Chapter 4

Transcription factors associated with the development of Barrett’s esophagus; in vivo expression and modulation by Retinoic Acid

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Abstract

**Background:** Barrett’s esophagus (BE) is a columnar metaplasia that is the main risk factor for esophageal adenocarcinoma. Altered expression of transcription factors that direct differentiation could be important in BE development, but little is known regarding their expression pattern and the signaling pathways that regulate them. Retinoic acid (RA) is a potent morphogen that has previously been implicated in the development of BE.

**Aim:** To investigate the expression of squamous (p63, SOX2), early (GATA6) and late (CDX2) columnar transcription factors in squamous epithelium and BE in vivo and study the effect of RA on the expression of these transcription factors in vitro.

**Methods:** The expression of the transcription factors p63, SOX2, GATA6 and CDX2 and the glycoprotein MUC2 was studied in formalin fixed and paraffin-embedded tissue biopsies of squamous epithelium (N=47), non-intestinal type (non-IM) BE (N=14) and intestinal-type (IM) BE (N=24). The effect of all-trans retinoic acid (ATRA) on the expression of squamous and columnar transcription factors was studied using the immortalized esophageal squamous cell line EPC2-hTERT.

**Results:** P63 and SOX2 were expressed in the majority of cells in squamous epithelium. P63 expression was lost completely (p<0.001) in BE. The percentage of SOX2 positive cells was strongly reduced in BE, but a minority of cells retained SOX2 expression (p<0.001). The number of GATA6 and CDX2 positive cells was very low in squamous epithelium and significantly increased in non-IM BE (p<0.001 for both transcription factors). The number of CDX2 positive cells further increased in IM BE (p<0.001), whereas the percentage of GATA6 positive cells remained constant in IM BE. The percentage of MUC2 positive cells was close to zero in squamous epithelium, low in non-IM, while the majority of the cells in IM BE were MUC-positive. In vitro, ATRA treatment induced downregulation of the p63 isoform ΔNp63α protein (p=0.037) but had no significant effect on p63 mRNA levels. ATRA treatment also induced significant upregulation of GATA6 and SOX9 mRNA expression (p=0.015 for both) but did not alter the expression levels of CDX2 or MUC2 mRNA.

**Conclusion:** Squamous epithelium and BE are each characterized by distinct patterns of transcription factor expression. RA can partially induce a columnar-type expression pattern of transcription factors in vitro.
Introduction

Barrett’s metaplasia of the esophagus (BE) is a precursor lesion of esophageal adenocarcinoma. BE is defined by the replacement of squamous epithelium in the distal end of the esophagus with columnar epithelium. Histologically, BE is broadly classified as metaplastic columnar epithelium without goblet cells (non-intestinal metaplasia, non-IM) or with goblet cells (intestinal metaplasia, IM). This distinction is important as non-IM is thought to precede IM during BE development (1). Persistent gastro-esophageal reflux disease (GERD) shows a strong association with BE and is thought to be the main driving factor in its development (2).

Several transcription factors, most notably CDX2, GATA6 and SOX9 have been associated with columnar differentiation of esophageal epithelium. However, no single columnar transcription factor is sufficient to drive a complete transdifferentiation of squamous epithelium towards a Barrett’s-like columnar metaplasia (3-5). Loss of transcription factors associated with squamous differentiation could be another necessary step for BE development. The transcription factors p63 and SOX2 are essential for the development and maintenance of squamous epithelium. Animal models showed that in mice lacking either p63 or SOX2 the esophagus is covered with a columnar epithelium resembling BE (6-8). The essential role of p63 in squamous epithelium is further supported by the finding that a single p63 isoform, ΔNp63α, is responsible for the maintenance of squamous stem cells in skin (9-11).

The signaling pathways responsible for the altered transcription factor expression during BE development are incompletely understood. Retinoic acid (RA) and its isoform All-Trans Retinoic Acid (ATRA) have been suggested as a possible inducer of columnar differentiation in the esophagus. ATRA is a known inducer of GATA6 during embryological development (13,14). In adult tissues ATRA deficiency induces a squamous metaplasia in the columnar lining of the trachea in rabbits and of the endocervix in mice (15,16). RA biosynthesis is increased in BE compared with normal squamous epithelium (17). In esophageal squamous epithelial cells RA induced the expression of MUC2, a glycoprotein that is a direct downstream target of CDX2 and is typically associated with IM, and ATRA caused loss of the squamous cytokeratin CK13 (18,19). While these data indicate that RA and its isoform ATRA play a role in inducing and maintaining columnar differentiation, it is unknown whether RA treatment has an effect on transcription factors associated with squamous or columnar differentiation of the esophagus.

In this study we hypothesized that the development of BE is associated with loss of the squamous transcription factors p63 and SOX2 and gain of columnar transcription factors GATA6, SOX9 and CDX2, and that ATRA can induce this shift in transcription factor expression in vitro. To test this hypothesis first investigated the expression of p63,
SOX2, CDX2 and MUC2 in tissue biopsies of squamous epithelium and BE. In addition, we studied the effect of ATRA on the expression of p63, miR-203, SOX2, GATA6, SOX9 and CDX2 in a squamous esophageal cell line.

Material and Methods

Tissue collection
A total of 73 paraffin-embedded biopsies containing samples of squamous epithelium (N=47), non-IM BE (N=14) and IM BE (N=24) was retrieved from the archives of the Department of Pathology, University Medical Center Groningen, the Netherlands. All biopsy samples were collected from patients undergoing gastro-esophageal endoscopy at the University Medical Center Groningen. The biopsy samples were fixed in formalin overnight and embedded in paraffin.

Immunohistochemistry
Slides were deparaffinized using xylene and rehydrated by a series of ethanol dilutions. Antigen retrieval was performed by heating the slides in 10mM citrate buffer (pH 6.0) for 15 minutes using a 400W microwave. Endogenous peroxidase blocking was performed by incubating the slides for 30 minutes with 0.3% H$_2$O$_2$ in phosphate-buffered saline (PBS). All primary antibodies, pan-p63 (Cat. # sc-8431, 1:500, Santa Cruz, USA), ΔNp63 (Cat. # 619001, 1:500 Biolegend, USA), SOX2 (Cat. # 4900, 1:400 Cell Signaling, USA), CDX2 (Cat. # ab76541, 1:500 Abcam, UK), MUC2 (Cat. # sc59859 1:100 Santa Cruz, USA) were diluted in PBS with 1% bovine serum albumin. Slides were incubated for 60 minutes at room temperature with the primary antibody. As a negative control slides were stained with an isotype-matched IgG. After washing, slides were incubated for 30 minutes at room temperature with horseradish peroxidase-conjugated secondary and tertiary antibodies (all from Dako, Denmark) diluted 1:50 in PBS with 1% bovine serum albumin and 1% human serum. Peroxidase was visualized using 3,3’diaminobenzidine (Sigma-Aldrich, USA) and slides were counterstained with hematoxilin. The percentage of positive cells was calculated from an average of three crypts. For squamous epithelium, one representative area was evaluated. The final percentage was calculated by averaging the estimates of two observers (KP and WB).

Cell culturing and ATRA treatment
The non-malignant squamous esophageal epithelial keratinocyte cell line EPC2-hTERT (kind gift from Dr. A.Rustgi, Division of Gastroenterology, University of Pennsylvania Perelman School of Medicine, Philadelphia, USA) was cultured in Keratinocyte Serum-Free Medium (KSFM) supplemented with KSFM Growth Supplement (Life Technologies,
Bleiswijk, The Netherlands) using plastic disposables from Greiner (Greiner Bio One, Alphen aan den Rijn, the Netherlands). Cells were maintained at a maximum of 80% confluency to avoid terminal differentiation. Cells were counted at low magnification under a brightfield microscope using trypan blue to distinguish living from dead cells. ATRA (Sigma-Aldrich, USA) was dissolved in 96% ethanol to a final concentration of $0.5 \times 10^{-2}$ M. For treatment ATRA was further diluted in KSFM to a concentration of 1 μM ATRA. The control vehicle consisted of 96% ethanol diluted in KSFM. EPC2-hTERT cells were harvested at 24, 48 and 72 hours after treatment with 1 μM ATRA or control. Data points for each experimental condition were gathered in duplicate and experiments were performed in triplicate.

**Western blotting**

5μg denatured protein from whole cell lysates was separated on a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, The Netherlands). ΔNp63α protein level was examined using two primary antibodies: the pan-p63 antibody (Cat. # sc8431 1:1000, Santa Cruz, USA) and the ΔNp63 antibody (Cat. # 619001 dilution 1:500 Biolegend, USA). Both antibodies showed a band corresponding with the molecular weight of the ΔNp63α isoform (~70 kDa). β-actin (IZN Pharmaceuticals, Zoetermeer, The Netherlands) was used as a loading control for quantification of the relative ΔNp63 protein levels. Densitometric analysis was performed using the ImageJ software 1.47 (U. S. National Institutes of Health, Bethesda, Maryland).

**RNA isolation, primers, PCR**

Total RNA was extracted using TRIzol (Invitrogen, Life Technologies, Bleiswijk, the Netherlands). RNA was reverse-transcribed using random primers (Invitrogen, Life Technologies, Bleiswijk, the Netherlands). For pan-p63, ΔNp63, SOX2 and GATA6 qRT-
PCR was performed on a Biorad thermocycler using Sybergreen (Life Technologies, Bleiswijk, The Netherlands). Since it is not possible to design a primer set that is specific for the ΔNp63α isoform, we used one set that recognizes all six p63 isoforms and a second primer pair that is specific for all three ΔNp63 isoforms. β-actin was used as a control. Primer pairs are listed in Table 1. Relative expression levels were calculated according to the $2^{-ΔΔCt}$ formula.

**Statistical analysis**
Mann Whitney test was used for the immunohistochemical data and cell number analysis and the Wilcoxon signed rank test was used for quantification of Western blot and PCR data. A p-value of $p<0.05$ was considered significant. Statistical analysis was performed using the Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

**Results**

*In vivo expression of squamous and columnar transcription factors*
We examined the expression pattern of squamous transcription factors in biopsies from squamous epithelium, non-IM and IM BE. P63 stained positive in 70% of the nuclei in squamous epithelium specimens, but was completely absent in non-IM and IM (p<0,001, Figure 1A and 1B). Similar to p63, nuclear SOX2 expression was present in 50% of the nuclei in squamous epithelium specimens (Figure 1C). In contrast to p63, we observed a subset of SOX2-expressing cells in both non-IM and IM BE. Compared to squamous epithelium the percentage of SOX2-positive cells was significantly lower (8%) in non-IM BE (p<0,001). The percentage of SOX2 positive cells decreased further in IM specimens where it was present in approximately 3% of cells (p=0,017, Figure 1D). Next, we studied the expression of GATA6 and CDX2, two known markers of columnar differentiation. GATA6 showed nuclear expression in a minority (10%) of squamous epithelial cells and the percentage of GATA6-positive cells increased in non-IM BE to 30% (p<0,001) while there was no further increase in IM (28% positive cells, p=0,834, Figure 1E and F). CDX2 was almost absent in squamous epithelium and the percentage of CDX2-positive cells was significantly higher in IM BE (90%) compared to non-IM BE (21%) (p<0,001, Figure 1G, H). MUC2 was absent in squamous epithelium, while 6% of the cells were MUC2-positive in non-IM BE specimens, and this increased to 71% IM BE specimens (Figure 1I, J).

*The in vitro effect of ATRA on cell proliferation and transcription factor expression*
We used the EPC2-hTERT as a model to explore the effects of ATRA. EPC2-hTERT cells showed a significant decrease in cell number at 48 and 72 hours compared to cells treated with the control vehicle (Figure 2A). ATRA did not induce cell death based on trypan blue
Figure 1: Expression of squamous and columnar transcription factors in patient biopsies

Representative images and quantification of immunohistochemical staining for pan-p63, SOX2, GATA6, CDX2 and MUC2. Both p63 and SOX2 show nuclear expression in basal and suprabasal layers of squamous epithelium. P63 is completely lost in Barrett’s esophagus in both the non-intestinal metaplasia (non-IM) as well as in intestinal-type metaplasia (IM, panel 1A, B). SOX2 is retained in a minority of the non-IM and IM BE cells (panel 1C,D). GATA6 is expressed in a minority of squamous cells, but the percentage of positive cells significantly increases in non-IM and IM as compared to squamous epithelium. However, the percentage of GATA6-positive cells does not differ between non-IM and IM (panel C,D). CDX2 expression is almost absent in squamous epithelium, but the percentage of positive cells increases progressively in non-IM and IM (panel G, H). The percentage of MUC2 positive cells mirrors that of CDX2, being absent in squamous epithelium and progressively increasing in non-IM and IM (panel I, J). The bars in the boxes indicate the mean and error bars denote the standard deviation.
Figure 2: ATRA treatment reduces cell proliferation and induces a shift in expression from squamous to columnar transcription factors

ATRA blocks cell proliferation (panel A). ΔNp63α protein expression was examined using two different antibodies, one recognizing all p63 isoforms (pan-p63 AB) and one specific for the ΔNp63 isoforms (ΔNp63 AB). For both antibodies, the lane corresponding to ΔNp63α was used for analysis. An example of a western blot is given in panel B and quantification of results in panels C and D. ATRA treatment reduced ΔNp63α protein level 72 hours after treatment (p=0.037 for the pan-p63 antibody and p=0.078 for the ΔNp63 antibody). ATRA treatment had no effect on pan-p63 or ΔNp63 mRNA levels (panel E and F). ATRA treatment had no effect on SOX2 mRNA levels (panel G), but increased GATA6 mRNA at 24 and 48 hours after treatment (panel H) and SOX9 mRNA at 72 hours after treatment (panel I). Control conditions are represented as white columns, and treated samples as black columns. The bars in the boxes indicate the mean and error bars denote the standard deviation. Data points for each experimental condition were gathered in duplicate and experiments were performed in triplicate.
Figure 2

C pan-p63 WB

D Δp63 WB

E pan-p63 PCR

F Δp63 PCR

G SOX2 PCR

H GATA6 PCR

I SOX9 PCR
dye exclusion, suggesting that the lower cell numbers were the result of an ATRA-induced block in proliferation (data not shown).

Next, we examined the effect of ATRA on the squamous transcription factors p63 and SOX2. We focused this analysis on the ΔNp63α isoform, since previous studies showed that this isoform was responsible for the induction and maintenance of squamous epithelium. ATRA treatment had little effect on ΔNp63α protein expression at 24 and 48 hours, but at 72 hours we observed a downregulation in ATRA-treated cells compared with control cells (p=0.037 for the pan-p63 antibody and p=0.078 for the ΔNp63α specific antibody, Figure 2C,D). Next, we studied mRNA levels of pan-p63 and ΔNp63 (all three isoforms) by qRT-PCR. No differences in pan-p63 or ΔNp63 mRNA levels were observed (Figure 2E, F). ATRA treatment had also no effect on SOX2 mRNA levels at all time points (Figure 2G).

Finally, we examined whether ATRA treatment could induce the expression of markers of columnar differentiation. Compared to time-matched controls, ATRA treatment significantly increased the mRNA expression of the columnar transcription factors GATA6 at 24 and 48 hours after treatment (p=0.002 and p=0.015 respectively, Figure 2H) and of SOX9 at 48 and 72 hours (p=0.015 and p=0.002 respectively, Figure 2I). However, ATRA treatment did not induce expression of the intestinal markers CDX2 or its downstream target MUC2 (data not shown).

Discussion

We have shown that p63 expression is lost completely in BE, while a minority of BE cells retain SOX2 expression. The number of GATA6 and CDX2-positive cells is strongly increased in BE compared to squamous epithelium. Interestingly, the percentage of CDX2 positive cells is higher in IM compared to non-IM specimens, while the percentage of GATA6-positive cells does not differ between these two subtypes of BE. In vitro, treatment of EPC2-hTERT keratinocytes with ATRA induces loss of ΔNp63α protein and upregulation of the columnar transcription factors GATA6 and SOX9.

RA signaling has long been implicated in columnar differentiation of various tissues including cervix, esophagus and bronchia (15,16,19,20). This is the first study that examined the effect of ATRA on a squamous and columnar transcription factors in an esophageal squamous epithelial cell line. ATRA downregulated ΔNp63α protein expression, but it had no effect on the mRNA level of p63 and SOX2. While we cannot exclude the possibility that ATRA affects SOX2 protein expression, the finding of a minority of SOX2-positive cells observed in BE biopsies suggests that loss of SOX2 expression is not essential for columnar differentiation. This is remarkable, since both p63 and SOX2 are necessary for the development of squamous epithelium during embryological development of the esophagus (6,8). The discrepancy between the observed levels of p63 protein and mRNA
might be explained by post-transcriptional regulation by for example miR-203, which has been shown to target the p63 transcript (21-23).

ATRA treatment enhanced the expression of GATA6 and SOX9 at the mRNA level. GATA6 is a zinc finger transcription factor that has been associated with proliferation and differentiation in intestinal epithelium (24-26), and a recent series of papers described GATA6 as a central regulator of stem cells in colon (27,28). While little is known about possible stem cells in BE, the crypt-based architecture of BE is suggestive of their presence, since it mimics the crypt structure of intestinal tissue that is known to be controlled by stem cells (29). SOX9 is another marker of intestinal stem cells and plays a role in intestinal homeostasis and Paneth cell differentiation (30-32). Previous studies showed that SOX9 and GATA6 expression are early events in BE development (4,5), suggesting that ATRA-treated EPC2 cells could provide a model for studying the early steps in BE development.

ATRA treatment did not alter CDX2 expression levels. CDX2 is required for intestinal differentiation and widely expressed in IM-type BE (1). However, forced CDX2 expression in squamous epithelium did not induce a columnar differentiation and co-expression of both SOX9 and CDX2 had the same phenotype as forced expression of SOX9 alone. This suggests that CDX2 is dispensable in the development of simple, non-intestinal columnar epithelium (5). In a previous study RA was reported to induce MUC2 mRNA expression after 24 hours of incubation in primary keratinocytes (33). Despite a longer incubation period, we did not detect MUC2 mRNA expression after ATRA treatment. This difference could possibly be explained by the fact that we used an established keratinocyte cell line as opposed to primary keratinocytes, but the lack of MUC2 expression is in line with the absence of CDX2 and the notion that our model (partially) may mimic the early stages of (non-IM) BE development. This is further supported by the finding that the percentage of positive cells of both CDX2 and MUC2 was low in non-IM type BE but significantly higher in IM-type BE.

While this study is the first to examine the in vitro effect of RA signaling on transcription factors implicated in BE development, it has several limitations. First, we noted that the in vitro model had significant variation with regard to transcription factor expression. Second, it is currently unclear whether the upregulation of the GATA6 and SOX9 is a direct consequence of ΔNp63α loss. In mouse models loss of p63 was associated with the development of columnar epithelium independent of RA signaling (6), suggesting that p63 loss induces a default columnar differentiation. Functional studies to further examine the relation between p63 and GATA6 and SOX9 were beyond the scope of the current study, but could be part of a further characterization of our in vitro model.

Antagonizing the effect of RA signaling could be a feasible therapeutic strategy to induce regression of BE towards normal squamous epithelium. Chang et al. used citral, a natural antagonist of RA signaling to successfully induce a columnar-to squamous
differentiation in explants of BE (17). Our finding that ATRA causes a loss of squamous and upregulation of columnar transcription factors provides mechanistic clues for further analysis of factors that drive the development of BE and could be used to study the therapeutic effect of RA-antagonists in vitro.

In conclusion, this paper shows for the first time that in contrast to p63, a minority of BE cells retain SOX2 expression and that ATRA treatment downregulates ΔNp63α expression, while inducing the expression of GATA6 and SOX9. These findings contribute to a better understanding of the molecular mechanisms involved in BE development, and offer a foundation for further studies aimed at BE chemoprevention through antagonizing RA signaling.
References


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