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CYTOKINE-INDUCED CHROMATIN MODIFICATIONS OF THE TYPE I COLLAGEN ALPHA 2 GENE DURING INTESTINAL ENDOTHELIAL-TO-MESENCHYMAL TRANSITION

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Abstract

Background—Fibrosis of the intestine is currently an irreversible complication of Inflammatory Bowel Disease yet little is understood of the underlying pathogenesis and anti-fibrotic strategies remain elusive. To develop effective therapies, knowledge of the mechanism of transcription and excessive deposition of type I collagen - a hallmark of fibrosis, is needed. We have shown previously that endothelial-to-mesenchymal transition (EndoMT) contributes to the pool of intestinal fibrotic cells and that a cytokine cocktail (IL1- β , TNF- α and TGF- β) induces Collagen I alpha 2 (*COL1A2*) mRNA and protein.

Methods—Chromatin immunoprecipitation assays on pure cultures of human intestinal mucosal endothelial cells undergoing EndoMT were performed with antibodies to specific histone modifications and RNA polymerase II. RT-PCR was used to quantify the levels of *Col1A2* and endothelial specific von Willebrand factor (vWF) mRNA.

Results—We show that cytokines induce selective chromatin modifications (histone 4 hyperacetylation and hypermethylation of histone 3) and phosphorylated RNA polymerase II at the *COL1A2* promoter. Hypoacetylated and hypomethylated histone 3 was detected on the repressed *vWF* gene. Prolonged exposure to cytokines (16 days) retained hyperacetylation of select lysines in H4 on the *COL1A2* promoter. Removal of cytokines after 16 days and continued culture for 10 days, showed persistent hyperacetylation at lysine 16 in histone H4.

Conclusion—This is the first study to show that *COL1A2* gene expression is associated with cytokine-induced, temporally ordered and persistent chromatin modifications and suggests that these are important determinants of gene expression in EndoMT and intestinal fibrosis.

Keywords

Cytokines; epigenetics; intestinal fibrosis; inflammatory bowel disease; type I collagen

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INTRODUCTION

Intestinal fibrosis is particularly common in Crohn's Disease (CD) where it is a progressive and serious consequence of chronic inflammation and represents a major cause of complications and hospitalizations^{1,2}. Surgical intervention is often required for inflammatory bowel disease (IBD) patients with the fibrostenosing phenotype which is characterized by transmural fibrosis and intestinal obstruction. Knowledge of the pathophysiology of intestinal fibrosis is incomplete and no specific anti-fibrotic therapies are available.

A prominent hallmark of fibrosis is the deposition of excessive collagen-containing extracellular matrix (ECM). Type I collagen is the most abundant component of the fibrotic ECM and consists of a heterotrimer of 2 alpha 1 (I) chains and one alpha 2 (I) chain (COL1A2)³. Knowledge of the mechanism by which inappropriate expression of type I collagen occurs is crucial to providing new targets by which deposition of excess collagen could be controlled and its detrimental consequences prevented.

The *COL1A2* gene is a widely used experimental model to study the molecular basis of collagen I biosynthesis⁴. Multiple studies have delineated the genomic region, cis-acting elements and trans-acting factors implicated in constitutive, cytokine-modulated and tissue-specific expression of *COL1A2* including five DNase I hypersensitive/open chromatin sites, a proximal promoter, far-upstream enhancer and downstream repressor. Despite this, a complete understanding of the mechanism by which type I collagen is induced and perpetuated, particularly in response to combinations of cytokines relevant to fibrosis remains elusive.

Changes to the structure of chromatin lie at the heart of alterations in gene transcription and differentiation and the contribution of such events to understanding the pathogenesis of IBD have only just begun to emerge⁵⁻⁸. The current general consensus is that histone modifications alter chromatin in a precise, gene- or cell-specific fashion probably in combination with other factors such as coregulators and transcription factors, to determine transcriptional, phenotypic and functional outcomes^{9,10}. Histone acetylation is the only histone modification for which evidence for its determining role in the activation of gene transcription has been demonstrated^{11,12}. Histone methylation has been linked with transcriptional activation or repression depending on the location of the specific residue targeted, and the degree of methylation, i.e., the number of methyl groups added^{9,10,13}. For example, silent genes are associated with distinctive patterns of histone modifications at promoters, e.g. tri methylation of lysine 27 in H3 (H3K27me3), di-methylation of H3K9, (H3K9me2) and trimethylation of H4K20 (H4K20me3). In contrast, active gene promoters contain trimethylated lysine 4 in histone H3 (H3K4me3) as well as hyperacetylated H3 and H4.

The traditional model of intestinal fibrosis is that chronic inflammation causes differentiation of mesenchymal cells to a fibrogenic or myofibroblastic phenotype. Recent human and murine studies including in our own lab using intestinal endothelial cells, have demonstrated that fibrogenic cells can arise from epithelial- and endothelial- to mesenchymal transition (EndoMT)¹⁴⁻¹⁷. The latter was originally described in experimental wound repair, in bovine endothelial cells from aortic or pulmonary arteries and in mouse models of cardiac, kidney and lung fibrosis¹⁸⁻²². Within the last few years, research in skin, kidney and lung fibrosis have identified a role for histone deacetylases (HDACs) and HDAC inhibitors in regulating the levels of collagen type I gene transcription, deposition, and fibrosis²³⁻²⁶. More recently, specific genome wide histone modifications (but intriguingly

not DNA methylation) have been shown to be altered by transforming growth factor β 1 (TGF β 1) during epithelial-to-mesenchymal transition of AML12 mouse hepatocytes *in vitro*²⁷.

We have demonstrated previously the induction of type I collagen and a variety of other fibrogenic genes in primary human intestinal mucosal endothelial cells (HIMEC) undergoing endothelial-to-mesenchymal-transition (EndoMT) *in vitro* following chronic exposure to supernatants from intestinal lamina propria mononuclear cells or a cytokine cocktail consisting of IL1 β , TNF α and TGF β . In the same study we demonstrated that this process occurs in the intestinal mucosa in models of experimental colitis *in vivo*¹⁷. Based on the concept that epigenetic changes are major contributors to phenotypic plasticity^{5, 6}, we hypothesized that there are select inflammation/cytokine induced histone modifications that are key regulators of the morphological and functional changes including the expression of Type I collagen observed during EndoMT and intestinal fibrosis. We demonstrate here for the first time that a cocktail of fibrosis relevant cytokines: TGF β , TNF α and IL1 β induces a gene- and temporally-specific pattern of histone modifications on the activated collagen type I gene during EndoMT. This supports the notion that chromatin factors regulate fibrotic gene transcription and has the potential to provide new targets by which the pathological expression of type I collagen could be modulated *in vivo*.

MATERIALS AND METHODS

Cell culture and treatments

Tissues were obtained from histologically normal large-bowel specimens from patients admitted for bowel resection due to non-malignant conditions, including benign polyps, and diverticulosis. Involved and non-involved CD and UC colonic tissues were also obtained. As shown in our previous studies¹⁷, there were no quantitative or qualitative differences in EndoMT when comparing HIMEC from involved, non-involved IBD tissue or normal control tissue and therefore cells from all these sources were used. All diagnoses were confirmed by clinical, radiologic, endoscopic and histologic criteria. The Institutional Review Board of the Cleveland Clinic Foundation approved the human tissue studies. Human intestinal mucosal endothelial cells (HIMEC) were cultured from intestinal resection specimens as previously described²⁸. HIMEC were cultured in MCDB131 media (Sigma) supplemented with 20% fetal bovine serum (Lonza), 2.5% penicillin/streptomycin/amphotericin B (Lonza), 90 μ g/ml heparin (Sigma) and 50 μ g/ml endothelial cell growth factor (Roche) in T75 flasks (Corning) or 6 well plates (Corning Costar) coated with 1 μ g/cm² fibronectin (Becton Dickinson). HIMEC cells at 50 % confluency were treated with a cytokine cocktail comprising: 5 ng/ml TGF- β 1, 5ng/ml, tumor necrosis factor- α (TNF- α , and 0.1 ng/ml interleukin-1 β (IL-1 β) (all Peprotech). The cytokines were replenished and the growth medium replaced every 3–4 days. HIMEC were monitored daily to identify endothelial-fibroblast transitions, and cells were harvested and divided for analysis of gene expression and preparation of chromatin for ChIP assays (see below).

Quantification of cell number

HIMEC were stained with 10 μ g/ml Bis-benzimide diluted in culture medium at 37°C for 15 min to allow nuclei to take up the stain and imaged on a Leica DMR 6000 inverted microscope. Cells were scanned in pre-warmed PBS and counted using a Well Plate Scanning Module. To exclude edge artifacts, a circular area comprising 722 sq. mm was selected from the middle of each well. A size exclusion filter was applied to reject any image artifact from the count.

Chromatin immunoprecipitations (ChIP)

a) Preparation of chromatin—ChIP assays to detect histone modifications on the *COL1A2* and *vWF* gene promoters were performed with the ChIP-It Express Enzymatic Kit (Active Motif) according to the manufacturer's instructions with the following modifications. Briefly, cells ($1-6 \times 10^6$) were trypsinized, counted, washed in PBS, pelleted, snap frozen in liquid nitrogen and stored at -80°C . To prepare chromatin, cell pellets were thawed on ice and lysed in Lysis Buffer (500 μl –1 ml, Active Motif) freshly supplemented with a protease inhibitor cocktail and PMSF (Active Motif) and with phosphatase inhibitors (I and II, Sigma) and 20 mM sodium butyrate (Sigma) to preserve histone acetylation. The cells were left in lysis buffer on ice for 30 min and homogenized 40 times (Kimble Kontes dounce homogenizer, pestle B) on ice to release nuclei from the lysed cells. Nuclei were pelleted at 2700g for 10 min at 4°C and resuspended in 25 $\mu\text{l}/10^6$ cells Digestion Buffer containing a protease inhibitor cocktail, PMSF (Active Motif kit), phosphatase inhibitors and sodium butyrate exactly as for the lysis buffer. The chromatin was sheared using enzymatic shearing cocktail (Active Motif) (1.2 $\mu\text{l}/10^6$ cells at 100U/ml) diluted in digestion buffer for 5 min at 37°C . The shearing reactions were stopped with 0.5 μl of 0.5M EDTA per 10^6 cells on ice for 10 min. Samples were then centrifuged at 20,000g, 4°C for 10 min and the resulting supernatant ("sheared chromatin") transferred to fresh eppendorf tubes, divided into single use aliquots, snap frozen in liquid nitrogen and stored at -80°C or used immediately.

b) ChIP reactions—ChIP reactions were set up using sheared chromatin from $2.5 - 4.5 \times 10^5$ cells/reaction. Proteinase and phosphatase inhibitors were included in all ChIP incubations. Rabbit IgG (Santa Cruz) was employed as a negative control. ChIP reactions with the following antibodies were performed (4 μg per antibody per ChIP): hyper-acetylated histone H4, acetylated histone H3, dimethyl K9 histone H3, trimethyl K20 histone H4, (Upstate/Millipore); trimethyl K4 histone H3, trimethyl K27 histone H3, acetyl Lys 5 histone H4 and acetyl Lys 12 histone H4 (Active Motif); and acetyl Lys 8 histone H4, acetyl Lys16 histone H4, RNA polymerase II phospho S2 and phospho S5, (Abcam). In each case, the specificity of the antibody and lack of crossreactivity with other similar histone modifications was demonstrated by the manufacturer either by dot blot or competition with the immunogen in the form of the specifically modified peptide. Protein magnetic Beads (25 $\mu\text{l}/\text{ChIP}$, Active Motif) were added in a total volume of 100 μl and reactions incubated on a rotator at 4°C overnight. The immunoprecipitated chromatin was captured on a magnetic stand (Promega) or 96-well magnetic plate (Active Motif). In all cases, immunoprecipitates were washed according to the manufacturer's instructions and eluted in 50 μl Elution Buffer (Active Motif) by rapidly resuspending the magnetic beads and eluting the DNA for 15–30 min on a rotator at room temperature. At the end of this incubation an equal volume of reverse crosslinking buffer (50 μl) was added to the ChIP samples. NaCl (0.1M) was added to 100 μl of the total non-immunoprecipitated or "input" chromatin (diluted 1:10). Both input and ChIP samples were then placed at 95°C for 15 min. The samples were allowed to cool to room temperature and chromatin was purified (PrepEase DNA clean up column purification kit, USB). The resulting samples (100 μl) were either stored at -20°C or analyzed immediately by PCR. Percent inputs were calculated; Ct values of input were subtracted from Ct values of sample IPs for each untreated and treated set. The $2^{-\Delta\text{Ct}} \times 100\%$ was calculated to give % inputs for each untreated and treated pair.

c) Crosslinking of Chromatin—For detection of RNA polymerase II association with the *COL1A2* gene promoter, HIMEC were crosslinked prior to lysis and shearing. Cells were trypsinized, counted and resuspended in PBS at a concentration of 1×10^6 cells/ml. Conditions for cross-linking chromatin to preserve the association of RNA polymerase II with DNA were optimized (data not shown). The cross-linking agent Disuccinimidyl

glutarate (DSG, Pierce) was freshly prepared and added to cells at a final concentration of 2mM²⁹. Samples were crosslinked for 30 min on a rotator at room temperature. Formaldehyde (Sigma) was then added to a final concentration of 0.5% for an additional 5 min at room temperature. To terminate the crosslinking reaction, glycine (0.125M) was added for a further 5 min. The samples were centrifuged (240g, 5 min), washed twice with ice cold PBS and centrifuged again. The final pellet was snap frozen and either stored at -80°C or used immediately to prepare chromatin see above. Crosslinked chromatin was sheared by sonication with the Misonix 3000 Sonicator and cup horn in 200 µl/1 × 10⁶ cells shearing buffer (150 mM NaCl, 5mM EDTA, 1% Triton X-100, 0.5% NP-40, 50 mM Tris-HCl pH 7.5, 1mM PMSF, 1X protease inhibitor cocktail, 20 mM sodium butyrate). Samples were sonicated in an ice bath (Output 8.0, 15 cycles of 5 sec on, 15 sec off, repeated 12 times). After sonication, the sheared chromatin was centrifuged (20,000g, 10 min, 4°C) and the resulting supernatants divided into single use aliquots and stored as before.

Quantitative Real Time PCR (qPCR)—For qPCR of ChIP samples reactions consisted of 5 µl undiluted ChIP samples or input chromatin (diluted 1:10), iQ SYBR Green Supermix (BioRad) and 250 nM primers. Reactions were performed on a iQ5 Cyclor (BioRad) using a 2 step amplification: 95°C for 3 min, followed by 40 cycles of 30 sec denaturation at 95°C plus combined annealing/extension at 60°C for 30 sec, followed by melt curve analysis. Data were analyzed with iCycler iQ version 3.1 software (BioRad). Two pairs of primers to amplify the *COL1A2* gene promoter region (NCBI reference sequence: AF004877.1) and a region upstream of the promoter were designed based on the available studies in fibroblasts [5,30]. The first pair amplified a region between -25bp to +63 bp in the proximal promoter³⁰: fwd: +2270-2285 5'-GGGCAGATCCGGGCTT-3' and rev: +2358-2344 5'-GCGCGGGCATGCAGT-3'. A second set of primers located upstream of the proximal promoter amplify a region that is -1599 to -1520 bp from the transcription start site³⁰ and is referred to here as the upstream enhancer region: fwd: +696-715: 5'-CTCAGGGAACACTACAGCCCAC-3''; rev: +775-757: 5'-CAGAAGTCATCGCAAAGGG-3'. For the von-Willebrand factor (*vWF*) gene promoter (NCBI reference sequence NG_009072.1) the endothelial specific region was amplified [33]: Fwd: +155-+184: 5'-GCTGAGAGCATGGCCTAGGGTGGGCGGCAC-3', and Rev: +247-+215: 5'-CCCCTGCAAATGAGGGCTGCGGCTATCTCCAAG-3'. Amplified products for all primer pairs were verified by sequencing prior to use in ChIP studies.

Reverse Transcriptase-PCR (RT-PCR)—For gene expression analysis, approximately 3 × 10⁵ cells were removed for qPCR prior to ChIP analysis. The cells were centrifuged, (240g, 5mins, 4°C) washed in PBS and centrifuged again before being snap frozen and stored at -80°C until use. RNA was extracted from cell pellets using an RNEasy mini kit (Qiagen) and quantitated on a Nanodrop Spectrophotometer (Thermo Scientific). RNA (250-500 ng) was used to prepare cDNA for PCR analysis with the iScript cDNA synthesis kit (BioRad) as per manufacturer's protocol. The resulting cDNA (2µl) was incubated with iQ SYBR Green Supermix and 250 nM primers, following the same parameters as for qPCR. The primer pairs for gene expression analysis were as follows: *COL1A2* 614 mRNA fwd: 5'-GCCCCCAGGCAGAGA-3', *COL1A2* 731 mRNA rev: 5'-CCAACTCCTTTTCCATCATACTGA-3', *vWF* 32 mRNA fwd: 5'-GTGGTTGGTGGATGTCACAG-3', *vWF* 270 mRNA rev: 5'-GCAAATCTGGCAGGAATCAT-3', GAPDH fwd mRNA: 5'-AACTTTGGTATCGTGGAAGGAC-3', and GAPDH rev mRNA: 5'-CAGTAGAGGCAGGGATGATGT T-3'. The products for all primer pairs were verified by sequencing prior to studies of gene expression in EndoMT. For gene expression studies, sample data were normalized to GAPDH as the reference gene using the Pfaffl correction of the 2^{-ΔΔCt} method³¹ to calculate the fold change in mRNA levels.

Statistical analysis—The mean $\pm$ Standard error of the data was calculated. Statistical analysis was performed with a two-way Student's paired T test to assess statistical significance. A p value < 0.05 was considered statistically significant. For the real time PCR data a two-way paired T test was performed on the Δ Ct values (Ct of *COL1A2* target gene – Ct of *GAPDH* reference gene) for cytokine-treated versus untreated cells and the fold change $\pm$ standard error of the mean were plotted.

ETHICAL CONSIDERATIONS

The Institutional Review Board of the Cleveland Clinic Foundation approved the human tissue studies.

RESULTS

Induction of *COL1A2* gene expression in EndoMT

Previous studies in our lab have defined conditions for rigorous establishment of intestinal EndoMT and the phenotypic and functional changes consistent with the acquisition of a mesenchymal-like phenotype¹⁷. These include induction of the mesenchymal specific *Col1A2*- and loss of the endothelial specific von Willebrand factor (*vWF*) mRNA and protein. We have extended these studies to identify in this system, the cytokine-induced chromatin modifications that mediate these changes in *COL1A2* gene expression. To this end, HIMEC were isolated and their absolute purity verified as published previously¹⁷. To induce EndoMT, cells at 50% confluency were incubated with either medium alone, or the cytokine cocktail consisting of IL-1 β , TNF α or TGF β 1, alone or in combination, for 0 – 20 days. At the end of the cytokine incubation period or the same period in cytokine free medium, cells were washed and the incubations continued in cytokine free medium for a further 10 days (Fig.1A and B). The transcript levels for *COL1A2* were quantified by qPCR and normalized to *GAPDH*. The fold change of *COL1A2* mRNA in cytokine treated cells or cells with cytokine removed (10 day wash off) compared with cells in cytokine free medium (baseline level of 1 indicating no change in *COL1A2* mRNA) is shown. At day 2, HIMEC undergoing EndoMT showed marked and statistically significant induction of *COL1A2* mRNA (Fig.1). Levels of *COL1A2* mRNA peaked between 6–8 days and remained significantly high compared to controls for 20 days (Fig.1).

COL1A2 gene expression persists in the absence of cytokine stimuli

In determining the levels of *COL1A2* mRNA during EndoMT we then investigated whether the induction of *COL1A2* persisted after removal of the cytokine cocktail from HIMEC/EndoMT cultures. The aim was to determine whether the observed cytokine induced changes in gene expression were permanent even in the absence of continued cytokine exposure as fibrotic gene expression can continue even when inflammation is inhibited *in vivo*³². At the end of each incubation with the cytokine cocktail, cells were washed with HIMEC growth medium and the cultures continued in the absence of cytokines for a further 10 days (wash off, Fig. 1) and *COL1A2* mRNA was quantified by RT-PCR. As shown in Fig. 1, statistically significant levels of *COL1A2* mRNA were maintained after 10, 16 and 20 days of cytokine incubation plus in each case a further 10 days in culture in the absence of cytokines. The longer the exposure to cytokines, the higher the level of *COL1A2* mRNA after cytokine removal and this increase continued in a time dependent manner for up to 20 days.

Cytokine-induced increase in histone modifications of the *COL1A2* gene promoter

Published evidence indicates that epigenetic changes that include chromatin/histone modifications are major contributors to differentiation and phenotypic variability and are

therefore strong candidate regulators of EndoMT^{6, 8}. Different combinations of histone modifications may therefore determine transcription outcome and levels of gene expression. We chose 6 classical histone modifications associated with activation and repression of gene transcription and asked whether any of these were induced by cytokines upon induction of the *COL1A2* gene in EndoMT or repression of the *vWF* gene¹⁷. ChIP assays were performed in HIMEC undergoing EndoMT after 5 days exposure to cytokines in the same conditions as for detection of *COL1A2* mRNA induction and *vWF* mRNA inhibition (Fig. 2A–G). As before, we verified that the cytokine cocktail (TGF- β 1, TNF- α) and IL-1 β) induced an increase in *COL1A2* mRNA (Fig.2G), and decreased expression of the *vWF* gene. EndoMT enhanced acetylation of histone H4 (H4Ac) on the *COL1A2* gene within the proximal promoter and upstream enhancer (Fig. 2A & C respectively) of the *COL1A2* gene respectively but had no effect on H3 acetylation. The increase in H4 acetylation occurred in parallel with cytokine-induced increase in *COL1A2* mRNA levels (Fig.2G), was specific to the *COL1A2* gene and was not detected on the down-regulated *vWF* gene (Fig. 2E & F). In contrast, a significant decrease in two other histone modifications, i.e., H3 acetylation (H3Ac) and H3K4me3, was found on the *vWF* gene promoter consistent with reduced levels of *vWF* mRNA (Fig.2E & G). The levels of H3K4me3 were not altered by cytokines on the *COL1A2* gene in the proximal promoter and upstream enhancer (Fig. 2A & C). Two of the three methylation modifications (H3K27me3 and H4K20me3) linked with transcriptional repression did not change on cytokine treatment on either the *COL1A2* (Fig. 2B & D) or the *vWF* gene (Fig.2F). However, H3K9me2, the third methylation modification found on repressed genes was statistically significantly increased compared to controls in both regions of the *COL1A2* gene (Fig. 2B & D) and in the *vWF* gene in cytokine-treated HIMEC undergoing EndoMT (Fig. 2F).

Recruitment of RNA polymerase II to the *COL1A2* promoter

Histone modification patterns that activate transcription can be functionally linked to the recruitment and phosphorylation of RNA polymerase II³³. To determine whether the combination of cytokines used here increased RNA polymerase II levels at the activated *COL1A2* gene promoter during EndoMT, we employed ChIP assays with antibodies specific for different modified forms of the enzyme. It is known that RNA polymerase II is recruited to promoters near the transcription start site with its carboxyl terminal domain (CTD) in a hypo-phosphorylated state and that once there, phosphorylation of the CTD is an important regulator of polymerase function. In the early stages of transcription, phosphorylation of Ser 5 in the CTD occurs but as transcription progresses, levels of phospho Ser 5 decline and Ser 2 phosphorylation increases as it is required for transcript elongation and the formation of stable elongation complexes. ChIP assays were performed as described before from cells undergoing EndoMT and the proximal promoter region was analyzed (Fig.3). HIMEC were crosslinked with DSG followed by formaldehyde and immunoprecipitations of the DNA-protein complexes performed with antibodies specific to Ser2P and Ser5P forms of RNA polymerase II and to the unphosphorylated form of the polymerase (8WG16). (Fig.3). We observed that the Ser2P form of RNA polymerase II on the *COL1A2* promoter was significantly enriched (2.9 fold) by the cytokine cocktail compared with control cells. However, no significant change in enrichment of Ser 5P RNA polymerase II and very low levels of the unphosphorylated form of the polymerase (at background levels) were detected.

Cytokine induced hyperacetylation of Histone H4 occurs at specific lysines on the *COL1A2* gene

The hyperacetylation of histone lysines is a well-recognized characteristic of actively transcribed genes⁷. Histone lysine acetylation makes DNA more accessible to transcription proteins through altering histone-DNA interactions. Further to our experiments shown in

Fig.2 that showed increased H4 acetylation on *COL1A2* during EndoMT, we determined which individual lysine was acetylated on H4 using native ChIP assays and antibodies that recognize 4 different forms of monoacetylated H4: lysines 5, 8, 12 or 16. The cytokine cocktail of IL-1 β , TNF α and TGF β significantly increased the levels of acetylated H4K16 on the *COL1A2* gene promoter after 1 day at both proximal promoter and upstream enhancer sites (Fig.4A & B respectively) in parallel with the observed cytokine-induced increase in *COL1A2* mRNA (Fig.4E). None of the other 3 acetylated lysines showed statistically significant changes in cytokine treated cells after 1 day (Fig.4A & B). At 5 days, acetylated H4K12 and H4K8 but not H4K5 were also statistically significantly increased after 5 days incubation with the cytokine cocktail in the proximal promoter region (Fig.4C) but did not reach statistical significance in the upstream enhancer region where only H4K16 acetylation was significantly increased (Fig. 4D).

Prolonged cytokine-induced COL1A2 gene expression is associated with persistent histone H4 hyperacetylation

We next tested whether the histone modifications statistically significant enriched at 5 days remain elevated on the *COL1A2* gene during prolonged incubation with the cytokines for 16 days and whether these, like Col1A2 mRNA levels (Fig.1), persisted after removal of the EndoMT cytokine cocktail over a further period of 10 days (Fig.5&6). The persistent induction of *COL1A2* gene expression after cytokine removal was confirmed prior to ChIP assays (Fig.6E). The results of our ChIP experiments demonstrated a significantly increased enrichment of acetylation at all 4 lysine residues H4K16Ac (Fig.5A,B), H4K12Ac (Fig. 5C,D), H4K8Ac (Fig.5E,F) and H4K5Ac (Fig.5G,H) after 16 days cytokine cocktail treatment at both proximal promoter and upstream enhancer regions of the *COL1A2* promoter. Of these, only H4K16Ac remained significantly enriched after wash off in the upstream enhancer region (Fig.5B). Interestingly, 16 day exposure to cytokines did not change the levels of dimethylated H3K9, that was elevated after 5 days (Fig.6A & B) or trimethylated H3K4, another modification associated with active genes (Fig.6C & D). These results indicate that the cytokine cocktail -induced selective enrichment of histone modifications in the form of H4 acetylated lysines 5,8,12 and 16 but not H3K9me2 or H3K4me3 on the *COL1A2* gene after prolonged exposure to cytokines. However, only H4K16Ac remained elevated above background levels after cytokine removal in the analyzed upstream enhancer region of the promoter. This demonstrates that the EndoMT cytokine cocktail induced a temporally ordered and differential acetylation of specific lysines in Histone H4 upon induction of the *COL1A2* gene during EndoMT (Summarized in Fig 7).

Quantification of cell number during wash off period

To determine whether differentiated HIMEC cells could undergo cell division in the absence of cytokines, we quantified the number of cells after incubation with the cytokine cocktail for 16 days and during the 10 day wash off (see *Materials and Methods*). On average, during this period, more than 40,000 cells were counted per condition. The cell number remained at a steady level, varying insignificantly by no more than 8% over 10 days indicating that the cells did not undergo cell division in the absence of cytokines.

DISCUSSION

The aim of these studies was to define the cytokine-induced changes in histone modifications that are strong candidate regulators of fibrotic gene expression during EndoMT, an important step in advancing current understanding of the pathogenesis of intestinal fibrosis induced by chronic inflammation in IBD. Intestinal EndoMT provides an excellent model¹⁷ in which to delineate the molecular mechanisms relevant to the regulation

of intestinal fibrosis and to other fibroproliferative disorders associated with tissue-specific inflammation. It allows the evaluation of chromatin modifications of fibrotic genes alongside precisely defined transcriptional and phenotypic changes in a system that is directly relevant to intestinal fibrosis. Hitherto, we have shown that human intestinal endothelial cells in response to a cocktail of cytokines (IL-1 β , TNF- α , TGF β) undergo EndoMT and adopt a mesenchymal-like morphology with loss of expression of endothelial specific markers, and *de novo* expression of an extensive range of mesenchymal genes including *collagen type I*, *vimentin* and α -SMA¹⁷. As in our previous experiments we use a cytokine cocktail to robustly induce endothelial cell differentiation, modifications to chromatin and changes in gene expression as this was used in our previous experiments in which EndoMT was characterized. The combination of cytokines also allows us to recapitulate some of the complex cytokine interactions that are elevated in experimental models of colitis and in human IBD-associated fibrosis^{17, 32}. The use of multiple inflammatory stimuli facilitates crosstalk of the signaling pathways that converge on the transcriptional machinery to determine cell type specific responses *in vivo*³⁴.

We report here our novel findings that the IL1 β , TNF α and TGF β combination of cytokines induced changes in *COL1A2* gene expression that were associated with unique temporally ordered enrichment of specific histone modifications within the promoter and close to the transcription start site of *COL1A2* gene and 1600bp upstream (upstream enhancer) in EndoMT (Fig.7). These sites are located 105bp and 700bp respectively from two DNaseI hypersensitivity sites described in fibroblasts that are sites of open chromatin and of key active regulatory elements and regions where histones modifications were likely to occur [30]. The upstream enhancer region is also located within the -3500 to -772bp region shown to dramatically reduce *COL1A2* promoter activity when deleted [5]. The induction of *COL1A2* mRNA began 1 day after cytokine incubation, was maintained over 5–20 days, and continued over 30 days even after complete removal of cytokine-induced stimulation. This provided a physiologically relevant *in vitro* model in which to study how chromatin modifications could maintain persistent fibrotic gene expression in the absence of inflammatory stimuli (see below). A cytokine-induced enrichment of acetylation of H4K16, K12 and K8 along with dimethylated H3K9 was associated with induction of gene expression at 5 days of EndoMT. Cytokine-induced enrichment of all 4 acetylated lysine residues in H4 (5,8,12,16) was detected over prolonged periods of cytokine incubation (16 days) but this was not the case for dimethylated H3K9 or trimethylated H3K4, another modification found on the promoters of active genes. In fact of the modifications we analyzed, only cytokine-induced acetylation of lysine 16 in histone 4 in the upstream enhancer region of the promoter was retained after cytokines were removed. The experiments described here provide the basis for further research to define the full complement and function of histone modifications in all regions of the *COL1A2* gene including the far upstream enhancer regions (-20kB) that maintain chronic type I Collagen gene expression.

Histone H4 acetylation has been shown to play a critical role in the transition between open/relaxed or a compact/folded chromatin state^{5, 35, 36}. Acetylation of H4K16 has been shown to be a causal event in transcriptional activation as it reduces the positive charge of histones and promotes a less stable and open chromatin structure that increases access of transcription factors and coregulators^{12, 36}. While distinct functions of individual acetylated lysines in Histone H4 have been reported, the precise contribution of acetylated H4K5, H4K12 and H4K8 to transcription is not known³⁷. Unlike H4K16 acetylation, none of the aforementioned H4 acetylated lysines seem to mediate chromatin compaction directly but have been reported as sites of acetylation in newly synthesized histones and may have a role in nucleosome assembly^{35, 37}. Further research is needed to determine the contribution of these individually acetylated sites to transcription of the *COL1A2* gene.

In parallel with the cytokine activated *COL1A2* promoter specific combination of histone modifications identified (H4K16Ac, H4K12Ac, H4K8Ac, and H3K9me2), elevated levels of the S2 phosphorylated form of RNA polymerase II were found to be present at the *COL1A2* promoter. This form of the enzyme mediates transcript elongation indicating a functional link between histone modifications and active transcription of the *COL1A2* gene. This is further supported by the increased levels of *COL1A2* mRNA (Fig.2G) and protein¹⁷ detected at this stage of EndoMT. The low level of binding of the antibody (8WG16, Abcam) specific to the non-phosphorylated form is consistent with other studies in human ES cells³³ and the lack of modulation of the S5 form of the polymerase by the EndoMT cytokine cocktail differs from published data in which high levels of phosphorylated Ser 5 in the carboxy terminal domain of RNA polymerase II have been reported on actively transcribed genes. The reason for this discrepancy may be the inaccessibility of residues due to another modification of the C terminal domain, or a conformational change in RNA polymerase II^{33, 38, 39} Alternatively, this may be due to promoter specific differences as ChIP studies in our lab in both EndoMT and human intestinal fibroblasts (not shown) revealed cytokine-induced enrichment of serine 5 phosphorylated RNA polymerase II at the IL-8 gene promoter.

Despite increasing numbers of studies identifying new histone modifications⁴⁰ considerable debate remains as to the relative contribution of specific histone modifications to gene expression^{41, 42}. To fully determine the mechanism by which the *COL1A2* gene is transcribed it is likely that histone modifications along with DNA methylation (reported on the *COL1A2* promoter in tumors³⁸), non-coding RNAs as well as other components of the chromatin/transcription machinery such as histone acetylases/deacetylases, other coregulators and their interactions with transcription factors⁵ via key regulatory *COL1A2* promoter elements will need to be identified (Fig.8).

Our experiments showing the selective persistence of acetylated H4K16 in the upstream enhancer region in the *COL1A2* promoter in the absence of cytokine stimulation suggests that this modification once added by cytokines, remains on the gene without requiring continuous cytokine stimulation. Given the length of these experiments, and lack of cell division in the differentiated cells during the wash off period, we hypothesize that acetylated H4K16 contributes to a chronic fibrogenic phenotype through promoting prolonged *COL1A2* gene transcription. Our observation is intriguing as it has the exciting potential to explain how fibrogenic gene expression and intestinal fibrosis can persist even when inflammation is suppressed. Interestingly, experiments in non-dividing glomerular podocytes have shown that alteration of a chromatin modification pathway (H3K4 methylation) affects stable gene expression patterns and promotes a chronic disease phenotype that resembles glomerular sclerosis⁴³.

Studies to define the functional relevance of H4K16 acetylation would require identification of the proteins that modify histone H4 at this site and/or that bind to it to regulate *COL1A2* gene transcription in these conditions. These are beyond the scope of the experiments reported here and are currently underway in our laboratory. Such strategies include histone peptide-pull down assays that have identified proteins associated with specific histone modifications in other systems.⁴⁴ We are also performing experiments to determine the chromatin modifications that are a permanent feature of type I collagen gene expression in primary human intestinal fibroblasts as these could identify potentially reversible pathways that could be exploited to prevent inappropriate expression of fibrotic genes.

Our studies provide important new insights into the regulation of type I collagen in EndoMT and demonstrate that a specific pattern of histone modifications is induced by a combination of three fibrosis-associated cytokines on the *COL1A2* gene promoter. These findings

provide the basis for identifying the key epigenetic contributors to the transcription status of pro-fibrotic genes in intestinal fibrosis and are likely to be applicable to fibrogenic cells in other organs commonly affected by fibrosis such as the lungs, skin and kidneys.

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REFERENCES

1. Rieder F, Fiocchi C. Intestinal fibrosis in IBD—a dynamic, multifactorial process. *Nat Rev Gastroenterol Hepatol*. 2009; 6:228–235. [PubMed: 19347014]
2. Lichtenstein GR, Olson A, Travers S, et al. Factors associated with the development of intestinal strictures or obstructions in patients with Crohn's disease. *Amer J Gastroenterol*. 2006; 101:1030–1038. [PubMed: 16606351]
3. Prockop DJ, Kivirikko KI. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem*. 1995; 64:403–434. [PubMed: 7574488]
4. Ramirez F, Tanaka S, Bou-Gharios G. Transcriptional regulation of the human alpha2(I) collagen gene (COL1A2), an informative model system to study fibrotic diseases. *Matrix Biol*. 2006; 25:365–372. [PubMed: 16815696]
5. Scarpa M, Stylianou E. Epigenetics: Concepts and relevance to IBD pathogenesis. *Inflamm Bowel Dis*. 2012; 18:1982–1996. [PubMed: 22407855]
6. Petronis A. Epigenetics as a unifying principle in the aetiology of complex traits and diseases. *Nature*. 2010; 465:721–727. [PubMed: 20535201]
7. Campos EI, Reinberg D. Histones: annotating chromatin. *Annu Rev Genet*. 2009; 43:559–599. [PubMed: 19886812]
8. Margueron R, Reinberg D. Chromatin structure and the inheritance of epigenetic information. *Nat Rev Genet*. 2010; 11:285–296. [PubMed: 20300089]
9. Heintzman ND, Stuart RK, Hon G, et al. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet*. 2007; 39:311–318. [PubMed: 17277777]
10. Wang Z, Zang C, Rosenfeld JA, et al. Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet*. 2008; 40:897–903. [PubMed: 18552846]
11. Nightingale KP, Wellinger RE, Sogo JM, Becker PB. Histone acetylation facilitates RNA polymerase II transcription of the *Drosophila* hsp26 gene in chromatin. *EMBO J* 1998; 17:2865–2876. *tes* RNA polymerase II transcription of the *Drosophila* hsp26 gene in chromatin. *EMBO J*. 1998; 17:2865–2876. [PubMed: 9582280]
12. Akhtar A, Becker PB. Activation of transcription through histone H4 acetylation by MOF, an acetyltransferase essential for dosage compensation in *Drosophila*. *Mol Cell*. 2000; 5:367–375. [PubMed: 10882077]
13. Barski A, Cuddapah S, Cui K, R, et al. High-resolution profiling of histone methylations in the human genome. *Cell*. 2007; 129:823–837. [PubMed: 17512414]
14. Bates RC, Mercurio AM. Tumor necrosis factor- α stimulates the epithelial-to-mesenchymal transition of human colonic organoids. *Mol Biol Cell*. 2003; 14:1790–1800. [PubMed: 12802055]
15. Bataille F, Rohrmeier C, Bates R, et al. Evidence for a role of epithelial mesenchymal transition during pathogenesis of fistulae in Crohn's disease. *Inflamm Bowel Dis*. 2008; 14:1514–1527. [PubMed: 18626977]
16. Flier SN, Tanjore H, Kokkotou EG, et al. Identification of epithelial to mesenchymal transition as a novel source of fibroblasts in intestinal fibrosis. *J Biol Chem*. 2010; 285:20202–20212. [PubMed: 20363741]

17. Rieder F, Kessler SP, West GA, et al. Inflammation-induced endothelial-to-mesenchymal transition: a novel mechanism of intestinal fibrosis. *Am J Pathol.* 2011; 179:2660–2673. [PubMed: 21945322]
18. Romero LI, Zhang DN, Herron GS, et al. Interleukin-1 induces major phenotypic changes in human skin microvascular endothelial cells. *J Cell Physiol.* 1997; 173:84–92. [PubMed: 9326452]
19. Frid MG, Kale VA, Stenmark KR. Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. *Circ Res.* 2002; 90:1189–1196. [PubMed: 12065322]
20. Zeisberg M, Bottiglio C, Kumar N, et al. Bone morphogenic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models. *Am J Physiol Renal Physiol.* 2003; 285:F1060–F1067. [PubMed: 12915382]
21. Zeisberg EM, Tarnavski O, Zeisberg M, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med.* 2007; 13:952–961. [PubMed: 17660828]
22. Hashimoto N, Phan SH, Imaizumi K, et al. Endothelial-mesenchymal transition in bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol.* 2010; 43:161–172. [PubMed: 19767450]
23. Rombouts K, Niki T, Greenwel P, et al. Trichostatin A, a histone deacetylase inhibitor, suppresses collagen synthesis and prevents TGF-beta(1)-induced fibrogenesis in skin fibroblasts. *Exp Cell Res.* 2002; 278:184–197. [PubMed: 12169274]
24. Hemmatazad H, Rodrigues HM, Maurer B, et al. Histone deacetylase 7, a potential target for the antifibrotic treatment of systemic sclerosis. *Arthritis Rheum.* 2009; 60:1519–1529. [PubMed: 19404935]
25. Glenisson W, Castronovo V, Waltregny D. Histone deacetylase 4 is required for TGFbeta1-induced myofibroblastic differentiation. *Biochim Biophys Acta.* 2007; 1773:1572–1582. [PubMed: 17610967]
26. Wang Z, Chen C, Finger SN, et al. Suberoylanilide hydroxamic acid: a potential epigenetic therapeutic agent for lung fibrosis? *Eur Respir J.* 2009; 34:145–155. [PubMed: 19224893]
27. McDonald OG, Wu H, Timp W, et al. Genome-scale epigenetic reprogramming during epithelial-to-mesenchymal transition. *Nat Struct Mol Biol.* 2011; 18:867–874. [PubMed: 21725293]
28. Binion DG, West GA, Ina K, et al. Enhanced leukocyte binding by intestinal microvascular endothelial cells in inflammatory bowel disease. *Gastroenterology.* 1997; 112:1895–1907. [PubMed: 9178682]
29. Nowak DE, Tian B, Brasier AR. Two-step cross-linking method for identification of NF-kappaB gene network by chromatin immunoprecipitation. *Biotechniques.* 2005; 39:715–725. [PubMed: 16315372]
30. Antoniv TT, De Val S, Wells D, et al. Characterization of an evolutionarily conserved far-upstream enhancer in the human alpha 2(I) collagen (COL1A2) gene. *J Biol Chem.* 2001; 276:21754–21764. [PubMed: 11279244]
31. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45. [PubMed: 11328886]
32. Wu F, Chakravarti S. Differential expression of inflammatory and fibrogenic genes and their regulation by NF-kappaB inhibition in a mouse model of chronic colitis. *J Immunol.* 2007; 179:6988–7000. [PubMed: 17982090]
33. Brookes E, Pombo A. Modifications of RNA polymerase II are pivotal in regulating gene expression states. *EMBO Rep.* 2009; 10:1213–1219. [PubMed: 19834511]
34. Fraser ID, Germain RN. Navigating the network: signaling cross-talk in hematopoietic cells. *Nature immunology.* 2009; 10:327–331. [PubMed: 19295628]
35. Dorigo B, Schalch T, Bystricky K, et al. Chromatin fiber folding: requirement for the histone H4 N-terminal tail. *J Mol Biol.* 2003; 327:85–96. [PubMed: 12614610]
36. Shogren-Knaak M, Ishii H, Sun JM, et al. Histone H4–K16 acetylation controls chromatin structure and protein interactions. *Science.* 2006; 311:844–847. [PubMed: 16469925]
37. Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem.* 2007; 76:75–100. [PubMed: 17362198]
38. Sengupta P, Xu Y, Wang L, et al. Collagen alpha1(I) gene (COL1A1) is repressed by RFX family. *J Biol Chem.* 2005; 280:21004–21014. [PubMed: 15788405]

39. Buratowski S. Progression through the RNA polymerase II CTD cycle. *Mol Cell*. 2009; 36:541–546. [PubMed: 19941815]
40. Tan M, Luo H, Lee S, et al. Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell*. 2011; 146:1016–1028. [PubMed: 21925322]
41. Gardner KE, Allis CD, Strahl BD. Operating on chromatin, a colorful language where context matters. *J Mol Biol*. 2011; 409:36–46. [PubMed: 21272588]
42. Henikoff S, Shilatifard A. Histone modification: cause or cog? *Trends Genet*. 2011; 27:389–396. [PubMed: 21764166]
43. Lefevre GM, Patel SR, Kim D, et al. Altering a histone H3K4 methylation pathway in glomerular podocytes promotes a chronic disease phenotype. *PLoS Genet*. 2010; 6:e1001142. [PubMed: 21060806]
44. Voigt P, Reinberg D. Histone tails: ideal motifs for probing epigenetics through chemical biology approaches. *Chembiochem : a European journal of chemical biology*. 2011; 12:236–252. [PubMed: 21243712]

Fig. 1. Induction and persistence of COL1A2 gene expression during EndoMT

HIMEC were seeded at 50 % confluency and were treated with a cytokine cocktail comprising: TGF- β 1 (5 ng/ml), TNF- α (5 ng/ml), and IL-1 β (0.1 ng/ml) or with cytokine-free growth medium alone for 0–20 days (A). HIMEC were monitored daily to identify endothelial-fibroblast transitions. The cytokines were replenished and the growth medium replaced every 3–4 days. At the end of the cytokine incubation period the cytokines were removed, the cells washed and the medium replaced (A). The cultures were continued for a further 10 days (“10 day wash off”). At the end of this period cells were harvested, RNA extracted and RT-PCR performed to determine the levels of *COL1A2* mRNA relative to *GAPDH* as a reference gene after incubation with the cytokine cocktail (gray bars) and after cytokine removal/“wash off” (black bars) (B). Data represent mean $\pm$ S.E.M. n = 4–5 experiments. * p<0.05.

Fig. 2. Cytokine-dependent enrichment of histone modifications on the *COL1A2* and *vWF* gene promoters during EndoMT

Chromatin was prepared from HIMEC cells that had undergone EndoMT after 5 days incubation with the cytokine cocktail as described. ChIP assays were performed with antibodies to 3 modifications linked to gene activation: (Panels A, C, E) H4 and H3 acetylation (H4Ac and H3Ac) and H3K4 trimethylation (H3K4me3) and 3 modifications characteristic of repressed genes (Panels B, D, F): H3K9 dimethylation (H3K9me2), H3K27 trimethylation (H3K27me3) and H4K20 trimethylation (H3K20me3). qPCR detected the *COL1A2* proximal promoter (A, & B), the *COL1A2* upstream enhancer (C & D) and *vWF* promoter endothelial specific region (E & F) respectively. Mean values $\pm$ SEM of % of total precipitated chromatin relative to input chromatin expressed as % input for each modification on each gene is shown. RNA was prepared from the same cells and RT-PCR performed to determine the level of *COL1A2* and *vWF* mRNA after 5 days (Panel G). Control (open bars) and cytokine-treated (gray bars) cells were compared. Data represent mean $\pm$ S.E.M of n=6–11 experiments. * $p < 0.05$.

Fig. 3. Cytokine-induced recruitment of S2 phosphorylated RNA polymerase II to the COL1A2 gene promoter

HIMEC were treated with TGF- β 1 (5 ng/ml), TNF- α (5 ng/ml), and IL-1 β (0.1 ng/ml) for 5 days as already described and cells were crosslinked with 2mM freshly prepared Disuccinimidyl glutarate (DSG) for 30 mins at room temperature followed by 0.5% v/v Formaldehyde (Sigma) for 5 min. To terminate the crosslinking reaction, glycine (0.125M) was added for a further 5 min. The crosslinked chromatin was sonicated and immunoprecipitated with antibodies to the S2, S5 and unphosphorylated forms of RNA polymerase II (polIIS2ph, polIIS5ph and PolII respectively). The proximal promoter region of the *COL1A2* promoter was analyzed. Mean values $\pm$ S.E.M of % of total precipitated chromatin relative to input chromatin expressed as % input for each form of the RNA polymerase is shown. Control (open bars) and cytokine-treated (gray bars) cells were compared. Data represent mean $\pm$ S.E.M of n=3 experiments. ** p<0.01.

Fig. 4. Cytokines increase the acetylation of Histone H4 at specific lysine residues on the COL1A2 gene

Chromatin was prepared from HIMEC cells that had undergone EndoMT after 1 day (Panel A & B) and 5 days (Panel C & D) incubation with the cytokine cocktail as described. ChIP assays were performed with antibodies to individual acetylated lysine residues in histone H4: H4K5Ac, H4K8Ac, H4K12Ac and H4K16Ac (Panels A – D). qPCR of the ChIP samples detected the *COL1A2* promoter (A & C) and the *COL1A2* upstream enhancer (B & D). Mean values $\pm$ S.E.M of % of total precipitated chromatin relative to input chromatin expressed as % input for each modification on each gene is shown. RNA was prepared from the same cells incubated with the cytokine cocktail for 1 day and RT-PCR performed to determine the level of *COL1A2* mRNA (Panel E). Control (open bars) and cytokine-treated (gray bars) cells were compared. Data represent mean $\pm$ S.E.M of n=8–11 experiments. * $p < 0.05$.

Fig. 5. Prolonged COL1A2 gene expression in EndoMT is associated with persistent histone H4 acetylation

Chromatin was prepared from HIMEC cells after 16 days incubation with growth medium (open bars), the cytokine cocktail (gray bars) as described and after removal of cytokines and a further 10 day incubation (wash off, black bars). ChIP assays were performed with antibodies to individual acetylated lysine residues in histone H4: H4K16Ac (Panels A & B), H4K12Ac (Panels C & D), H4K8Ac (Panels E & F), H4K5Ac (Panels G & H). qPCR of the ChIP samples was performed to detect the proximal promoter (Panels A,C,E,G) and upstream enhancer region (Panels B,D,F,H) of the *COL1A2* gene promoter. Mean values $\pm$ S.E.M of % of total precipitated chromatin relative to input chromatin expressed as % input for each modification on each gene is shown (Panels A–H). Data represent mean $\pm$ S.E.M of n=3 experiments. * $p < 0.05$.

Fig. 6. Prolonged COL1A2 gene expression in EndoMT is associated with persistent acetylation of H4K16

Chromatin was prepared from HIMEC cells after 16 days incubation with growth medium (open bars), the cytokine cocktail (gray bars) as described and after removal of cytokines and a further 10 day incubation (wash off, black bars). ChIP assays were performed with antibodies to H3K9me2 (Panels A & B) and H3K4me3 (Panels C & D). qPCR of the ChIP samples was performed to detect the proximal promoter (Panels A & C) and upstream enhancer (Panels B & D) of the *COL1A2* gene promoter (A & C) and upstream enhancer (B & D). Mean values $\pm$ S.E.M of % of total precipitated chromatin relative to input chromatin expressed as % input for each modification on each gene is shown (Panels A–D). Fold increase in *Col1A2* mRNA (Panel E) over background is shown after incubation with the cytokine cocktail (open bars), and after cells treated with cytokine cocktail for 16 days

followed by an additional 10 days of culture after the removal of cytokines (washoff, black bars) were compared. Data represent mean $\pm$ S.E.M of n=3 experiments. * $p<0.05$.

Fig.7. Schematic summary of cytokine-induced, time dependent changes in histone acetylation and methylation at the *COL1A2* promoter

Induction of *COL1A2* gene expression after 5 days of cytokine (IL-1 β , TNF α and TGF β ,) treatment is associated with acetylation (Ac) of H4 lysines 8, 12 and 16 and dimethylated H3K9 (Me) at the regions encompassing the proximal promoter and upstream enhancer regions (Panel A). H4 acetylation at the 3 aforementioned sites is retained during prolonged expression of *COL1A2* following 16 day cytokine treatment whereas dimethylated H3K9 is lost and acetylation at lysine 5 of histone H4 is enhanced over this time period (Panel B). Cytokine treatment for 16 days followed by removal of the cytokines for a further 10 days resulted in cytokine dependent retention of H4K16 acetylation at the upstream enhancer region of the *COL1A2* promoter (Panel C).

Fig.8. Schematic model of cytokine-induced modification of chromatin and regulation of COL1A2 gene transcription

IL-1 β , TNF α and TGF β induce H4 acetylation, H3K9 methylation and RNA polymerase II (PolII) binding on induction of COL1A2 gene expression. The increase in histone acetylation and methylation are likely to be markers of increased activity of specific chromatin modifying enzymes e.g. increased activity of specific histone acetylases (HATs) or decreased activity of histone deacetylases (HDAC). Hyperacetylated and hypermethylated histones are also potential binding sites for the recruitment of transcription factors (TF) and other coregulator proteins. These contribute to the formation of an active transcription complex responsible for the induction and prolonged expression of *COL1A2*.