNPs. The allele proportion of 50:50 on DNA level for valid pyrosequencing assays was expected. Total RNA was extracted using the Direct-zol RNA Mini Prep Kit (Zymo Research) according to the manufacturer’s instructions. 200 ng total RNA was used to synthesise complementary DNA (cDNA) using Superscript IV reverse transcriptase (Invitrogen). cDNA was amplified by RT-PCR, and allele expression for selected SNPs was analysed by pyrosequencing using the Pyromark Q24 system (Qiagen). Pyrosequencing primers were designed using the PyroMark Assay Design Software 2.0 (Qiagen). Allele expression differences at individual SNPs were evaluated using Students t-test.

### Primers
- **The authors will provide all primer sequences used in this study on request.**

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Author Contributions
K.F., T.E., M.S., D.S., A.K., A.S. designed, analysed and interpreted data and wrote the manuscript; K.E., T.F., M. A., C. W., A.W., A.H.C., C. W., A.P., K. F. carried out molecular genetic experiments; D.S. performed colonoscopy and polyp biopsy assisted by K.F., T.E., A.K.; A.H.C., T.F. performed immunohistochemistry; H.X., H.P., D.E., R.F. performed bioinformatic analyses.
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