Comparative Analysis of Gene Expression Patterns for Oral Epithelium-Related Functions with Aging

J. L. Ebersole, L. Orraca, M. J. Novak, S. Kirakodu, J. Gonzalez-Martinez, and O. A. Gonzalez

Introduction

The majority of agents that cause infections in humans gain access through the mucosal surfaces of the body. As such, the epithelium and epithelial cells have evolved to provide an array of features to protect from pathogenic challenge. These include barrier functions in which the epithelial cells rapidly mature and are sloughed from the surface while maintaining tight junctions enhancing exclusion of deleterious agents at luminal surfaces (Parrish 2017; Yu et al. 2012). In addition to these mechanical barriers, recent evidence has supported the capacity of epithelial cells to constitutively synthesize an array of innate immune protective molecules, as well as a range of cell communication factors providing an "early warning system" to the host inflammatory and immune armamentarium (Pardo-Camacho et al. 2018; Partida-Rodriguez et al. 2017; Ahluwalia et al. 2017). Moreover, a number of these protective signaling molecules are induced through engagement of microbial-associated molecular patterns (e.g., MAMPs, PAMPs) and danger-associated molecular patterns (DAMPs) (Rajaee et al. 2018; Patel 2018; De Lorenzo et al. 2018; Stocks et al. 2018; Walsh et al. 2013; Olive 2012). The functions of the epithelial cells continue to emerge as critical determinants of maintaining host integrity from challenge with pathogenic bacteria, viruses, and fungi, which includes receptor recognition and engagement resulting in specific intracellular signaling pathways leading to antimicrobial activities in the local mucosal environment (Jin and Weinberg 2018; Guncu et al. 2015; Sukhithasri et al. 2013; Ho et al. 2013; McCormick and Weinberg 2010).

The oral cavity is somewhat unique in the properties of its epithelium. While other mucosal

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G. N. Belibasakis et al. (eds.), *Oral Mucosal Immunity and Microbiome*, Advances in Experimental Medicine and Biology 1197, https://doi.org/10.1007/978-3-030-28524-1_11

sites in the body consider it a substantial benefit to maintain the integrity of the barrier function, in the oral cavity, the epithelium is routinely deliberately breached from about 6 months to 21 years of age with eruption of the deciduous and permanent dentition. This developmental anomaly of innate immune protection has fostered the development of a unique junctional epithelium that covers connective tissue cells and a collagen matrix that attaches the erupted teeth to the underlying alveolar bone. This junctional epithelial lining of the subgingival sulcus, in health, is attached to the cementoenamel junction of the teeth (Tsukamoto et al. 2012; Hatakeyama et al. 2006; Bosshardt and Lang 2005). Interestingly, in health, this junctional epithelium is somewhat leaky and allows that passage of a low protein fluid transudate in the gingival crevice that mechanically aids in rinsing colonizing bacteria into the saliva, which is swallowed approximating 1 L/day. Accompanying accumulation of bacterial deposits supra- and subgingivally, the gingival tissue reacts with an inflammatory response with the classic signs of acute inflammation. This inflammation, termed gingivitis, is considered a reversible process that responds rapidly to removal of the bacterial insult (Tonetti et al. 2015; Chapple et al. 2015). An inability to clear this stimulus can lead to a persistent immunoinflammatory lesion, i.e., periodontitis, with ulceration of the epithelium, influx of an array of inflammatory cells, breakdown of connective tissue and collagen, vasculitis, and net resorption of alveolar bone at the localized site of the microbial challenge (Tonetti et al. 2015). While substantial strides are being made in the area of tissue regeneration to reestablish normal function for the periodontium following disease, periodontitis remains considered as irreversible once tissue destruction has occurred.

Age-dependent variations in epithelial barrier function have been previously described in different tissues (e.g., skin, lung, intestine, and kidney) of humans and animal models. A common finding is an impaired cell–cell adhesion mediated by tight junctions consistent with aging-increased permeability (Parrish 2017). Additionally, this decline in epithelial barrier function and repair seems to be associated with an alteration in epithelium stem cells niches (Doles et al. 2012; Moorefield et al. 2017). Nevertheless, the molecular mechanisms associated to these observations remain unclear. Thus, as the epithelial cells and functions of the epithelium are critical to the health of the oral cavity, we used a nonhuman primate model to profile the transcriptome of gingival tissues in health across the lifespan. It was hypothesized that in younger animals, epithelial genes related to functions of a more rigid, less developmentally flexible tissue would be decreased, enabling these young animals to respond to the microbial burden by enhanced signaling pathways associated with rapid wound healing, anti-inflammatory/inflammation resolution, maintaining an effective barrier. In contrast, in older animals, these patterns would differ creating epithelial cells highly responsive to the surrounding environment and less able to modulate and resolve the noxious challenge from the bacteria in the absence of some collateral damage of the periodontal tissues and enhancing the long-term risk for initiation and progression of periodontitis.

Methods

Nonhuman Primate Model and Oral Clinical Evaluation

Rhesus monkeys (*Macaca mulatta*) (n = 23; 10 females and 13 males) housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, Puerto Rico, were used in these studies. Healthy animals (5–7/group) were distributed by age into four groups: ≤3 years (young), 3–7 years (adolescent), 12–16 years (adult), and 18–23 years (aged). The nonhuman primates are typically fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad). The diet is supplemented with fruits and vegetables, and water is provided ad libitum in an enclosed corral setting.

A protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico enabled anesthetized animals to be examined for clinical

measures of periodontal including probing pocket depth (PD) and bleeding on probing (BOP) as we have described previously (Ebersole et al. 2008).

Tissue Sampling and Gene Expression Microarray Analysis

A buccal gingival sample from healthy tissues from the premolar/molar maxillary region of each animal was taken using a standard gingivectomy technique and maintained frozen in RNAlater solution. Total RNA was isolated from each gingival tissue using a standard procedure as we have described, and tissue RNA samples submitted to the microarray core to assess RNA quality analyze the transcriptome using the GeneChip® Rhesus Macaque Genome Array (Affymetrix) (Meka et al. 2010; Gonzalez et al. 2011). Individual samples were used for gene expression analyses.

Data Analysis

Normalization of values across the chips was accomplished through signal intensity standardization across each chip using Affymetrix PLIER algorithm. The GeneChip® Rhesus Macaque Genome Array contained matched and mismatched pairs allowing the MAS 5 algorithm to be used. For each gene, we first determined differences in expression across the groups using ANOVA (version 9.3, SAS Inc., Cary, NC). The healthy-aged tissues were then compared among the age groups using a *t*-test and accepting a *p*-value ≤ 0.05 for significance. Because of the cost of these types of nonhuman primate experiments and availability of primates of the various ages, we did not have sufficient samples to identify if the relationship between age and gene expression could be treated using a linear model; thus, the subjects were classified and ANOVA was used for analysis. Correlations with aging and clinical parameters in healthy tissues were determined using a Spearman Rank correlation analysis. A *p*-value ≤ 0.05 was used to evaluate the significance of the correlation. The data have been uploaded to http://www.ncbi.nlm.nih.gov/geo/info/submission.html.

Results

Epithelium Gene Transcriptome in Healthy Gingival Tissues

Using the microarray results, we examined 336 genes that are linked to epithelium and epithelial cells functions (Table 1). The set of genes were categorized into 9 broad functional groups: extracellular matrix and cell structure; extracellular matrix remodeling enzymes; cell adhesion molecules, cytoskeleton regulation; inflammatory response; growth factors; kinases/cell signaling; cell surface receptors; junction associated molecules; autophagy/apoptosis; antimicrobial peptides; and transcription factors.

Figure 1a–d summarizes the level of expression of genes in which the normalized signal was >100 in gingival tissues from any of the 4 groups of animals that included 255 genes. From these data, we identified a group of genes that were altered in younger and aged animals when compared to expression levels in the adult tissues, which included selected extracellular matrix components (e.g., KRT2, KRT4, MMP1, MMP9, TIMP1, F13A1, SERPINF1, CTSK, FBN1, LAD1, CHI3L1), cytoskeleton regulators (e.g., ACTN1, TAGLN, ZYX, DES), cell surface receptors and adhesion molecules (e.g., SELL, ICAM2, ITGAL, SPP1, ITGB2, ITGA8, SELP, ITGAM, ICAM1, ITGAX), and host response genes (e.g., PPBP, CAMP, DEFB4, CXCL11).

Aging Effects on Epithelium Gene Transcriptome

Within the subset of 255 genes, Fig. 2a–c provides volcano plot visualization of the distribution of altered responses and significant differences in the young, adolescent, and aged animals versus healthy adult levels that were considered to be the normal expression level. From these analyses, it appeared that a lower number of genes were significantly different in the young

Table 1 Targeted gene for functions of epithelium

Gene ID	Product, Fxn Group	Gene ID	Product, Fxn Group	Gene ID	Product, Fxn Group
COL17A1	Collagen, ECM structure	ITGB1	Integrin, cell adhesion	RIPK1	Ser/thr, kinases
COL1A1	Collagen, ECM structure	ITGB2	Integrin, cell adhesion	VPS13A	Vacuolar protein, kinases
COL1A2	Collagen, ECM structure	ITGB3	Integrin, cell adhesion	GSK3B	Glycogen synthase, kinases
COL3A1	Collagen, ECM structure	ITGB4	Integrin, cell adhesion	AGER	Glycation end products, receptors
COL5A1	Collagen, ECM structure	ITGB5	Integrin, cell adhesion	CD36	Scavenger, receptors
COL7A1	Collagen, ECM structure	ITGB6	Integrin, cell adhesion	CD44	Hyaluronic acid, receptors
FBLN5	Fibulin, ECM structure	LGALS3	Galectin, cell adhesion	CD59	C′ mediated lysis, receptors
FBN1	Fibrillin, ECM structure	MSN	Moesin, cell adhesion	EGFR	Epidermal growth factor, receptors
FN1	Fibronectin, ECM structure	PVRL1	Poliovirus receptor related, cell adhesion	ESR1	Estrogen, receptors
HSPG2	Heparin sulfate proteoglycan, ECM structure	PVRL2	Poliovirus receptor related, cell adhesion	F2R	Thrombin, receptors
KRT1	Keratin, ECM structure	PVRL3	Poliovirus receptor related, cell adhesion	IL9R	Interleukin, receptors
KRT10	Keratin, ECM structure	PVRL4	Poliovirus receptor related, cell adhesion	PECAM1	Platelet/endothelial receptors
KRT12	Keratin, ECM structure	SELL	Selectin, cell adhesion	PROCR	Protein C, receptors
KRT13	Keratin, ECM structure	SELP	Selectin, cell adhesion	THBD	Thrombomodulin, receptors
KRT14	Keratin, ECM structure	SPP1	Secreted phosphoprotein, cell adhesion	TNFRSF1A	TNF family, receptors
KRT15	Keratin, ECM structure	VTN	Vitronectin, cell adhesion	TNFRSF6B	TNF family, receptors
KRT16	Keratin, ECM structure	VWF	Von Willebrand factor, cell adhesion	TRAF1	TNF associated, receptors
KRT17	Keratin, ECM structure	ACTN1	Actin, cytoskeleton regulators	TRAF2	TNF associated, receptors
KRT18	Keratin, ECM structure	ACTN2	Actin, cytoskeleton regulators	CDSN	Corneodesmosin, junction proteins
KRT19	Keratin, ECM structure	ACTN3	Actin, cytoskeleton regulators	DSC1	Desmocollin, junction proteins
KRT2	Keratin, ECM structure	ACTN4	Actin, cytoskeleton regulators	DSC2	Desmocollin, junction proteins
KRT20	Keratin, ECM structure	ATP2C1	ATPase secretory pathway, cytoskeleton regulators	DSC3	Desmocollin, junction proteins
KRT23	Keratin, ECM structure	ATP2C2	ATPase secretory pathway, cytoskeleton regulators	DSG1	Desmoglein, junction proteins
KRT24	Keratin, ECM structure	CCDC19	Cilia/flagella associated protein, cytoskeleton regulators	DSG2	Desmoglein, junction proteins
KRT25	Keratin, ECM structure	DNM1	Dynamin, cytoskeleton regulators	DSG3	Desmoglein, junction proteins
KRT27	Keratin, ECM structure	ENTPD1	EctoATPase, cytoskeleton regulators	DSP	Desmoplakin, junction proteins
KRT28	Keratin, ECM structure	FLNA	Filamin, cytoskeleton regulators	EVPL	Envoplakin, junction proteins
KRT3	Keratin, ECM structure	FLNB	Filamin, cytoskeleton regulators	F11R	F11 receptor, junction proteins

KRT35	Keratin, ECM structure	PDGFRB	Platelet-derived growth factor receptor, cytoskeleton regulators	GJA1	Gap junction, junction proteins
KRT37	Keratin, ECM structure	RAC1	Ras family GTPase, cytoskeleton regulators	GJA3	Gap junction, junction proteins
KRT38	Keratin, ECM structure	SMURF1	Ubiquitin ligase, cytoskeleton regulators	GJA4	Gap junction, junction proteins
KRT4	Keratin, ECM structure	STX5	Syntaxin, cytoskeleton regulators	GJA5	Gap junction, junction proteins
KRT5	Keratin, ECM structure	TAGLN	Transgelin, cytoskeleton regulators	GJA8	Gap junction, junction proteins
KRT6A	Keratin, ECM structure	TIAM1	T-cell lymphoma invasion/metastases, cytoskeleton regulators	GJB1	Gap junction, junction proteins
KRT6B	Keratin, ECM structure	TLN1	Talin, cytoskeleton regulators	GJB2	Gap junction, junction proteins
KRT6C	Keratin, ECM structure	TLN2	Talin, cytoskeleton regulators	GJB3	Gap junction, junction proteins
KRT7	Keratin, ECM structure	VCL	Vinculin, cytoskeleton regulators	GJB4	Gap junction, junction proteins
KRT71	Keratin, ECM structure	WAS	Wiscott-Aldrich syndrome, cytoskeleton regulators	GJB5	Gap junction, junction proteins
KRT72	Keratin, ECM structure	WASF1	Wiscott-Aldrich syndrome, cytoskeleton regulators	GJC2	Gap junction, junction proteins
KRT73	Keratin, ECM structure	WASL	Wiscott-Aldrich syndrome, cytoskeleton regulators	GJC3	Gap junction, junction proteins
KRT74	Keratin, ECM structure	ZYX	Zyxin, cytoskeleton regulators	GJD2	Gap junction, junction proteins
KRT75	Keratin, ECM structure	ALOX5	Lipoxygenase, inflammation	JAM2	Junctional adhesion, junction proteins
KRT76	Keratin, ECM structure	APOH	Apolipoprotein, inflammation	JAM3	Junctional adhesion, junction proteins
KRT77	Keratin, ECM structure	CCL2	MCP-1, inflammation	JAM3	Junctional adhesion, junction proteins
KRT78	Keratin, ECM structure	CCL5	RANTES, inflammation	JUP	Plakoglobin, junction proteins
KRT79	Keratin, ECM structure	CCL7	MCP-3, inflammation	MAGI1	Guanylate kinase, junction proteins
KRT8	Keratin, ECM structure	CXCL10	IP-10, inflammation	MAGI2	Guanylate kinase, junction proteins
KRT80	Keratin, ECM structure	CXCL11	I-TAC, inflammation	OCLN	Occludin, junction proteins
KRT84	Keratin, ECM structure	CXCL17	DC/monocyte chemokine, inflammation	PKP1	Plakophilin, junction proteins
KRT85	Keratin, ECM structure	CXCL2	MIP-2α, inflammation	PKP2	Plakophilin, junction proteins
KRT9	Keratin, ECM structure	CXCL5	ENA-78, inflammation	PKP3	Plakophilin, junction proteins

(continued)

Table 1 (continued)

Gene ID	Product, Fxn Group	Gene ID	Product, Fxn Group	Gene ID	Product, Fxn Group
LAD1	Ladinin, ECM structure	IKBKB	NFαB inhibitor, inflammation	PKP4	Plakophilin, junction proteins
LAMA3	Laminin, ECM structure	IL1RN	IL-1 receptor antagonist, inflammation	PLEC1	Plectin, junction proteins
LAMA5	Laminin, ECM structure	IL23A	Cytokine, inflammation	PNN	Pinin, junction proteins
LAMB3	Laminin, ECM structure	LIF	Leukemia inhibitory factor, inflammation	PPL	Periplakin, junction proteins
LAMC2	Laminin, ECM structure	NFKB1	NFκB, inflammation	TJAP1	Tight junction associated, junction proteins
PRELP	Prolargin proteoglycan, ECM structure	NFKBIA	NFκB, inflammation	TJP1	Tight junction, junction proteins
SPARC	Osteonectin, ECM structure	OSM	Oncostatin M, inflammation	TJP2	Tight junction, junction proteins
VCAN	Versican, ECM structure	PTGS2	Cox2, inflammation	ATG10	Autophagy/apoptosis
VIM	Vimentin, ECM structure	TNF	Tumor necrosis factor, inflammation	ATG12	Autophagy/apoptosis
CHI3L1	Chitinase, ECM remodeling	ARHGEF2	Microtubule regulated, growth factors	ATG13	Autophagy/apoptosis
CTSG	Cathepsin, ECM remodeling	BMP1	Bone morphogenetic protein, growth factors	GABARAP	ATG8A, autophagy/apoptosis
CTSK	Cathepsin, ECM remodeling	BMP2	Bone morphogenetic protein, growth factors	GABARAPL2	ATG8C, autophagy/apoptosis
ELA2	Elastase, ECM remodeling	BMP7	Bone morphogenetic protein, growth factors	BCL2	Apoptosis regulator, autophagy/apoptosis
F13A1	Coagulation factor XIII, ECM remodeling	CTGF	Connective tissue, growth factors	BIRC2	Apoptosis inhibitor, autophagy/apoptosis
F3	Thromboplastin, ECM remodeling	EGF	Epidermal, growth factors	BIRC3	Apoptosis inhibitor, autophagy/apoptosis
LOX	Lysyl oxidase, ECM remodeling	FGF10	Fibroblast, growth factors	CASP3	Caspase, autophagy/apoptosis
MMP1	Matrix metalloproteinase, ECM remodeling	FGF7	Fibroblast, growth factors	DAXX	Death domain, autophagy/apoptosis
MMP2	Matrix metalloproteinase, ECM remodeling	GNG11	G-protein, growth factors	FAS	Death receptor, autophagy/apoptosis
MMP7	Matrix metalloproteinase, ECM remodeling	PPBP/CXCL7	Connective tissue, growth factors	PERP	TP53 effector, autophagy/apoptosis
MMP9	Matrix metalloproteinase, ECM remodeling	PPP2CA	Microtubules, growth factors	CAMP	Cathelicidin, AMPs
PLAT	Plasminogen activator, ECM remodeling	PTEN	Tumor suppressor, growth factors	DEFA1	Defensins, AMPs
PLAU	Plasminogen activator, ECM remodeling	PTP4A1	Phosphatase, growth factors	DEFA4	Defensins, AMPs

PLAUR	Plasminogen activator receptor, ECM remodeling	RHOA	Ras homolog, growth factors	DEFA5	Defensins, AMPs
PLOD1	Lysyl hydroxylase, ECM remodeling	TGFB1	Transforming, growth factors	DEFA6	Defensins, AMPs
PLOD2	Lysyl hydroxylase, ECM remodeling	TGFB2	Transforming, growth factors	DEFB1	Defensins, AMPs
SERPINE1	PAI-1, ECM remodeling	TGFB3	Transforming, growth factors	DEFB103A	Defensins, AMPs
SERPINF1	Alpha-2 antiplasmin, ECM remodeling	TMEFF1	EGF-like, growth factors	DEFB104A	Defensins, AMPs
SERPINF2	Alpha-2 antiplasmin, ECM remodeling	TSPAN13	Tetraspanin, growth factors	DEFB105A	Defensins, AMPs
TIMP1	Metallopeptidase inhibitor, ECM remodeling	VEGFA	Vascular, growth factors	DEFB106A	Defensins, AMPs
CAV1	Calveolin, cell adhesion	WISP1	Connective, growth factors	DEFB108B	Defensins, AMPs
CAV2	Calveolin, cell adhesion	WNT5B	Adipogenesis, growth factors	DEFB118	Defensins, AMPs
CAV3	Calveolin, cell adhesion	AKT1	Ser/thr, Kinases	DEFB119	Defensins, AMPs
CDH1	Cadherin, cell adhesion	CHUK	IKK-α, kinases	DEFB121	Defensins, AMPs
CDH2	Cadherin, cell adhesion	CSNK2A1	Casein, kinases	DEFB122	Defensins, AMPs
CDH3	Cadherin, cell adhesion	CSNK2A2	Casein, kinases	DEFB123	Defensins, AMPs
CDH4	Cadherin, cell adhesion	DBF4	Zinc finger, kinases	DEFB125	Defensins, AMPs
CDH5	Cadherin, cell adhesion	JAG1	Notch signaling, kinases	DEFB126	Defensins, AMPs
CTNNA1	Catenin, cell adhesion	MAP2K1	Mitogen activated, kinases	DEFB127	Defensins, AMPs
CTNNA2	Catenin, cell adhesion	MAP2K3	Mitogen activated, kinases	DEFB129	Defensins, AMPs
CTNNA3	Catenin, cell adhesion	MAP2K4	Mitogen activated, kinases	DEFB132	Defensins, AMPs
CTNNAL1	Catenin, cell adhesion	MAP2K6	Mitogen activated, kinases	DEFB4	Defensins, AMPs
CTNNB1	Catenin, cell adhesion	MAP2K7	Mitogen activated, kinases	GZMA	Granzyme, AMPs
CTNNBIP1	Catenin, cell adhesion	MAP3K1	Mitogen activated, kinases	PLA2G2A	Phospholipase, AMPs
CTNNBL1	Catenin, cell adhesion	MAP3K14	Mitogen activated, kinases	PLUNC	Palate/lung/nasal, AMPs
CTNND1	Catenin, cell adhesion	MAP3K5	Mitogen activated, kinases	CEBPA	Leu zipper, transcription factors
CTNND2	Catenin, cell adhesion	MAPK1	Mitogen activated, kinases	EHF	ETS homologous, transcription factors

(continued)

Table 1 (continued)

Gene ID	Product, Fxn Group	Gene ID	Product, Fxn Group	Gene ID	Product, Fxn Group
DES	Desmin, cell adhesion	MAPK13	Mitogen activated, kinases	ETS1	Proto-oncogene, transcription factors
ICAM1	Intracellular adhesion, cell adhesion	MAPK14	Mitogen activated, kinases	JAK3	Janus kinase, transcription factors
ICAM2	Intracellular adhesion, cell adhesion	MAPK3	Mitogen activated, kinases	JUNB	Proto-oncogene, transcription factors
ITGA1	Integrin, cell adhesion	MAPK8	Mitogen activated, kinases	KRAS	Proto-oncogene, transcription factors
ITGA2	Integrin, cell adhesion	NOTCH1	Transmembrane EGF, kinases	MITF	Melaninogenesis, transcription factors
ITGA3	Integrin, cell adhesion	NOTCH2	Transmembrane EGF, kinases	RAF1	Proto-oncogene, transcription factors
ITGA4	Integrin, cell adhesion	NOTCH3	Transmembrane EGF, kinases	TCF3	Ig, transcription factors
ITGA5	Integrin, cell adhesion	NOTCH4	Transmembrane EGF, kinases	TWIST1	bHLH, transcription factors
ITGA6	Integrin, cell adhesion	PIK3C3	Lipid, kinases	ZEB1	Zinc finger homeobox, transcription factors
ITGA7	Integrin, cell adhesion	PIK3R4	Lipid, kinases	ZEB2	Zinc finger homeobox, transcription factors
ITGA8	Integrin, cell adhesion	PRKAA1	AMP activated, kinases		
ITGA9	Integrin, cell adhesion	PRKAA2	AMP activated, kinases		
ITGAL	Integrin, cell adhesion	PRKCG	PKC, kinases		
ITGAM	Integrin, cell adhesion	PRKCZ	PKC, kinases		
ITGAV	Integrin, cell adhesion	PRKD1	PKD, kinases		
ITGAX	Integrin, cell adhesion	PTK2	Tyr, kinases		

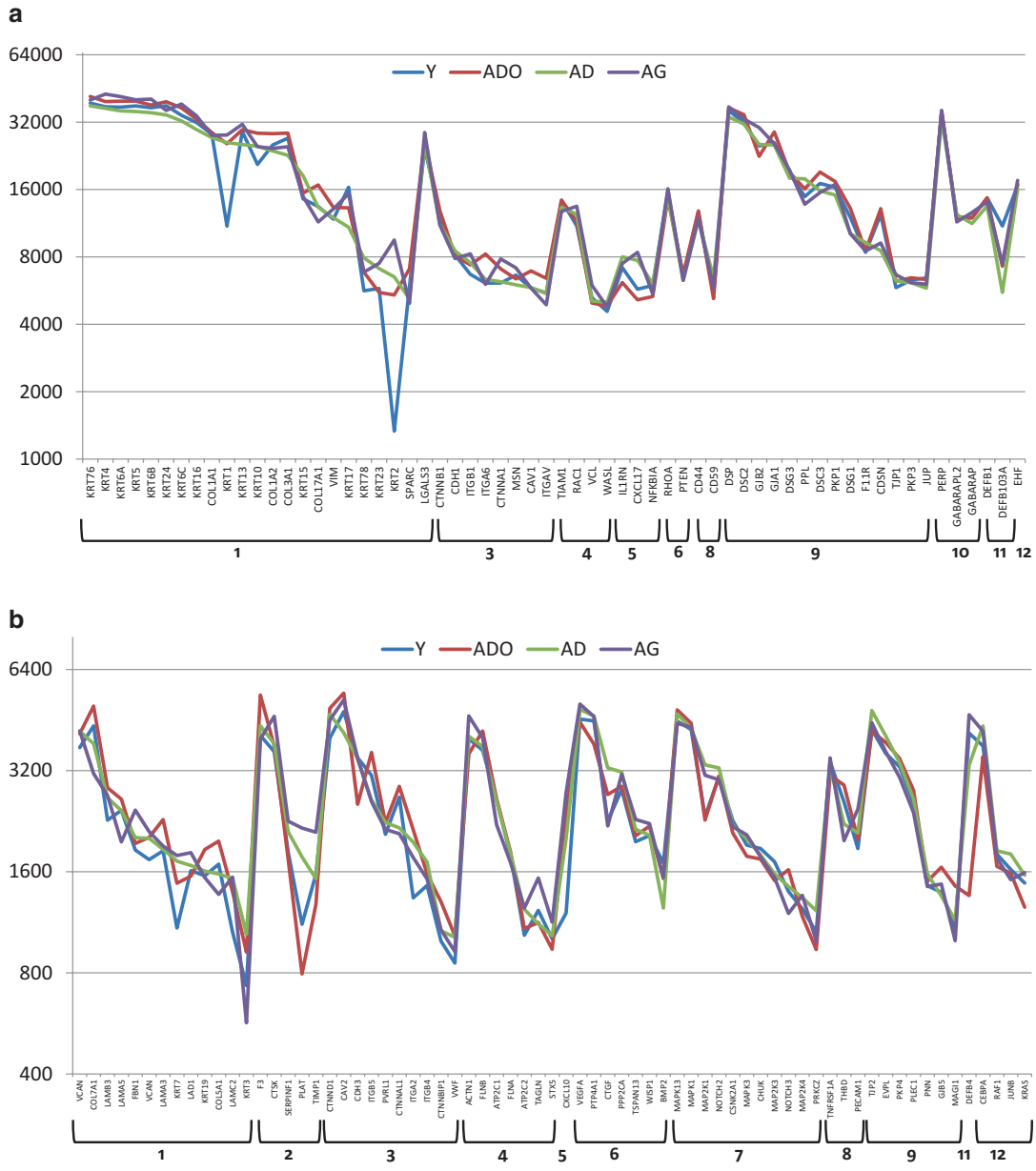

Fig. 1 (**a**–**d**): Gene expression levels in gingival tissues reflecting epithelium/epithelial cell functions. The lines represent the mean normalized signal level for each age group on animals. The genes are stratified into general functional categories and grouped in the graphs based upon the magnitude of signal (1: extracellular matrix components; 2: extracellular matrix enzymes; 3: cell adhesion molecules; 4: cytoskeleton regulators; 5: inflammatory cytokines/chemokines; 6: growth factors; 7: kinases/cell signaling; 8: cell surface receptors; 9: junction associated proteins; 10: autophagy/apoptosis; 11: antimicrobial molecules; 12: transcription factors)

animals versus the other groups, where 10–20% of the genes varied from healthy adult tissues.

Interrogating this dataset more specifically, Fig. 3 provides a heatmap representation of the fold increase or decrease in gene expression in healthy young, adolescent, and aged tissues compared to adults. Additionally, the genes were classified into 9 categories across their range of functions for the epithelium and epithelial cells. The results showed that the extracellular matrix

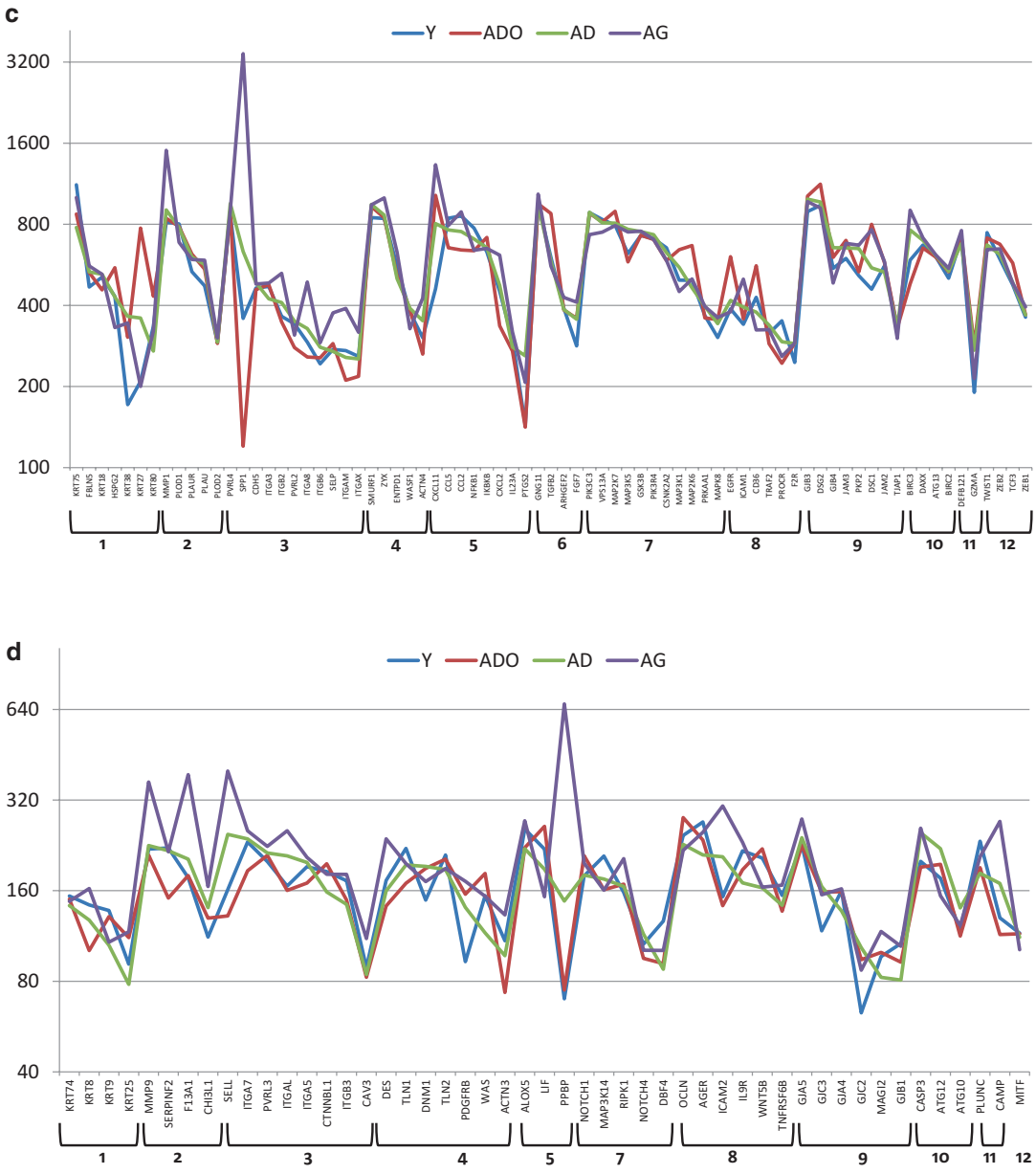

Fig. 1 (continued)

and cell structure, cell adhesion molecules, and cell surface receptor categories appeared to be most frequently different. Several extracellular matrix structural and cell adhesion molecules were elevated in the aged tissues, with generally decreased levels in the young tissue samples. The cell surface receptors were, generally, increased across the different age groups versus the adult levels. Also of interest was the lack of effect on the array of molecules related to epithelium junctions, transcription factors, kinases, and genes linked to autophagy/apoptosis. Of note, specific genes such as SPP1 and PPBP showed and aging-related increase. Table 2 provides a pathway analysis to assess biologic processes that were enriched in the set of genes that were significantly

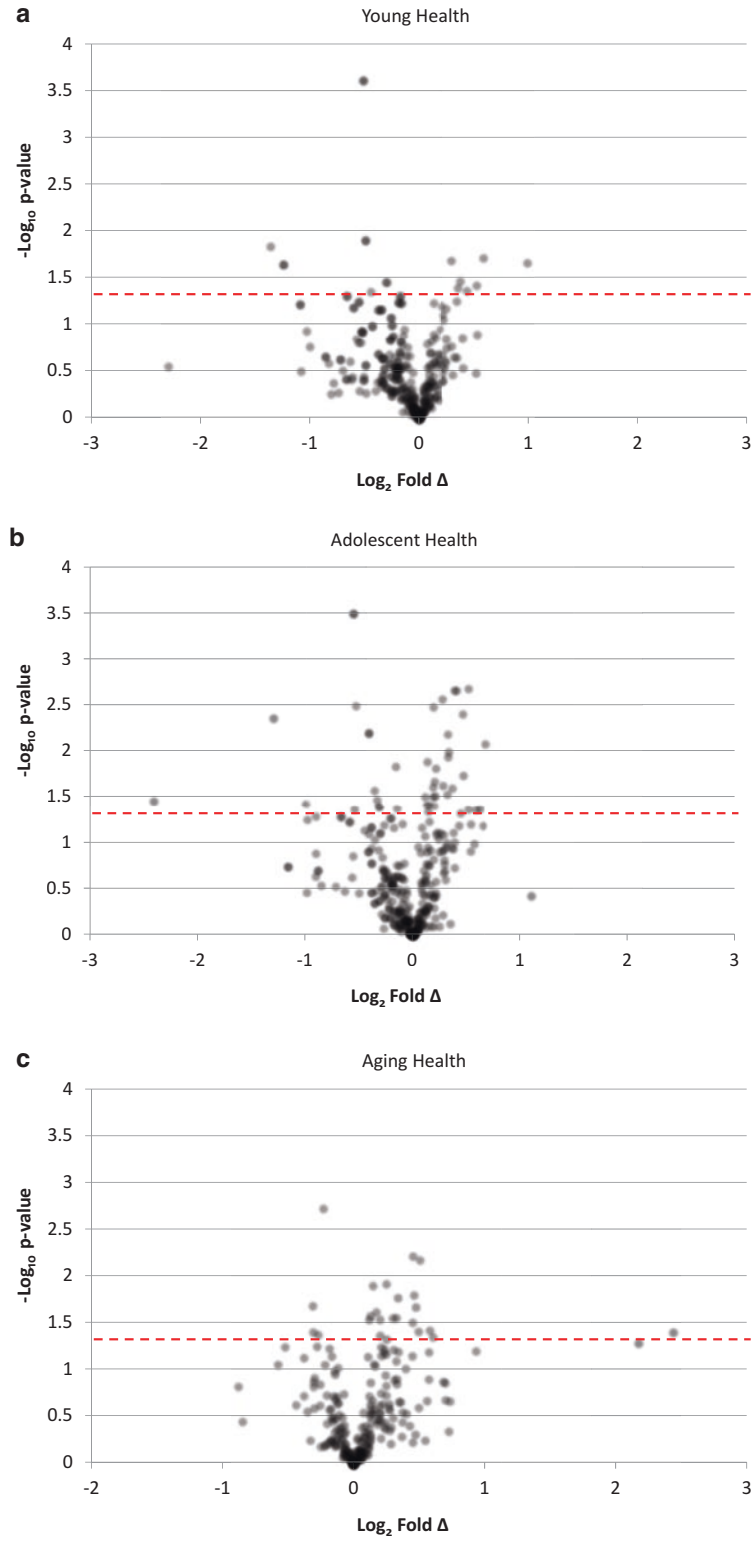

Fig. 2 (**a**–**c**) Volcano plots of gene expression levels in young, adolescent, and aged animals compared to the healthy adult tissue levels. Each point denotes a gene related to fold and statistical difference from adult levels. The red dashed line signifies a *p*-value <0.05

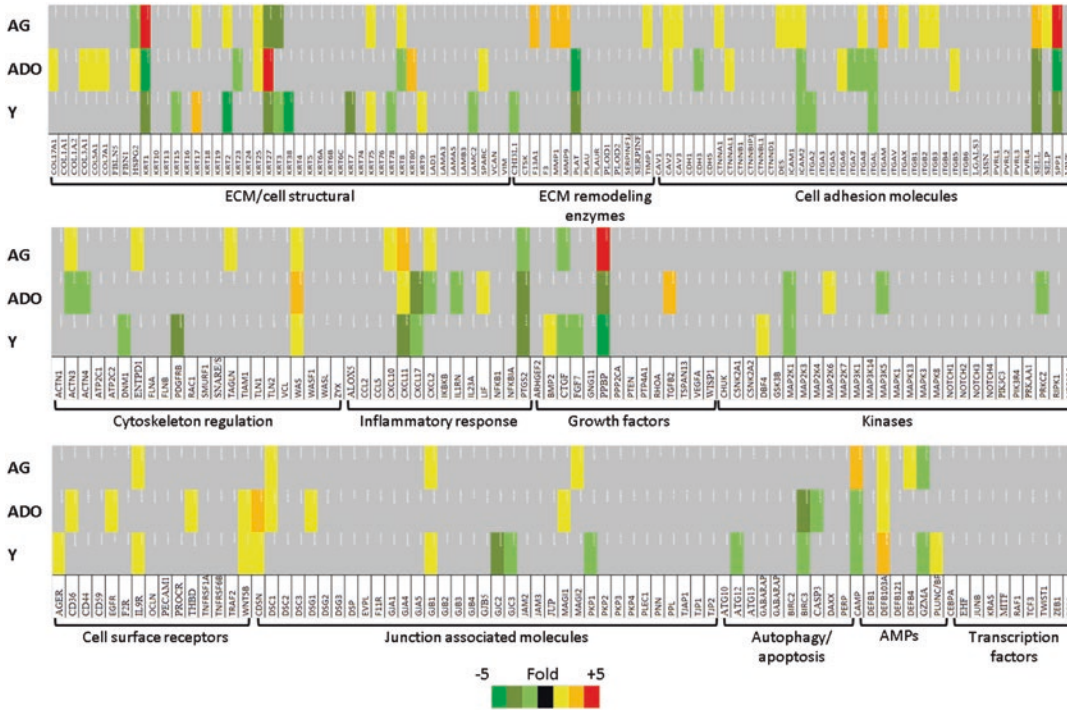

Fig. 3 Heatmap of fold differences in gene expression in young, adolescent, and aged animals compared to health adults. Genes are grouped into the 9 major categories, and the coloration reflects the mean differences in gene levels for the age group

Table 2 Major pathways of epithelial gene up-regulation in healthy gingival tissues

PANTHER biological processes	*M. mulatta* Genome #	Tissue #	Expected	Fold enrichment	Raw*P*-value	FDR
Cell–matrix adhesion	46	5	0.16	31.16	9.18E−07	5.06E−05
Cell adhesion	308	18	1.07	16.75	4.50E−17	1.10E−14
Cell–cell adhesion	143	7	0.50	14.03	9.09E−07	7.39E−05
MAK cascade	333	7	1.16	6.03	1.79E−04	5.47E−03
Signal transduction	2088	21	7.28	2.88	6.37E−06	3.11E−04
Cell communication	2399	21	8.37	2.51	5.16E−05	1.80E−03
Regulation of phosphate metabolic processes	509	8	1.78	4.51	4.24E−04	1.15E−02
Cell differentiation	503	7	1.75	3.99	1.96E−03	4.78E−02
Developmental process	1412	16	1.93	3.25	2.54E−05	1.03E−03

and/or >1.25-fold-regulated. As was seen in the heatmap, cell–matrix, cell–cell adhesion, and differentiation were enriched. While the heatmap did not provide a clear visualization of alterations in MAPK signaling pathway genes, these were enriched in the pathway analysis evaluation.

Figure 4a and b focuses on the details of altered expression of the array of keratins that are critical for epithelial cell functions. The results showed that approximately 20 of the keratins were expressed at high levels in the gingival tissues. Keratins 2, 5, 6B, 13, 16, 17 were all significantly increased in healthy-aged tissues versus adults. In contrast, keratins 1 and 2 were significantly decreased and keratin 17 increased in tissue from young animals compared to healthy adults. An additional set of molecules critical for communication of the epithelial cells are the

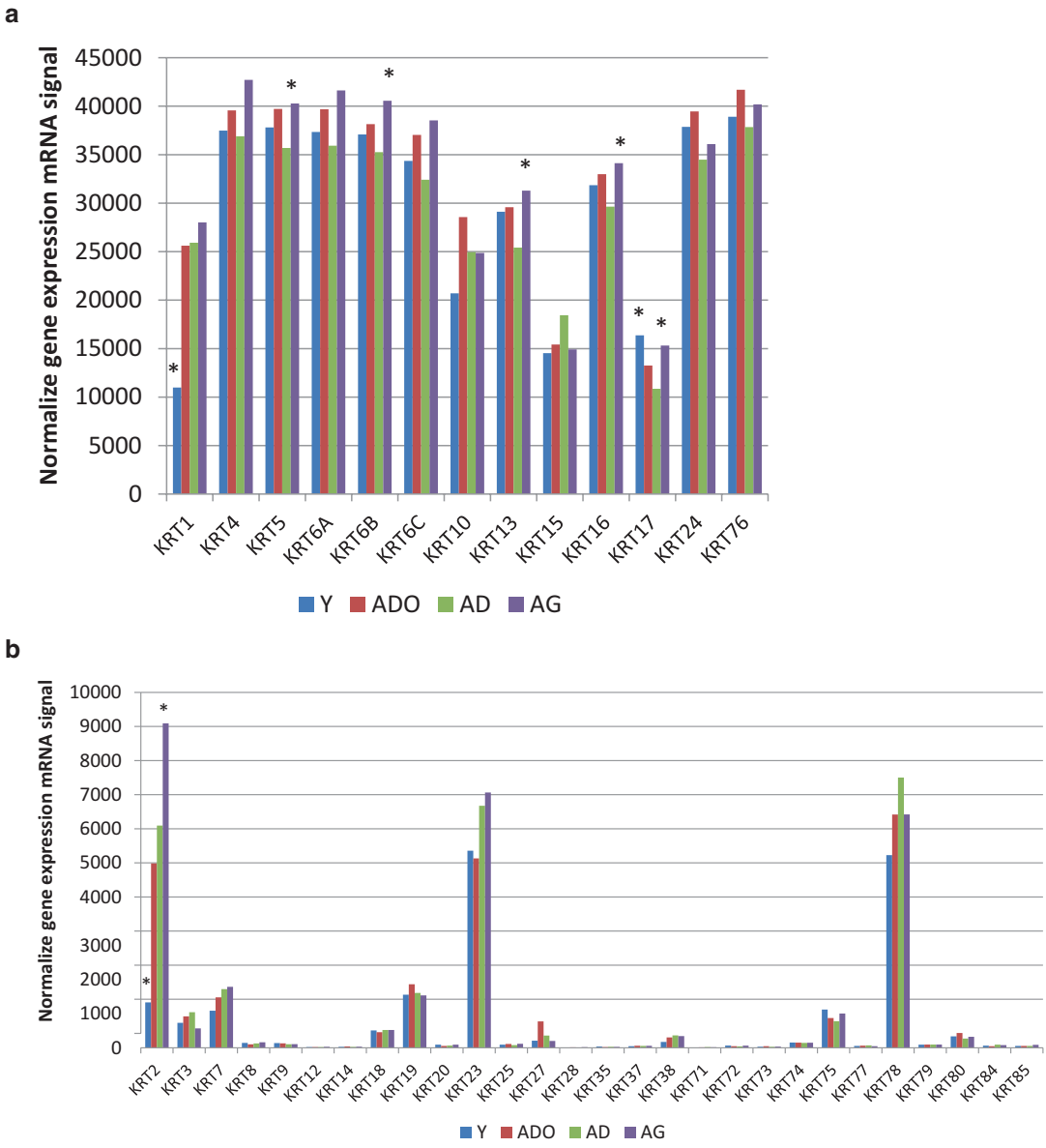

Fig. 4 (**a** and **b**) Normalized gene expression levels for keratins in the 4 age groups. The bars denote mean group levels. The asterisk (∗) signifies statistically different than other groups at $p < 0.05$

array of integrin surface receptors. Figure 5 provides an overview of these response profiles across the age groups. Approximately 15 of these integrins are highly expressed in the gingival tissues across the age groups. Only ITGA8, ITGAM (CD11b), and ITGB2 were significantly increased in the aged tissues compared to adults, with no difference in the younger animals. ITGB2 is a component portion of integrins that bind ICAMs, VCAM, and even complement components. ITGAM/ITGB2 is particularly implicated in interactions of monocytes, macrophages, and granulocytes and the uptake of complement-coated particles. Thus, while these integrins can be related to epithelial cell biology, their role in these complex oral tissues may be more related to

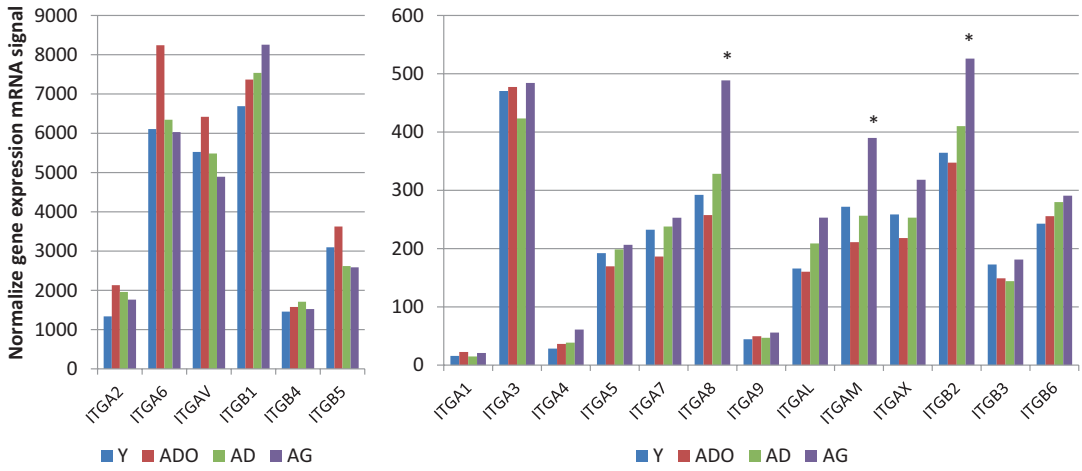

Fig. 5 Normalized gene expression levels for integrins in the 4 age groups. The bars denote mean group levels. The asterisk (∗) signifies statistically different than other groups at $p < 0.05$

the physiologic inflammation of the gingiva and reflect tissue maintenance by inflammatory cell responses in these tissues. Lastly, we focused on the array of biomolecules related to epithelial junctions including desmosomal and hemidesmosomal proteins (Fig. 6a, b). As was noted from the heatmap, few of these proteins were significantly altered across the age groups, with only CDSN (corneodesmosin) being increased in younger animals versus adults, and COL7A1 (collagen) and LAMA5 (laminin) decreased in the aged animal tissues.

The data were also analyzed beyond an age categorization (young, adolescent, adult, aged) by evaluating correlations of the gene expression profiles with age as a continuous variable (Fig. 7a). The results demonstrated about 10% of the genes demonstrated significant correlations ($p < 0.01$) with similar numbers positively and negatively correlated. While those positively correlated genes represented a range of functions, of interest was the number of collagen and integrin genes that were significantly decreased with aging even in healthy tissues. Figure 7b, c provides a similar type of assessment, relating gene profiles to clinical features of the periodontium in the healthy animals (bleeding on probing—BOP; mean probing pocket depth—PPD). In contrast to the correlations with age, fewer relationships were observed with either of the clinical parameters, with only PLAU, SMURF1, and MAP 3 K5 genes positively correlated and KRT17 and BMP2 negatively correlated with both BOP and PPD.

Discussion

Within the paradigms of gingivitis and periodontitis that affect the global population, there remain some observations that have yet to be understood at the molecular level. First, while gingivitis is generally considered to presage to periodontal lesions, identified populations have long-standing, florid gingival inflammation and never progress to periodontitis (Loe et al. 1986; Lang et al. 2009). Second, many cases of localized aggressive periodontitis that tend to occur in younger individuals associated with infection with *Aggregatibacter actinomycetemcomitans* demonstrate substantial rapid localized bone loss in the absence of gross inflammatory changes in the gingival tissues (Kinane and Hodge 2001; Jenkins and Papapanou 2001; Bimstein et al. 2002). Third, in children and adolescents, there is a high incidence of gingivitis that increases in prevalence and severity through puberty, in the absence of progressing to periodontitis (Albandar and Tinoco 2002; Modeer and Wondimu 2000; Bimstein et al. 2013; Bimstein and Ebersole 1989).

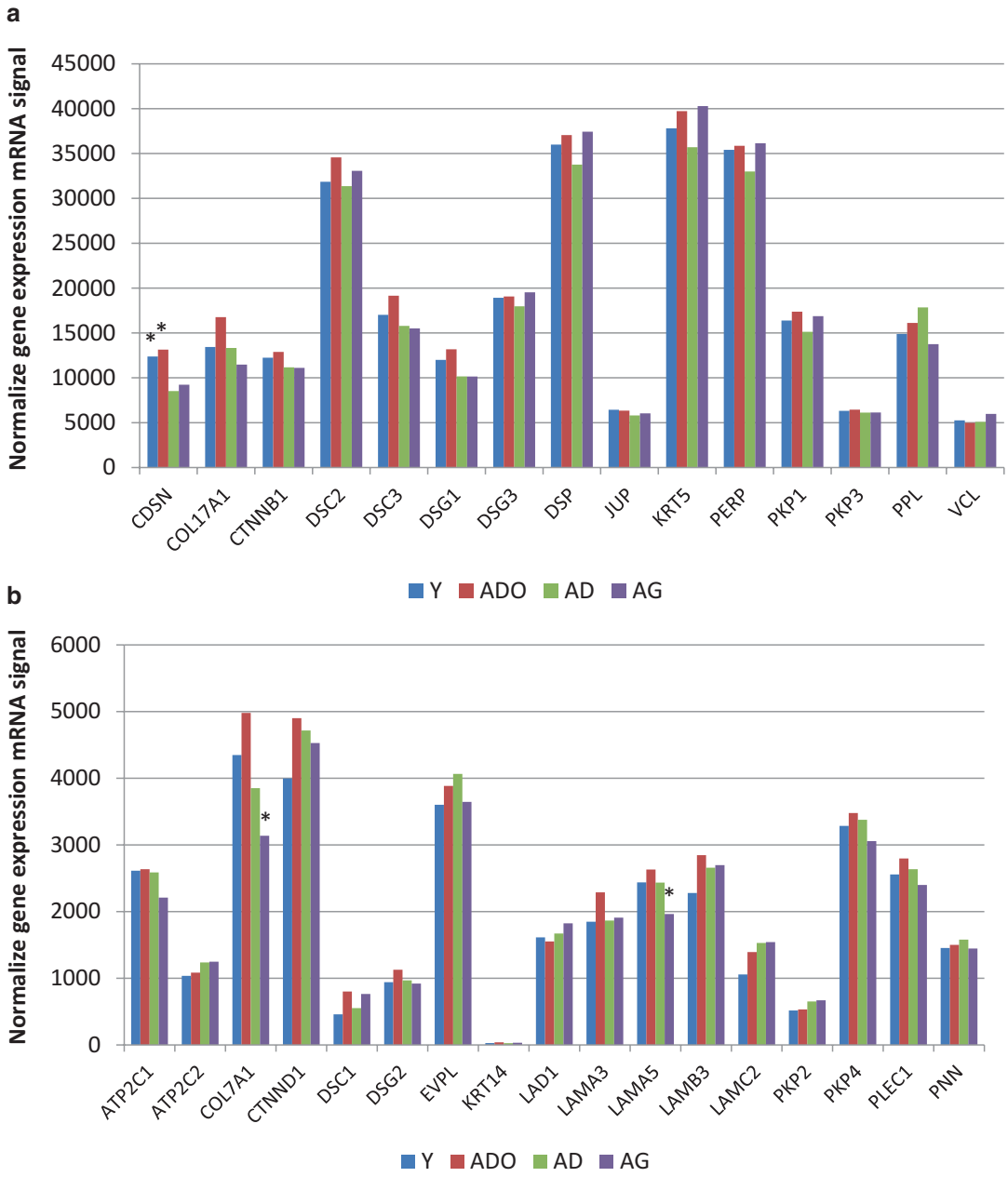

Fig. 6 (**a** and **b**) Normalized gene expression levels for desmosomal and hemidesmosomal genes involved in cell junction formation in the 4 age groups. The bars denote mean group levels. The asterisk (∗) signifies statistically different than other groups at $p < 0.05$

Fourth, during pregnancy, subsets of women can develop rather severe pregnancy-associated gingivitis that has been suggested to be linked to hormonal changes that could influence the oral microbial ecology, although there remains sparse data documenting the molecular features of this unique gingivitis that does not progress to periodontitis (Gumus et al. 2016; Gursoy et al. 2014; Barak et al. 2003). Finally, periodontitis has long been described as a disease of aging with sub-

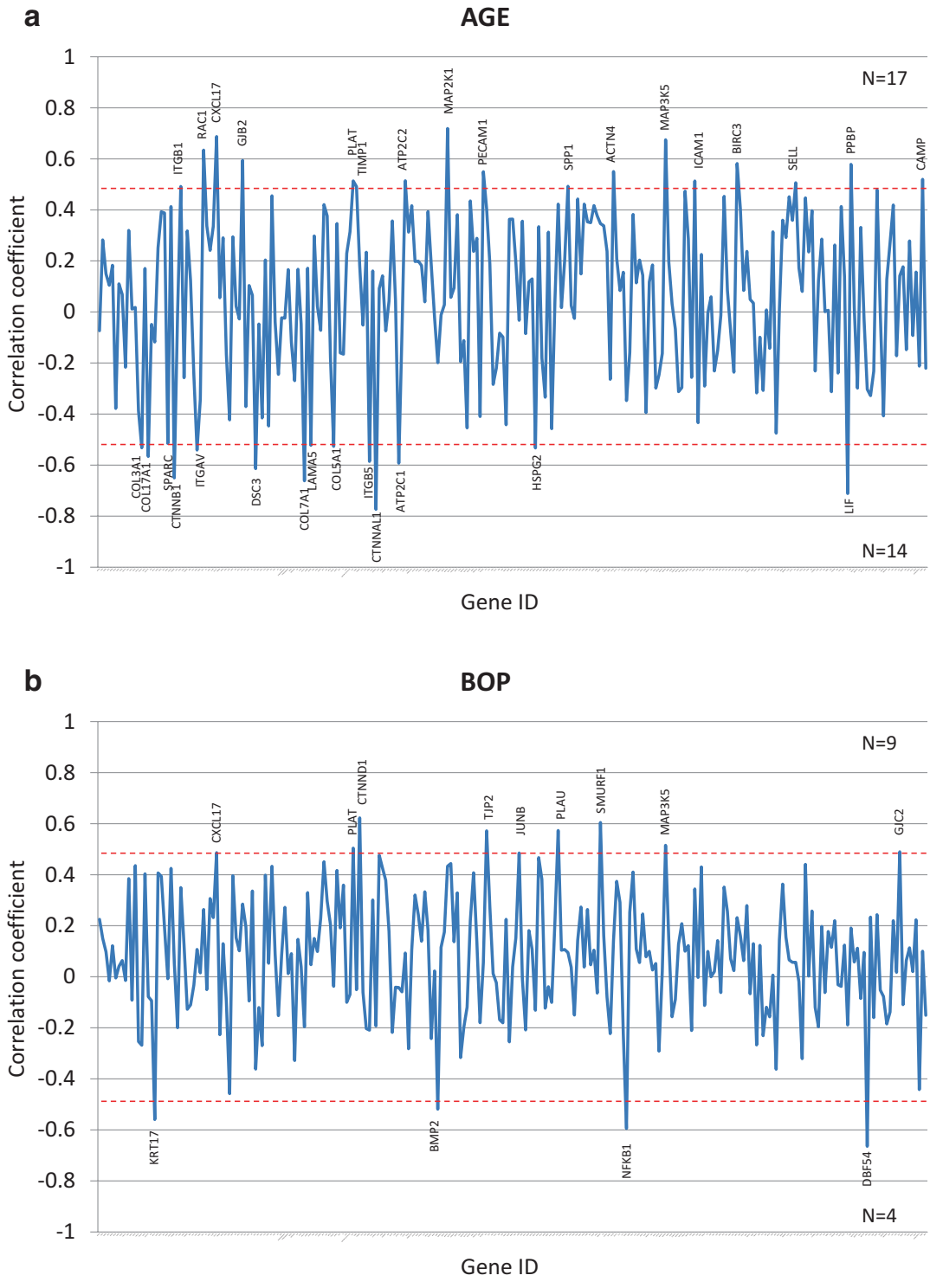

Fig. 7 Correlation analysis of gene expression levels with age (**a**) and clinical parameters of mean bleeding on probing (**b**) and mean probing pocket depth (**c**). The red dashed lines denote significance level at $p < 0.01$

stantial increases in incidence and severity in aging populations, and thought to be related to a lifetime accumulation of noxious challenge to the gingival tissues (Papapanou and Susin 2017; Wu et al. 2016; Lamster et al. 2016; Hajishengallis 2014; Huttner et al. 2009). Thus, there remains a need to better understand the underlying molecular biology of the range of cells in gingival tissues and how their functions can dictate variation in disease expression across the lifespan.

This study used a nonhuman primate model to focus in the biology of the epithelium and epithelial cells in gingival tissues to test a hypothesis that alterations in the transcriptome representing a range of functions of these cells/tissues would be altered with aging even in clinically healthy sites. We had previously reported on rather dramatic changes in various immune and inflammatory cells in gingival tissues in this model. There were clear alterations even in healthy-aged tissues with regard to lymphocyte classes (Ebersole et al. 2014, 2016a), apoptosis (Gonzalez et al. 2011, 2013), macrophage function and antigen recognition and presentation (Gonzalez et al. 2014, 2015, 2018), hypoxia (Ebersole et al. 2018), and inflammasome characteristics (Ebersole et al. 2016b). However, while there were some differences in the epithelial-related gene expression profiles in periodontal health with aging, the number of genes affected with a fold-change >1.25 was only about 30% and only 8% >1.5-fold. These alterations were also focused on a more limited functional activity of the epithelium/epithelial cells with extracellular matrix structural components, cell adhesion molecules, and cell surface receptors appearing to be most greatly affected.

Drilling down into these categories, multiple collagen and keratin gene levels were lower in young versus aged tissues, which were confirmed with correlation analysis related to aging. These findings suggested that these altered structural components in healthy aging could either reflect a physiological adaptation with aging that helps to maintain healthy tissues, or potentially these changes reflect altered epithelium characteristics that could increase the risk for initiation of periodontitis. Clear histopathological results demonstrate a breakdown in epithelium integrity accompanying the chronic inflammation of periodontitis (Bosshardt and Lang 2005; Dale 2002; Van der Velden 1984). It is accepted that these microulcerations enhance access of the microbiome components (e.g., bacteria, bacterial structures) into deeper tissues contributing to activating the local inflammatory response responsible for tissue destruction. Additionally, this process is considered as part of the feature allowing bacteria to traverse the gingival tissues and enter into the systemic circulation (Cardoso et al. 2018; Abbayya et al. 2015; Maddi and Scannapieco 2013; Kumar 2013). However, examination of the genes related to cell–cell interactions and cell–matrix interactions (desmosomes, hemidesmosomes) did not show a substantial impact of aging on the expression of these molecules. Thus, how these patterns reflect aging processes in health and risk for disease remains ill-defined, and further studies will be required to discriminate between these options.

An array of genes for cell adhesion molecules including cadherins, integrins, caveolins, and selectins were increased with aging. These molecules are critical for maintaining homeostasis of the epithelium in the septic environment of the oral cavity. Thus, since the tissue samples were from clinically healthy sites in the aged animals, this type of response profile may signify an effective healthy aging process in the tissues from these animals. Of note, remarkably elevated gingival expression levels of SPP (osteopontin) and PPBP (pro-platelet basic protein:CXCL7:NAP-2) were observed with aging. SPP has been shown to play important roles in wound healing seemingly through inhibiting apoptosis and modulating the expression of MMPs (Icer and Gezmen-Karadag 2018). From an epithelial cell function viewpoint, PPBP as a heterodimer with other chemokines is involved in glycosaminoglycan interactions with cells via the CXCR2 receptor (Brown et al. 2017). It is a chemoattractant for neutrophils and has some antimicrobials activities. This chemokine has been associated with the pathogenesis of chronic diseases, such as cancer and arthritis (Yeo et al. 2016; Desurmont et al. 2015). It is also identified as one of a group of

platelet-associated chemokines that were systemically elevated in patients with antiphospholipid syndrome (Patsouras et al. 2015), which has also been linked to the microbiome in periodontitis (Schenkein et al. 2003). Finally, a recent study by Shusterman et al. (2017) combining data from murine studies and an existing human dataset identified a gene cluster of platelet factor 4 (PF4:CXCL4)/PPBP/CXCL5 (neutrophil activating peptide 78: ENA-78) being significantly associated with aggressive periodontitis. These variations are consistent with previous reports demonstrating the persistence of inflammatory cells in diseased gingiva that may results from decreased apoptotic responses and/or enhanced transmigration of neutrophils into the inflammatory lesion with aging (Gonzalez et al. 2013; Wael Youssef 2018; Xia et al. 2017; Zhang et al. 2016; Jang et al. 2015; Sakai et al. 1999). Since this study showed elevations in "clinically healthy" aging tissues, there is a potential that this profile describes an enhanced risk of exhibiting disease initiation in the aged individuals.

While considerable effort has been delivered in attempting to delineate the microbiome and host response parameters that drive the disease process, there remains much less information defining, at the molecular level, what tissue responses are required to help maintain health. Recently, understanding the characteristics of the bacteria that constitute a healthy microbiome and the metabolic functions for these commensal bacteria has come under increasing scrutiny as both an explanatory variable in determining the population variation in disease and as a potential therapeutic target for more biologically oriented treatment strategies (Nassar et al. 2017; Ebersole et al. 2017; Hajishengallis and Lamont 2016; Lamont and Hajishengallis 2015; Wade 2013). However, much less is known regarding the host features controlling the periodontal microbiome in health. As an example, there is limited literature that the expression of various epithelial genes/proteins can be regulated by microbial biofilms and that members of the "red complex" can alter components of the epithelial junctions, particularly desmosomal components (Belibasakis et al. 2015). However, if age-associated alterations in these epithelial functions can affect the characteristics of the subgingival microbiome in moving from health to disease related remains unknown.

As noted, our previous examination of the gingival transcriptome in healthy nonhuman primates with aging, as well as with naturally occurring periodontitis demonstrated significant differences in gene profiles that supported innate and adaptive immune responses, inflammation, and cellular senescence changes occur in aging gingival tissues even when clinically healthy. These findings suggested that a basis for increased periodontitis in the human population with age may be linked to inherent changes in the biology of the gingival tissues during aging decreasing the capacity of the tissues to respond to local environmental changes, including alterations in the pathogenic capacity of the microbiome (Belibasakis 2018). The findings from this study suggested some changes in the functional activities of the epithelium and epithelial cells with aging; however, these differences were considerably less that noted with aging effects on immune system components. These more marginal changes in healthy aging will need to be evaluated in the context of the changes taking place in naturally occurring periodontitis, as well as the dynamics of epithelial responses in the gingival during ligature-induced periodontitis using this human-like disease model. Therefore, a more clear understanding of the fundamental biologic responses of the epithelium should provide insight into disease variation related to increased susceptibility or resistance to periodontitis across the population.

Acknowledgements This work was supported by National Institute of Health grants P20GM103538 and UL1TR000117. We express our gratitude to the Caribbean Primate Research Center (CPRC) supported by grant P40RR03640, and the Microarray Core of University Kentucky for their invaluable technical assistance. We thank M. Kirakodu for data management support.

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