Transcript Profiling Identifies Dynamic Gene Expression Patterns and an Important Role for Nrf2/Keap1 Pathway in the Developing Mouse Esophagus

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Abstract

Background and Aims: Morphological changes during human and mouse esophageal development have been well characterized. However, changes at the molecular level in the course of esophageal morphogenesis remain unclear. This study aims to globally profile critical genes and signaling pathways during the development of mouse esophagus. By using microarray analysis this study also aims to determine how the Nrf2/Keap1 pathway regulates the morphogenesis of the esophageal epithelium.

Methods: Gene expression microarrays were used to survey gene expression in the esophagus at three critical phases: specification, metaplasia and maturation. The esophagi were isolated from wild-type, Nrf2+/−/−, Keap1−/−, or Nrf2+/−/− Keap1−/− embryos or young adult mice. Array data were statistically analyzed for differentially expressed genes and pathways. Histochemical and immunohistochemical staining were used to verify potential involvement of the Wnt pathway, Pparγ/δ and the PI3K/Akt pathway in the development of esophageal epithelium.

Results: Dynamic gene expression patterns accompanied the morphological changes of the developing esophagus at critical phases. Particularly, the Nrf2/Keap1 pathway had a baseline activity in the metaplasia phase and was further activated in the maturation phase. The Wnt pathway was active early and became inactive later in the metaplasia phase. In addition, Keap1−/−/− mice showed increased expression of Nrf2 downstream targets and genes involved in keratinization. Microarray and immunostaining data also suggested that esophageal hyperkeratosis in the Keap1−/−/− mice was due to activation of Pparγ/δ and the PI3K/Akt pathway.

Conclusions: Morphological changes of the esophageal epithelium are associated with dynamic changes in gene expression. Nrf2/Keap1 pathway activity is required for maturation of mouse esophageal epithelium.

Introduction

Morphological changes in developing human organs require coordinated activation of gene transcription and signaling pathways [1]. The epithelial cells lining the human esophagus transform from simple columnar into ciliated epithelium at an early phase. The ciliated epithelium is then gradually replaced by a squamous epithelium until a non-keratinized stratified squamous epithelium. Morphological changes during human esophageal development have been well-characterized for several decades [2,3]. However, the molecular mechanisms underlying these morphological changes remain largely unknown.

Studies using mouse genetic models provided initial insights into the roles of transcription factors and signaling pathways for the morphogenesis of the esophagus [4,5]. The esophagus is specified from the foregut tube at embryonic day E9.5 in mice, and at four weeks in humans. In mouse embryo, the esophagus is completely separated from the trachea at E11.5. Mutation of genes encoding transcription factors (e.g., Sox2 and Tgfα) and signaling molecules (e.g., Noggin, Shh) disrupts the separation process, leading to the formation of esophageal atresia [6]. From E11.5 to E15.5 the esophageal epithelium is transformed from a simple columnar epithelium to a multiple-layered epithelium. Towards the end of
this phase the epithelium starts to lose columnar cell differentiation markers and express squamous cell markers. From E15.5 to birth, columnar features are almost lost and the epithelium is further stratified. From postnatal day 7 (P7) onwards, the top layer of the stratified squamous epithelium starts the matriculating process and forms a keratin layer which is not present in the human esophagus [5,7,8,9].

According to these morphological changes, the development of the esophagus can be divided into three phases: specification phase (E9.5–11.5), metaplasia phase (E11.5–P7), and maturation phase (P7-adult). Our previous studies showed that Bmp signaling plays a two-stage role in the developing esophagus [4]. During the early metaplasia phase (E11.5–15.5), the Bmp pathway is inhibited by Noggin to allow stratification to occur. Subsequently, the Bmp pathway must be activated to promote squamous differentiation of the top layers of the stratified epithelium [4]. Interestingly, other signaling pathways including Wnt pathway and Shh pathway are active in the separating esophagus at the early specification phase (reviewed by Morrisey and Hogan [10]). Nevertheless, it is unknown whether these signaling molecules assume a dynamic change of expression pattern similar to Bmps. In our previous study on human Barrett’s esophagus, a metaplastic condition in which the stratified squamous epithelium of the lower esophagus is replaced by stratified columnar epithelium, we found that several transcription factors such as Nf2 (nuclear factor erythroid derived 2 like 2), and Nf2(22) and small Maf proteins (MafF, MafG) were enriched in the normal human esophagus as compared with Barrett’s esophagus [11]. As a major cellular defense pathway, the Nrf2/Keap1 (kelch-like ECH-associated protein 1) pathway is known to regulate expression of enzymes involved in detoxification and anti-oxidative stress response [12]. Nrf2 forms heterodimers with small Maf proteins and binds to the antioxidant-responsive elements of target genes when cells are exposed to oxidative stress or xenobiotics. Keap1 regulates the function of Nrf2 by returning Nrf2 in the cytoplasm under normal physiological conditions, and by allowing nuclear translocation of Nrf2 under stress conditions. Certain Keap1 mutants have a dominant-negative effect on wild-type Keap1 [13]. In addition to its function in stress response, the Nrf2/Keap1 pathway is known to participate in wound healing, inflammation resolution, apoptosis, and keratinocyte differentiation [14]. Nf2(−/−) mice developed normally. Keap1(−/−) mice died within three weeks after birth, probably due to malnutrition as a result of hyperkeratosis in the esophagus and forestomach. In the esophageal epithelium, Keap1(−/−) mice expressed higher levels of Kit1, Kit6 and Lar and lower levels of Kit13 and Itw than the wild-type mice. These phenotypes were due to superactivation of Nf2 with the help of small Maf proteins because both Nf2(−/−) Keap1(−/−) and MafF/MafG/Keap1(−/−) rescued the Keap1(−/−) phenotype [15,16]. These studies clearly indicate that the Nrf2/Keap1 pathway plays a critical role in the development of esophageal epithelium.

In this study, we examined gene expression in the esophagi of wild-type and mutant mice (Nf2(−/−), Keap1(−/−) and Nf2(−/−)Keap1(−/−)) using gene microarrays. Our goal was to survey gene expression during the development of mouse esophageal epithelium, and to better understand the role of the Nrf2/Keap1 pathway in the process.

Materials and Methods

Animals

Wild-type C57BL/6j mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Nf2(−/−) and Keap1(−/−) mice on C57BL background were obtained from the Experimental Animal Division, RIKEN Biosource Center (Tsukuba, Japan) [15]. BAT-GAL and TOP-GAL mouse lines were purchased from the Jackson Laboratory, and they were maintained on C57BL/6J and CD1 background, respectively [17,18].

These mice were bred in-house to generate embryos and offspring with proper genotypes. Mice were PCR-genotyped according to protocols provided by the original developers. Esophagi of E11.5, E15.5, P0, P7, and adult (8 weeks old) mice were dissected and snap-frozen for future extraction of total RNA. Part of each esophagus was fixed in 10% buffered formalin or frozen for future use in histology. Three esophageal samples from each group at each time point were harvested. The following tissue samples were harvested for gene expression profiling: (1) wild-type esophagi at E11.5, E15.5, P0, P7; (2) wild-type, Nf2(−/−), Keap1(−/−) and Nf2(−/−)Keap1(−/−) esophagi at P7; (3) wild-type and Nf2(−/−)-adult esophageal epithelium (see Figure 1A for the sampling scheme). All animal experiments were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Rochester and North Carolina Central University (protocol number XC-12-03-2008).

RNA isolation and quality check

Total RNA was extracted from individual mouse esophagi (E11.5, E15.5, P0, P7 and adult) with an RNeasy Fibrous Tissue Mini Kit (Qiagen; Valencia, CA). These RNA samples were checked for their quality using gel electrophoresis, and their concentrations were measured using spectrophotometry. Their quality (RIN>7) was further checked with Bioanalyzer (Agilent Technologies; Santa Clara, CA) at the Genomics Core Facility, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill.

Microarray data collection, data pre-processing and probe annotation

Microarray experiments were performed at the Genomics Core Facility with Agilent two-channel mouse 4×44k microarrays. Red channel (Cy5) was used for esophageal samples, and green channel (Cy3) for mouse universal reference RNA (provided by the Genomics Core Facility). Hybridization was performed according to the standard protocol of “Two-Color Microarray-Based Gene Expression Analysis” for Agilent Gene Expression Oligo microarrays Version 5.0.1. Briefly, a 2× target mix was generated containing 125 ng cyanine 3- labeled cRNA, 125 ng cyanine 5-labeled cRNA, appropriate amounts of labeled synthetic target, and 25 μl of Agilent’s 10× control solution in a final volume of 125 μl. The sample was then fragmented by the addition of 5 μl 25X fragmentation buffer followed by incubation at 60°C for 30 minutes. Samples were moved to ice, and fragmentation was stopped by addition of 125 μl of Agilent’s 2X in situ hybridization buffer. Microarrays were hybridized in Agilent Microarray Hybridization Chambers for 17 hours at 60°C with mixing on an Agilent Rotator in a Robbin’s Scientific Hybridization Oven. After hybridization, the arrays were scanned by an Axon GenePix 4000B scanner (Axon Instruments; Foster City, CA). The images were analyzed using GenePix Pro 5.0 software (Axon Instruments). Gene expression values were quantified by log base 2 ratio of red channel intensity (mean) and green channel intensity (mean), followed by Lowess normalization to remove the intensity-dependent dye bias. The raw data was submitted to NCBI’s GEO database (Series GSE34278).

Data pre-processing was carried out via the UNC Microarray Database for quality filtering and data normalization. UNC Microarray Database (https://genome.unc.edu/) provides the
service for microarray data storage, retrieval, analysis, and visualization to registered UNC-Chapel Hill researchers and their collaborators. Agilent array data was extracted on the probe level. For probes spotted multiple times, the mean expression value was computed and retained. All probe sequences were BLAT against the NCBI database [19] and were annotated with Entrez ID. When multiple probes were targeted on the same gene (with the same Entrez ID), these data were collapsed onto the Entrez ID, and mean values were computed as the gene expression value.

Obtaining differentially expressed gene (DEG) and multivariate analyses

Pre-processed data were used to construct a series of data matrix files for further analysis. For a given data matrix, the rows were excluded if more than 40% of missing values were observed. The rest of missing data was imputed with a K-nearest neighbor (k = 9) approach. DEGs were obtained from two-class and multi-class statistical modeling using SAM (R package samr v.1.25) [20]. DEGs were obtained based on the corrected p-value ≤ 0.05. When SAM was performed with Excel, DEGs were generated with the median number of false positives less than 1. To perform hierarchical clustering analysis [1,21,22], a data matrix with DEGs only was extracted, row median-centered and column-standardized. Clustering analysis was also performed with R (2.10.0). A separate principal component analysis (PCA) was further performed on each DEG dataset using the R bio3d package. PCA plots on the first three components were reported, and a scree plot was reported showing the accumulated variability explained by the first three principal components.

Extraction of gene expression patterns

In order to show overall trends of the gene expression profile across the metaplasia phase, a pattern extraction method, the EPIG process, was applied [23]. Preprocessed and normalized data matrix and experimental design files were loaded into ExP software [24], the expression profile of each gene was compared exhaustively against all other genes, and statistically significant “profile patterns” were self-extracted and stored. Then the genes whose expression profiles supported the “profile patterns” were retained in their corresponding profile pattern gene lists and reported. Expression data matrices of the significant gene expression patterns obtained from EPIG were loaded into GeneSpring (Agilent Technologies) for pattern visualization.

Gene set analysis (GSA)

GSA was carried out using R (GSA package). Curated gene sets in three major categories - canonical pathway (CP; 880 gene sets), transcription factor targets (TF; 615 gene sets), and Gene Ontology (GO; C5, 1,454 gene sets) - were downloaded from the GSEA web portal and used in this study (http://www.broadinstitute.org/gsea/index.jsp). Both two-class unpaired and multi-class comparisons were implemented based on the experimental design. 100 permutations were applied to generate a null distribution for statistical testing, and significantly enriched gene sets were obtained at a false discovery rate cutoff of 0.05–0.5. To ensure the validity of the analysis, in addition to the recommended GSA analysis, each analysis was repeated 100 times and the gene sets that showed in <10% of the repetitive studies were excluded from the final report. When GSA was performed in Excel,
significantly enriched gene sets were obtained with a false discovery rate ≤0.5.

In addition, Fisher’s exact test was performed against ten knowledge-based gene sets. These knowledge-based gene sets were manually collected from the literature. These genes are related to the structure of keratinized stratified squamous epithelium (i.e., basal lamina, basal layer, granular layer, spinous layer and keratinized layer), the epidermal differentiation complex (EDC), P63 target genes, Sox2 target genes, Pax9 target genes, and Nrf2/Keap1 target genes (Excel S1). P-values were reported based on the hypergeometric distribution, and gene sets with p-value≤0.05 were reported as significantly enriched gene sets within the DEG list.

Analysis of archival data from the public database

Differential gene expression between E8.25 definitive endoderm and E11.5 esophagus has been studied previously using an Illumina Ambion microarray [8]. Raw microarray data of this study were downloaded from the Gene Expression Omnibus (GSE13040) under the accession numbers GSM326633–35 (E8.25) and GSM326642–44 (E11.5). Only probes which were significantly different from the background were used (p-value<0.05). To generate ratio data, the intensity of each probe on a single array was divided by the average intensity of the same probe on the rest of the arrays. Entrez ID was also used in Illumina Ambion microarray data. For probes without Entrez ID, GenBank accession numbers were used and then converted to Entrez ID.

Real-time PCR

cDNA was prepared from DNase-treated total RNA using the Advantage RT-for-PCR Kit (Clontech; Mountain View, CA). TaqMan® Gene Expression Assays (FAM™ dye-labeled) with pre-designed primers for each target gene were obtained from Applied Biosystems (Foster City, CA). The six target genes were: Pox9 (paired box gene 9, Assay ID: Mm00440629_m1); Calm4 (calmodulin 4, Assay ID: Mm00490975_s1); Shh (suprabasin; Assay ID: Mm00525057_m1); Ppard (peroxisome proliferator receptor delta, Assay ID: Mm0083184_m1); Pten (phosphatase and tensin homolog, Assay ID: Mm00477208_m1); Akt2 (thymoma viral proto-oncogene 2; Mm00206778_g1). 18S (18S ribosomal RNA)hypothetical LOC799064, Assay ID: Mm03928990_g1) was used as the endogenous control. Relative quantitative real-time PCR was performed using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) with SDS v2.3 software. The real-time data exported from RQ Manager 1.2 were further analyzed by DataAssist 3.0 (Applied Biosystems) to generate the RQ Plot.

Histochemical and immunohistochemical staining

Tissues were routinely processed for paraffin sectioning (5 μm). H&E staining was carried out using a standard protocol. For X-Gal staining, mouse esophagi were isolated and fixed in 4% paraformaldehyde at 4°C for 20 min on ice. Staining and subsequent sample processing were performed as previously described [4,6].

For immunohistochemical staining, the deparaffinized sections were submerged in methanol containing 0.3% hydrogen peroxide for 15 min at RT to inhibit endogenous peroxidase activity. Antigen retrieval was done prior to incubation with rabbit polyclonal anti-Nrf2 (#PA1-38312, 1:40; Thermo Scientific, Waltham, MA), or rabbit polyclonal anti-Pparβ/δ (#Ab5312, 1:1000; LifeSpan Biosciences, Seattle, WA), or rabbit polyclonal anti-pAkt(Ser473) (#3840, 1:25; Cell Signaling Technology, Danvers, MA), overnight at 4°C. Tissue sections were then washed again in PBS and incubated with peroxidase-conjugated secondary antibodies for 30 minutes at 37°C. Detection of the antibody complex was done using the streptavidin-peroxidase reaction kit with DAB as a chromogen (ABC kit; Vector Labs, Burlingame, CA). To ensure the specificity of the primary antibody, control tissue sections were incubated in the absence of primary antibodies.

Results

In this study, we divided the developmental process of mouse esophageal epithelium into three phases based on morphological changes (Figure 1A): (a) The specification phase is defined as the phase during which the definitive endoderm differentiates into the esophagus. Two time points, E8.25 and E11.5, were chosen to represent this phase. (b) The metaplasia phase is defined as the phase during which the simple columnar epithelium in the esophagus undergoes metaplastic changes (stratification, squamation and keratinization) into a keratinized stratified squamous epithelium. Four time points, E11.5, E15.5, P0 and P7, were selected to represent this phase. (c) The maturation phase is defined as the phase during which the keratinized stratified squamous epithelium continues to thicken and finally forms the esophageal epithelium in adults. Two time points, P7 and adult, were selected to represent this phase. In the maturation phase, the esophagus is covered by a simple columnar epithelium surrounded by a well-defined but undifferentiated mesenchyme at E11.5 (Figure 1B, F). At E15.5, it becomes stratified, consisting of ~3 cell layers, with well-defined submucosa and muscle (Figure 1C, G). At P0, epithelial cells lose columnar features and appear squamous. The esophagus is covered by a stratified squamous epithelium with 5–5 cell layers surrounded by a mesenchyme consisting of thicker muscle (Figure 1D, H). At P7, a keratinized layer has clearly formed at the surface of the epithelium, and the base membrane and submucosal papillae are well-formed (Figure 1E, I).

1. Gene expression profiles during the development of wild-type mouse esophagus

a. Specification phase. Two-class SAM analysis identified 1,612 genes up-regulated and 1,303 genes down-regulated in E11.5 esophagi as compared with E8.25 definitive endoderm (Excel S2). Hierarchical clustering analysis and PCA analysis showed that E8.25 definitive endoderm and E11.5 esophagus were clustered separately (Figure S1). Among the up-regulated genes, Ift6, Sox21, Nfib, Upk2, Hoxa3, Sox2, P63, Foxq1, Hoxa2, Hoxa4, Osx2, Enp1, Lhfp, Kiemen2, Twist1, Rarb, Hoxd4, Nfj2, Isf and Hox6 were reported exclusively or highly expressed in E11.5 esophagus as compared to other definitive endoderm-derived organs [8]. Furthermore, several signaling pathway-related genes, such as Nfj5 (TGFβ signaling), Shh and FoxA2 (Hedgehog signaling), and β-catenin (Wnt signaling), were up-regulated in E11.5 esophagus as compared with E8.25 definitive endoderm, suggesting these pathways were likely involved in esophageal specification.

GSA analysis identified multiple enriched gene sets in the categories of canonical pathway, gene ontology and transcription factor (Excel S2). For example, GO_629 (morphogenesis of an epithelium), GO_727 (epidermis development), and GO_1049 (ectoderm development) were enriched in E11.5 esophagus. However, using Fisher’s exact test, only P63 target genes were significantly different between E8.25 definitive endoderm and E11.5 esophagus (Excel S2).

b. Metaplasia phase. Multi-class SAM analysis identified 2,076 DEGs at this phase (Excel S3). Hierarchical clustering and
PCA analysis clearly showed that three samples at each time point were clustered together (Figure S2). As expected, E11.5 and E15.5 were separated from P0 and P7. Among the 2,076 DEGs, many genes are known to be involved in differentiation and function of keratinocytes; these processes include keratinization (Cbfal, Cnmd, Esp1, Fgfl10, Krt10, Krt17, Krt36, Krt79, Krt80, Krt91, Plk, Ptch1, Tgfβ2), gap junction (*Cda*, *Gjb2*, *Gjb3*, *Gja4* *Gja5*, *Gja6*), muscle development (*Myh12*, *Myh3*, *Myb1*, *Myml1*, *Myo2*, *Myo3*, *Myo1b*, *Myo5e*, *Mym1*, *Bmp4*), blood vessel development (*Tgfβ3*, *Tgfβ2*, *Fgfl10*, *Gcsln1*, *Edn1*, *Edn2*, *Eps11*, *Agf*), and neuron development (*Bdcf*, *Canog4*, *Dbp2*, *Dmd*, *Epha7*, *Ebb3*, *Hexb*, *Ntn5*).

Eighteen expression patterns were extracted from DEGs (Excel S4). Pattern 1 showed 763 genes up-regulated from E11.5 to P0, and Pattern 2 showed 369 genes down-regulated from E11.5 to P0. These genes were associated with metaplasia. Pattern 4 (188 genes), Pattern 11 (65 genes), Pattern 13 (33 genes), Pattern 15 (13 genes), and Pattern 16 (7 genes) were up-regulated from E11.5 to E15.5 and stayed at the same level or were down-regulated after E15.5. Genes in Pattern 5 (12 genes), Pattern 12 (15 genes), and Pattern 17 (5 genes) were down-regulated from E11.5 to E15.5 and stayed at the same level afterwards. These genes were probably involved in stratification of columnar epithelial cells. From E11.5 to P0, Pattern 3 (180 genes), Pattern 8 (40 genes), Pattern 10 (37 genes) and Pattern 15 were up-regulated, and Pattern 5 (79 genes), Pattern 11, Pattern 13 and Pattern 16 (7 genes) were down-regulated. These genes were probably involved in squamation. From P0 to P7, Pattern 6 (51 genes), Pattern 12 (18 genes), Pattern 14 (16 genes), and Pattern 18 (5 genes) were up-regulated, and Pattern 9 (18 genes), Pattern 10, Pattern 11, and Pattern 15 were down-regulated. These genes were probably involved in keratinization, as supported by the fact that genes in Pattern 1, Pattern 3, Pattern 4, Pattern 6 were generally up-regulated from E11.5 to P7. As expected, these genes (Muc4, Ppi, Arg1, Ocln, Bmp6, Tchh, Timp3, Les, Krt5, Spro5) were known to be associated with keratinized stratified squamous epithelium.

We collated ten knowledge-based gene sets from the literature (Excel S1). These gene sets are associated with differentiation of the skin, the esophagus and the tongue, all of which are covered by keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia. Fisher’s exact test of our data showed that nine gene sets were significantly associated with keratinized stratified squamous epithelia.
Table 1. Differential expression of knowledge-based gene sets in the mouse esophagus in the metaplasia phase.

| Samples | Knowledge-based gene set | No. of genes in the gene set | No. of genes in array dataset | No. of DEGs | P value |
|---------|--------------------------|------------------------------|-------------------------------|-------------|---------|
| Wild-type E11.5 vs E15.5 vs P0 vs P7 Basal lamina genes | 40 | 29 | 2 | 0.222 |
| Basal layer genes | 14 | 10 | 1 | 0.267 |
| Spinous layer genes | 11 | 7 | 0 | 1.000 |
| Granular layer genes | 16 | 10 | 1 | 0.267 |
| Keratinized layer genes | 39 | 14 | 4 | 6.9E-4 |
| EDC genes | 58 | 21 | 3 | 0.029 |
| Nrf2/Keap1 pathway genes | 281 | 181 | 31 | 1.8E-14 |
| P63 target genes | 59 | 39 | 4 | 0.031 |
| Pax9 target genes | 23 | 13 | 3 | 0.007 |
| Sox2 target genes | 141 | 80 | 6 | 0.036 |
| Wild-type vs Keap1−/− at P7 Basal lamina genes | 40 | 29 | 16 | 3.0E-7 |
| Basal layer genes | 14 | 10 | 4 | 0.041 |
| Granular layer genes | 16 | 10 | 4 | 0.043 |
| Spinous layer genes | 11 | 7 | 1 | 0.661 |
| Keratinized layer genes | 39 | 14 | 9 | 2.6E-5 |
| EDC genes | 58 | 21 | 9 | 0.001 |
| Nrf2/Keap1 pathway genes | 281 | 181 | 37 | 0.019 |
| P63 target genes | 59 | 39 | 21 | 8.9E-9 |
| Pax9 target genes | 23 | 11 | 9 | 1.1E-6 |
| Sox2 target genes | 141 | 80 | 20 | 0.008 |

Keap1−/− esophagi (Excel S6). In agreement with these data, hierarchical clustering analysis and PCA analysis clearly demonstrated separation of the Keap1−/− esophagi from others (Figure S4). Between wild-type and Nrf2−/− esophagi, only one gene (Rbm45) was up-regulated, and three genes including Nrf2 were down-regulated in Nrf2−/− esophagi. Between wild-type and Nrf2−/− Keap1−/− esophagi, 19 genes were up-regulated and 10 genes including Nrf2, Upk3a and Krt17 were down-regulated in Nrf2−/− Keap1−/− esophagi. In contrast, 309 genes were up-regulated and 346 genes down-regulated in Keap1−/− esophagi as compared with wild-type esophagi. Among the up-regulated genes, many classical Nrf2 target genes were enriched, such as Nqo1, Gelm, Gclc, Gits, Cat, Cyps, Mgs, Alhds, Cess and Abccs, indicating Nrf2 superactivity. Keratinization-related genes such as Spr2h, Krt84, Ptg2, Casp14 and Ppard were also up-regulated in Keap1−/− esophagi. These data suggest that although the Nrf2/Keap1 pathway was involved in the metaplasia phase as shown above, Nrf2−/− did not have any significant impact on gene expression in the esophagus. This observation may be explained by compensation by other genes or a low baseline activity in this phase. However, hyperactive Nrf2 due to Keap1−/− activated the Nrf2/Keap1 pathway in the esophagus, and hence up-regulated downstream target genes.

Among the 10 knowledge-based gene sets, Fisher’s exact test identified six gene sets significantly different in Keap1−/− esophagi as compared to wild-type esophagi: keratinized layer genes, EDC genes, P63 target genes, Pax9 target genes, Sox2 target genes and Nrf2/Keap1 pathway genes (Table 1). As expected, the Nrf2/Keap1 pathway genes were highly significant (p = 1.8 × 10^{-14}). The keratinized layer genes, EDC genes and Pax9 target genes were known to be associated with keratinization of stratified squamous epithelium. These data were consistent with the phenotype of esophageal hyperkeratosis in Keap1−/− mice.

An interesting question is why Keap1−/− mice developed esophageal hyperkeratosis. Two-class GSA analysis was performed to identify gene sets associated with the Keap1−/− esophagi as compared with wild-type esophagi. Among the enriched gene sets (Excel S6), the Nrf2/Keap1 transcription factors were significantly up-regulated in Keap1−/− esophagi (Nfe2, Nrf2, Srebp1), as well as Nrf2-relevant metabolism GO gene sets (GO_666, GO_1221, GO_1333, GO_1374, GO_1408, GO_1418) and canonical pathway gene sets (CP_29, CP_67, CP_68, CP_71, CP_429, CP_530, CP_625, CP_626). In addition to these, Ppar pathway (CP_80 and CP_101) and PI3K/Akt pathway (CP_629 and...
CP_435) were also up-regulated in Keap1−/− esophagi, suggesting potential roles of these pathways in superactive Nrf2-induced esophageal hyperkeratosis in Keap1−/− mice. Real-time PCR showed that Pparδ was up-regulated and Pten down-regulated in Keap1−/− esophagus, while keratinization-associated genes (Calm4 and Sbsn) were up-regulated (Figure S6).

Among three Ppar isoforms, Pparδ/b/d activation is known to cause terminal differentiation of keratinocytes [25], and Pparδ/b was up-regulated in Keap1−/− esophagi as compared with wild-type esophagi (Excel S6). Keratinocyte-specific deficiency of Pten caused Akt activation, and subsequently resulted in postnatal death due to esophageal hyperkeratosis [26]. Here we examined expression of Nrf2, Pparδ/b/d and pAkt in the P7 esophageal epithelium of wild-type and Keap1−/− mice. Consistent with the expression pattern reported in the literature [27], Nrf2 was found to translocate into the nuclei of esophageal epithelial cells in Keap1−/− mice (Figure 3E–F). Corresponding to Nrf2 activation, Pparδ/b and pAkt were also overexpressed in the cytoplasm and nuclei (Figure 3H, I, K, L). These data suggested that hyperactive Nrf2 might promote esophageal hyperkeratosis in Keap1−/− mice through activation of the Pparδ/b and PI3K/Akt pathway.

b. Maturation phase. Further analysis of adult wild-type and Nrf2−/− esophagi showed that 11 genes were up-regulated and 25 down-regulated (including Nrf2 and its target genes), in Nrf2−/− esophagi (Excel S7). Among these 25 genes, Aldh1b8, Nqo1, Gstm3, Nrf2, Gsta3, Gstm1 and Gcl are known as classical Nrf2 target genes. Hierarchical clustering and PCA analysis clustered wild-type and Nrf2−/− esophagi separately (Figure S5). Based on three lines of evidence, we concluded that Nrf2 was mainly involved in maturation phase from P7 to adulthood: (1) there was little difference in gene expression between wild-type and Nrf2−/− esophagus at P7; (2) Nrf2/Keap1 pathway genes were differentially expressed between P7 and adult esophagus of wild-type mice; (3) Nrf2/Keap1 pathway genes were differentially expressed between adult wild-type and Nrf2−/− mice. These genes are known to function in detoxification and anti-oxidative defense.

Discussion

This study clearly demonstrated a complex mechanism involving many genes and pathways at each phase during the development of mouse esophageal epithelium. There was a baseline activity of the Nrf2/Keap1 pathway in the metaplasia phase, and a higher activity in the maturation phase. Hyperactive Nrf2 in Keap1−/− mice resulted in esophageal hyperkeratosis, probably through activation of the Pparδ/b and PI3K/Akt pathway.

Our data were consistent with previous studies on mouse esophageal development. P63 and Sox2 were expressed in the mouse esophagus prior to E11.5, suggesting their critical roles in esophageal specification [6]. Pax9 was expressed in the mouse esophagus at E13.5 [28] and was essential for expression of...
multiple genes in the keratinized layer or EDC of mouse tongue [29]. The Wnt pathway promoted respiratory progenitor identity in the mouse foregut, and continuous activation of the Wnt pathway resulted in the reprogramming of esophagus and stomach to a lung endoderm progenitor fate [30,31]. This explains why the Wnt pathway became inactive in the esophagus later in the metaplasia phase (Figure 2). The NFκB pathway, especially IKKα, played an important role in keratinocyte differentiation [32]. Hedgehog pathway participated in esophageal development by signaling from the endoderm to the mesoderm [33,34]. Bmp pathway was inhibited between E10.5 and E14.5 to allow metaplasia to take place. After E14.5–E15.5, active Bmp signaling is required for further differentiation of esophageal epithelium [4].

Our main goal in this study was to determine the role of the Nrf2/Keap1 pathway in the development of esophageal epithelium. Using gene microarray analysis with wild-type mouse samples, we found that the Nrf2/Keap1 pathway was likely uninvolved in the specification phase (Excel S2). Starting from the metaplasia phase, there was a baseline activity of the Nrf2/Keap1 pathway. However, Nrf2−/− did not have a significant impact on gene expression and morphology of esophageal epithelium at P7 (Excel S6). We believe that the Nrf2/Keap1 pathway is mainly involved in the development of esophageal epithelium in the specification phase (Excel S5). As compared with wild-type adult mice, Nrf2−/− reduced expression of multiple downstream genes whose major functions are detoxification and anti-oxidative defense (Excel S7).

It is unknown why hyperactive Nrf2 in Keap1−/− mice caused hyperkeratosis of the esophageal epithelium at P7. Similar to the esophagus, the skin was also hyperkeratic in Keap1−/− mice [15], suggesting similar mechanisms of hyperkeratosis in the skin and the esophagus. Mechanistically, Nrf2 is known to regulate Krt16/Krt17 expression through MAP kinases [35]. In this study, GSA analysis identified two potential candidate pathways responsible for hyperkeratosis: Ppar signaling and PI3K/Akt pathway (Excel S6). Although Pparγ is a direct transcriptional target of Nrf2 [36], Pparβ/δ is more likely to be the isoform involved among the three Ppar isoforms because Pparβ/δ agonists were known to cause terminal differentiation of keratinocytes in vitro [25,37] and dermal hyperkeratosis in vivo [38]. While Pparβ/δ−/− inhibited epidermal keratinization, transgenic overexpression promoted epidermal hyperkeratosis [39,40]. Several Nrf2 target genes (Add1α, Gtm3, Gto1, Gstad1, Add1α1) were also known to be regulated by Pparβ/δ [41]. In this study, we confirmed overexpression of Pparβ/δ in Keap1−/− esophageus relative to wild-type esophageus at P7. Adult esophageus also expressed a higher level of Pparβ/δ than P7 esophageus, which is less keratinized (Figure 3 H, I, J). These data supported the hypothesis that Keap1−/− might produce esophageal hyperkeratosis through activation of Pparβ/δ.

Table 2. Pathway changes in the three phases of mouse esophageal development.

| Signaling pathway | Developmental phase |
|-------------------|---------------------|
|                   | Specification       | Metaplasia | Maturation |
| Wnt               | ↑                   | ↑         | ↓         |
| Hedgehog          | ↑                   | ↓         |           |
| TGFβ              | ↑                   | ↓         |           |
| BMP6              | ↓                   | ↑         |           |
| NFκB              | ↑                   | ↓         |           |
| Notchb            | ↑                   |           |           |
| Nrf2/Keap1        | ↑                   |           |           |

Note: ↑ and ↓ indicate up- or down-regulation, respectively.
Reference [4].
Reference [43].
doi:10.1371/journal.pone.0036504.t002

In some of these cases, for example, retinoic acid is known to inhibit Nrf2 [45]. Vitamin A deficiency may cause Nrf2 hyperactivity and esophageal hyperkeratosis. In addition to a mechanistic understanding of human esophageal disease, manipulation of the Nrf2/Keap1 pathway may provide a novel way of enhancing the protective barrier of the esophageal epithelium. The keratinized layer is the major protective layer against physical stress and chemical injuries [46]. Terminally differentiated keratinocytes express proteins which can provide protection by quenching reactive oxygen species [47]. In fact, sulforyphane, a chemical activator of Nrf2, restores skin integrity in an epidermolysis bullosa simplex model (created by Kit5 or Kit14 mutation) by activating Kit7 expression [48]. Similarly, Pparβ/δ activation can also enhance the epidermal permeability barrier [25,38].

This study has many potential implications for future studies. Several developmental pathways involved in esophageal development were found to be active at an early time point and then became inactive later on (Table 2). However, these pathways are known to be involved in esophageal diseases such as Barrett’s esophagus and esophageal cancer, suggesting that tight spatiotemporal regulation of these pathways is critical for both development and disease [49,50,51,52,53,54]. Further understanding of these pathways during development will shed light on molecular mechanisms of esophageal diseases.

Supporting Information

Figure S1 Hierarchical clustering analysis and PCA analysis of gene expression array data of wild-type mouse definitive endoderm (E8.25) and esophagi (E11.5): (A) clustering analysis; (B) PCA analysis. (TIF)

Figure S2 Hierarchical clustering analysis and PCA analysis of gene expression array data of wild-type mouse esophagi (E11.5, E15.5, P0, P7): (A) clustering analysis; (B) PCA analysis. (TIF)

Figure S3 Real-time PCR analysis of mRNA expression in wild-type mouse esophagei: relative mRNA levels of Pax9 and its target genes (Shox, Calm4) in mouse esophageal epithelium of E15.5, P0, P7 and adult mice. (TIF)
PCA analysis. Sbsn gene sets (E11.5 vs E15.5 vs P0 vs P7).

expression array data after data pre-processing (E11.5, E15.5, P0 during wild-type mouse esophageal development: (1) Raw gene expression array data after data pre-processing (E8.25 endoderm vs E11.5 esophagus). Differential gene expression in the specification phase

Differential gene expression in the maturation phase during the development of wild-type mouse esophageal epithelium: (1) Raw gene expression array data after data pre-processing (P7 and adult esophagus); (2) SAM analysis of differentially expressed genes (P7 vs adult esophagus); (3) GSA analysis of differentially expressed gene sets (P7 vs adult esophagus); (4) Fisher’s exact test of knowledge-based gene sets (P7 vs adult esophagus).

Differential gene expression at P7 between wild-type mice, Nrf2−/− mice, Keap1−/− mice, and Nrf2−/− Keap1−/− mice: (1) Raw gene expression array data after data pre-processing (wild-type P7, Nrf2−/− P7, Keap1−/− P7, Nrf2−/− Keap1−/− P7); (2) SAM analysis of differentially expressed genes (wild-type P7 vs Nrf2−/− P7 vs Keap1−/− P7 vs Nrf2−/− Keap1−/− P7); (3) GSA analysis of differentially expressed gene sets (wild-type P7 vs Keap1−/− P7).

Hierarchical clustering analysis and PCA analysis of gene expression array data of mouse esophagi (wild-type adult, Nrf2−/− adult); (A) clustering analysis; (B) PCA analysis.

Hierarchical clustering analysis and PCA analysis of gene expression array data of mouse esophagi (wild-type, Nrf2−/−, Keap1−/−, Nrf2−/− Keap1−/−); (A) clustering analysis; (B) PCA analysis. (TIF)

Real-time PCR analysis of mRNA expression in wild-type and Keap1−/− mouse esophagi; relative mRNA levels of Pase9, Skiln, Calm4, Ppard, Pen and Akt2 in the whole esophagi of wild type and Keap1−/− mice at P7. (TIF)

Differential gene expression in the specification phase during wild-type mouse esophageal development: (1) Raw gene expression array data after data pre-processing (E8.25 endoderm and E11.5 esophagus); (2) SAM analysis of differentially expressed genes (E8.25 endoderm vs E11.5 esophagus); (3) GSA analysis of differentially expressed gene sets (E8.25 endoderm vs E11.5 esophagus); (4) Fisher’s exact test of knowledge-based gene sets (E8.25 endoderm vs E11.5 esophagus). (XLSX)

Differential gene expression in the maturation phase during wild-type mouse esophageal development: (1) Raw gene expression array data after data pre-processing (E11.5, E15.5, P0 and P7); (2) SAM analysis of differentially expressed genes (E11.5 vs E15.5 vs P0 vs P7); (3) GSA analysis of differentially expressed gene sets (E11.5 vs E15.5 vs P0 vs P7). (XLSX)

Differential gene expression of eighteen gene expression patterns and nine knowledge-based gene sets in the metaplasia phase. (XLSX)

Differential gene expression analysis and PCA analysis of gene expression array data of P7 mouse esophagi (wild-type, Nrf2−/−, Keap1−/−, Nrf2−/− Keap1−/−); (A) clustering analysis; (B) PCA analysis. (TIF)

Hierarchical clustering analysis and PCA analysis of gene expression array data of mouse esophagi (wild-type adult, Nrf2−/− adult); (A) clustering analysis; (B) PCA analysis. (TIF)

Hierarchical clustering analysis and PCA analysis of gene expression array data of mouse esophagi (wild-type adult, Nrf2−/− adult); (A) clustering analysis; (B) PCA analysis. (TIF)

Hierarchical clustering analysis and PCA analysis of gene expression array data of mouse esophagi (wild-type adult, Nrf2−/− adult); (A) clustering analysis; (B) PCA analysis. (TIF)

Differential gene expression analysis and PCA analysis of gene expression array data of P7 mouse esophagi (wild-type, Nrf2−/−, Keap1−/−, Nrf2−/− Keap1−/−); (A) clustering analysis; (B) PCA analysis. (TIF)

Hierarchical clustering analysis and PCA analysis of gene expression array data of mouse esophagi (wild-type adult, Nrf2−/− adult); (A) clustering analysis; (B) PCA analysis. (TIF)

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