Rectal epithelial cell mitosis and expression of macrophage migration inhibitory factor are increased 3 years after Roux-en-Y gastric bypass (RYGB) for morbid obesity: implications for long-term neoplastic risk following RYGB

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ABSTRACT

Background Rectal epithelial cell mitosis and crypt size, as well as expression of proinflammatory genes including *macrophage migration inhibitory factor* (*MIF*), are increased 6 months after Roux-en-Y gastric bypass (RYGB) in morbidly obese patients. Tests were carried out to determine whether these putative colorectal cancer risk biomarkers remained elevated long term after RYGB, and the mechanistic basis, as well as the functional consequences, of *Mif* upregulation in intestinal epithelial cells was investigated.

Methods Rectal mucosa and blood were obtained a median of 3 years after RYGB from the original cohort of patients with RYGB (n=19) for crypt microdissection, real-time PCR, immunohistochemistry for MIF and immunoassay of proinflammatory markers. Immunohistochemistry for Mif and bromodeoxyuridine labelling were performed on $AhCre^{+}$ mouse and $Apc^{Min/+}$ mouse (with and without functional *Mif* alleles) intestine, respectively.

Results Rectal epithelial cell mitosis and crypt size remained elevated 3 years after RYGB compared with preoperative values (1.7- and 1.5-fold, respectively; $p<0.05$). There was a 40-fold (95% CI 13 to 125) increase in mucosal *MIF* transcript levels at 3 years associated with increased epithelial cell MIF protein levels. Conditional *Apc* loss in $AhCre^{+}$ mice led to increased epithelial cell Mif content. *Mif* deficiency in $Apc^{Min/+}$ mice was associated with a combined defect in intestinal epithelial cell proliferation and migration, which was reflected by the longitudinal clinical data.

Conclusions Mucosal abnormalities persist 3 years after RYGB and include elevation of the protumorigenic cytokine MIF, which is upregulated following *Apc* loss and which contributes to intestinal epithelial cell homeostasis. These observations should prompt clinical studies of colorectal neoplastic risk after RYGB.

Significance of this study

What is already known about the subject?

- Roux-en-Y gastric bypass (RYGB) is increasingly performed in morbidly obese patients.
- Six months after RYGB, there is increased rectal mucosal crypt size and epithelial cell mitosis, as well as increased mucosal proinflammatory gene expression (including cyclooxygenase-2 (COX-2) and macrophage migration inhibitory factor (MIF)).
- It is not known whether these changes represent a short-term postoperative phenomenon or persist in the long term.
- Rectal crypt parameters such as mitosis frequency may be biomarkers of future colorectal cancer risk, and several proinflammatory factors such as cyclooxygenase-2 (COX-2) and MIF have protumorigenic properties.

What are the new findings?

- Rectal crypt parameters remain persistently abnormal at least 3 years after RYGB.
- Rectal epithelial cell expression of MIF is elevated long term.
- *Mif* is upregulated following loss of the *Adenomatous polyposis coli* (*Apc*) tumour suppressor gene in mouse intestinal epithelial cells.
- Mif contributes to mouse intestinal epithelial cell homeostasis, and the effect of *Mif* deletion in the $Apc^{Min/+}$ mouse is compatible with our clinical association of increased mitosis frequency and MIF upregulation.

How might it impact on clinical practice in the foreseeable future?

- Persistent rectal mucosal changes in putative biomarkers of future CRC risk at least 3 years after RYGB should prompt clinical evaluation of colorectal adenoma incidence post-RYGB.
- MIF is a potential target for chemoprevention of CRC using small molecule inhibitors.

INTRODUCTION

Considerable epidemiological evidence links obesity and increased risk of colorectal cancer (CRC).[1] The RR of CRC in overweight (defined as a body mass index (BMI) of 25–29.9 kg/m^2) and obese (BMI >30 kg/m^2) individuals is ~1.5–2.5 compared with normal weight (BMI 18.5–25 kg/m^2) controls,[1] with a similar RR for colorectal adenoma.[2–5] Recent forecasts predict a continued rise in the prevalence of obesity in the UK to >30%

by 2012, hence the burden of colorectal neoplasia linked to excess body weight is likely to increase.[6]

The mechanisms linking obesity and colorectal carcinogenesis remain incompletely understood.[7] Obesity is recognised as a state of chronic, low-grade systemic inflammation.[8] As long-standing chronic mucosal inflammation predisposes to a number of gastrointestinal cancers, in particular CRC,[9] one plausible hypothesis which links obesity and CRC is that there is also subclinical mucosal inflammation in obese individuals, which then drives colorectal carcinogenesis.[7 10] A linked concept is that weight reduction should decrease systemic and mucosal inflammation, leading to lower CRC risk.

We have previously measured rectal mucosal epithelial cell mitosis and apoptosis, as well as levels of serum biomarkers of inflammation and mucosal proinflammatory gene expression, in normal weight individuals and morbidly obese (BMI >40 kg/m^2) patients before and after Roux-en-Y gastric bypass (RYGB), in order to determine whether differences in crypt epithelial cell turnover were associated with obesity and whether rapid weight loss altered biomarkers of inflammation.[11] Rectal crypt cell mitosis frequency and crypt size were significantly higher in morbidly obese patients compared with normal weight individuals.[11] Unexpectedly, mitosis frequency and crypt size were actually increased following weight loss 6 months after RYGB.[11] Furthermore, although serum levels of inflammatory markers, including C-reactive protein (CRP), were lower after RYGB, mucosal proinflammatory gene transcript levels (including cyclooxygenase-2 (COX-2), interleukin 6 (IL-6) and macrophage migration inhibitory factor (MIF)) were raised, supporting a local proinflammatory response following this form of bariatric surgery.[11]

Herein, we test the hypothesis that rectal crypt morphometric parameters and mucosal proinflammatory gene expression remain elevated in the long term (3 years) after RYGB, rather than being short-term, self-limiting perioperative phenomena. We report that rectal epithelial cell mitosis frequency and crypt size, as well as proinflammatory gene expression, are indeed persistently elevated post-RYGB, with a marked increase in mucosal expression of the protumorigenic cytokine *MIF*.[12 13]

We also reveal that *Mif* controls intestinal epithelial cell proliferation and migration in mouse intestine and that *Mif* expression is upregulated following loss of the tumour suppressor gene *Adenomatous polyposis coli* (*Apc*) in murine intestinal epithelial cells.

Persistent elevation of putative rectal mucosal biomarkers of CRC risk and increased expression of the protumorigenic cytokine MIF suggest that increased long-term risk of colorectal neoplasia needs to be excluded by longitudinal, cohort studies of patients after RYGB.

METHODS

Ethics approval

All aspects of the study were approved by the Leeds (East) Research Ethics Committee. Written, informed consent was obtained from all participants prior to recruitment.

Patients undergoing follow-up after RYGB

All patients (n=26) who had participated in our previous study were contacted ~3 years after RYGB surgery.[11] Patients were interviewed in addition to their routine postoperative review in the Outpatient Clinic. We collected the same patient data and biological samples as before.[11] We determined current drug use, including non-steroidal anti-inflammatory drug (NSAID; including low-dose aspirin) and supplementary calcium intake and enquired about recent acute illness or antibiotic use. Height and weight were measured before a blood sample was collected for serum analysis. Six rectal mucosal biopsies were obtained from the posterior wall of the unprepared rectum, 10 cm from the dentate line, using a rigid sigmoidoscope and 2.2 mm jaw, single-use biopsy forceps (Boston Scientific, Miami, Florida, USA).

Mucosal biomarker analysis

Rectal crypt epithelial cell mitosis frequency, branching and area were measured by a single operator (PK) blinded to the identity of each sample as described.[11] Interobserver agreement for mitosis frequency scoring between PK and AS (the previous blinded operator)[11] was excellent (Spearman r=0.79; p<0.01; n=10 crypts). Zonal analysis of mitotic epithelial cells was performed by dividing each crypt into five zones from the crypt base (zone 1) to the top of the crypt as it joins the mucosal surface (zone 5).[11] Immunohistochemistry (IHC) for neo-cytokeratin 18 (CK18) was performed as described.[11] Rectal mucosal gene transcript expression was measured by real-time reverse transcription–PCR (RT–PCR) and quantified by the ΔC_t method.[11]

IHC for MIF

IHC for MIF was performed on formalin-fixed, paraffin-embedded (FFPE) rectal mucosal sections using a modification of an established, in-house protocol.[12] Tissue sections from 3 years post-RYGB were used together with freshly cut sections from archival tissue blocks from before RYGB and 6 months post-RYGB.[11] MIF immunoreactivity was visualised using the Dako EnVision+ System (DakoCytomation, Ely, UK). Omission of the primary antibody, incubation with an equivalent concentration (2.5 μg/ml) of isotype-control mouse immunoglobulin G (IgG) antibody (DakoCytomation) and preabsorption of anti-MIF antibody with recombinant human MIF (15 μg/ml) at 4°C overnight; R&D Systems, Abingdon, UK) served as negative controls. Sections from the three time points for each individual were stained together. Two internal control sections were also included in all IHC experiments to confirm lack of inter-assay variation in staining intensity. MIF immunoreactivity in luminal surface epithelium and crypt epithelial cells, as well as in the lamina propria, was scored separately on a scale of 0–3 (0, no staining; 1, weak, patchy staining; 2, moderate and widespread (>50% of cells) staining; 3, intense and uniform staining) by two independent observers (PK and MAH) blinded to the identity of each section. Scores from both observers for each compartment were combined to give a total score ranging from 0 to 6. Scores for the luminal and crypt epithelium were combined to give a total 'epithelial' MIF score ranging from 0 to 12.

Serum inflammatory biomarkers

Serum levels of MIF, tumour necrosis factor α (TNF α) and IL-6 were measured using immunoassays from R&D Systems.[11] Serum CRP was measured by high-sensitivity immunoassay (Advia 1650/2400, Bayer HealthCare Diagnostics, Newbury, UK).[11]

Mouse studies

All studies and procedures involving mice were carried out under licence and in accordance with the UK Home Office Animal Scientific Procedures Act 1986.

C57Bl/6 $Apc^{Min/+}$ and C57Bl/6 $Apc^{Min/+} \times Mif^{-/-}$ mice were bred and housed in isolator conditions as described.[12] At 30 days of age, mice were injected subcutaneously with 250 µl of bromodeoxyuridine (BrdU; GE Healthcare, Amersham, UK) and then sacrificed either 2 or 24 h later. The whole of the small intestine was opened longitudinally and washed with phosphate-buffered saline (PBS). Intestinal mounts were fixed in methacarn (4:2:1 methanol:chloroform:glacial acetic acid) overnight and then stored as 'Swiss rolls' prior to embedding in paraffin. IHC for BrdU and scoring of the crypt cell position and number of BrdU-positive enterocytes blind to genotype was performed as described.[14]

IHC for Mif was performed on FFPE sections of $AhCre^{+}$ $Apc^{fl/fl}$ and $AhCre^{+}$ $Apc^{+/+}$ mouse small intestine obtained 4 days after β-naphthoflavone induction[15] as described, using affinity-purified goat polyclonal anti-MIF antibody (R&D Systems).[12] *Mif*-null intestine acted as a negative control.

Statistical analysis

Normally distributed data are expressed as the mean and the SEM or the 95% CI with comparison using Student paired t test or one-way analysis of variance (ANOVA). Non-parametric data are expressed as the median and the IQR with comparison by the Wilcoxon signed rank test. The percentage of patients with branching crypts was compared using the McNemar test. The difference between the distribution of BrdU-positive epithelial cells in crypts was analysed using the Kolmogorov–Smirnov test. Statistical significance was assumed if $p \geq 0.05$.

RESULTS

Clinical outcomes 3 years after RYGB

Nineteen of the original 26 patients who underwent RYGB were studied at a median of 36 months (range 34–39 months) post-RYGB. The remaining seven patients did not give consent for further participation. Four of 19 individuals had undergone abdominoplasty in the intervening period. Twelve of 19 patients had continued regular calcium supplementation (10 on calcium alone; 2 on calcium and vitamin D). Six of 19 individuals were taking a statin prior to RYGB, but only two continued to do so 3 years later. Statin therapy was not started in any other patient during follow-up. No patients were taking a proton pump inhibitor, NSAID or low-dose aspirin on a regular basis. No patients reported recent, acute illness or antibiotic use. Patients had no other significant co-morbidity including vascular disease. Preoperative and 6 month post-RYGB data were analysed for the 19 patients who underwent assessment 3 years after RYGB.

At ~3 years after surgery, there was further loss of weight loss compared with values at 6 months post-RYGB (table 1 and Supplementary figure 1). However, 18 of 19 patients were still obese, with a mean BMI (±SEM) of 39.1±1.7 kg/m^2 3 years post-RYGB, which represents a mean (±SEM) excess weight loss of 46.2±3.9% since RYGB.[16]

In all cases, the endoscopic appearance of the rectal mucosa was normal.

Rectal crypt cell mitosis frequency and crypt size remain elevated 3 years after RYGB

The total number of mitoses per crypt 3 years after RYGB remained significantly 1.7-fold (95% CI 1.3 to 2.0) higher than values obtained before surgery ($p<0.05$, Wilcoxon signed rank test; figure 1A and table 1). There was no significant difference between mitosis frequency per crypt at 6 months and 3 years after surgery (figure 1A and table 1).

The mean rectal crypt area remained higher 3 years after RYGB compared with preoperative and 6 month post-RYGB values (figure 1B and table 1). At 3 years, there was a 1.5-fold (95% CI 1.3 to 1.7; $p<0.01$; Wilcoxon signed rank test) increase in crypt size compared with values obtained in the same patients before RYGB.

Consistent with the 6 month post-RYGB data, there was no significant difference in crypt branching (fission) 3 years post-RYGB (table 1).

The frequency of neo-CK18-positive epithelial cell apoptosis was increased compared with paired values obtained at 6 months after RYGB, but values did not 'normalise' to preoperative values (table 1).

These data suggested that increased epithelial cell proliferation and larger crypt size persist long term after RYGB. Therefore, we next investigated whether the increased mucosal levels of proinflammatory gene mRNAs that we observed 6 months after surgery also persisted up to 3 years following RYGB.

Marked upregulation of MIF in rectal mucosa 3 years after RYGB

We observed a striking 40.5-fold (95% CI 13.1 to 125.4; $p<0.001$; Student paired t test) increase in *MIF* transcript levels compared with baseline pre-RYGB values (figure 2A and table 1). In 18 out of 19 cases, the MIF transcript level was higher at 3 years than the corresponding value at 6 months post-RYGB (figure 2A).

COX-1 and COX-2 mRNA levels also remained significantly elevated compared with baseline expression levels, but were similar to values 6 months post-RYGB (table 1).

In contrast, there was a significant decrease in mucosal *TNFα* mRNA levels 3 years after RYGB compared with preoperative values (mean 4.0-fold (95% CI 2.2 to 7.1) reduction; $p<0.001$; table 1). There was a significant inverse correlation between individual changes in *MIF* and *TNFα* mRNA levels from baseline to 3 years post-RYGB (Spearman $r=-0.47$, $p=0.04$).

Mucosal IL-6 and IL-1β transcript levels at 3 years post-RYGB remained slightly (but not significantly) elevated compared with preoperative values (table 1).

The marked increase in transcript levels of MIF, a proinflammatory cytokine with known roles in innate and acquired immunity,[13] prompted detailed histological analysis of H&E-stained FFPE mucosal sections by a Consultant Histopathologist (NS), who was blinded to the identity of each section. No histological abnormality of mucosal biopsies taken 3 years after RYGB was detected, ruling out development of a microscopic colitis. In particular, there was no evidence of an increased mononuclear cell mucosal infiltrate in any specimen.

MIF protein levels are increased in colorectal epithelial cells 3 years after RYGB

Next, we performed IHC for MIF in order to determine the cellular localisation of MIF in rectal mucosa from our cohort of obese individuals. We, and others, have previously described predominant epithelial cell localisation of MIF protein in histologically normal colorectal mucosa.[12 18] The pattern of expression of MIF in rectal mucosa from morbidly obese patients before RYGB was similar, with MIF immunoreactivity prominent in luminal, surface epithelial cells (figure 3A,C,E). Intense staining for MIF was also present in some crypt epithelial cells, which were mainly in, but not restricted to, putative crypt stem cell and transit amplifying zones (figure 3A,C,G). The majority of MIF-positive lamina propria cells had morphological characteristics of plasma cells, consistent with the previous literature (figure 3A, G).[12] The absence of staining in negative control sections confirmed the specificity of the MIF IHC technique (figure 3B).

Table 1 Outcome measures before and after RYGB

	Pre-RYGB	Post-RYGB	
		6 months	3 years
Weight (kg)*	151.9±8.0	118.6±6.8	110.7±6.7
BMI (kg/m^2)*	54.5±2.3	42.0±2.1	39.1±1.7§
% Excess weight loss* †	—	39.2±2.9	46.2±3.9
Rectal crypt measurements			
Mitosis frequency (per crypt)	4.4 (3.5—6.5)	9.7 (5.9—12.5)	7.0 (6.1—8.9)‡
Crypt area (mm^2)	0.034 (0.032—0.045)	0.042 (0.038—0.050)	0.054 (0.050—0.059)§
Crypt branching			
No. of branching crypts/patient	1.0 (0.25—2)	1.5 (0.5—2)	1.5 (0.5—2.5)
% of patients with ≥1 branching crypt	73.7	94.7	84.2
Apoptosis			
M30-positive crypts/patient	0.45 (0.24—0.64)	0.17 (0.15—0.40)	0.27 (0.20—0.46)
% patients with M30-positive crypts	42.1	15.8	31.6
Mucosal mRNA (fold increase from pre-RYGB level)¶			
MIF	—	1.2 (−3.1 to 4.7)	40.5 (13.1—125.4)§
COX-1	—	7.8 (1.2—51.2)	7.7 (1.5—38.4)‡
COX-2	—	3.5 (1.2—10.1)	5.8 (2.2—15.4)‡
TNFα	—	1.1 (−1.8 to 2.2)	−4.0 (−7.1 to −2.2)‡
IL-6	—	3.1 (1.1—8.8)	2.4 (−1.3 to 7.4)
IL-1β	—	1.8 (−1.3 to 4.2)	2.0 (−1.1 to 4.3)
Epithelial cell MIF immunoreactivity	6 (4.5—7)	6 (5—7.5)	9 (7.5—11)§
Systemic inflammatory markers			
CRP (mg/l)	6.5 (2.3—10.7)	2.8 (1.4—5.1)	0.3 (0.1—1.6)§
MIF (ng/ml)	8.7 (7.2—10.9)	10.9 (8.3—13.8)	5.6 (4.6—7.1)§
TNFα (pg/ml)	1.7 (1.5—1.9)	1.9 (1.8—2.7)	1.6 (1.4—2.0)
IL-6 (pg/ml)	4.2 (3.6—6.4)	2.8 (2.4—3.6)	1.1 (0.9—1.5)§

*Values indicate the mean±SE. All other values are stated as the median (IQR). All data are from the 19 RYGB patients who underwent assessment at 3 years.
†% Excess weight loss is determined from an 'ideal weight' (equivalent to a BMI of 22).[16]
‡Indicates a significant difference compared with pre-RYGB values; p≤0.05.
§Indicates a significant difference compared with both pre-RYGB and 6 month post-RYGB values; p≤0.05.
¶Fold increase and 95% CI are converted from the mean ΔΔCt (from pre-RYGB values).[17]
BMI, body mass index; COX, cyclooxygenase; CRP, C-reactive protein; IL, interleukin; MIF, macrophage migration inhibitory factor; RYGB, Roux-en-Y gastric bypass; TNFα, tumour necrosis factor α.

The same cellular distribution of MIF immunoreactivity was evident in rectal mucosa 3 years after RYGB, with prominent surface and crypt epithelial cell staining, as well as lamina propria MIF positivity (figure 3D,F,H). MIF immunoreactivity was increased in surface epithelium (figure 3D,F), and the number of MIF-positive crypt epithelial cells was higher in rectal mucosa 3 years after RYGB compared with paired preoperative tissue (figure 3D,H). Semiquantitative analysis of the extent and intensity of MIF protein staining in rectal mucosa was consistent with the mucosal MIF transcript data (figure 2B). Increased rectal epithelial MIF protein at 3 years post-RYGB compared with pre-RYGB expression was evident in 17 of 19 patients (figure 2B). Overall, there was a significant increase in epithelial cell MIF immunoreactivity in rectal mucosa 3 years after RYGB (median MIF score 9 (IQR 7.5—11)) compared with preoperative values (6 (4.5—7); p<0.01; Wilcoxon signed rank test; figure 2B). In contrast, there was no significant difference in lamina propria cell MIF staining over the same time period (data not shown).

Serum markers of inflammation continue to decrease during long-term follow-up after RYGB

MIF is expressed throughout the gastrointestinal tract and is present at high levels in the anterior pituitary, from which it contributes to the acute stress response in an endocrine manner.[19] Therefore, we investigated whether increased rectal mucosal MIF expression post-RYGB was part of a more generalised effect on MIF expression or was restricted to the colorectum. Serum MIF levels 3 years after RYGB were actually lower than values before and 6 months after RYGB (table 1 and Supplementary figure 2), suggesting that mucosal upregulation of MIF expression is a local phenomenon rather than part of a systemic MIF response.

Serum CRP and IL-6 levels were also significantly lower than corresponding values at 6 months post-RYGB, consistent with further weight loss between 6 months and 3 years after surgery (table 1 and Supplementary figure 2). There was no significant difference in serum TNFα levels between time points (table 1).

Mechanisms driving MIF upregulation in colorectal epithelial cells

Next, we investigated potential mechanisms driving increased epithelial cell MIF expression in the colorectum. Intestinal bypass increases the colorectal mucosal surface exposure to primary and secondary bile acids, which are believed to be procarcinogenic.[20] Recently, it has been reported that the secondary bile acid deoxycholic acid (DCA) increased MIF protein levels in human squamous oesophageal cancer cells.[21] Therefore, we tested the hypothesis that DCA promotes colorectal epithelial cell MIF expression. However, we did not observe any change in MIF protein expression by HT-29 or SW480 human CRC cells exposed to DCA for 24 h (Supplementary figure 3).

Dramatic upregulation of MIF mRNA and protein in human and *Apc*$^{Min/+}$ mouse intestinal adenomas[12] suggests that MIF expression may be linked to loss of function of the tumour suppressor gene *APC* during initiation of intestinal

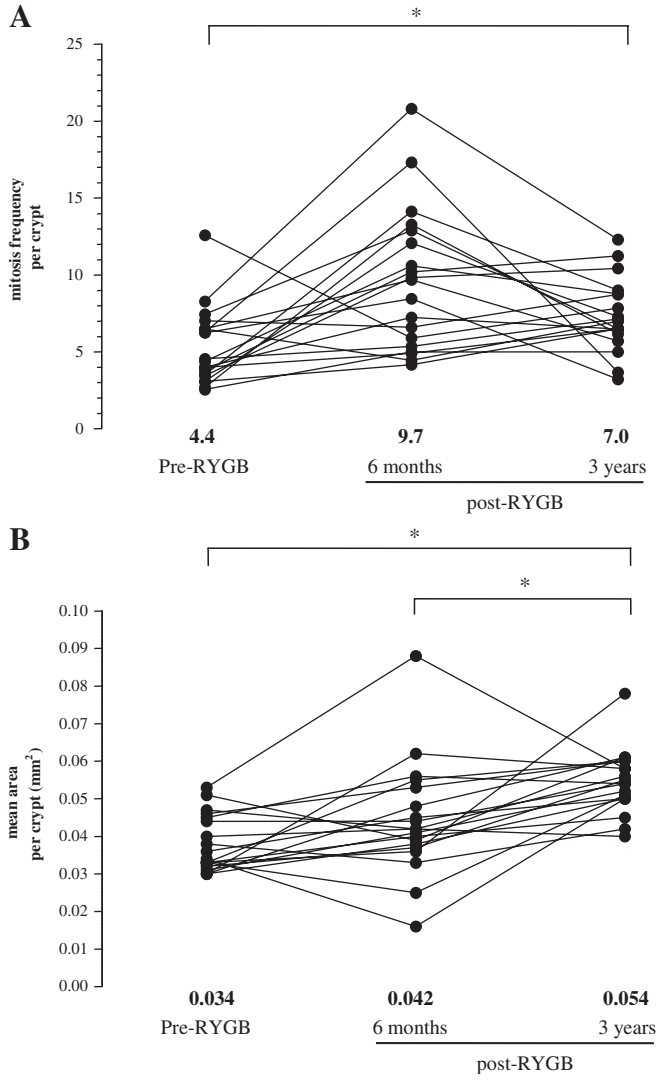

Figure 1 Rectal mucosal biomarkers in morbidly obese individuals before and after Roux-en-Y gastric bypass (RYGB). (A) The mean total number of mitoses per crypt. (B) Mean crypt area. Linked data points represent individual patients before, 6 months after and 3 years after RYGB (n =19). Values below the graph represent the median value for each group. *p<0.05 compared with 6 months post-RYGB and pre-RYGB values (Wilcoxon signed rank test). Pre-RYGB and 6 months post-RYGB data are included in order to aid comparison.[11]

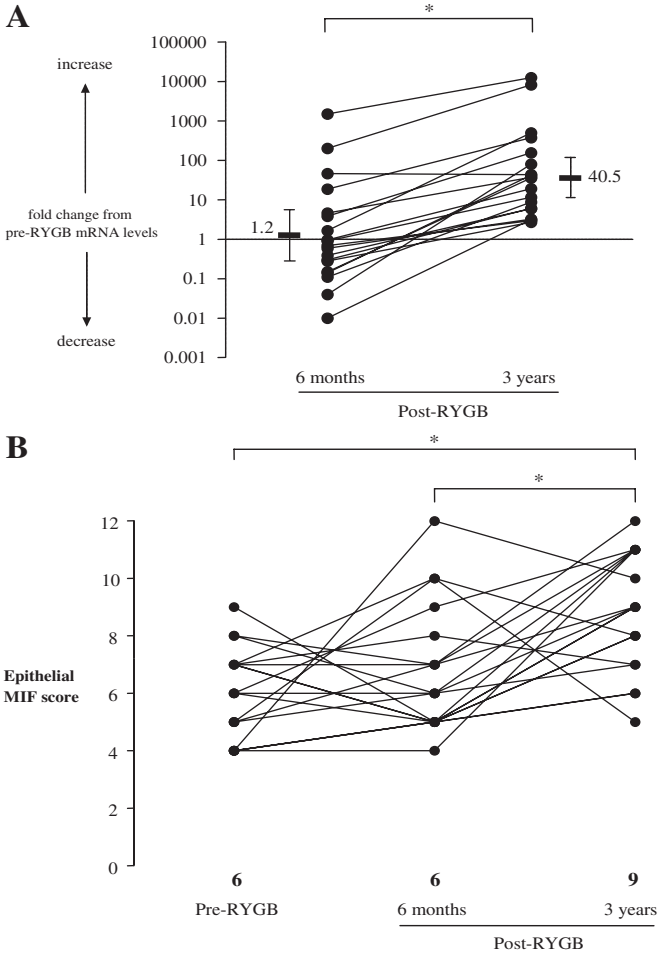

Figure 2 Rectal mucosal macrophage migration inhibitory factor (MIF) expression is increased in morbidly obese patients 3 years after Roux-en-Y gastric bypass (RYGB). (A) The fold change in *MIF* mRNA level normalised to the *GAPDH* (glyceraldehyde phosphate dehydrogenase) mRNA level measured by reverse transcription–PCR and quantified by the $\Delta\Delta C_t$ method.[17] Data points represent the fold difference from the preoperative level in each individual (n=19). Six month post-RYGB data are from our previous study.[11] Bars represent the mean fold change, with error bars denoting the 95% CI. *p<0.05, Wilcoxon signed rank test. (B) Semiquantitative analysis of MIF immunoreactivity in FFPE (formalin-fixed, paraffin-embedded) rectal mucosal sections. Linked data points represent the combined luminal epithelial and crypt epithelial scores from 19 patients before, 6 months after and 3 years after RYGB. Pre-RYGB and 6 months post-RYGB data were from our previous study.[11] Values below the data points are the median scores for each time point. *p<0.05 compared with pre-RYGB and 6 month post-RYGB values; Wilcoxon signed rank test.

tumorigenesis. Therefore, we formally tested whether *Mif* is upregulated in intestinal epithelial cells following deletion of *Apc* using the conditional *AhCre*$^+$ *Apc*$^{fl/fl}$ mouse model, in which β-naphthoflavone administration induces *Apc* loss in crypt intestinal epithelial cells.[15] In *AhCre*$^+$ *Apc*$^{+/+}$ small intestine, Mif protein was present uniformly in villus intestinal epithelial cells and in individual cells in the crypt base (figure 4B,C), consistent with our previously published data and MIF localisation in human intestine.[12] IHC for lysozyme on sections adjacent to those stained for Mif did not demonstrate cellular co-localisation (Supplementary figure 4), implying that basal crypt cells containing Mif protein were not Paneth cells. The presence of Mif-positive epithelial cells in the base of mouse colonic crypts (which do not contain Paneth cells) is also consistent with the fact that Mif protein expression is not restricted to Paneth cells (Supplementary figure 4).

Four days after β-naphthoflavone induction, Mif immunoreactivity was dramatically increased in abnormal 'floxed' intestinal epithelial cells in expanded crypts of *AhCre*$^+$ *Apc*$^{fl/fl}$ mice (figure 4D,F). Upregulation of Mif protein in intestinal epithelial cells following *Apc* loss was particularly apparent at the border between 'floxed' cells emerging from the crypt and 'normal' intestinal epithelial cells approximately one-third of the way up the crypt–villus unit at day 4 postinduction (figure 4E). Therefore, it can be concluded that loss of Apc function leads to Mif upregulation in mouse intestinal epithelial cells.

Mif controls intestinal epithelial cell proliferation and migration

We then investigated a possible role for *Mif* in the abnormal phenotype of *Apc*-deficient intestinal epithelial cells by comparing intestinal epithelial cell proliferation and migration

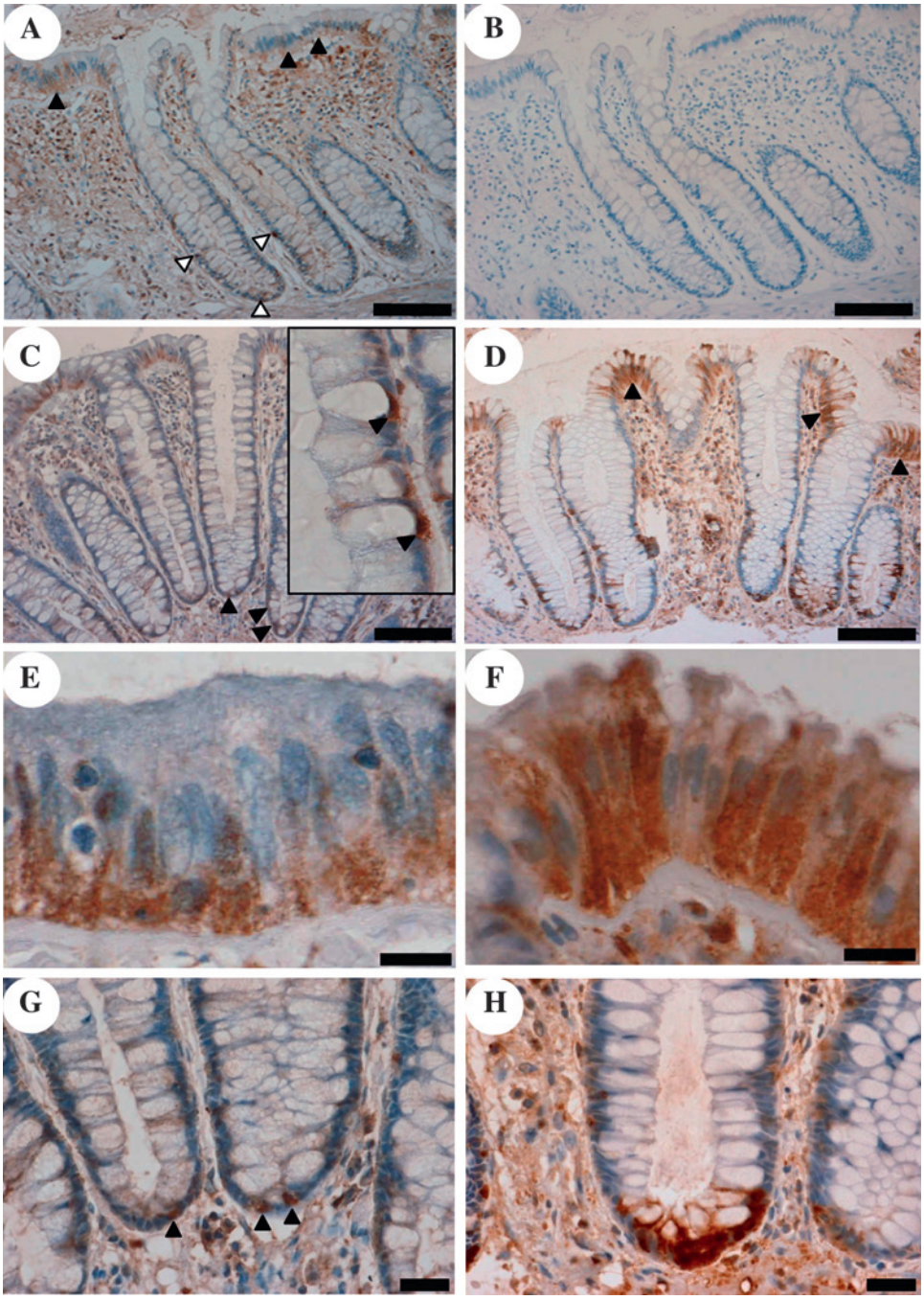

Figure 3 Macrophage migration inhibitory factor (MIF) protein localisation in human rectal mucosa from morbidly obese individuals before and 3 years after Roux-en-Y gastric bypass (RYGB). (A) Section of rectal mucosa before RYGB. Brown staining indicates localisation of MIF in surface epithelial cells (black arrowheads), crypt epithelial cells (white arrowheads) and lamina propria cells. Total epithelial MIF score 4. Size bar 50 μm. (B) Negative control section adjacent to (A) after omission of the primary antibody. Size bar 50 μm. Incubation with an equivalent concentration of mouse immunoglobulin G_1 (IgG_1) isotype control antibody (DakoCytomation) or with the primary antibody preabsorbed with recombinant human MIF (R&D Systems) overnight at 4°C (15 μg/ml) did not give specific staining (data not shown). (C) MIF localisation in rectal mucosa before RYGB. Diffuse staining of surface epithelium and MIF immunoreactivity in individual epithelial cells in the lower third of the crypt (black arrowheads). Total epithelial MIF score 5. Size bar 50 μm. The inset shows a higher magnification view of columnar epithelial cells with basolateral MIF staining (black arrowheads) below mucus granules. (D) Rectal mucosa 3 years after RYGB. There is intense staining in surface epithelial cells (black arrowheads) and an increased number of MIF-positive epithelial cells in the base and lower third of crypts. Total epithelial MIF score 8. Size bar 50 μm. (E) Basolateral MIF staining of surface epithelial cells in rectal mucosa from before RYGB. Surface epithelial MIF score 2. Size bar 10 μm. (F) Intense surface epithelial cell staining in rectal mucosa from a patient 3 years after RYGB. Note the variable staining intensity with lighter staining cells interspersed by epithelial cells with intense basolateral and apical immunoreactivity. Surface epithelial MIF score 6. Size bar 10 μm. (G) MIF staining in the crypt base from rectal mucosa before RYGB. Individual MIF-positive cells are highlighted by black arrowheads. Note the presence of scattered lamina propria cells adjacent to crypts. Crypt epithelial MIF score 2. Size bar 20 μm. (H) MIF staining in the crypt base from rectal mucosa 3 years after RYGB. Intense staining of crypt base cells. Crypt epithelial MIF score 4. Size bar 20 μm.

in *Mif*-null and wild-type *Mif* mice on an $Apc^{Min/+}$ background. We analysed intestinal epithelial cell BrdU incorporation 2 h after (as a 'snap-shot' measure of the number of cells in the S phase of the cell cycle at that time) and 24 h after (providing a measure of a combination of ongoing intestinal epithelial cell proliferation and migration along the crypt–villus unit) BrdU administration.

There was no significant difference in the number of BrdU-positive epithelial cells per crypt–villus unit in *Mif*-null versus wild-type *Mif* animals 2 h after BrdU injection (figure 5A). However, analysis of intestinal tissue 24 h after BrdU injection revealed a significantly lower number of BrdU-positive epithelial cells per crypt–villus unit in mice lacking *Mif* compared with wild-type *Mif* animals (figure 5A). This implies a role for Mif in control of epithelial cell proliferation and is consistent with our observation that elevated MIF protein expression by epithelial cells in human rectal mucosa was associated with increased mitotic frequency in microdissected human rectal crypts (see table 1).

Analysis of epithelial cell position within the crypt–villus unit demonstrated that the number of BrdU-positive cells in each segment of the crypt–villus unit was lower in *Mif*-null mice than in wild-type *Mif* animals (figure 5B), but also revealed that there was a 'left-shift' in the distribution of BrdU-positive

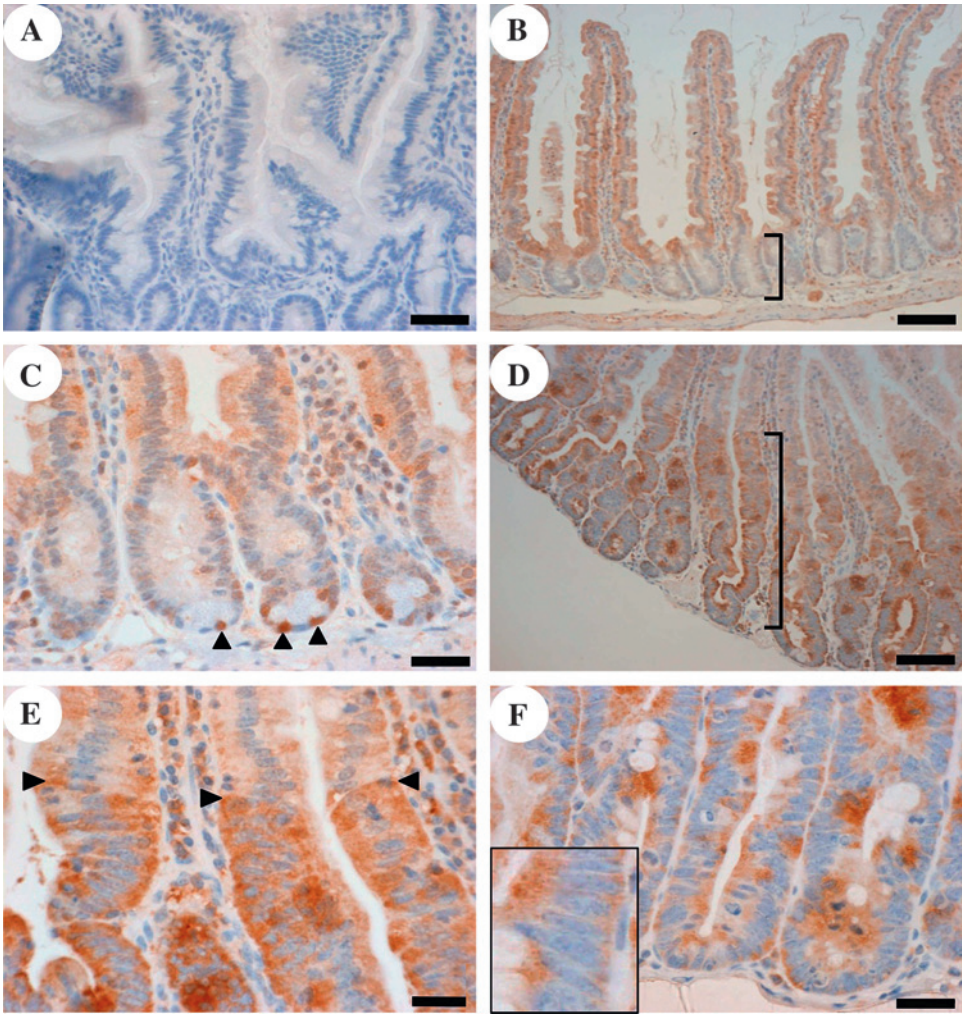

Figure 4 Macrophage migration inhibitory factor (Mif) protein expression in mouse small intestinal mucosa. (A) *Mif*-null small intestinal mucosa acting as a negative control for Mif immunohistochemistry. Size bar 30 μm. (B) Mif immunoreactivity in *AhCre*$^{+}$ *Apc*$^{+/+}$ mouse intestine. There is diffuse Mif immunoreactivity in villus epithelial cells but only occasional Mif positivity in epithelial cells in the crypt compartment (bracket). Size bar 50 μm. (C) Distinct Mif staining in basal crypt epithelial cells (black arrowheads) in *AhCre*$^{+}$ *Apc*$^{+/+}$ mouse intestinal crypts. Size bar 20 μm. (D) Mif protein expression in *AhCre*$^{+}$ *Apc*$^{fl/fl}$ mouse intestine 4 days after β-naphthoflavone induction. There is a marked increase in Mif immunoreactivity in 'floxed' epithelial cells in expanded crypt compartments (bracket). Size bar 20 μm. (E) Higher power view of (D) demonstrating the clear demarcation between 'floxed' intestinal epithelial cells with increased Mif staining (below the arrows) and normal intestinal epithelial cells (above the arrows). Size bar 20 μm. (F) Mif staining in crypts from *AhCre*$^{+}$ *Apc*$^{fl/fl}$ mouse intestine 4 days after β-naphthoflavone induction. In contrast to (C), there is widespread cytoplasmic staining for Mif in all crypt epithelial cells following *Apc* loss. Size bar 20 μm.

epithelial cells in *Mif*-null animals (figure 5B). *Mif*-null epithelial cells failed to migrate along the villus at the same rate as wild-type *Mif* cells at 24 h ($p<0.05$ for the difference in distribution of BrdU-positive epithelial cells, Kolmogorov–Smirnov test; figure 5B). Therefore, Mif plays a complex role in the fate of intestinal epithelial cells in mouse intestine, with effects on both proliferation and migration.

In order to determine whether the findings in mouse intestine were consistent with our human observational data, we carried out a similar positional analysis of mitotic cells in human rectal crypts taken 3 years after RYGB (a state of high epithelial cell MIF expression), in comparison with equivalent data from the same individuals before RYGB (a state of lower epithelial cell MIF expression). The frequency of mitotic epithelial cells in crypt zones 1–4 was higher in crypts from mucosa 3 years after RYGB compared with mucosal crypts before RYGB, with the difference being statistically significant in deeper crypt zones 1 and 2 (figure 6). The increase was particularly prominent in zone 2, with increased mitotic frequency higher up the rectal crypt (figure 6). Therefore, we can conclude that the association between increased epithelial cell MIF levels and epithelial cell mitosis in human crypts is consistent with the functional consequences of *Mif* deficiency in our mouse model.

DISCUSSION

We have demonstrated that changes in rectal mucosal epithelium and proinflammatory gene expression persist long term (at least 3 years) following RYGB. This is strong evidence that changes in rectal mucosal crypt morphometry and gene expression are not due to a postoperative 'tissue repair' or a stress response after gastrointestinal bypass, particularly as some parameters (crypt size and MIF expression) actually increased between 6 months and 3 years after surgery.

In the absence of a prospective cohort study, there is still debate as to whether increased epithelial cell 'proliferation' (as measured by mitosis frequency in whole crypts or Ki-67/proliferating cell nuclear antigen (PCNA) positivity in FFPE sections) is a biomarker of future CRC risk.[22] However, given the association between increased epithelial cell 'proliferation' and the presence of colorectal adenoma,[23] we argue that increased mitosis frequency, in combination with increased mucosal proinflammatory (and protumorigenic) gene expression, is sufficient to prompt clinical studies of future colorectal neoplastic risk in patients after RYGB.

Three studies has previously addressed CRC incidence following gastric bypass surgery.[24–26] Adams and colleagues performed a large, retrospective cohort study with a mean follow-up period of 12.3 years for incidence data.[24] There was a non-significant HR of 0.7 (95% CI 0.4 to 1.2) for CRC incidence in the surgery group compared with morbidly obese controls.[24] The other smaller, retrospective cohort study, in which only 81% of cases underwent RYGB, had a shorter follow-up time (5 years) and reported an RR of CRC of 0.3 (95% CI 0.08 to 1.3).[25] However, the long natural history of colorectal

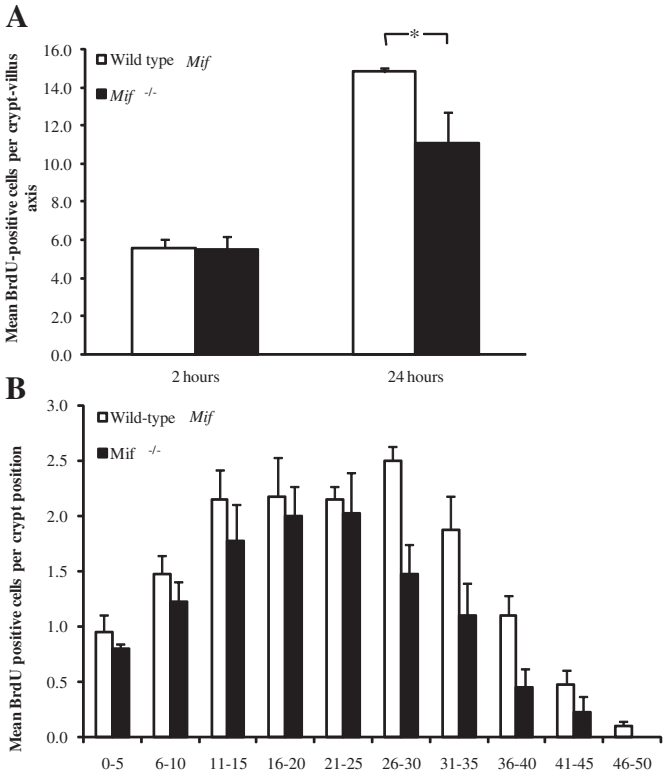

Figure 5 Analysis of bromodeoxyuridine (BrdU)-positive epithelial cells in mouse small intestine. (A) The number of BrdU-positive cells per crypt–villus unit in either $Apc^{Min/+}$ mice with wild-type *Mif* (migration inhibitory factor) alleles (open bars) or *Mif*-null ($Mif^{-/-}$) animals (solid bars) 2 h (n=4 and n=3, respectively) and 24 h (both n=4) post-BrdU injection. Columns represent the mean and bars represent the SEM. (B) The distribution of BrdU-positive epithelial cells along the crypt–villus axis in either $Apc^{Min/+}$ mice with wild-type *Mif* alleles (open bars) or *Mif*-null ($Mif^{-/-}$) animals (solid bars) 24 h (both n=4) post-BrdU injection. Cell position is numbered from 0 (base) upwards towards the lumen. Columns represent the mean and bars represent the SEM. *p<0.05, Komolgorov–Smirnov test.

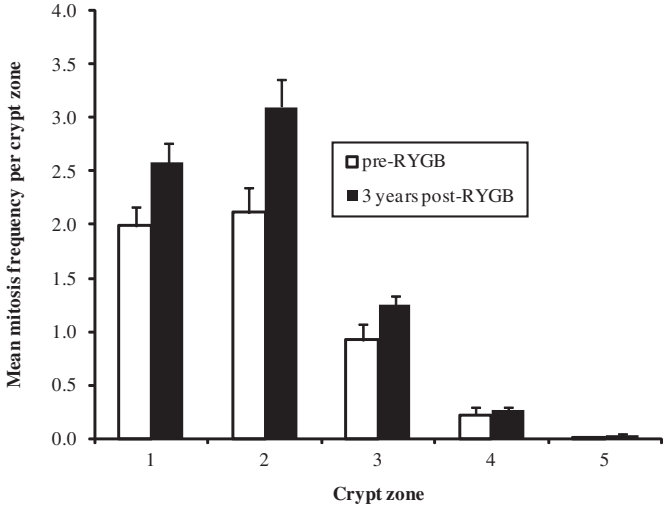

Figure 6 Zonal analysis of mitotic epithelial cells in microdissected human rectal crypts. Columns represent the mean rectal epithelial mitosis frequency in individual zones of crypts from rectal mucosa from patients before Roux-en-Y gastric bypass (RYGB) and from 3 years postsurgery. Bars represent the SEM. p<0.05, analysis of variance.

carcinogenesis means that incident cancers in both these studies are likely to have arisen from colorectal adenomas or asymptomatic cancers already present around the time of surgery rather than from de novo tumorigenesis after RYGB. Recently, a Swedish population-based cohort study has been published that reported longer follow-up after bariatric surgery.[26] This study demonstrated a time-dependent increase in CRC risk with a standardised incidence ratio of 2.13 (95% CI 1.33 to 3.22) >10 years after surgery compared with age- and sex-matched population controls.[26] This is the first tentative clinical evidence that CRC risk may be increased following bariatric surgery. There was no obese, non-surgical control group in this cohort study so the relative contributions of obesity-related CRC risk versus a postsurgical effect could not be determined. Further cohort studies of patients after RYGB should use colonoscopic colorectal adenoma incidence as a surrogate end point for CRC risk in comparison with both obese and normal weight individuals who have not undergone bypass surgery.

A striking finding was that epithelial cell MIF content in rectal mucosa was consistently higher 3 years after RYGB compared with MIF expression before surgery and was increased significantly compared with findings at 6 months after RYGB. The lack of any significant change in circulating MIF levels, combined with the persistent increase in mucosal *COX-1* and *COX-2* mRNA levels 3 years after RYGB compared with preoperative values, argues strongly for localised mucosal MIF upregulation after gastric bypass rather than as part of a prolonged systemic stress response to gastric bypass.

We addressed potential mechanisms for RYGB-induced epithelial cell MIF upregulation. Increased epithelial cell bile acid exposure following intestinal bypass was a strong hypothesis, particularly in the light of the recent report that DCA induced MIF protein expression in human squamous oesophageal cancer cells.[21] However, the absence of any MIF induction in our in vitro studies with human CRC cells treated with DCA did not lend support to this hypothesis. The other hypothesis that we addressed, namely that MIF upregulation occurs downstream of loss of *APC* function, was based on the marked upregulation of MIF observed in dysplastic epithelial cells at the earliest stages of intestinal tumorigenesis in both the $Apc^{Min/+}$ mouse and humans.[12] Moreover, Mif levels (measured by tautomerase assay) in non-neoplastic intestinal mucosa have been noted to be higher in $Apc^{Min/+}$ mice compared with wild-type animals, suggesting that *Apc* haploinsufficiency, prior to tumour initiation, may be sufficient to drive *Mif* expression.[12] We now provide incontrovertible evidence that *Apc* loss leads to Mif upregulation in mouse intestinal epithelial cells using the established $AhCre^{+}$ mouse model.[15] Ongoing studies in our laboratory will now investigate the *APC* status of microdissected rectal epithelium from FFPE tissue obtained before and 3 years after RYGB in order to address the hypothesis that *APC* haploinsufficiency in epithelial cells develops after gastric bypass. Putative mouse and human *MIF* promoters do not contain consensus T cell factor–β-catenin binding elements so *MIF* is unlikely to be a classical direct Wnt target gene. An alternative hypothesis that now warrants testing is that *Mif* upregulation following *Apc* loss is a *Myc*-dependent process.[27]

Other testable hypotheses can be put forward to explain the persistent upregulation of MIF (and COX-2) that we have observed, including changes to the gut microbiota secondary to RYGB.[28] However, the observation by Maaser and colleagues that the presence of *Salmonella* organisms promoted release of MIF protein but did not drive MIF synthesis in CaCo-2 human CRC cells is not consistent with the hypothesis that an

altered bacteria–host relationship is responsible for our clinical observations.

The negative association between the changes in mucosal MIF and TNFα transcript levels following RYGB is intriguing. Several mouse models of colitis have demonstrated a positive association between upregulation of MIF and TNF.[29] A possible causal negative relationship between these two pivotal cytokines in non-colitic human colorectal tissue certainly warrants further investigation.

Another novel finding is that MIF, a cytokine with established roles in innate and acquired immunity,[13] as well as with protumorigenic properties,[12] contributes to murine intestinal epithelial cell fate, with a complex role in control of proliferation and migration. The dual findings of a migratory defect in *Mif*-null mice and the expanded mitotic epithelial cell zone in crypts from patients with increased mucosal MIF levels following RYGB are consistent and together suggest that MIF may contribute to the increased mitotic frequency in crypt epithelial cells observed in patients after RYGB. In fact, MIF has previously been implicated in induction of cell proliferation and resistance to apoptosis in epithelial (HEK293) and non-epithelial (fibroblasts, macrophages) cells.[30] We have previously described that MIF promotes resistance to apoptosis in human colorectal adenoma cells.[12] This work has recently been extended by Maharshak and colleagues, who have reported that MIF promotes survival of normal mouse colorectal epithelial cells in a CD74-dependent manner.[31] These data are consistent with our findings that MIF regulates intestinal epithelial cell fate.

In summary, we have combined long-term, longitudinal clinical observations and mechanistic insights from mouse models in order to characterise long-term changes in rectal epithelial cells and mucosal gene expression that should prompt evaluation of future neoplastic risk in patients who have undergone RYGB surgery, the majority of whom remain obese. MIF is amenable to direct inhibition by small-molecule drugs[32] and so should be considered as a potential CRC chemoprevention target, particularly in patient groups with high mucosal MIF expression.

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Competing interests None.

Ethics approval This study was conducted with the approval of the Leeds (East) Research Ethics Committee.

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Rectal epithelial cell mitosis and expression of macrophage migration inhibitory factor are increased 3 years after Roux-en-Y gastric bypass (RYGB) for morbid obesity: implications for long-term neoplastic risk following RYGB

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