Gene Expression Profiling of Early-Phase Sjögren's Syndrome in C57BL/6.NOD-*Aec1Aec2* Mice Identifies Focal Adhesion Maturation Associated with Infiltrating Leukocytes

Ammon B. Peck,^{1,2,3} Benjamin T. Saylor,¹ Linh Nguyen,⁴ Ashok Sharma,⁵ Jin-Xiong She,⁵ Cuong Q. Nguyen,^{1,3} and Richard A. McIndoe⁵

PURPOSE. Despite considerable efforts, the molecular and cellular events in lacrimal gland tissues initiating inflammatory responses leading to keratoconjunctivitis sicca (KCS), autoimmunity, and Sjögren's syndrome (SjS) have yet to be defined. To determine whether altered glandular homeostasis occurs before the onset of autoimmunity, a temporal transcriptome study was carried out in an animal model of primary SjS.

METHODS. Using oligonucleotide microarrays, gene expression profiles were generated for lacrimal glands of C57BL/6.NOD-*Aec1Aec2* mice 4 to 20 weeks of age. Pairwise analyses identified genes differentially expressed, relative to their 4-week expression, during the development of SjS-like disease. Statistical analyses defined differentially and coordinately expressed gene sets. The PANTHER (Protein ANALysis THrough Evolutionary Relationships) classification system was used to define annotated biological processes or functions.

RESULTS. Temporal transcript expression profiles of C57BL/6.NOD-*Aec1Aec2* lacrimal glands before, or concomitant with, the first appearance of inflammatory cells revealed a highly restricted subset of differentially expressed genes encoding interactive extracellular matrix proteins, fibronectin, integrins, and syndecans. In contrast, genes encoding interepithelial junctional complex proteins defined alterations in tight junctions (TJ), adherens, desmosomes, and gap junctions, suggesting perturbations in the permeability of the paracellular spaces between epithelial barriers. Correlating with this were gene sets defining focal adhesion (FA) maturation and Ras/Raf-Mek/Erk signal transduction. Immunohistochemically, FAs were as-

sociated with infiltrating leukocytes and not with lacrimal epithelial cells.

CONCLUSIONS. For the first time, FA maturations are implicated as initial biomarkers of impending autoimmunity in lacrimal glands of SjS-prone mice. Changes in TJ complex genes support an increased movement of cells through paracellular spaces. (*Invest Ophthalmol Vis Sci.* 2011;52:5647-5655) DOI: 10.1167/iovs.11-7652

The lacrimal functional unit (LFU) is an integrated system that includes the orbital lacrimal glands, the accessory lacrimal glands, the ocular surface, the meibomian glands, and the connecting sensory and motor neurons.¹ The lacrimal glands, composed primarily of acinar, ductal, myoepithelial, and neural cells, are the major sources of the aqueous fluid coating the ocular surface. Lacrimal gland secretions contain lubricating substances, multiple antibacterial and antiviral proteins, and growth factors that contribute to the health and integrity of the ocular surface.² Reduced lacrimal gland secretions represent a leading cause of aqueous-deficient dry eye syndrome, especially as observed in Sjögren's syndrome (SjS), an autoimmune exocrinopathy with an increasing prevalence in the general population, especially in postmenopausal women.

SjS is classified as a chronic progressive autoimmune rheumatic disease primarily of the lacrimal and salivary glands that results in hypolacrimation and hyposalivation, respectively.^{3,4} Exocrine gland dysfunction in SjS is thought to result from a combination of proinflammatory cytokine production capable of inducing cellular apoptosis, synthesis of autoantibodies reactive with the muscarinic acetylcholine and adrenergic receptors capable of inhibiting parasympathetic neural stimulation of secretion, and the direct cytotoxic action of infiltrating T cells (possibly including CD4⁺ T_H17 cells).^{5,6} This multicomponent attack leads to a chronic, progressive loss of acinar cell mass with ductal epithelial cell hyperplasia and, in the human disease, severe fibrosis. Despite widespread efforts to define the genetic, environmental, and immunologic bases of SjS, the underlying etiology of this disease remains ill defined.

To study the immunopathophysiological nature of SjS, a variety of mouse models have been developed representing different facets of the human disease.^{7,8} Based on results of studies using nonobese diabetic (NOD) mice and various single-gene knockout (KO) congenic partner strains of NOD, we have postulated that the development and onset of autoimmune exocrinopathy can be divided into a series of distinct consecutive, temporal, yet overlapping phases.^{7,9,10} In brief, the earliest phase, seen between 6 to 10 weeks of age, is characterized by aberrant genetic, physiological, and biochemical activities, resulting presumably from retarded gland devel-

From the ¹Department of Oral Biology and the ³Center for Orphaned Autoimmune Diseases, College of Dentistry, the ²Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, and the ⁴Department of Chemistry, College of Liberal Arts, University of Florida, Gainesville, Florida; and the ⁵Center for Biotechnology and Genomic Medicine, Georgia's Health Sciences University, Augusta, Georgia.

Supported by Public Health Service Grants K99 DE018958 (CQN), DE014344, and AI081952 (ABP) from the National Institutes of Health, and funds from the Center for Orphaned Autoimmune Diseases, University of Florida, Gainesville, Florida.

Submitted for publication March 29, 2011; revised May 2, 2011; accepted May 11, 2011.

Disclosure: A.B. Peck, None; B.T. Saylor, None; L. Nguyen, None; A. Sharma, None; J.-X. She, None; C.Q. Nguyen, None; R.A. McIndoe, None

Corresponding author: Ammon B. Peck, Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida, Box 100424, Gainesville, FL 32610; peck@pathology.ufl.edu.

opment and increased acinar cell apoptosis. In the subsequent phase, occurring around 10 to 16 weeks of age, exocrine gland pathology is observed in conjunction with the appearance of leukocytic infiltrates and the formation of lymphocytic foci consisting mostly of T and B cell aggregates. In the late phase, usually detected after 18 weeks of age, an overt clinical disease is seen defined by measurable loss of salivary and lacrimal gland secretory function,^{7,11} postulated to be antibody mediated. These latter two stages of disease tend to demarcate innate versus adaptive immune responses.

Although the exact factors driving the early physiological changes that initiate the innate and subsequent autoimmune response in SjS remain unknown, altered glandular homeostasis with associated pathologic changes are hypothesized to be the basis for why autoreactive T cells eventually attack exocrine gland tissues.¹² Aberrant proteolytic activity, elevated apoptosis, downregulated EGF gene expression, and upregulated expression of multiple genes associated with tissue homeostasis are commonly observed before and independent of detectable autoimmunity.^{13–15} Thus, unique gene sets, signaling pathways, and various molecular networks reflecting these early pathologic alterations are hypothesized to correlate with temporal changes in expression during the earliest stages of SjS. This concept has been strongly supported by our recent microarray studies of differentially expressed genes in the salivary and lacrimal glands, defining the development and onset of sicca syndrome in the NOD-derived C57BL/6.NOD-*Aec1Aec2* mouse model of primary SjS.^{16–18} However, a serious gap remains in our understanding of the earliest glandular and cellular events associated with initiating inflammation that evolves into chronic autoimmunity. Therefore, the goal of the present work has been to define early glandular events by identifying gene networks exhibiting coordinated early-stage changes in expression. We hypothesize that such differential gene expression will provide an in-depth snapshot of molecular events associated with the pre-autoimmune or early autoimmune phase of SjS. Results of the present study point directly to activations of interepithelial junctional complexes and to the concomitant maturation of focal adhesions (FAs) expressed by leukocytes infiltrating lacrimal glands.

MATERIALS AND METHODS

Materials

C57BL/6.NOD-*Aec1Aec2* and C57BL/6J mice were bred and maintained under specific pathogen-free conditions within the Department of Pathology's Mouse Facility with oversight by Animal Care Services at the University of Florida, Gainesville. RNA purification kits (RNeasy Mini-Kits; Qiagen, Valencia, CA) were purchased, and hybridizations were performed (GeneChip Mouse Genome 430 2.0 Arrays; Affymetrix Carlsbad, CA). Reverse transcriptase (M-MuLV) was purchased from New England Biolabs (Ipswich, MA), supermix (iQ SYBR Green) was purchased from Bio-Rad (Hercules, CA), PCR primers were purchased from Integrated DNA Technologies (Coralville, IA), and antiphosphorylated paxillin (sc-101774) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Blocking reagent (CytoQ Background Buster) was obtained from (Innovex Biosciences, Richmond, CA), whereas rabbit serum, goat serum, avidin/biotin blocker, diaminobenzidine substrate, biotinylated goat anti-rabbit IgG, biotinylated horseradish peroxidase, and ABC kits (Vectastain) were all purchased from Vector Laboratories (Burlingame, CA).

Methods

Animals. Although SjS in humans is most common in postmenopausal women, male mice were used exclusively in the present study because we have noted a qualitatively stronger disease in the lacrimal glands of male C57BL/6.NOD-*Aec1Aec2* mice over age-matched female

mice. Both C57BL/6.NOD-*Aec1Aec2* and C57BL/6J mice were maintained on a 12-hour light/12-hour dark schedule and were provided food and acidified water ad libitum. Mice were euthanized at 4, 8, 12, 16, or 20 weeks of age by cervical dislocation after deep anesthetization with isoflurane. There are no indications that this procedure affects the preparation of tissues or RNA. Both the breeding and the use of mice for the present studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the studies were approved by the University of Florida's Institutional Animal Care and Use Committee (protocols 201004820 and 2008011756).

Preparation of RNA for Detection of Differentially Expressed Genes in Microarray Analyses. As detailed elsewhere,^{17,18} lacrimal glands were freshly excised from individual male mice ($n = 5$ per age group) at 4, 8, 12, 16, or 20 weeks of age, snap-frozen in liquid nitrogen, and stored at -80°C until all glandular samples were obtained. Using one lacrimal gland from each mouse, without any lymph nodes if present, all 25 samples of total RNA from the five age groups of C57BL/6.NOD-*Aec1Aec2* mice were isolated concurrently using the RNA purification kit (RNeasy Mini-Kit; Qiagen), in accordance with the manufacturer's protocol. Hybridizations were carried out with each of the 25 individual RNA samples using mouse arrays (GeneChip Mouse Genome 430 2.0 Arrays; Affymetrix) in accordance with the manufacturer's instructions, providing five data sets per age group. Each GeneChip contains 45,000 probe sets that analyze the expression level of more than 39,000 transcripts and variants from more than 34,000 well-characterized mouse genes. Microarray data have been deposited with Gene Expression Omnibus, accession number GSE15640.

Differential Gene Expression Analysis. Microarray data were normalized using the "robust multiarray average" algorithm and analyzed using the LIMMA (Linear Models for Microarray Analysis) package from the R Development Core Team (The R Project for Statistical Computing, <http://www.r-project.org>) to perform differential expression analyses. LIMMA takes into account the correlation between replicates and uses the empiric Bayes approach, which gives stable inferences for relatively small numbers of arrays.¹⁹ The "fdr" method to adjust the P values for multiple testing was used to control the false-discovery rate.²⁰ Pairwise comparisons between the five groups were made to assess the genes as differentially expressed at 8, 12, 16, and/or 20 weeks of age compared with the 4-week expression.

Verification of Selected Gene Expression by Real-Time Polymerase Chain Reaction Analysis. Total RNA samples were prepared from lacrimal glands isolated from 8-week-old C57BL/6J or 5- and 9-week-old C57BL/6.NOD-*Aec1Aec2* male mice, as described. Aliquots of each RNA preparation were used to synthesize cDNA, and the cDNAs were quantified by spectrophotometry. Real-time PCR was carried out (iCycler with iQ SYBR Green Supermix; Bio-Rad, Hercules, CA) in accordance with the manufacturer's instructions. Sequences of the primers used are listed in the legend to Supplementary Fig. S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7652/-/DCSupplemental>). Cycling parameters of amplification were as follows: 3 minutes at 95°C , 50 cycles of 95°C for 30 seconds, and 63°C for 30 seconds. This was followed by melting curves, with 100 cycles starting at 60°C for 10 seconds with an increase of 0.4°C per cycle. Results shown in Supplementary Figure S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7652/-/DCSupplemental>) are comparisons between real-time PCR and microarray data.

Indirect Immunohistochemical Staining. Lacrimal glands, explanted from mice at the times indicated in the text, were embedded in paraffin and sectioned to a thickness of $5\ \mu\text{m}$. Antigen retrieval was performed in 25 mM Tris/EDTA buffer, pH 9.1, followed by incubation with blocking reagent (CytoQ Background Buster; Innovex Biosciences). After sections were blocked with goat serum and avidin/biotin blocker, each was incubated overnight at 4°C with antiphosphorylated paxillin. Isotype controls were performed using rabbit IgG. Slides were washed for 5 minutes and incubated for 30 minutes in biotinylated goat anti-rabbit IgG followed by biotinylated horseradish peroxidase using

ABC kits (Vectastain; Vector Laboratories). Staining was developed by the addition of diaminobenzidine substrate and was counterstained with hematoxylin. Images were taken using a Zeiss (Oberkochen, Germany) microscope fitted with a camera (AxioCam MRc5; Zeiss).

RESULTS

Changes in Lacrimal Gland Homeostasis of C57BL/6.NOD-*Aec1Aec2* Mice during the Pre-Autoimmune Phase of SjS-like Disease Is Defined by a Highly Restricted Number of Differentially Expressed Genes Encoding ECM Molecules

Epithelial tissues rely heavily on the extracellular matrix (ECM) to maintain structure, function, integrity, and homeostasis. ECM encompasses the connective tissue (the stroma) and the basement membrane (sometimes referred to as the basal lamina). Epithelial cells bind to several products in the basal lamina, many of which are secreted by the epithelial cells per se, while others are secreted by cells residing in the stroma. As such, changes in ECM molecules should indicate the first signs of homeostatic changes in epithelial tissues of the lacrimal glands. Major proteins of the basement membrane include fibrillar, nonfibrillar, associated, and transmembrane collagens (e.g., collagen I, collagen IV, collagen VI, and collagen XVII, respectively), microfibrillar proteins (e.g., the fibulins, fibrillins, emilins, and elastin), hyaluronan and the hyalectans (e.g., aggrecan, brevican, neurocan, and versican), proteoglycans (e.g., biglycan, decorin, agrin, bamacan, dystroglycan, and perlecan), and noncollagenous glycoproteins (e.g., fibronectins, laminins, tenascins, thrombospondins, and entactins).²¹ In addition, a subset of integrins is associated with ECM, bridging epithelial cells with basement membrane proteins.

Previously published histology of lacrimal glands in C57BL/6.NOD-*Aec1Aec2* mice revealed a normal appearance in glandular architecture at 4 weeks of age, equivalent to parental C57BL/6J mice.^{22,23} Based on these observations, we selected the 4-week time point as the baseline for temporal analyses in this study. Therefore, genes differentially expressed after 4 weeks of age, yet earlier than 16 weeks of age, should correlate with one or more manifestations of aberrant glandular homeostasis representing a definable pathologic process. Temporal microarray analyses of lacrimal glands from C57BL/6.NOD-*Aec1Aec2* mice between 4 and 20 weeks of age indicate that the vast majority of genes encoding ECM components showed either static or reduced expression over the entire time frame examined (Supplementary Fig. S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7652/-/DCSupplemental>). For example, of 38 genes encoding either a collagen or a collagen subunit, only three (*Col6a3*, *Col18a1*, and *Col9a3*) were upregulated, and then only weakly. Genes encoding the other 35 collagen subunits exhibited either no temporal changes in expression compared with the 4-week time period or decreased expression. Similarly, of the genes encoding hyalectins, proteoglycans, and microfibrillar proteins, only *Bgn* (encoding biglycan), *Agrn* (encoding agrin), and *Cspg6* (encoding chondroitin sulfate proteoglycan, subunit 6) were found to be temporally upregulated. Of the many genes encoding noncollagenous glycoproteins, only *Fn1* (encoding fibronectin), *Tbbs1* (encoding thrombospondin-1), and 2 of the 4 profilin-encoding genes (*Pfn1* and *Pfn2*) showed a temporal differential expression, whereas no changes in expression were observed for the lamins, tenascins, or nidogens. Lastly, of the 18 alpha and 8 beta subunits that make up the 24 unique integrins,²⁴ only three beta subunit genes (*Itgb2*, *Itgb5*, and *Itgb4*) plus two alpha subunit genes (*Itgav* and *Itgax*) exhibited differential expression. Although these differentially expressed integrin subunit genes could encode theoretically for six integrins, only

two, $\alpha\beta2$ and $\alpha\beta5$, are known functional integrins. Integrin molecule $\alpha\beta2$, also known as CR4,²⁵ is involved in phagocytosis of apoptotic cells, whereas integrin molecule $\alpha\beta5$ is involved in cell adhesion to ECM, focal adhesion development and possibly phagocytosis. The major ligands for $\alpha\beta5$ include vitronectin and fibronectin involved in cell adhesion, plus thrombospondin-1,²⁶ a molecule that can bridge integrin $\alpha\beta5$ to phosphatidylserine (PS) on apoptotic cells. Overall, these data suggest that only minimal structural changes involving the interface between the ECM and the acinar epithelium are occurring in the lacrimal glands, and this corresponds to the observation that, at most, only subtle changes in the architecture of lacrimal glands are detected by immunohistologic analyses.

Temporal Changes in the Expression of Genes Encoding Interepithelial Junctional Complex-Associated Proteins

The interepithelial junctional complexes in epithelium, whose role is to maintain functional barriers and to regulate junction-associated signaling, consist of protein complexes forming tight junctions (TJs), gap junctions (GJs), adherens, desmosomes, and FAs.^{27,28} These complexes have been shown to be highly dynamic, controlling the movement of solutes and cells through the paracellular spaces. It is not surprising then that these complexes are important, not only for mechanical adhesions and for linking cytoplasmic structures between adjacent cells but also for growth, differentiation, morphogenesis, migration, and extrusion of apoptotic cells, the latter of which is considered important in light of the elevated acinar cell apoptosis seen in the exocrine glands of SjS patients and animal models.

The most apical junctional complexes in epithelium are the TJs. TJs can consist of zona occludens (ZO) protein complexes, PALS1-ALS-associated protein complexes, and/or partitioning-defective (PAR-3/PAR-6) protein complexes. Recent studies²⁹ have reported that human salivary glands express primarily the ZO complexes consisting of ZO-1, JAM-A, Occludin-1, and Claudins-1, -2, -3, -4, and -16. TJ proteins present in the mouse include occludin-1 (encoded by *Ocln1*), 20 claudins (encoded by *Cldn1* up to *Cldn23*), three junction adhesion molecules (encoded by *Jam2*, *Jam3*, and *Igsf5*), the membrane-associated guanyl kinase inverted proteins (*Magi1-Magi3*), multi-PDZ domain protein, Mupp-1 (encoded by *Mpdz*), the partitioning-defective proteins (*Par1-Par6*), the protein-associated with Lin-7, Pals-1 (encoded by *Mpp5*), the Pals-associated tight junction protein, Patj (encoded by *Inadl*), scribble (encoded by *Scrib*), the zonal occludins ZO-1 and ZO-2 (encoded by *Tjp1* and *Tjp2*), and the recently described lacrimal gland-associated Angiomotin-like protein-1 (*Amotl1* encoded by *Amotl1*). Of these, only *Tjp1*, *Tjp2*, *Amotl1*, *Igsf5*, *Cldn3*, *Cldn7*, *Cldn8*, and *Cldn12* were differentially expressed in a temporal fashion in C57BL/6.NOD-*Aec1Aec2* lacrimal glands (Fig. 1A). These eight TJ proteins strongly suggest changes in the ZO protein complexes in the absence of changes in either the Pals1-Als or Par-3/Par-6 complexes.

Connexins, encoded by *Gpa* and *Gjb* gene families, are membrane-spanning proteins that make up gap junctions, or channels that link the cytoplasm of adjacent cells.³⁰ Of the 15 GJ-associated connexin genes represented on the microarray, only three exhibited temporally upregulated expression in the lacrimal glands of C57BL/6.NOD-*Aec1Aec2* mice: *Gja1*, with a strong optimal expression at 16 weeks of age, plus *Gjb1* and *Gjb2*, with weaker optimal expression around 12 weeks of age (Fig. 1B). Neither *Gjb3* nor *Gjb4* showed any changes in expression.

FIGURE 1. Temporal changes in gene expression in the lacrimal glands of C57BL/6.NOD-*Aec1Aec2* male mice for select sets of genes encoding intraepithelial junctional complexes. (a) Tight junctions. (b) Connexins. (c) Adherins. (d) Desmosomes.

Adherens junctions and desmosomes are intercellular structures that connect actin cytoskeletons and intermediate filaments, respectively, between adjacent cells.^{31,32} Molecules of the adherens junctions (composed of the cadherin family of proteins, such as the catenins, procadherins, and cadherins) can interact directly with the proteins of TJs, whereas desmosomes can interact indirectly with integrins through the intermediate filaments to initiate intracellular signaling. Of the >60 genes encoding cadherin family proteins, only *Cdh1* (encoding cadherin-1), *Pcdh1* (encoding procadherin-1), *Ctnna1* (encoding α -catenin), and *Ctnnd1* (encoding δ -catenin) exhibited upregulated expression in the lacrimal glands of C57BL/6.NOD-*Aec1Aec2* mice between 8 and 12 weeks of age (Fig. 1C). *Pcdh9*, which showed no temporal changes in gene expression, is representative of the other cadherin family genes.

Lastly, desmosomes consist of two interdigitating plasma membrane proteins, desmoglein, encoded by *Dsg*, and desmocollin, encoded by *Dsc*. Each of these molecules attaches to the *Jup*-encoded protein, plakoglobin, which also binds with plakophilin, encoded by 1 of 4 separate *Pkp* genes, and desmoplakin, encoded by *Dsp*. Plakophilin, in turn, interacts with the intracellular intermediate filaments, especially keratin-10 (encoded by *Krt10*) or keratin-14 (encoded by *Krt14*). Of the five different protein families that form the desmosome complexes, genes encoding at least one member of each family were upregulated (Fig. 1D). Interestingly, *Krt14*, but not *Krt10*, was also upregulated.

Differential Gene Expression in Lacrimal Glands of C57BL/6.NOD-*Aec1Aec2* Mice Implicate FA Maturation, Assembly, and Disassembly

As described, integrins such as $\alpha\beta5$ are considered major transmembrane components of focal adhesion, yet they inter-

act with numerous other transmembrane molecules such as members of the tetraspan proteins of the Tm4sf protein family, the integrin-associated protein CD47, and the syndecan molecules. CD47 appears to regulate cell migration through its ligands, the thrombospondins,³³ whereas the syndecans not only bind to ECM molecules such as fibronectin and have a propensity to bind growth factors, they also participate in regulating PKC α , important in orchestrating FA formation/maturation and activation of signaling pathways.³⁴ Syndecan-4, when overexpressed, can induce cortical actin to rearrange into stress fibers. Two additional membrane-associated molecules of interest are calreticulin, a chaperone protein that regulates integrin-dependent adhesions,³⁵ and calnexin, a protein that facilitates heterodimerization of integrin subunits in FAs.³⁶ Importantly, the genes encoding tetraspan protein Tmem51 (*Tmem51*), CD47 (*Cd47*), calreticulin (*Calr*), and calnexin (*Canx*) and the transmembrane proteoglycans syndecan-1, -3, and -4 (*Sdc1*, *Sdc3*, and *Sdc4*) were all coordinately upregulated in the lacrimal glands of C57BL/6.NOD-*Aec1Aec2* mice during the early stage of pathology, 8 to 12 weeks of age, similar to integrin $\alpha\beta5$ (Fig. 2A). *Sdc2* was neither upregulated nor exhibited differential expression.

Proteins that interact with cytoplasmic domains of integrins, especially Itg β subunits in FAs, include a number of cytoskeletal proteins, among them talin (encoded by *Tln*), α -actinin (encoded by *Actn1*), and α -filamin (encoded by *Flna*),³⁷ plus several regulatory proteins, especially FAK (encoded by *Plk2*), Src kinase (encoded by *Src*), Pip2 (encoded by *Pk3c* genes), PKC α (encoded by *Prka*), paxillin (encoded by *Pxxn*), vinculin (encoded by *Vcn*), and synectin (encoded by *Gipc1*), synbindin (encoded by *Trappc4*), tensin-1 (encoded by *Tenc1*), and zyxin (encoded by *Zyx*).³⁸ Similarly, one protein that interacts specifically with cytoplasmic domains of the

FIGURE 2. Temporal changes in gene expression in the lacrimal glands of C57BL/6.NOD-*Aec1Aec2* male mice for genes encoding FA-associated proteins. (a) Integrin-binding transmembrane proteins. (b) Integrin-binding cytoplasmic proteins. (c) Functional proteins associated with migration and phagocytosis.

Itga subunit is the calcium- and integrin-binding protein Cib1 (encoded by *Cib1*).³⁹ As presented in Figure 2B, a majority of these genes show coordinated and upregulated expression in the lacrimal glands of C57BL/6.NOD-*Aec1Aec2* mice between 8 to 12 weeks of age, whereas a small set is differentially expressed between 12 to 16 weeks of age. It is well documented that a stepwise recruitment of integrins to FAs, together with specific sets of these diverse integrin-associated proteins, occurs after oligomerization of syndecan molecules.²⁸

Anchored to FAs in migrating cells are actin filaments (microfilaments) that, along with actin cross-linking proteins and myosin motors, make up stress fibers. In fact, Pasapera et al.⁴⁰ recently showed that the recruitment and association of FAK, vinculin, α -actinin, and zyxin to FAs requires myosin II contractility. Myosin motors move along the actin filaments to exert their tension and are critical for mediating the phosphorylation of paxillin. Controlling the formation and maintenance of stress fibers are several actin-regulating proteins, including gelsolin (encoded by *Gelsolin*), gelsolin-like capping protein (encoded by *Capg*), radixin (encoded by *Rdx*), and guanine nucleotide binding protein- β -polypeptide 2-like (or activated protein kinase C receptor encoded by *Gnb2l1*). As shown in Figure 2C, the genes for this set of proteins are coordinately upregulated in the lacrimal glands at 8 to 12 weeks of age. These capping proteins, when removed, permit the activation of actin-polymerization involving cofilin and profilin, two molecules that enhance the disassembly and reassembly, respectively, of actin monomers. Also binding to stress fibers and interacting with vinculin and zyxin at the leading edge of spreading lamellipodia of migrating cells is Vasp (vasodilator-stimulator phosphoprotein encoded by *Vasp*), whose interaction with profilin underlies recruitment of the actin monomers to growing filaments.⁴¹ Temporal analysis of *Vasp* indicates that this gene is also upregulated between 8 and 12 weeks of

age (Fig. 2C). Lastly, immunohistochemical staining of lacrimal gland sections for phosphorylated paxillin, used as a surrogate for FA assembly, revealed that FAs are not only associated with resident mononuclear leukocytes at 4 and 9 weeks in C57BL/6.NOD-*Aec1Aec2* mice, they are also at the leading edges of infiltrating leukocytes at later time points (Fig. 3).

FA Activation of the Ras/Raf-MAPK Downstream Signal Transduction Pathways

If the integrin-syndecan receptor system is associated with homeostatic changes observed in lacrimal gland tissue of SjS-susceptible C57BL/6.NOD-*Aec1Aec2* mice, it is reasonable to expect the activation of downstream signal transduction pathways. One important pathway activated by FA maturation involves Ras/Raf-Mapk signaling. Thus, genes encoding Grb2 (*Grb2*), Sos (*Sos2*), Ras (*Ras-GTP*), Raf (*Raf1*), Mek (*Mapk2k1*), and Erk1/2 (*Mapk3* and *Mapk1*, respectively) should exhibit a coordinately upregulated differential expression profile. As presented in Figure 4 (left), temporal expression profiles for these genes revealed that each was markedly upregulated in the lacrimal glands between 8 and 12 weeks of age. These data are consistent with our previous findings that the genes of this signaling pathway are upregulated in the salivary glands of C57BL/6.NOD-*Aec1Aec2* mice compared with salivary glands of age-matched C57BL/6J mice.^{16–18}

Erk1/2 can activate one of the nuclear Rsk factors, which, in turn, interacts with the CBP-p300 complex, leading to the subsequent phosphorylation and activation of c-Fos. Alternatively, Erk1/2 can phosphorylate c-Fos directly, because both molecules can associate with the intermediate filament Lamin A/C (encoded by *Lmna*). Such direct phosphorylation of c-Fos results in the release of c-Fos from the intermediate filament permitting the activation of AP-1, the consequence of which is

FIGURE 3. Immunohistochemical staining for phosphorylated-paxillin in lacrimal glands of C57BL/6.NOD-Aec1Aec2 mice. (a) Lacrimal glands from 4-, 9-, and 24-week-old C57BL/6.NOD-Aec1Aec2 mice were stained with hematoxylin and eosin (left) or immunohistochemically stained with phosphorylated-paxillin (right), showing the temporal influx of paxillin-positive infiltrating cells (magnification, 400 $\times$). (b) Early leukocytic focus in a lacrimal gland of a C57BL/6.NOD-Aec1Aec2 mouse (left) magnified (right) showing phosphorylated-paxillin to be at the leading edge of infiltrating cells, suggesting the directional movement.

the transcription of *c-Fos* and *c-Jun*. As presented in Figure 4 (right), *Lmna*, *c-Fos*, and *c-Jun*, but not *Rsk* (encoded by *Rps6Ka3*), CBP (encoded by *Crebbp*), or p300 (encoded by *E1abp300*), exhibited strongly upregulated expression at 8 weeks of age. In contrast, *c-Fos*, *c-Jun*, *Rps6Ka3*, *Crebbp*, and *E1abp300* expression was upregulated at 16 weeks of age. Considered in total, these data suggest a highly restricted activation of transcription in the lacrimal glands of C57BL/6.NOD-Aec1Aec2 mice in the preautoimmune phase, and two distinct signaling pathways are involved, each at a different time point.

DISCUSSION

In the present study, we used microarray transcriptome technology to identify genes whose temporal expression was differentially upregulated during the early stages of SjS-like disease development (i.e., between 4 and 16 weeks of age) occurring within the lacrimal glands of C57BL/6.NOD-Aec1Aec2 mice, a model of primary human SjS. In addition, we took advantage of the fact that the development of SjS-like disease in C57BL/6.NOD-Aec1Aec2 mice progresses through well-defined sequential, yet continuous, covert pathologic stages that result eventually in the onset of overt clinical manifestations, including hypolacrimation and hyposalivation.^{7,11}

This latter feature permits correlating changes in gene expression identified by microarray, a technology that can potentially provide both false-positive and false-negative data, with changes in glandular histopathology and the aberrant biochemical and physiological manifestations already well defined in the glands of these SjS-susceptible mice,^{17,18} thereby providing general validations for the differential gene expression.

Results presented here reveal temporal changes in gene expression that, when considered in total, identify a set of specific, highly linked cellular processes associated with changes in interepithelial TJ complexes concomitant with an apparent induction of FAs and Ras/Raf-Mapk signal transduction pathway activation. Interestingly, changes in TJ complexes have been described in salivary glands of SjS patients, providing a logical means for exposure of the acinar cells to proinflammatory cytokines.⁴² The fact that our temporal microarray gene profiles also demonstrate that an overwhelming majority of genes are not differentially expressed in the lacrimal glands of C57BL/6.NOD-Aec1Aec2 mice during the early development of SjS adds even greater significance to the few genes identified as temporally (and coordinately) upregulated. Most significant, however, is the fact that these sets of differentially expressed genes point to critical pathophysiological changes that provide both a comprehensive molecular basis for early development of a chronic inflammatory response and the foundation on which to generate molecular modeling that can now be investigated in greater detail at the epigenetic and protein levels.

Based on the limited sets of genes differentially expressed in the lacrimal glands, we propose that alterations occurring during the preautoimmune phase involve interepithelial TJ complexes and appearance of FAs; however, the relationship between changes in TJ complexes and activation of genes encoding proteins associated with FAs remains to be determined. Although we favor the concept that both these events identify glandular changes associated with infiltration of recruited macrophages and dendritic cells, known to occur in the exocrine glands of C57BL/6.NOD-Aec1Aec2 mice between 8 and 12 weeks of age, we have yet to rule out other possibilities, such as the extrusion of apoptotic cells with concomitant glandular cell hyperplasia. Similarly, we are confronted with an activation of the Ras/Raf-Mapk signaling pathway, a pathway identified as upregulated in the salivary glands¹⁶ and simultaneously used by multiple cellular receptors in signaling. Even though this signaling pathway can be activated by a variety of cellular events, we now suspect that its activation in the lacrimal glands results from the induction of FAs. However, unlike what occurs in the salivary glands, in which Ras/Raf-Mapk signaling appears to flow directly through Erk1/2 to *c-Fos*/*c-Jun*, this signaling pathway appears to flow first directly

FIGURE 4. Temporal changes in gene expression in the lacrimal glands of C57BL/6.NOD-Aec1Aec2 male mice for genes encoding the FAK-Src/Mapk signaling pathway associated with FA function. Temporal expression of genes encoding the proteins of the Ras/Raf-Mapk (left) and transcription factor AP-1 (right) pathways.

FIGURE 5. Proposed model of early pathologic events occurring in the lacrimal glands of C57BL/6.NOD-Aec1Aec2 male mice defined by changes in gene expression. (a) Pre-autoimmune phase turnover of interepithelial junctions of lacrimal glands, with the migration of leukocytes into the glands to form lymphocytic foci, a hallmark biomarker of SjS. The interepithelial junctions, consisting of tight junctions, gap junctions, adherens, desmosomes, and focal adhesions, maintain cell-cell homeostasis and tissue integrity and at the same time tightly regulate solute movement through the paracellular spaces. Cellular emigration between cells requires changes in the interepithelial junctions, opening a pathway for cells to move through the tissues. The present study has shown changes in gene expression for proteins within each of the molecular complexes composing the interepithelial junctions. Of special interest for future studies are changes in the desmosomes and ZO molecules because of their associated signaling pathways. (b) Model of FA maturation during the early changes in lacrimal gland homeostasis of SjS-predisposed C57BL/6.NOD-Aec1Aec2 mice. Integrins represent the principal cell surface adhesion receptors mediating cell-ECM adhesion plus lateral association with cell surface proteins that, in turn, provide bidirectional signaling across membranes. Integrin clustering occurs through ECM contact, an event that initiates intracellular aggregation of signaling molecules that then activate nonreceptor tyrosine kinases and increased tyrosine phosphorylation of proteins subsequently recruited to the integrin-ECM adhesion. The specific makeup of the integrin-binding proteins such as talin, adapter molecules such as paxillin or vinculin, and enzymes such as Src that are subsequently recruited, determines the downstream function. In the present study, the differentially expressed genes suggest cell emigration and contractility, consistent with the immunohistochemical staining of lacrimal gland tissue during the development of disease.

through Erk1/2 to AP-1 (8 weeks of age), followed by a second activation through the Rps6Ka3/CBP-p300 pathway (16 weeks of age). Sorting out these pathways will require further studies.

Although transcriptome data suggested that only two beta integrin subunits, Itgβ2 and Itgβ5, exhibit temporal differential expression, it is important to note that Itgβ1 is constitutively expressed at high levels in the lacrimal glands (as shown in Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7652/-DCSupplemental>). One function of Itgβ2 (or LFA-1) is the adherence of macrophages to ICAM-1 on epithelial cells, whereas one function of integrin αvβ5 involves the activation of FAs, even though Itgβ5 is not known to localize to FAs.⁴³ Because FAs are initiated through activation of the extracellular regions of various integrins by means of cytoplasmic tail interactions with the vinculin- and actin-binding molecule talin, one might predict that Itgβ1 is also involved in this process. In any event, recruitment of the adaptor molecule paxillin appears to be critical in initiating clustering of integrins into FAs, which, in turn, recruits actin-bundling of α-actinin that, in conjunction with talin, forms bonds between the integrins and the actin cytoskeleton. Myosin II transmits tension along stress fibers as part of the α-actinin/actin network to the integrin-ECM linkage, promoting the elongation of adhesion-associated actin bundles and zyxin accumulation. Myosin II-dependent recruitment of vinculin to FAs is a result of

FAK/Src-mediated phosphorylation of paxillin, thereby promoting the recruitment and accumulation of vinculin that further propels FA maturation. This sequence is depicted in Figure 5, summarizing the results of the present study. The fact that *Tln* is optimally expressed at 8 weeks of age, while *Vcl* and *Actn* are optimally expressed at 16 weeks of age, is consistent with FA-activation associated with two events, but this too will require further study. Nevertheless, we believe that phosphorylated-paxillin represents a surrogate molecule for identification of FA maturation and assembly, and immunohistochemical staining clearly revealed that the appearance of FAs is associated with the infiltrating leukocytes.

As early as 1999, Sondermann et al.⁴⁴ reported that the molecular composition of FAs can be modified by both the environment and the physical state of the ECM, even to the point that quantitative differences will exist in FA components. Assembly and disassembly of FAs occur in a stepwise process involving primarily β-integrin subunits, with sequential association of a wide range of various FA-assembling proteins.^{28,45–47} FA assembly involves the recruitment of multiple molecules that can activate the Ras/Raf-Mapk signaling pathway and/or the formation of stress fibers. At the same time, FA disassembly, which also involves the phosphorylation of junctional plaques, is achieved through the Cdc42/Rac1 effectors, two factors whose genes are highly upregulated in both the lacrimal and the salivary glands of

C57BL/6.NOD-*Aec1Aec2* mice.^{17,18} This cycling depicts a molecular turnover that occurs during cell motility,²⁸ leading us to hypothesize that the temporal profile of differentially expressed genes in the lacrimal glands of C57BL/6.NOD-*Aec1Aec2* mice represent, at least, the influx of leukocytes in response to glandular injury. From this perspective, then, the different temporal expression of *Flna*, *Tln*, *Acta2*, and *Tpm1* (encoding tropomyosin) compared with that of *Vcl*, *Pxn*, *Actn*, *Tpm2*, and *Tpm3* may represent two separate molecular events, perhaps in different cell populations. In light of the argument presented earlier, we surmise that the upregulated gene expression profiles of the coronin family of proteins are indicative of the FA maturation associated with the appearance of phagocytic cells because these actin-associated proteins are known to interact with actin during phagocytosis, with coronin-1 being important for phagosome formation.

In summary, temporal microarray transcriptome analyses can provide a snapshot of the changing biological processes involved in the development and onset of complex diseases, such as SjS, based on coordinated gene expression at any chosen time point in relation to the overall progression of the disease state. Modeling these biological processes based on known immunohistopathology and disease progression focuses attention on possible pathophysiological changes defining the roles of the targeted tissue compared with the host response. However, it must also be kept in mind that such genetic studies as described herein require further investigation to ensure that the gene products of interest are, in fact, functioning consistently with any proposed model based on gene expression as shown by phosphorylated paxillin. Nevertheless, the temporal changes in gene expression during the preimmune period are consistent with our published concept that the lacrimal gland tissues of the C57BL/6.NOD-*Aec1Aec2* mice are actively being infiltrated first with cells of the innate immune system and then with cells of adaptive immunity. The fact that the very first detectable sign of the impending autoimmune disease in this mouse model of primary SjS appears to be activation of genes defining FAs and interepithelial junctional complex changes may represent not only the earliest measurable biomarkers of disease but also a critical point for possible future intervention therapies.

References

1. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye Workshop. (2007). *Ocul Surf.* 2007;5(2):75-92.
2. Stern ME, Gao J, Siemasko KF, Beuerman RW, Pflugfelder SC. The role of the lacrimal functional unit in the pathophysiology of dry eye. *Exp Eye Res.* 2004;78(3):409-416.
3. Fox RI. Sjögren's syndrome. *Lancet.* 2005;366(9482):321-331.
4. Delaleu N, Jonsson MV, Appel S, Jonsson R. New concepts in the pathogenesis of Sjögren's syndrome. *Rheum Dis Clin North Am.* 2008;34(4):833-845, vii.
5. Hansen A, Lipsky PE, Dörner T. Immunopathogenesis of primary Sjögren's syndrome: implications for disease management and therapy. *Curr Opin Rheumatol.* 2005;17(5):558-565.
6. Fox PC, Speight PM. Current concepts of autoimmune exocrinopathy: immunologic mechanisms in the salivary pathology of Sjögren's syndrome. *Crit Rev Oral Biol Med.* 1996;7(2):144-158.
7. Nguyen CQ, Cha SR, Peck AB. Sjögren's syndrome (SjS)-like disease of mice: the importance of B lymphocytes and autoantibodies. *Front Biosci.* 2007;12:1767-1789.
8. Chiorini JA, Cihakova D, Ouellette CE, Caturegli P. Sjögren syndrome: advances in the pathogenesis from animal models. *J Autoimmun.* 2009;33(3-4):190-196.
9. Nguyen CQ, Peck AB. Unraveling the pathophysiology of Sjögren syndrome-associated dry eye disease. *Ocul Surf.* 2009;7(1):11-27.
10. Robinson CP, Cornelius J, Bounous DE, Yamamoto H, Humphreys-Beher MG, Peck AB. Characterization of the changing lymphocyte populations and cytokine expression in the exocrine tissues of autoimmune NOD mice. *Autoimmunity.* 1998;27(1):29-44.
11. Cha S, Peck AB, Humphreys-Beher MG. Progress in understanding autoimmune exocrinopathy using the non-obese diabetic mouse: an update. *Crit Rev Oral Biol Med.* 2002;13(1):5-16.
12. Robinson CP, Yamamoto H, Peck AB, Humphreys-Beher MG. Genetically programmed development of salivary gland abnormalities in the NOD (nonobese diabetic)-scid mouse in the absence of detectable lymphocytic infiltration: a potential trigger for sialoadenitis of NOD mice. *Clin Immunol Immunopathol.* 1996;79(1):50-59.
13. Humphreys-Beher MG, Peck AB. New concepts for the development of autoimmune exocrinopathy derived from studies with the NOD mouse model. *Arch Oral Biol.* 1999;44(suppl 1):S21-S25.
14. Cha S, van Blockland SC, Versnel MA, et al. Abnormal organogenesis in salivary gland development may initiate adult onset of autoimmune exocrinopathy. *Exp Clin Immunogenet.* 2001;18(3):143-160.
15. Cha S, Brayer J, Gao J, et al. A dual role for interferon-gamma in the pathogenesis of Sjögren's syndrome-like autoimmune exocrinopathy in the nonobese diabetic mouse. *Scand J Immunol.* 2004;60(6):552-565.
16. Killedar SJ, Eckenrode SE, McIndoe RA, et al. Early pathogenic events associated with Sjögren's syndrome (SjS)-like disease of the NOD mouse using microarray analysis. *Lab Invest.* 2006;86(12):1243-1260.
17. Nguyen CQ, Sharma A, She JX, McIndoe RA, Peck AB. Differential gene expressions in the lacrimal gland during development and onset of keratoconjunctivitis sicca in Sjögren's syndrome (SjS)-like disease of the C57BL/6.NOD-*Aec1Aec2* mouse. *Exp Eye Res.* 2009;88(3):398-409.
18. Nguyen CQ, Sharma A, Lee BH, She JX, McIndoe RA, Peck AB. Differential gene expression in the salivary gland during development and onset of xerostomia in Sjögren's syndrome-like disease of the C57BL/6.NOD-*Aec1Aec2* mouse. *Arthritis Res Ther.* 2009;11(2):R56.
19. Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics.* 2005;21(9):2067-2075.
20. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B.* 1995;57:289-300.
21. Järveläinen H, Sainio A, Koulu M, Wight TN, Penttinen R. Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacol Rev.* 2009;61(2):198-223.
22. Brayer J, Lowry J, Cha S, et al. Alleles from chromosomes 1 and 3 of NOD mice combine to influence Sjögren's syndrome-like autoimmune exocrinopathy. *J Rheumatol.* 2000;27(8):1896-1904.
23. Cha S, Nagashima H, Brown VB, Peck AB, Humphreys-Beher MG. Two NOD Idd-associated intervals contribute synergistically to the development of autoimmune exocrinopathy (Sjögren's syndrome) on a healthy murine background. *Arthritis Rheum.* 2002;46(5):1390-1398.
24. Barczyk M, Carracedo S, Gullberg D. *Integrins Cell Tissue Res.* 2010;339(1):269-280.
25. Paidassi H, Tacnet-Delorme P, Arlaud GJ, Frachet P. How phagocytes track down and respond to apoptotic cells. *Crit Rev Immunol.* 2009;29(2):111-130.
26. Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol.* 2006;16(4):189-197.
27. Steed E, Balda MS, Matter K. Dynamics and functions of tight junctions. *Trends Cell Biol.* 2010;20(3):142-149.
28. Petit V, Thiery JP. Focal adhesions: structure and dynamics. *Biol Cell.* 2000;92(7):477-494.
29. Baker OJ. Tight junctions in salivary epithelium. *J Biomed Biotechnol.* 2010;2010:278948.
30. Brink PR, Valiunas V, Moore L, Birzgalis A, Walcott B. The role of gap junctions in lacrimal acinar cells: the formation of tears. *Adv Exp Med Biol.* 2002;506(pt A):109-113.

31. Green KJ, Getsios S, Troyanovsky S, Godsel LM. Intercellular junction assembly, dynamics, and homeostasis. *Cold Spring Harb Perspect Biol.* 2010;2(2):a000125.
32. Meng W, Takeichi M. Adherens junction: molecular architecture and regulation. *Cold Spring Harb Perspect Biol.* 2009;1(6):a002899.
33. Wang XQ, Frazier WA. The thrombospondin receptor CD47 (IAP) modulates and associates with alpha2 beta1 integrin in vascular smooth muscle cells. *Mol Biol Cell.* 1998;9(4):865-874.
34. Oh ES, Woods A, Couchman JR. Syndecan-4 proteoglycan regulates the distribution and activity of protein kinase C. *J Biol Chem.* 1997;272(13):8133-8136.
35. Rojiani MV, Finlay BB, Gray V, Dedhar S. In vitro interaction of a polypeptide homologous to human Ro/SS-A antigen (calreticulin) with a highly conserved amino acid sequence in the cytoplasmic domain of integrin alpha subunits. *Biochemistry.* 1991;30(41):9859-9866.
36. Lenter M, Vestweber D. The integrin chains beta 1 and alpha 6 associate with the chaperone calnexin prior to integrin assembly. *J Biol Chem.* 1994;269(16):12263-12268.
37. Choi Y, Kim S, Lee J, et al. The oligomeric status of syndecan-4 regulates syndecan-4 interaction with alpha-actinin. *Eur J Cell Biol.* 2008;87(10):807-815.
38. Ballestrem C, Erez N, Kirchner J, Kam Z, Bershadsky A, Geiger B. Molecular mapping of tyrosine-phosphorylated proteins in focal adhesions using fluorescence resonance energy transfer. *J Cell Sci.* 2006;119(pt 5):866-875.
39. Naik UP, Patel PM, Parise LV. Identification of a novel calcium-binding protein that interacts with the integrin alphaIIb cytoplasmic domain. *J Biol Chem.* 1997;272(8):4651-4654.
40. Pasapera AM, Schneider IC, Rericha E, Schlaepfer DD, Waterman CM. Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. *J Cell Biol.* 2010;188(6):877-890.
41. Rottner K, Behrendt B, Small JV, Wehland J. VASP dynamics during lamellipodia protrusion. *Nat Cell Biol.* 1999;1(5):321-322.
42. Ewert P, Aguilera S, Allende C, et al. Disruption of tight junction structure in salivary glands from Sjögren's syndrome patients is linked to proinflammatory cytokine exposure. *Arthritis Rheum.* 2010;62(5):1280-1289.
43. Borradori L, Sonnenberg A. Hemidesmosomes: roles in adhesion, signaling and human diseases. *Curr Opin Cell Biol.* 1996;8(5):647-656.
44. Sondermann H, Dogic D, Pesch M, Aumailley M. Targeting of cytoskeletal linker proteins to focal adhesion complexes is reduced in fibroblasts adhering to laminin-1 when compared to fibronectin. *Cell Adhes Commun.* 1999;7(1):43-56.
45. Yamada KM, Miyamoto S. Integrin transmembrane signaling and cytoskeletal control. *Curr Opin Cell Biol.* 1995;7(5):681-689.
46. Miyamoto S, Teramoto H, Coso OA, et al. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J Cell Biol.* 1995;131(3):791-805.
47. Danen EH, Lafrenie RM, Miyamoto S, Yamada KM. Integrin signaling: cytoskeletal complexes, MAP kinase activation, and regulation of gene expression. *Cell Adhes Commun.* 1998;6(2-3):217-224.