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Hypomethylation of *NANOG* promoter in colonic mucosal cells of obese patients: a possible role of NF-κB‡

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Abstract

Obesity and particularly central obesity are the main risk factors of colon cancer. All intestinal cell populations including stem cells, their progenitors and differentiated colonocytes seem to be the origin of colorectal cancer. However, recent data support the role of differentiated cells as cancer origin especially during inflammation. Based on Yamanaka's seminal work, re-expression of few transcription factors in terminally differentiated cells creates stemness properti'es. Although these transcription factors are involved in tumorigenesis, they are epigenetically repressed in adult tissues. We proposed that obesity might regulate methylation of stemness genes in colonocytes via inflammatory signalling. Obesity-associated inflammation was analysed using Western blot analysis of phospho-IκB (inhibitor of NF-κB). Methylation-sensitive high-resolution melting analysis was performed on colonic mucosal samples of twenty obese and twenty normal-weight men to analyse promoter methylation of *POU5F1* (OCT4), *NANOG*, *MYC* and *CDKN2A*. TNF-treated HT-29 cells were used to recapitulate the effect of NF-κB activation on stemness genes methylation. Our results showed that colonic phosphorylation of IκB, as a signal of NF-κB activation, was higher in obese subjects compared with their normal-weight counterparts. Moreover, promoter methylation of *NANOG* was likely to be lower in obese subjects and correlated with central obesity. HT-29 cells incubated by TNF-α showed hypomethylation of *POU5F1* and *MYC* genes in addition to the *NANOG*. These results suggest that obesity-induced inflammation might be involved in the regulation of DNA methylation of oncogenic genes such as *NANOG* in differentiated colonocytes and thus predispose them to later oncogenic alterations.

Key words: Stemness genes: DNA methylation: NF-κB: Ten-eleven translocation enzymes: Obesity

Despite all controversies in molecular cause and cellular origin of cancer, there is a relative agreement that some physiological and pathological states including ageing, obesity and type 2 diabetes are associated with increased risk of many types of cancer[1]. Based on epidemiological studies, obesity correlates with the increase in incidence of several cancer types including the colon, breast, endometrium, oesophagus, pancreas and liver[2]. Among them, colorectal cancer (CRC) is the third most common cancer in men and women[3]. Although some mechanisms such as hyperinsulinaemia and high level of insulin-like growth factor-1 are proposed to link adiposity and cancer[4], obesity-induced chronic inflammation has been further mentioned. The high prevalence of cancer in inflammatory diseases including chronic pancreatitis, ulcerative colitis and liver infectious diseases further confirm the probable involvement of inflammation in obesity-induced tumorigenesis[5].

It was believed that conversion of stem cells into specific type of differentiated cells is a unidirectional way. However, Yamanaka's pioneering study indicated that artificial expression of few stemness transcription factors (TF), including SOX-2, OCT-4, KLF-4, NANOG, LIN-28A and c-Myc in differentiated somatic cells, is sufficient to reprogram them into pluripotent stem cells[6].

Although, based on the prevalent paradigm, intestinal stem cells are the main origin of CRC stem cells[7], it has been accepted that differentiated epithelium can also acquire precancerous properties (reviewed in Huels & Sansom[8]). Intestinal stem cells are located in mucosal crypt base and undergo

Abbreviations: CpG, cytosine–phosphate diester–guanine; CRC, colorectal cancer; HRM, high-resolution melting; IκB, inhibitor of NF-κB; IQR, interquartile range; TET, ten-eleven translocation; TF, transcription factor; WC, waist circumference.

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‡ The original version of this article was published with the incorrect author name. A notice detailing this has been published and the error rectified in the online and print PDF and HTML copies.

self-renewal and differentiation to provide all intestinal cell population. Early observation of physicians indicated that adenomatous polyps, as a preneoplastic lesion, appear at the top of colonic crypts[9]. Furthermore, differentiated intestinal epithelium transforms into neoplastic state in top-down manner during inflammatory bowel disease[10]. Differentiated cells need to re-enter cell cycle, overcoming tumour suppressor barriers and expressing stemness genes to generate stem-like properties and transform into neoplastic cells[11].

TF control cell identity and final fate through transcriptional network of specific cell lineage[12]. It has been proposed that NF-κB transcription factor, as main regulator of inflammation, provides mechanistic link between chronic inflammatory conditions and tumorigenesis. Signals that induce inhibitor of NF-κB (IκB) phosphorylation and subsequent degradation, such as proinflamatory cytokines and DNA damage, cause nuclear translocation of NF-κB dimer such as p65/p52[13]. Nuclear NF-κB promotes the expression of a subset of genes including those involved in inflammation process. Recently, it has been shown that NF-κB is the main culprit in inflammation-associated epithelial-to-mesenchymal transition and stemness[14].

Epigenetic landscape of somatic cells is dramatically altered during dedifferentiation. Impairment in epigenetic alterations can disturb cellular dedifferentiation and reprogramming[15]. Master stemness factors usually are suppressed in adult somatic tissues[16]. It has been previously revealed that inflammatory stimuli remarkably induce alteration in DNA methylation[17]. We assume that obesity-associated inflammation and NF-κB activation might overcome epigenetic barriers of stemness genes' expression to reproduce stem-like properties in differentiated epithelial cells. Methylation of cytosine–phosphate diester–guanine (CpG) on transcriptional *cis*-regulatory elements, namely promoters and enhancers, often suppresses transcription (reviewed in Han[18]). DNA methyltransferases (DNMT) are responsible for DNA methylation, and ten-eleven translocation (TET) enzymes are part of a DNA demethylation mechanism[19,20].

In this study, we proposed that NF-κB transcription factor might epigenetically control expression of genes involved in dedifferentiation. To this purpose, we analysed methylation of CpG within gene promoters of *POU5F1*, *NANOG* and *MYC* nearby NF-κB binding sites in obese human colonic tissues. In parallel, promoter methylation of *CDKN2A* was analysed to show DNA methylation status of a tumour suppressor in response to obesity. Moreover, HT-29 cells treated by TNF-α were exploited to show the role of NF-κB in epigenetic alteration of stemness factors. Although there is evidence for the role of NF-κB in dedifferentiation and reprogramming, its contribution in obesity-induced hypomethylation of stemness genes has not been addressed. Here, we provide evidence regarding the probable obesity-related NF-κB activation on hypomethylation of *NANOG*.

Methods

Subjects

In this hospital-based, case–control study, twenty obese men and twenty sex- and age (with a 5-year interval)-matched lean controls were recruited between February 2015 and April 2016 to ambulatory endoscopy clinic of Moddares hospital. Inclusion criteria were as follows: age 20–60 years old, BMI ≥30 kg/m^2 for obese cases and BMI <25 kg/m^2 for lean controls; lack of any acute or chronic illness; and no daily medication. Individuals with a prior history of colonic neoplasia, colitis, polyposis, previous colon resection and CRC or any other cancers were excluded.

In all, 3 d before the colonoscopy, subjects were on a special diet which included low-fibre foods containing no whole grains, nuts, seeds, dried or raw fruits and vegetables. The day before the procedure, no solid food was allowed and subjects were asked to only consume clear liquids such as clear broth, black tea and coffee, or clear juices (apple, white grape), while on the day all were fasted. Standard optical colonoscopy was performed on all participants by the attending gastroenterologist. In all, four rectal pinch biopsies of colon epithelium were obtained from the mid-rectum using jumbo biopsy forceps. The biopsies were immediately snap-frozen in liquid N_2, transported to the laboratory and stored at −80°C until further sample processing.

Weight and height were measured using standard protocols, and BMI was calculated by dividing weight in kg by the square of height in metres. Moreover, to calculate waist:hip ratio, waist circumference (WC) and hip circumference were measured using a non-stretchable tape measure. The standard cut-off values for abdominal obesity in adults were WC ≥102 cm in men[21]. Participants were personally interviewed based on a structured questionnaire to obtain information on age, family history of CRC in a first-degree relative, family history of other cancers, education, occupation, smoking habit, chronic or acute disease history, consumption of multivitamin or other nutritional supplement, and list of medications. The ethics committee at the National Nutrition and Food Technology Research Institute of Shahid Beheshti University of Medical Sciences approved the study protocol (code of ethics committee: IR.SBMU.NNFTRI.REC.1395.50). In addition, written informed consents were obtained from all participants before enrolment.

Cell culture and treatment

The HT-29 human colon adenocarcinoma cells were purchased from the National Cell Bank of Pasteur Institute, Iran. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Life Technologies) supplemented with 10 % fetal bovine serum (Gibco, Life Technologies), 1 % penicillin/streptomycin (Sigma) and 2 mM glutamine (Sigma). Cells were kept in 37°C, 5 % CO_2 in a humidified incubator until reaching approximately 80 % confluency. For TNF treatment experiment, the HT-29 cells were seeded in twelve-well plates (500×10^5 cells/well). After 24 h, medium was renewed, and the cells were treated with human recombinant TNF (Invitrogen) at two different concentrations of 10 and 25 ng/ml and incubated for 48 and 72 h.

DNA extraction and sodium bisulphite modification

Total genomic DNA was extracted from the cultured cells and tissue samples using EZ-10 spin column animal DNA mini-preps kit (Bio Basic Inc.) according to the manufacturer's protocol. In the next step, the total genomic DNA samples were modified by sodium bisulphite conversion using an EZ DNA methylation-gold kit (Zymo Research). The purpose was to convert unmethylated

cytosines to uracils while leaving methylated cytosines unmodified. The modified DNA samples were stored immediately at –80°C.

Primer design

High-resolution melting (HRM) primer pairs were designed for specific CpG islands in promoter sequences of each gene according to the HRM primer design guidelines (Table 1). In addition, we aimed to design primers for a given DNA sequence based on their proximity to NF-κB binding site. NF-κB proteins act as transcriptional activators or repressors by binding to the consensus DNA sequence (5-GGGRNYYYCC-3) known as the kB site. We analysed the putative NF-κB binding sites within the promoter region of human *POU5F1*, *NANOG* and *p-16* promoter using Softberry (http://www.softberry.com/berry.phtml?topic=nsite&group=programs&subgroup=promoter) and MatInspector (http://www.genomatix.de) programs.

Methylation-sensitive high-resolution melting

HRM was used to assess the methylation status of the *NANOG*, *POU5F1*, *MYC* and *CDKN2A* genes. PCR was carried out in a 20 µl total volume containing 13 µl of double-distilled water, 4 µl of 5× hot FIREPOL Eva Green HRM mix-Rox kit (Solis BioDyne), primers at final concentration of 0·3 pmol and 1 µl of bisulphite modified template. Each reaction was performed in duplicate. HRM was performed using the following protocol: (1) PCR amplification protocol including denaturation for 15 min at 95°C for one cycle, denaturation for 15 s at 95°C, appropriate annealing temperature for each primer set (Table 1) for 15 s and extension for 20 s at 72°C for forty-five cycles; followed by (2) HRM protocol including 95°C for 1 min, 40°C for 1 min, 74°C for 5 s and continuous acquisition to 90°C at twenty-five acquisitions per 1°C (step one plus; Applied Biosystems). Unconverted DNA (not treated with the bisulphite reagent) served as a negative control. Human methylated and unmethylated DNA sets from Zymo Research were used as 100 % methylated and 0 % unmethylated controls, respectively. The percentages of methylation of 0, 25, 50, 75 and 100 % were used to draw the standard curve for the *POU5F1*, *NANOG* and *CDKN2A* genes, Whereas the standards of 0, 5, 10, 25, 50, 75 and 100 % were used for the *MYC*. Melting curves were normalised relative to two normalisation regions before and after major decrease in fluorescence. This indicated the melting region of the PCR product using the HRM version 2.2 software (ThermoFisher Scientific).

Western blotting analysis

Frozen colon tissue samples were homogenised in radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology), incubated for 30 min on ice and centrifuged for 20 min at 15 000 g. Protein assay was performed using the Bradford method. Equal amount of proteins (50 µg) were subjected to SDS-PAGE and then electroblotted onto a polyvinylidene difluoride membrane. The membranes were blocked with 5 % bovine serum albumin for 1 h at room temperature and incubated with a primary mouse pIκB antibody (B9, Santa Cruz Biotechnology, Inc.) at 4°C for overnight, followed by incubation with a secondary antibody (Santa Cruz Biotechnology Inc.). Subsequently, the signal was detected by chemiluminescence (Amersham ECL kit; GE Healthcare, Life Sciences). In addition, equal loading and constant protein expression of β-actin were determined and served as the normalising control.

Statistical analysis

Statistical tests were performed using SPSS software (version 16.0), and a two-sided *P*-value ≤0·05 was considered significant. χ^2 Tests or independent-samples *t* tests were applied for categorical and continuous variables, respectively. Where the distribution of the continuous variables was not normal, the Mann–Whitney *U* test was used. To assess the relation between obesity (BMI >30 kg/m^2) and percentage methylation of the *POU5F1*, *NANOG* and *MYC* logistic regression models were applied for estimating OR with 95 % CI adjusting for age and smoking. Gene methylation was assessed as medians based on the distribution among the controls. We also assessed the relation between methylation status and WC using regression analysis.

Results

Characteristics of cases and controls

Demographic and lifestyle characteristics of participants are presented in Table 2. By design, age was similar in both groups (50·8 *v.* 52·5 years in controls and cases, respectively). However, the cases were more likely to have a history of former smoking ($P \leq 0{\cdot}05$). No significant differences were observed in terms of cancer and particularly colon cancer family history, non-steroidal anti-inflammatory drug use, marital status and educational level between cases and matched controls.

Table 1. Methylation-sensitive high-resolution melting primers and amplicon information

Gene name	Sequence (5′ → 3′)	T_a (°C)	Accession number	Number of CpG-sites/amplicon length (bp)
POU5F1	F-GGGTTAGGTTTTGAGGTGTG R-TCGAAACTCACTTACCTCCT	60	NC_000006.12	11/227
NANOG	F-GTTGTTTAGGTTGGAGTATCGT R-CACCCCTATAATCCCAATAAATT	60	NC_000012.12	8/201
MYC	F-GCGTTTTGGGAAGGGAGATT R-TGCCGAAATAAACAAAATAACCTC	56	NC_000008.11	16/166
CDKN2A	F-TCGGAGGAAGAAAGAGGAG R-ACCGTAACCAACCAATCAAC	60	NC_000009.12	14/166

T_a, appropriate annealing temperature; CpG, cytosine–phosphate diester–guanine.

British Journal of Nutrition

Table 2. Selected baseline characteristics of cases (twenty) and controls (twenty), Iranian case–control study, 2016–2017
(Mean values and standard deviations; medians and interquartile ranges (IQR); numbers and percentages)

	Controls		Cases		
Variables	*n*	%	*n*	%	*P**
Number	20		20		
Weight (kg)					<0·001
Mean	70·1		95·0		
SD	9·7		8·9		
Height (cm)					0·77
Median	172·5		172·5		
IQR	10·7		7·7		
BMI (kg/m^2)					<0·001
Median	24·8		32·5		
IQR	2·7		3·0		
Waist (cm)					<0·001
Mean	83·0		107·0		
SD	15·4		9·5		
Hip (cm)					<0·001
Mean	99·1		112·1		
SD	1·7		5·0		
WHR					<0·001
Median	0·88		0·95		
IQR	0·06		0·06		
Age (years)					0·60
Mean	50·8		52·5		
SD	10·1		10·6		
Cancer family history (yes)	11	55	14	70	0·33
Colon cancer family history (yes)	4	20	7	35	0·28
NSAID use (yes)	3	15	6	30	0·25
University education	14	70	16	80	0·14
Marital status: married	17	85	20	100	0·07
Smoking status					0·01
Never	16	80	9	45	
Current	4	20	5	25	
Former	0	0	6	30	

WHR, waist:hip ratio; NSAID, non-steroidal anti-inflammatory drugs.
* Student's *t* test or Mann–Whitney test was used for continuous variables with normal and non-normal distribution, respectively, χ^2 test was used for categorical variables.

Phosphorylation of inhibitor of NF-κB in colon epithelial tissue

Due to the small size of obtained colon tissues and the importance of DNA methylation analysis of stemness genes, only phosphorylation of IκB could be analysed as the indicator of subsequent nuclear translocation and activation of NF-κB. As shown in Fig. 1, three distinct immunoblotting analyses on the lysate of the colon tissue samples revealed that IκB phosphorylation was remarkably higher in obese samples in comparison with normal-weight controls. Higher level of IκB phosphorylation was observed across all cases.

Hypomethylation of NANOG gene in colon epithelial tissue

Based on software analysis, we found that *POU5F1*, *NANOG* and *MYC* promoter regions include a NF-κB binding site (*POU5F1*: +247/257, *NANOG*: −485/−475, *MYC:* +288/298s from transcription start site) whereas *CDKN2A* lacks NF-κB binding site in its promoter. Preliminary results indicated that methylation of the *CDKN2A* promoter was rare: 0 % in both the cases and controls. The median methylation levels of the *POU5F1*, *NANOG* and *MYC* in the controls were 50 % interquartile range (IQR: 25), 62·5 % (IQR: 25) and 5 % (IQR: 5), respectively, whereas the levels were 50 % (IQR: 25), 50 % (IQR: 25) and 10 % (IQR: 5) in the obese cases, respectively. Statistical analysis confirmed that the methylation (%) level of the *NANOG* was significantly different ($P < 0·05$; Mann–Whitney *U* test) between obese cases and lean controls.

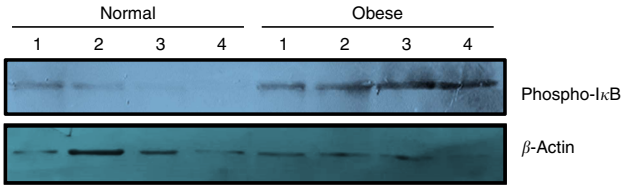

Fig. 1. Phosphorylation of inhibitor of NF-κB (IκB) increases in the colon tissue samples of obese subjects. Frozen colon sample tissues were lysed by radioimmunoprecipitation assay buffer and an equal amount of protein was subjected to Western blot using primary antibodies against total phospho-IκB (Ser 32). Antiactin antibody was used as the loading control. Results are representative of three independent experiments.

The OR from binary logistic regression models of the association between obesity and gene methylation by median categories of the control groups are presented in Table 3. We did not find any association between obesity and *POU5F1* and *MYC* promoter methylation. However, obesity was more likely to be associated with hypomethylation of *NANOG* in both crude and adjusted models (crude OR = 0·25, 95 % CI 0·06, 1·00; adjusted OR = 0·18, 95 % CI 0·04, 0·86). Following categorising the subjects into two groups according to WC (≥102 cm as an indicator of central obesity), we found an inverse association between WC and *NANOG* methylation but only after adjusting for age and smoking (adjusted OR = 0·08, 95 % CI 0·008, 0·90).

We also examined the association between gene methylation and the characteristics of the study subjects. *NANOG* methylation status was inversely associated with former smoking (OR = 0·07, 95 % CI 0·006, 0·98) after adjusting for age and case–control status (Table 4). We found no other significant associations between genes methylation and other characteristics of the study subjects.

Regulation of gene methylation in HT-29 cells by TNF treatment

To mimic the role of NF-κB in hypomethylation of *NANOG* in the colon epithelial tissue of the obese patients, TNF-α was used as the best known NF-κB stimulator. Methylation-sensitive-HRM assessment of methylation percentage after TNF-α treatment in the HT-29 cell line is shown in Table 5. Two different concentrations of TNF (10, 25 ng/ml) for 48 and 72 h were used. As expected in HT-29 CRC cell line and due to lack of NF-κB binding site in its promoter, *CDKN2A* was totally hypermethylated (100 %) before and after treatment with TNF-α. The methylation percentages in the control group were stable at all time points. Treatment with 10 ng/ml TNF for 48 h did not affect the methylation status (%) of the *POU5F1*, *NANOG* and *MYC*. However, their methylation (%) was reduced when exposure time increased to 72 h, with the same concentration. Further

Table 3. Association of obesity (BMI ≥30 kg/m^2), central obesity and genes methylation by median categories of control group in a case–control study
(Odds ratios and 95% confidence intervals)

	NANOG		*POU5F1*		*MYC*	
	OR	95 % CI	OR	95 % CI	OR	95 % CI
Obesity (yes/no)						
Crude OR	0·25*	0·06, 1·00*	0·65	0·18, 2·35	2·27	0·63, 8·1
Adjusted OR†	0·18*	0·04, 0·86*	0·49	0·11, 2·18	2·60	0·57, 11·7
Central obesity‡						
Crude OR	0·31	0·07, 1·4	1·0	0·27, 3·69	2·6	0·71, 9·95
Adjusted OR†	0·08*	0·008, 0·90*	0·85	0·20, 3·67	1·06	0·98, 1·13

* The association is statistically significant ($P \leq 0{\cdot}05$).
† Adjusted for age and smoking.
‡ Central obesity defined as waist circumference ≥102 cm in men and subjects categorised into two groups based on it.

Table 4. Association of *NANOG*, *POU5F1* and *MYC* methylation status with selected demographic and lifestyle factors of the study subjects*
(Odds ratios and 95 % confidence intervals)

	NANOG		*POU5F1*		*MYC*	
Characteristics	OR	95 % CI	OR	95 % CI	OR	95 % CI
Cancer family history†						
No	1		1		1	
Yes	2·01	0·29, 13·8	12·8	0·08, 86·2	0·30	0·57, 1·57
Colon cancer family history†						
No	1		1		1	
Yes	0·78	0·11, 5·10	2·27	0·47, 10·81	0·57	0·10, 3·06
NSAID use†						
None	1		1		1	
Yes	0·57	0·05, 5·99	1·87	0·33, 10·38	0·44	0·07, 2·62
Smoking status‡						
Never	1		1		1	
Former	0·07§	0·006, 0·98	0·89	0·16, 4·96	1·09	0·20, 5·89
Current	1·32	0·11, 15·5	2·49	0·30, 20·6	0·56	0·66, 4·82

NSAID, non-steroidal anti-inflammatory drugs.
* Genes methylation categorised into two groups based on median categories of control groups.
† Adjusted for age, smoking and case–control status.
‡ Adjusted for age and case–control status.
§ The association is statistically significant ($P \leq 0{\cdot}05$).

Table 5. Methylation status of *POU5F1*, *NANOG* and *MYC* before and after TNF treatments*

	Methylation (%)			
HT-29 cell line	*POU5F1*	*NANOG*	*MYC*	*CDKN2A*
Control-48	75	25	10	100
T-48-[10]	75	25	10	100
T-48-[25]	25	0	5	100
Control-72	75	25	10	100
T-72-[10]	25	0	5	100
T-72-[25]	25	0	5	100

* Control-48 and HT-29 cells were cultured for 48 h in basic medium alone served as the control group. T-48-[10] and HT-29 cells were cultured in basic medium treated with 10 ng/ml TNF for 48 h. T-48-[25] and HT-29 cells were cultured in basic medium treated with 25 ng/ml TNF for 48 h. Control-72 and HT-29 cells were cultured for 72 h in basic medium alone served as the control. T-72-[10] and HT-29 cells were cultured in basic medium treated with 10 ng/ml TNF for 72 h. T-72-[25] and HT-29 cells were cultured in basic medium treated with 25 ng/ml TNF for 72 h.

decline in DNA methylation (%) was observed when 25 ng/ml TNF-α was administered for 48 and 72 h compared with the untreated controls.

Discussion

Today, it is widely accepted that the cell plasticity allows differentiated cells to revert back to stem cell state. The differentiation states of cells depend on its transcriptional network and related lineage-specific transcription factors. Our study suggested that obesity might attenuate DNA methylation of *NANOG* in human intestinal mucosa. *NANOG* plays several roles in promoting tumorigenesis as an oncogene. *NANOG* is not expressed in normal adult tissues but is highly expressed in some human cancers[22]. Obesity-induced *NANOG* hypomethylation may overcome epigenetic barrier of its ectopic expression in colon epithelium. WC is recommended as a stronger risk factor for colon cancer than BMI, as the risk of colon cancer significantly increased with higher levels of central obesity in both men and women[23]. Interestingly, our results showed that there is a probable positive correlation between central obesity and hypomethylation of *NANOG*. NF-κB is the master TF in regulation of inflammation in cells. Recent findings support the idea that obesity and high-fat diet promote intestinal inflammation[24]. The association between visceral adiposity and Crohn's disease has also been reported[25].

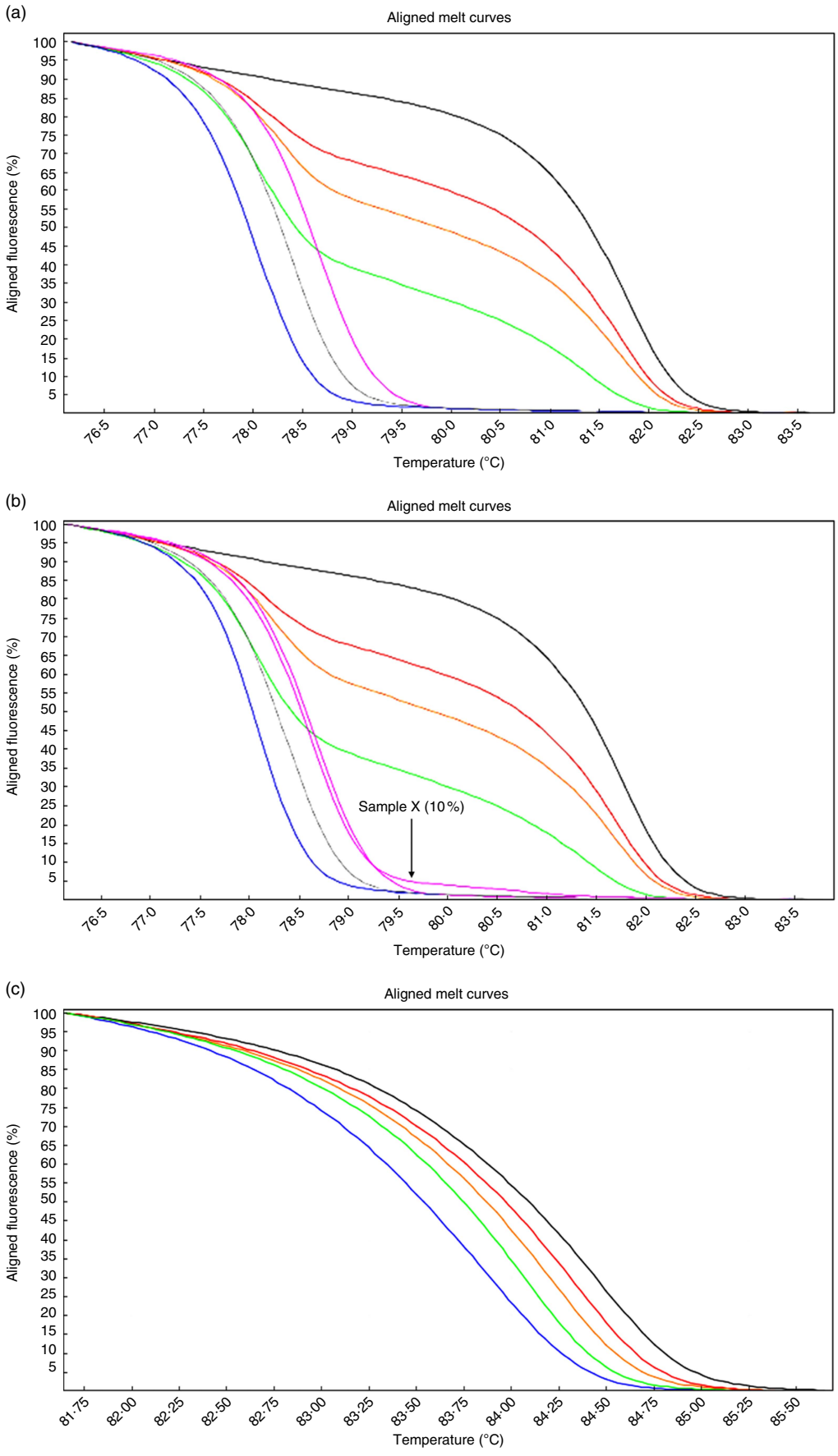

Fig. 2. *(Continued on following page).*

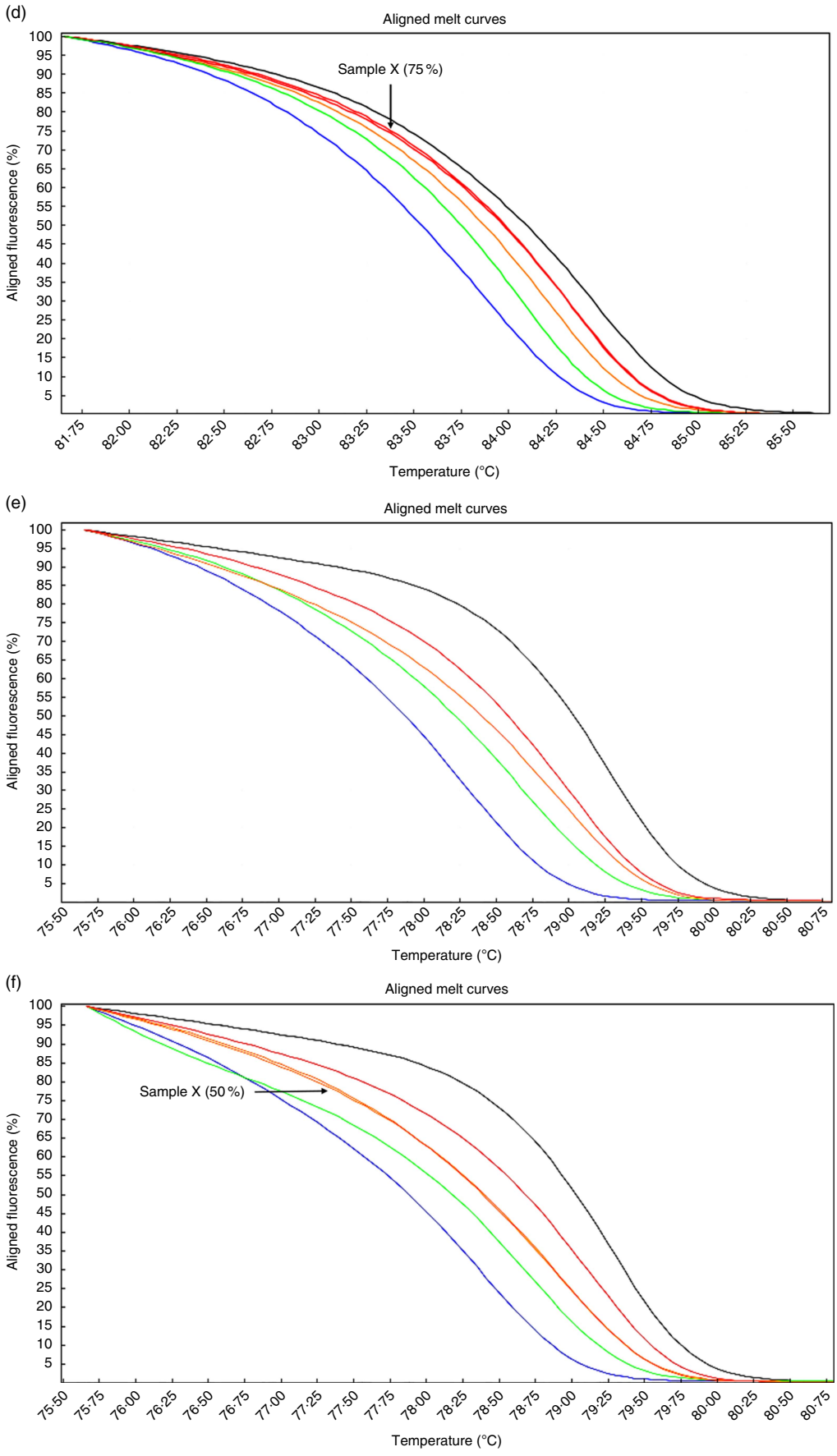

Fig. 2. *(Continued on following page).*

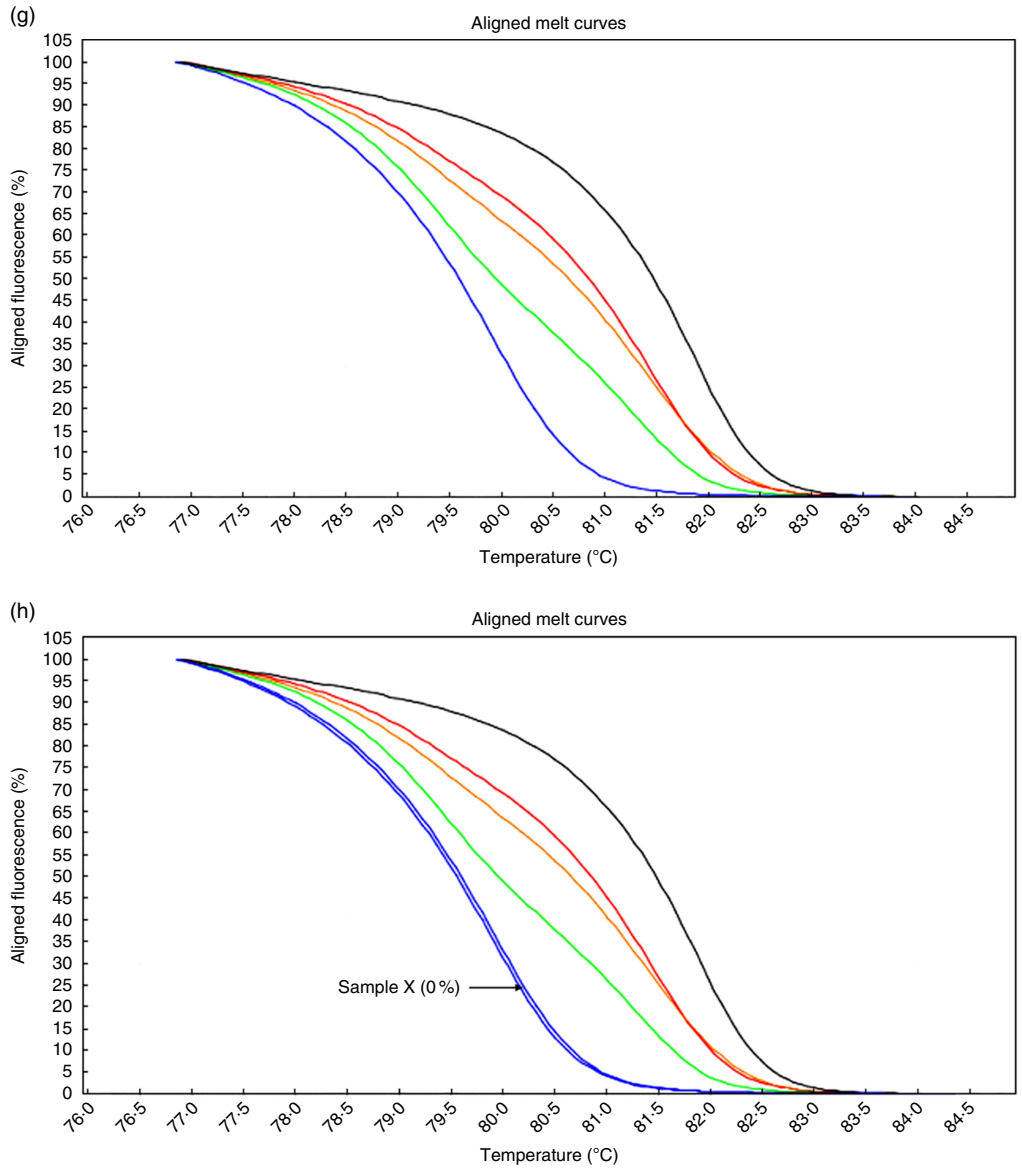

Fig. 2. *(Continued from previous page)* High-resolution melting aligned melt curves for the *MYC, POU5F1, NANOG and CDKN2A* methylation. Control curves (a) and one sample curves (b) for the *MYC* gene. Control curves (c) and one sample curves (d) for the *POU5F1* gene. Control curves (e) and one sample curves (f) for the *NANOG* gene. Control curves (g) and one sample curves (h) for the *CDKN2A* gene. Standards: 100 %, black line; 75 %, red line; 50 %, orange line; 25 %, green line; 10 %, pink line; 5%, grey line and 0%, blue line.

Accordingly, higher level of phospho-IκB – as an indicator of NF-κB activation – in colonic tissue samples of obese cases, could justify obesity as the cause of inflammation in intestine.

Cutting-edge studies have revealed the role of NF-κB activation in regulation of differentiation. It has been shown that proinflammatory cytokines TNF-α and IL-17 impair osteogenic differentiation of mesenchymal stem cells[26]. Schwitalla *et al.*[27] showed that intestinal epithelium can be reprogrammed into cancer stem cells through an inflammatory signalling and involvement of Wnt activation. Also, NF-κB has a key role in *in vivo* dedifferentiation and generation of tumour-initiating cells in intestinal tumorigenesis. Consistently, there is an evidence that NF-κB plays a major role in maintaining the undifferentiated state of human-inducible pluripotent stem cells through up-regulation of *NANOG* and *POU5F1*[28]. However, these studies do not provide evidence about the role of NF-κB in transactivation of genes which epigenetically were suppressed in normal tissues.

TET methylcytosine dioxygenases are the main part of active DNA demethylation mechanism[29]. TET are not site-specific enzymes to remove methyl moiety from particular promoter; therefore, it might be plausible to assume a molecular guide directing them to specific target genes. Transcription factors seem to be good candidates to do this mission. There are evidences showing some TF regulate DNA methylation through recruitment of DNMT and TET as co-regulators of TF[30]. In addition, differentially methylated regions of DNA were often nearby TF binding sites[31]. It has been shown that inactivation

of repressor element 1-silencing transcription factor (REST) promotes DNA methylation in CpG, which are normally near the REST binding sites. Re-expression of REST induces hypomethylation of these regions. Recently, one study indicated that REST recruits TET3 and subsequently promotes its hydroxylase activity[32]. Although the results of the present study did not show direct interaction between NF-κB and TET in demethylation of *NANOG* or other stemness genes, TNF-α-induced hypomethylation of the *NANOG*, *POU5F1* and *MYC* genes in the HT-29 cell persuaded us to suggest a role for NF-κB in this process. Further studies are in progress in our laboratory to show the direct interaction between NF-κB and TET.

Hypermethylation of tumour suppressors is a major step in tumorigenesis[33]. As mentioned, *CDKN2A* lacks NF-κB binding sites in its promoter, accordingly TNF-α had no effect on methylation of hypermethylated *CDKN2A* promoter in HT-29 cells. Considering the fact that our colonic biopsy samples in obese and normal-weight control were non-cancerous, it seems reasonable that in both groups, *CDKN2A* was completely unmethylated.

TF have pharmacological inhibitors and activators; the suggested role for transcription factors such as NF-κB in DNA methylation regulation provides a novel perspective for developing epigenetic drugs, which may preferentially alter DNA methylation of related genes.

To assess the complex interactions between potential aetiologic factors, molecular alterations and disease evolution, 'molecular pathology' and 'epidemiology' have recently become integrated, creating the interdisciplinary field of 'molecular pathological epidemiology' (MPE). MPE takes a comprehensive approach to incorporate multiple intersecting pathways such as genomics, epigenetics, energetics, inflammation, microbiome, microbiota, immunity and so on into an integrated analysis of aetiologies, evolution and progression of cancers and all other human diseases[34,35]. Our results regarding obesity-driven DNA methylation alterations of *NANOG* gene in normal colon tissues could be applied in the context of the MPE of cancer epigenetics to identify the possible risk factors contributing to CRC development.

In analysing results, it is important to pay more attention to the level of statistical significance. In the present study, we considered statistical significance at *P*-value ≤0·05, which was in accordance with most previous studies assessing specific genes[36,37]. Recently, it has been suggested that changing the default *P*-value threshold for statistical significance from 0·05 to 0·005 is needed for improving the reproducibility of scientific research[38]. However, it seems that reducing *P*-value level would be more applicable in microarray analysis when multiple tests are conducted on 1000's of transcripts, in order to reduce chance of false positives[39]. We just assessed the methylation status of four specific genes, and therefore, lower *P*-value might not be necessary for interpretation our findings.

In conclusion, our findings suggest that obesity and particularly central obesity altered methylation of the *NANOG* gene in epithelium of the colon. Hypomethylation of this gene occurred in promoter regions close to NF-κB binding sites, suggesting the involvement of this transcription factor in epigenetic regulation. Also, incubation of the HT-29 cells with TNF-α promoted hypomethylation of CpG in the promoter of *NANOG*, *POU5F1* and *MYC* nearby NF-κB binding sites.

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F. S. performed the main experiments. M. C., K. P., L. T. T., A. M. and R. T. assisted in all the experiments. S. A. H. assisted with project design. H. Z. designed the study and wrote the manuscript.

The authors declare that there are no conflicts of interest.

References

1. Berstein LM (2012) Metformin in obesity, cancer and aging: addressing controversies. *Aging (Albany NY)* **4**, 320–329.
2. Basen-Engquist K & Chang M (2011) Obesity and cancer risk: recent review and evidence. *Curr Oncol Rep* **13**, 71–76.
3. Siegel RL, Miller KD & Jemal A (2018) Cancer statistics, 2018. *CA Cancer J Clin* **68**, 7–30.
4. Cohen DH & LeRoith D (2012) Obesity, type 2 diabetes, and cancer: the insulin and IGF connection. *Endocr Relat Cancer* **19**, F27–F45.
5. Deng T, Lyon CJ, Bergin S, *et al.* (2016) Obesity, inflammation, and cancer. *Ann Rev Pathol* **11**, 421–449.
6. Okita K, Ichisaka T & Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. *Nature* **448**, 313–317.
7. Sell S (2010) On the stem cell origin of cancer. *Am J Pathol* **176**, 2584–2494.
8. Huels DJ & Sansom OJ (2015) Stem vs non-stem cell origin of colorectal cancer. *Br J Cancer* **113**, 1–5.
9. Cole JW & McKalen A (1963) Studies on the morphogenesis of adenomatous polyps in the human colon. *Cancer* **16**, 998–1002.
10. Shih I-M, Wang T-L, Traverso G, *et al.* (2001) Top-down morphogenesis of colorectal tumors. *Proc Natl Acad Sci* **98**, 2640–2645.
11. Soufi A & Dalton S (2016) Cycling through developmental decisions: how cell cycle dynamics control pluripotency, differentiation and reprogramming. *Development* **143**, 4301–4311.
12. Yamamizu K, Piao Y, Sharov Alexei A, *et al.* (2013) Identification of transcription factors for lineage-specific ESC differentiation. *Stem Cell Reports* **1**, 545–559.
13. Luo J-L, Kamata H & Karin M (2005) IKK/NF-κB signaling: balancing life and death – a new approach to cancer therapy. *J Clin Invest* **115**, 2625–2632.
14. Zhou C, Liu J, Tang Y, *et al.* (2012) Inflammation linking EMT and cancer stem cells. *Oral Oncol* **48**, 1068–1075.
15. Papp B & Plath K (2011) Reprogramming to pluripotency: stepwise resetting of the epigenetic landscape. *Cell Res* **21**, 486–501.
16. De D, Jeong M-H, Leem Y-E, *et al.* (2014) Inhibition of master transcription factors in pluripotent cells induces early stage differentiation. *Proc Natl Acad Sci U S A* **111**, 1778–1783.

17. Ligthart S, Marzi C, Aslibekyan S, *et al.* (2016) DNA methylation signatures of chronic low-grade inflammation are associated with complex diseases. *Genome Biol* **17**, 255.
18. Han H (2011) DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. *Hum Mol Genet* **20**, 4299–4310.
19. Jin B & Robertson KD (2013) DNA methyltransferases, DNA damage repair, and cancer. In *Epigenetic Alterations in Oncogenesis*, pp. 3–29 [AR Karpf, editor]. New York, NY: Springer New York.
20. Wu H & Zhang Y (2011) Mechanisms and functions of TET protein-mediated 5-methylcytosine oxidation. *Genes Dev* **25**, 2436–2452.
21. Klein S, Allison DB, Heymsfield SB, *et al.* (2007) Waist circumference and cardiometabolic risk. *Diabetes Care* **30**, 1647–1652.
22. Kim J, Liu Y, Qiu M, *et al.* (2015) Pluripotency factor Nanog is tumorigenic by deregulating DNA damage response in somatic cells. *Oncogene* **35**, 1334–1340.
23. Moore LL, Bradlee ML, Singer MR, *et al.* (2004) BMI and waist circumference as predictors of lifetime colon cancer risk in Framingham Study adults. *Int J Obes Relat Metab Disord* **28**, 559–567.
24. Ding S & Lund PK (2011) Role of intestinal inflammation as an early event in obesity and insulin resistance. *Curr Opin Clin Nutr Metab Care* **14**, 328–333.
25. Peyrin-Biroulet L, Chamaillard M, Gonzalez F, *et al.* (2007) Mesenteric fat in Crohn's disease: a pathogenetic hallmark or an innocent bystander? *Gut* **56**, 577–583.
26. Chang J, Liu F, Lee M, *et al.* (2013) NF-κB inhibits osteogenic differentiation of mesenchymal stem cells by promoting β-catenin degradation. *Proc Natl Acad Sci U S A* **110**, 9469–9474.
27. Schwitalla S, Fingerle Alexander A, Cammareri P, *et al.* (2013) Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* **152**, 25–38.
28. Takase O, Yoshikawa M, Idei M, *et al.* (2013) The role of NF-κB signaling in the maintenance of pluripotency of human induced pluripotent stem cells. *PLOS ONE* **8**, e56399.
29. Rasmussen KD & Helin K (2016) Role of TET enzymes in DNA methylation, development, and cancer. *Genes Dev* **30**, 733–750.
30. Brenner C, Deplus R, Didelot C, *et al.* (2005) Myc represses transcription through recruitment of DNA methyltransferase corepressor *EMBO J* **24**, 336–346.
31. Oda M, Kumaki Y, Shigeta M, *et al.* (2013) DNA methylation restricts lineage-specific functions of transcription factor Gata4 during embryonic stem cell differentiation. *PLoS Genet* **9**, e1003574.
32. Perera A, Eisen D, Wagner M, *et al.* (2015) TET3 is recruited by REST for context-specific hydroxymethylation and induction of gene expression. *Cell Rep* **11**, 283–294.
33. Waki T, Tamura G, Tsuchiya T, *et al.* (2002) Promoter methylation status of E-cadherin, hMLH1, and p16 genes in nonneoplastic gastric epithelia. *Am J Pathol* **161**, 399–403.
34. Ogino S, Chan AT, Fuchs CS, *et al.* (2011) Molecular pathologic epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. *Gut* **60**, 397–411.
35. Ogino S, Nowak JA, Hamada T, *et al.* (2018) Integrative analysis of exogenous, endogenous, tumour and immune factors for precision medicine. *Gut* **67**, 1168–1180.
36. Johnson KC, Koestler DC, Cheng C, *et al.* (2014) Age-related DNA methylation in normal breast tissue and its relationship with invasive breast tumor methylation. *Epigenetics* **9**, 268–275.
37. Crujeiras AB, Morcillo S, Diaz-Lagares A, *et al.* (2018) Identification of an episignature of human colorectal cancer associated with obesity by genome-wide DNA methylation analysis. *Int J Obes* (epublication ahead of print version 1 May 2018).
38. Benjamin DJ, Berger JO, Johannesson M, *et al.* (2018) Redefine statistical significance. *Nat Hum Behav* **2**, 6–10.
39. Mudge JF, Martyniuk CJ & Houlahan JE (2017) Optimal alpha reduces error rates in gene expression studies: a meta-analysis approach. *BMC Bioinformatics* **18**, 312.

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