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Review

Lacritin and the tear proteome as natural replacement therapy for dry eye☆

Roy Karnati [a], Diane E. Laurie [b], Gordon W. Laurie [c,d,*]

[a] School of Life Sciences, University of Hyderabad, Hyderabad, India
[b] Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic and State University, Blacksburg, VA, USA
[c] Department of Cell Biology, University of Virginia, P.O. Box 800732, UVa Health System, Charlottesville, VA 22908-0732, USA
[d] Department of Ophthalmology, University of Virginia, Charlottesville, VA, USA

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ABSTRACT

Tear proteins are potential biomarkers, drug targets, and even biotherapeutics. As a biotherapeutic, a recombinant tear protein might physiologically rescue the ocular surface when a deficiency is detected. Such a strategy pays more attention to the natural prosecretory and protective properties of the tear film and seeks to alleviate symptoms by addressing cause, rather than the current palliative, non-specific and temporary approaches. Only a handful of tear proteins appear to be selectively downregulated in dry eye, the most common eye disease. Lacritin and lipocalin-1 are two tear proteins selectively deficient in dry eye. Both proteins influence ocular surface health. Lacritin is a prosecretory mitogen that promotes basal tearing when applied topically. Levels of active monomeric lacritin are negatively regulated by tear tissue transglutaminase, whose expression is elevated in dry eye with ocular surface inflammation. Lipocalin-1 is the master lipid sponge of the ocular surface, without which residual lipids could interfere with epithelial wetting. It also is a carrier for vitamins and steroid hormones, and is a key endonuclease. Accumulation of DNA in tears is thought to be proinflammatory. Functions of these and other tear proteins may be influenced by protein–protein interactions. Here we discuss new advances in lacritin biology and provide an overview on lipocalin-1, and newly identified members of the tear proteome.

1. Introduction

Tears accumulate on the avascular corneal epithelium, and vascularized conjunctiva, as a translucent film rich in proteins, lipids and metabolites. The tear proteome is estimated to comprise 1543 proteins (Zhou et al., 2012), with over half designated as 'intracellular' (Table 1) by Gene Ontology, implying that cell death from normal epithelial renewal may be a contributor. Beyond its capacity to lubricate the lid, tears are essential for the refraction of light (Montés-Micó, 2007). Equally important and irreplaceable by drugs or drops is the role of tears in promoting corneal epithelial health for when tears are chronically insufficient, the epithelium becomes stressed and releases inflammatory cytokines that further exacerbate the situation (Massingale et al., 2009). Dry eye affects 5–6% of the general population, rising to 6–9.8% and as high as 34%, respectively in postmenopausal women (Schaumberg et al., 2003) and the elderly (Lin et al., 2005).

Relatively few tear proteins appear to be selectively down- or upregulated in dry eye (Table 1). Appreciating which are bioactive and at what molar levels would be insightful. The only growth factor-like molecule downregulated in mild to severe aqueous deficiency was lacritin (Srinivasan et al., 2012). Lacritin promotes basal tearing when added topically in rabbits (Samudre et al., 2011). Also decreased was lipocalin-1 (Srinivasan et al., 2012), that cleanses the ocular surface of lipids that would otherwise interfere with ocular surface wetting (Glasgow and Gasymov, 2011). Lacritin was the most severely downregulated protein in contact lens-related dry eye (Nichols and Green-Church, 2009) – perhaps in part because it is readily adsorbed on contact lenses (Green-Church and Nichols, 2008). It is also deficient in blepharitis (Koo et al., 2005), a common inflammation of the eyelid, associated with evaporative dry eye (Mathers, 1993). Two studies did not note any lacritin change in dry eye using mass spectrometry coupled with liquid chromatographic cationic separation followed by reverse phase separation (Zhou et al., 2009; Boehm et al., 2013), although one observed a decrease of lipocalin-1 (Zhou et al., 2009). 2-D SDS PAGE prior to mass spectrometry (Koo et al., 2005; Nichols and

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* Corresponding author. Department of Cell Biology, The University of Virginia, P.O. Box 800732, UVa Health System, Charlottesville, VA 22908-0732, USA. Tel.: +1 434 924 5250; fax: +1 434 982 3912.
E-mail address: glaurie@virginia.edu (G.W. Laurie).

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Table 1
Proteins in the normal human tear 'proteome' that are predicted to be extracellular according to Gene Ontology (GO), with underline and bold text indicating a respective decrease or increase in dry eye.

Gene symbol	Protein	Function (as per locust link, OMIM or source)
		(i) Angiogenesis
ANG	Angiogenin, ribonuclease, RNase A family, 5[5]	Promotes angiogenesis
AAMP	Angio-associated migratory cell protein[4,a]	Promotes angiogenesis
BAI3	Brain-specific angiogenesis inhibitor 3[3]	Possible angiogenesis inhibitor
ECGF1	Endothelial cell growth factor 1[2]	Promotes angiogenesis
EFEMP1	Fibulin 3 isoform 1[4,a]	Inhibits angiogenesis
SERPINF1	Serp. pep. inhib., cl. F (α-2 antiplas., PEDF), mem. 1[2]	Promotes neurodifferent. and inhibits angiogenesis
		(ii) Biosynthesis
ATP5A1	ATP synth, H+ transp, mitoch F1 complex, α subunit 1[4,a]	Catalyzes ATP synthesis in mitochondrion
ATP5B	ATP synth, H^+ transp., mitoch. F1 complex, βpolypep.[2]	Catalyzes ATP synthesis in mitochondrion
B4GALT1	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase[2]	Polypep, glycoconjugate and lactose biosynthesis
PDIA6	Protein disulfide isomerase family A, member 6[2]	Predicted electron transport and protein folding roles
		(iii) Calcium
AHSG	Alpha-2-HS-glycoprotein[2]	Calcification inhibitor
ANXA2	Annexin A2 Ca^{2+} depend. phospholip. binding prot.[2]	Osteoclast formation and bone resorption
ANXA5	Annexin A5 Ca^{2+} depend. phospholip. binding prot.[2,7]	Promotes Ca^{2+} channel activity
CALR	Calreticulin[2]	Ca^{2+} binding protein in ER and nucleus; SS assoc.
CALU	Calumenin[2]	Ca^{2+} binding protein in ER, protein folding/sorting
CALML5	Calmodulin like skin protein[4,a]	Ca^{2+} binding protein and keratinocyte differentiation
CANT1	CANT1[2]	Ca^{2+} activated nucleotidase
GAS6	Growth arrest specific 6[4,a]	Ca^{2+} channel regulator and cell communication
MCFD2	Multiple coagulation factor deficiency protein 2[4,a]	Ca^{2+} binding protein and protein transport
NUCB1	Nucleobindin 1[2]	Golgi and peripheral membrane Ca^{2+} binding protein
NUCB2	Nucleobindin 2[2]	Peripheral membrane Ca^{2+} binding protein
PPIB	Peptidylprolyl isomerase B (cyclophilin B)[4,a]	Ca^{2+} flux, ERK phosphorylation and chemotaxis
PPIC	Peptidylprolyl isomerase C (cyclophilin C)[2]	Protein folding, binds cyclosporin A
		(iv) Carbohydrate
AGL	Amylo-1, 6-glucosidase, 4-alphaglucanotransferase[2]	Glycogen degradation
AMY1C	Amylase alpha 1C[4,a]	Starch degradation
B3GAT3	Beta-1,3-glucuronyltransferase 3[4,a]	Proteoglycan biosynthesis
CES1	Carboxylesterase 1[4,a]	Xenobiotic metabolism
CHI3L2	Chitinase 3-like 2[2]	Glycan but not heparin binding
ENO1	Enolase 1, (alpha)[2]	Glycolytic enzyme
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase[2,7]	Carbohydrate metabolism
LGALS3	Lectin, galactoside-binding, soluble, 3, galectin[2,7]	Galactose-specific lectin
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein[2]	Binds Mac-2 and galectin 1
MANBA	Mannosidase, beta A[2]	Lysosomal N-linked oligosaccharide catabolism
MDH2	Malate dehydrogenase mitochondrial[4,a]	NADPH oxidation
PKM2	Pyruvate kinase, muscle[2]	Carbohydrate degradat., binds bacterial Opa protein
		(v) Carrier/binding protein/steroid assoc.
ALB	Albumin*[1,2,3]	Carrier protein
AFM	Afamin[4,a]	Vitamin E binding protein
APCS	Serum amyloid P-component[4,a]	Protein complex assembly
ARTS-1	Type 1 TNFR shedding aminopeptidase regulator[2]	Binds TNFR1 to promote shedding
AZGP1	Alpha-2-glycoprotein 1#[1,2,7]	Zinc-binding, lipid degrad., cell adhesion
CD14	CD14 molecule[2]	Binds LPS binding protein (LBP) and apoptotic cells
DMBT1	Deleted in malignant brain tumors 1[1,2,3,7]	Scavenger receptor, binds surfactant protein D
DSP	Desmoplakin[2]	Key component of desmosomes
GC	Group-specific component (vitamin D binding protein)[2]	Carrier protein for vitamin D and metabolites
HBB	Hemoglobin beta chain[4,a]	Oxygen transport
HP	**Haptoglobin#**[1,2]	Hemoglobin binding, turnover to diminish iron loss
HPX	Hemopexin[1,2]	Heme binding, turnover
HSPG2	Heparan sulfate proteoglycan 2 (perlecan)[2,7]	Growth factor binding, filtration, matrix polymerization
IGFBP1	Insulin-like growth factor binding protein 1[5]	Slows turnover of IGF's
IGFBP2	Insulin-like growth factor binding protein 2[5]	Slows turnover of IGF's
KPNB1	Karyopherin (importin) beta 1[2]	Nuclear transport
LCN1	Lipocalin 1 (tear prealbumin) Δ#$¥§[1,2,13]	Hydrophobic prot. binding, cyst. protease. inhibitor
M6PRBP1	Mannose-6-phosphate receptor binding protein 1[2]	Endosome-to-golgi transport
PEBP4	Phosphatidylethanolamine-binding protein 4[2]	?
PLIN3	Perilipin3[4,a]	Endosome-to-golgi transport
SCGB1D1	Secretoglobin, family 1D, member 1†#[1,2,3,7]	In complex that binds steroids, including androgen
SCGB2A1	Secretoglobin, family 2A, member 1#[1,2,3,7]	Possibly binds steroids, including androgen
SCGB2A2	Secretoglobin, family 2A, member 2 (mammaglobin 2)#[7]	?
TCN1	Transcobalamin I (vitamin B12 bind., R bind. family)#[2,7]	Binds and helps move vitamin B12 into cells
TF	Transferrin[1,2,3]	Iron binding and transport to proliferating cells
TTR	Transthyretin (prealbumin, amyloidosis type I)[1,2]	Thyroxine binding and transport
		(vi) Cell adhesion/motility/structure
ACTB	Actin, beta[1]	Cell structure, motility
ACTA1	Actin, alpha 1, skeletal muscle[7]	Formation of filaments
CDH1	E-cadherin[4,a]	Cell–cell adhesion, migration and cell communication
CFL1	Cofilin 1[4,a]	Cytoskeleton organization
CSTA	Stefin a[4,a]	Cell–cell adhesion, keratin organization
DAG1	Dystroglycan[4,a]	Cell-matrix adhesion and cell communication

Table 1 (*continued*)

Gene symbol	Protein	Function (as per locust link, OMIM or source)
FGA	Fibrinogen alpha chain[1,2]	Cell adhesion, spreading, mitogenic, chemotactic
FGB	Fibrinogen beta chain[4,a]	Cell adhesion, spreading, mitogenic, chemotactic
FGG	Fibrinogen gamma chain[2]	Cell adhesion, spreading, mitogenic, chemotactic
FLRT3	Fibronectin leucine rich transmembrane protein 3[3]	Possibly cell adhesion, receptor signaling
FN1	Fibronectin 1[2,7]	Cell adhesion, migration, blood coagulation
GSN	Gelsolin (amyloidosis, Finnish type)[2]	Blocks actin monom. exchange or promotes nucleat.
ICAM1	Intercellular adhesion molecule 1[4,a]	Cell–cell adhe., positive regulator of vasoconstric.
IGFALS	Insulin like growth fac. bind. protein, acid labile subunit[4,a]	Cell adhesion and cell communication
JUP	Catenin, gamma[4,a]	Cell–cell adhesion, cell migration and proliferation
KRT17	Keratin, type I cytoskeletal 17[4,a]	+ve Regul. of hair follicle dev. and inter. filament org.
KRT18	Keratin18[4,a]	Cytoskeletal intermediate filaments
LAMA3	Laminin, alpha 3[13]	Cell adhesion and differentiation
LGALS9	Galectin 9[4,a]	Cell–cell adhesion and cell communication
LUM	Lumican[4,a]	Fibril organization and spacing
MFGE8	Milk fat globule-EGF factor 8 protein[2]	Cell adhesion, rotavirus binding/inhibition
MIF	Macrophage migration inhibitory factor[4,a]	Chemo attractant and cell communication
MMP10	Matrix metalloproteinase 10[4,a]	Cell migration
MSLN	Mesothelin[2,3]	Possible cell adhesion activity
PFN1	Profilin 1[2]	Regulator of actin polymerization and cytoskeleton
SERPINF2	Alpha 2 antiplasmin[4,a]	Collagen fibril organization
SLIT3	Slit homolog 3 (Drosophila)[2]	Cell migration
THBS1	Thrombospondin 1[2]	Cell–cell and cell-matrix adhesion
TLN1	Talin 1[2]	Actin filament assembly and cell spreading
TNFAIP2	Tumor necrosis factor alpha induced protein 2[4,a]	Promotes cell migration
VIM	Vimentin[2]	Cytoskeletal intermediate filaments
		(vii) Cell growth
ANGPTL1	Angiopoietin-like 1[2,7]	May inhibit cell growth
DKK4	Dickkopf homolog 4[4,a]	Negative regulator of WNT pathway
EHD4	EH domain containing protein 4[4,a]	Protein trafficking
EGF	Epidermal growth factor (beta-urogastrone)[5]	Prosecretory mitogen
FAM3C	predicted osteoblast protein[4,a]	Cell communication and development
GDNF	Glial cell derived neurotrophic factor[5]	Dopaminergic neuron survival, differentiation
GRN	Granulin[4,a]	Growth factor and cell signaling
HDGF	Hepatoma derived growth factor[4,a]	Mitogenic and cell communication
HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)[5]	Serine protease-activated mitogen
KRT8	Keratin 8[4,a]	Cell growth and maintenance
LACRT	Lacritin*Δ†#£¥[1,2,13,5,7]	Prosecretory mitogen
MUC4	Mucin 4[2]	Epithelial cell proliferation and differentiation
NTF3	Neurotrophin 3[5]	Sensory neuron survival
NTF5	Neurotrophin 5[5]	Peripheral sensory sympathetic neuron survival
PDAP1	PDGF alpha associated protein 1[4,a]	Cell communication
PDGFC	Platelet derived growth factor C[4,a]	Cell proliferation and wound healing
PHGDH	Phosphoglycerate dehydrogenase[4,a]	Cell proliferation, metabolism, energy pathways
PTPRF	Protein tyrosine phosphatase, receptor type, F[4,a]	Pro apoptotic
QSOX1	(QSCN6) quiescin Q6 sulfhydryl oxidase 1[2]	Growth regulation
RNASET2	Ribonuclease 6[4,a]	Tumor suppressor
SERPINB5	Serpin peptidase inhibit., clade B (ovalbum), member 5[2]	Blocks mammary tumor growth
TES	Testin[4,a]	Tumor supp., cytoskel. and adhe. complex organi.
TFF1	Trefoil factor 1[4,a]	Growth factor and protein binding
TPM3	Tropomyosin 3[4,a]	Muscle dynamics and cell growth
		(viii) Cytoprotective/anti-apoptotic
CASP14	Caspase 14[4,a]	Apoptosis induction
CLU	Clusterin[1,2,3,7]	Inhibits apoptosis
MUC16	Mucin 16[2]	Cytoprotective, hydrophillic
MUC5AC	Mucin 5AC[2]	Mucus/gel-forming, cytoprotective, hydrophilic
NAMPT	Pre b cell colony enhancing factor 1[4,a]	Anti-apoptotic
PARK7	Oncogene DJ1[4,a]	Nucleotide metabolism, oxidat. stress, transformation
PHB	Prohibitin[4,a]	energy metab., fat utili., anti apop. and cell commu.
PIP	Prolactin-induced protein*$#§[1,2,3,7]	Inhibitor of T-cell apoptosis, aspartyl protease (?)
PRB1	Proline-rich protein BstNI subfamily 1[7]	?
PROL1	Proline rich, lacrimal 1#[1,2,13,3,7]	Possible ocular protective function
PRR4	Proline rich 4 (lacrimal)Δ†#[1,2,13,3,7]	Possible ocular protective function
SERPINB2	Urokinase inhibitor[4,a]	Regulates proteolysis, wound healing, anti-apoptotic
TFF3	Trefoil factor 3[4,a]	Chemotaxis, anti apoptosis, migration
TPT1	Tumor protein, translationally-controlled 1[4,a]	Anti-apoptotic and stem cell maintenance
		(ix) Extracellular matrix
COL6A1	Collagen, type VI, alpha 1[2]	Microfibril component
FBLN1	Fibulin 1[4,a]	Extracellular matrix organization
MUCL1	Mucin-like 1[3]	?
SPARCL1	SPARC-like 1 (mast9, hevin)[2,7]	Reg. of collagen assembly and decorin secretion
TGFBI	Transforming growth factor, beta-induced, 68 kDa variant[4,a]	Reg. of cell adh. and extracellular matrix organi.
		(x) Immune
ATRN	Attractin[2]	Receptor or clustering of immune cells
C1S	Complement component 1, subcomponent S[4,a]	Complement activation and innate immunity

(*continued on next page*)

Table 1 (*continued*)

Gene symbol	Protein	Function (as per locust link, OMIM or source)
C1QB	Complement C1q subcomponent, B chain[4,a]	Complement activation and innate immunity
C1QC	Complement C1q subcomponent subunit C[4,a]	Negative regul. of granul. and macrop. differentiation
C1R	Complement component 1, subcomponent R[4,a]	Complement activation and innate immunity
C3	**Complement component 3#**[1,2,7]	Complement activation
C4A	Complement component 4A (Rodgers blood group)[2]	Cleaved to a trimer for complement activation
C4B	Complement component 4B[4,a]	Similar and greater activity than C4A
C8A	Complement component C8 alpha[4,a]	Complement activation and innate immunity
C8B	Complement component C8 beta[4,a]	Complement activation and innate immunity
C8G	Complement component C8 gamma[4,a]	Complement activation and innate immunity
CCL2	**Chemokine (C–C motif) ligand 2**€[5]	Monocyte, basophil specific chemotaxis
CCL4	Chemokine (C–C motif) ligand 4[5]	Inflammatory, chemokinetic
CCL8	chemokine (C–C motif) ligand 8[5]	Monocyte, basophil, eosinphil, lympho. chemotaxis
CCL11	Chemokine (C–C motif) ligand 11[5]	Eosinophil specific chemotaxis
CCL22	Chemokine (C–C motif) ligand 22[5]	NK cell, dendritic, monocyte chemotaxis
CCL24	Chemokine (C–C motif) ligand 24[5]	Resting T cell chemotaxis
CD55	Decay accelerating factor for complement[4,a]	Complement activation and innate immunity
CD59	CD59 glycoprotein[4,a]	Protects leukocytes from homolog. complement
CFI	Complement component I[4,a]	Complement activation and innate immunity
CFB	Complement factor B[1,2]	CFD cleaved to: prolif. serine protease & antiprolif.
CFH	Complement factor H[1,2,7]	Restricts complement activation to microbial defense
CSF1	Colony stimulating factor 1 (macrophage)[5]	Prod'n, different, function of macrophages
CSF2	Colony stimulating factor 2 (granulocyte–macrophage)[5]	Prod'n, different, function of granulocytes, macroph.
CSF3	Colony stimulating factor 3 (granulocyte)[5]	Prod'n, different, function of granulocytes, macroph.
CXCL5	**Chemokine (C-X-C motif) ligand 5**€[5]	Inflammatory cytokine, neutrophil activation
CXCL10	Chemokine (C-X-C motif) ligand 10[5]	T cell, monocyte chemotaxis
CXCL1	Chemokine (C-X-C motif) ligand 1 (MGS activity, alpha)[5]	Neutrophil chemotaxis
ELANE	Neutrophil elastase[4,a]	Phagocytosis and leukocyte migration
FAM3B	Fam3b[4,a]	? Cytokine
FCRL5	Fc receptor-like 5[13]	Possible mature B cell inhibitory co-receptor
HLA-B	hla-B[4,a]	MHC, class I, receptor activity
HLA-G	hla-G[4,a]	MHC, class I, receptor activity
HRG	Histidine rich glycoprotein[4,a]	Inhibit T cell proliferation, complement activation
HSPD1	60 kDa heat shock protein, mitochondrial[4,a]	B cell, T cell and macroph. activ. and protein fold.
IGHD	FLJ00382 protein[4,a]	+ve regul. of B cell prolif. and immune respon.
IL6	Interleukin 6 (interferon, beta 2)[6]	B cell, nerve cell differentiation
IL8	Interleukin 8[5,6]	Inflammatory response mediator, angiogenic
IL10	Interleukin 10[5]	Inhibit activated macrophage cytokine synthesis
IL12	interleukin 12€[5]	Activated T, NK cell mitogen
IL15	Interleukin 15[5]	T cell proliferation
IL16	**Interleukin 16 (lymphocyte chemoattractant factor)** €[5]	CD4+ lymphocyte, monocyte, eosinophil migration
IL18	Interleukin 18[4,a]	Regul. angiog., cell adhe. and immune respon.
IFNG	Interferon, gamma[5]	Immune-regulatory, antiviral
LGALS8	Putative uncharacterized protein LGALS8[4,a]	Plasma cell differentiation and T cell stimulation
ORM1	Orosomucoid 1[1,2]	Apparent modulator of acute-phase immune activity
ORM2	Orosomucoid 2[4,a]	Apparent modulator of acute-phase immune activity
PGLYRP2	Peptidoglycan recognition protein 2[4,a]	Innate immunity
PPBP	Pro-platelet basic prot. (chemokine (C-X-C motif) lig. 7)[5]	Neutrophil chemoattractant and prosecretory
SERPING1	Serpin peptid. inhibitor, clade G (C1 inhib), member 1[2]	Complement activation regulator
TGFB2	Transforming growth factor, beta 2[5]	Suppress IL-2 T cell growth
TNF	Tumor necrosis factor (TNF superfamily, member 2)[5]	Autoimmune, pyrogen, mitogen, differentiation
TNFSF13	Tumor necrosis factor ligand superfamily, member 13[4,a]	Cell proliferation and immune response
		(xi) Lipid/cholesterol
ANPEP	Alanyl (membrane) aminopeptidase[2]	Aminoprotease
ANXA1	**Annexin A1**§[1]	Regulate phospholipase a2 activity
APOA1	Apolipoprotein A-I[1]	Cholesterol transport
APOA2	Apolipoprotein A-II[2]	In high density lipoprotein particles
APOA4	Apolipoprotein A-IV[1]	In HDL and chylomicrons
APOB	Apolipoprotein B[2]	In chylomicrons and low density lipoproteins
APOC2	Apolipoprotein C-II[4,a]	Lipoprotein trans., −ve regulator of VLDL clearance
APOC3	Apolipoprotein C-III variant 1[4,a]	Lipid transport
APOD	Apolipoprotein D[4,a]	VLDL transport
APOE	Apolipoprotein E[4,a]	VLDL transport
APOH	Apolipoprotein H[1,2]	Lipoprotein metabolism, coagulation
APOL1	Apolipoprotein L1[4,a]	Lipid transport
APOM	Apolipoprotein M[4,a]	HDL particle assembly and clearance
GM2A	GM2 ganglioside activator protein[4,a]	Lipid metabolism
PAFAH1B2	PAFAH beta subunit[4,a]	Lipid metabolism
PAM	Isof. 1 of peptidyl-glyc. α-amidating monooxy.[4,a]	Protein and fatty acid metabolism
PLA2G2A	Phospholipase A2, group IIA[3]	Membrane phospholipid metabolism
PLTP	Phospholipid transfer protein[2,3]	Cholesterol metabolism
PON1	Paraoxonase 1[4,a]	HDL trans. and cholesterol metab. and antioxidant
PSAP	Prosaposin[2,7]	Enzyme stimulator in glycosphingolipid metabolism
SAA4	Serum amyloid A4[4,a]	HDL transport
		(xii) Other/unknown
AGT	Angiotensinogen[2]	Cleaved to angiotensin I, blood pressure
APOA1BP	Apolipoprotein A-1 binding protein[4,a]	?

Table 1 (*continued*)

Gene symbol	Protein	Function (as per locust link, OMIM or source)
A1BG	Alpha 1b glycoprotein[4,a]	?
B2M	Beta-2-microglobulin[1,2,3,7]	Loss assoc. with hypercatabolic hypoproteinemia
BTD	Biotinidase[4,a]	Nitrogen compounds metabolism
CLEC3B	Tetranectin[4,a]	Stimulates activation plasminogen to plasmin
COCH	Cochlin[4,a]	?
EVC2	Ellis van Creveld syndrome 2 (limbin)[13]	Mutation assoc. wdith Ellis-van Creveld syndrome
F12	Coagulation factor xii[4,a]	Blood coagulation regulator
GPX3	Glutathione peroxidase 3[4,a]	Regulates oxidative-reductive process
HSPA4	Heat shock 70 kDa protein 4[2]	Heat shock protein
HSP90AA1	Heat shock protein HSP 90-alpha[4,a]	Protein folding
KNG1	Kininogen[4,a]	Blood coagulation regulator
LIN7C	Protein lin-7 homolog C[4,a]	Neurotransmitter secretion
LRG1	Leucine-rich alpha-2-glycoprotein 1[2]	?
QDPR	Quinoid dihydropteridine reductase[4,a]	Tetrahydrobiopterin metabolism
RNASE4	Ribonuclease 4[4,a]	Nucleotide and nucleic acid metabolism
RPL7A	Ribosomal protein L7A[4,a]	Ribosome biogenesis
SFRP1	Secreted frizzled-related protein 1[2]	Possible role in polarity of retinal photoreceptor cells
SMR3A	Submax. gland androg. reg. prot. 3 homol. A (mouse)[1,3]	?
SMR3B	Submax. gland androg. reg. prot. 3 hom. B (mouse)[1,3,7]	?
SOD1	Superoxide dismutase 1[4,a]	Antioxidant
SOD3	Superoxide dismutase 3[4,a]	Binds heparin sulfate, anti oxidant
SULF2	Isoform 2 of extracellular sulfatase Sulf-2[4,a]	Sulfur compound metabolism
TJP1	Isoform short of tight junction protein ZO-1[4,a]	Blastocyst formation
TXNRD1	Thioredoxin reductase 1[4,a]	Cell redox homeostasis
VMO1	Vitelline membrane outer layer protein 1 homolog[4,a]	Vitelline membrane formation
VTN	Vitronectin[4,a]	Cytolysis and blood coagulation regulator
YARS	Tyrosyl-tRNA synthetase[4,a]	Protein metabolism
XDH	Xanthene dehydrogenase[4,a]	Purine metabolism
		(xiii) Phosphatase/kinase/GTPase/other enzyme
ACPP	Acid phosphatase[2]	Phosphatase
ARHGAP1	Rho GTPase activating protein[2]	GTPase activator of Rho, Rac and Cdc42
CA2	Carbonic anhydrase II[2]	Hydration of carbon dioxide
CP	Ceruloplasmin (ferroxidase)[1,2,7]	Peroxidation of Fe(II)transferrin, binds copper
F2	Coagulation factor II (thrombin)[1]	Fibrinogen to fibrin conversion
F5	Coagulation factor V[2]	Prothrombin/thrombin conversion with coag. factor X
GNPTG	N-acetylglucosamine-1-phosphate transf., γsubunit[2]	Targeting of lysosomal hydrolases to lysosomes
HTRA1	HtrA serine peptidase 1[2]	Cleaves IGF-binding proteins
MMP9	Matrix metallopeptidase 9[2]	Matrix collagen IV and V degradation
NME1	Nucleoside diphosphate kinase A[4,a]	Cell different., develop. and metastasis suppres.
NENF	Neudesin[4,a]	Positive regulator of MAPK cascade
S100A8	**S100 calcium binding protein A8 S**§[1,2]	Possible cytokine and inhibitor of casein kinase
S100A9	**S100 calcium binding protein A9 S**§[1,2]	Possible inhibitor of casein kinase
TGM2	Transglutaminase 2[2]	Protein crosslinker
TXN	Thioredoxin[2]	Catalyzes dithiol-disulfide exchange and redox rxns
USP5	Ubiquitin specific peptidase 5 (isopeptidase T)[2]	Cellular protein degradation
		(xiv) Protease/Inhibitor/Antimicrobial
A2M	Alpha-2-macroglobulin[2,7]	Protease inhibitor, cytokine transporter
AMBP	Alpha-1-microglobulin/bikunin precursor[2]	Inhibits trypsin, plasmin, elastase
ARG1	Arginase[4,a]	Hydrolysis of L-arginine
AZU1	Azurocidin 1 (cationic antimicrobial protein 37)[2]	Antibacterial and monocyte chemoattractant
CLN5	Ceroid-lipofuscinosis neuronal protein 5[4,a]	Lysosome organization
CST1	Cystatin SN§[1,2,3]	Cysteine protease inhibitor
CST2	Cystatin SA[3]	Thiol protease inhibitor
CST3	Cystatin C[1,3]	Abundant cysteine protease inhibitor
CST4	Cystatin S#§[1,2,3,7]	Cysteine protease inhibitor
CST5	Cystatin D[3]	Cysteine protease inhibitor
CST6	Cystatin M[4,a]	Cysteine protease inhibitor
CTSB	Cathepsin B[2]	Lysosomal cysteine protease
CTSD	Cathepsin D[2]	Lysosomal aspartyl protease
CTSG	Cathepsin G[2]	Chymotrypsin C-like protease, antimicrobial
CTSS	Cathepsin S[4,a]	Hydrolase and protease activity
DCD	Dermcidin[2]	C-terminal antibacterial, N-terminal prosurvival
DEFA3	Defensin, alpha 3, neutrophil-specific[1]	Anti-bacterial, -viral, -fungal
DPP4	Dipeptidyl-peptidase 4 (CD26)[2]	Intrinsic membrane serine exoprotease
ELA2	Elastase 2, neutrophil[1]	Matrix hydrolysis, antibacterial
GBP1	Interferon-induced guanylate-binding protein 1[4,a]	Anti-viral
HABP2	Hyaluronan-binding protein 2 isoform 2[4,a]	Hyaluronan binding
IDE	Insulin degrading enzyme[4,a]	Insulin degradation
IGHA1	Immunoglobulin heavy constant alpha 1[1,2,13,3,7]	Microbial and foreign antigen defense
IGHA2	Immunoglob. heavy constant alpha 2 (A2m marker)[3]	Microbial and foreign antigen defense
IGHM	Immunoglobulin heavy constant mu[1,2,3]	Microbial and foreign antigen defense
IGJ	Immunoglobulin J polypeptide[1,3]	Microbial and foreign antigen defense
IGKC	Immunoglobulin kappa constant[3]	Microbial and foreign antigen defense
IGLC2	Immunoglobulin lambda constant 2#[3]	Microbial and foreign antigen defense

(*continued on next page*)

Table 1 (*continued*)

Gene symbol	Protein	Function (as per locust link, OMIM or source)
IGLV1-40	Immunoglobulin lambda variable 1−40[2]	Microbial and foreign antigen defense
ITIH1	Inter-alpha (globulin) inhibitor H1[2]	Hyaluronan bp/carrier, pred. serine protease inhib.
ITIH2	Inter-alpha (globulin) inhibitor H2[2]	Hyaluronan bp/carrier, pred. serine protease inhib.
ITIH3	Inter alpha trypsin inhibitor, heavy chain 3[4,a]	Hyaluronan metabolism
ITIH4	Inter-alpha (globulin) inhibitor H4[2]	Predicted serine protease inhibitor
KLKB1	Plasma kallikrein[4,a]	Fibrinolysis and proteolysis
LCN2	Lipocalin 2 (oncogene 24p3)[2]	MMP9 binding, bacteriostatic, growth factor-like
LPO	Lactoperoxidase[2,7]	Antibacterial
LTF	Lactotransferrin $#§[1,2,13,3,7]	Iron metabolism, antibacterial
LYZ	Lysozyme (renal amyloidosis)*$#[1,2,13,3,7]	Hydrolase, antibacterial
MPO	Myeloperoxidase[2]	Antimicrobial
MUC7	Mucin 7, secreted[1]	Antibacterial, antifungal
PH4B	Protein disulfide isomerase[4,a]	Breaking and formation of disulphide bonds
PI3	Elafin[4,a]	Elastase inhibitor
PIGR	Polymeric immunoglobulin receptor#[1,2,3,7]	Antibacterial, polymeric Ig transcellular transport
PLG	Plasminogen[2]	Activates urokin.-type plasmin. activat., collagenas.,
PRB4	Proline-rich protein BstNI subfamily 4[3]	Possible bacterial binding (lost in point mutant)
RNPEP	Arginyl aminopeptidase[4,a]	Exoprotease (removes arginine and/or lysine)
S100A7	S100 calcium binding protein A7[4,a]	Chemotactic, anti-bacterial
SCPEP1	Retinoid-inducible serine carboxypeptidase[4,a]	Carboxyprotease activity
SERPINA1	Serpin peptid. inhibit., clade A (alpha-1), member 1*[1,2]	Serine protease inhibitor, anti-inflammatory
SERPINA3	Serpin peptidase inhibit., clade A (alpha-1), member 3[2]	Serine protease inhibitor
SERPINA4	Kallistatin[4,a]	Kallikrein inhibitor
SERPINB8	Serpin B8[4,a]	Prohormone convertase inhibitor
SERPINA6	Corticosteroid binding globulin[4,a]	Serine protease inhib. and cortisol binding and trans.
SERPINA7	Thyroxine binding globulin[4,a]	Hormone binding and serine protease inhibitor
SERPINC1	Serpin peptid. inhibit., clade C (antithrom.), member 1[1,2]	Blood coagulation cascade regulator
SLPI	Secretory leukocyte peptidase inhibitor[1,3,7]	Acid-stable protease inhib., antibacterial
SPINT1	Serine peptidase inhibitor, kunitz type 1[4,a]	Protease inhib. and extra cellular matrix organi.
TIMP1	TIMP metallopeptidase inhibitor 1[5,2]	Metalloprotease inhibitor
TIMP2	TIMP metallopeptidase inhibitor 1[5]	Metalloprotease inhibitor, inhib. endothelial prolif.
WFDC2	WAP four disulfide core domain 2[4,a]	Serine protease inhibitor
		(xv) Receptor/channel/transport
ATP5J	ATP synth.-coup. factor 6, mitochon. isoform B precursor[4,a]	Proton and ion transport
CLIC2	Chloride intracellular channel 2[3]	Potential chloride ion channel
MFI2	Melanoma associated antigen p97[4,a]	Iron ion transport and homeostasis
MT1X	Metallothionein[4,a]	Cellular metal ion homeostasis
PRSS8	Prostasin[4,a]	Channel regulator and protease
RBP4	Retinol binding protein 4[4,a]	Retinol binding and transport
SLC7A4	Solute carrier family 7, member 4[3]	Cationic amino acid transport
SLC12A2	Solute carrier family 12, member 2[4,a]	Ion transport
STX7	Syntaxin 7[4,a]	Vesicle mediated transport

The list is derived from published reflex tear (1, 4), closed eye tear (2), open and closed eye tear (3), Meibomian gland secretion (14), open and closed eye tear capture ELISA or antibody array (5, 6) and lacrimal gland EST (7) analyses. Not listed are the numerous cytoplasmic proteins that are also detected in tears (2, 4). *,Δ,†,$,#,€,£,¥,§ suggested to be less or **more** than normal in tears from patients suffering from *blepharitis (8), $#€dry eye (9, 10,11), ΔSjögren's syndrome(12), †contact lens related dry eye (13) £climatic droplet keratopathy (15), ¥fusarium keratitis (16) or §dry eye and meibomian gland dysfunction (17).
(1-Zhou et al., 2004; 2-de Souza et al., 2006; 3-Sack et al., 2007; 4-Zhou et al., 2012; 5-Sack et al., 2005; 6-Ozyildirim et al., 2005; 7-Green-Church et al., 2008; 8-Koo et al., 2005; 9-Zhou et al., 2009; 10-Srinivasan et al., 2012; 11-Na et al., 2012; 12-McKown et al., 2009; 13-Nicholas and Green-Church, 2009; 14-Tsai et al., 2006; 15-Lei et al., 2009; 16-Ananthi et al., 2013; 17-Soria et al., 2013).

[a] Updated from Table 1 of "Laurie GW, Olsakovsky LA, Conway BP, McKown RL, Kitagawa K, Nichols JJ. Dry eye and designer ophthalmics. Optom Vis Sci 2008; 85:643-52. ©The American Academy of Optometry 2008".

Green-Church, 2009; Srinivasan et al., 2012) is necessary to distinguish monomeric lacritin from inactive multimeric lacritin (Velez et al., 2013), and likely also the inactive lacritin-c splice variant (McKown et al., 2009). Exploration of lacritin cell targeting and signaling mechanisms has revealed a network of interdependent molecules, each necessary for lacritin activity. New evidence is suggesting that some of these are also decreased in dry eye. Here we review recent advances in our understanding of lacritin, and provide an overview of lipocalin-1 whose eye specific expression parallels that of lacritin. We also update our current understanding of the tear proteome.

2. Lacritin

2.1. Structure and expression

The discovery of lacritin indirectly emerged from a screen for novel factors capable of promoting tear protein secretion, with cDNA cloning out of a human lacrimal gland library (Sanghi et al., 2001). The lacritin gene, LACRT, is one of the most eye specific (Sanghi et al., 2001) and resides on 12q13, within ~1.24 Mb of the AAAS gene associated with alacrima (Kumar et al., 2002). Human lacritin is coded by a 417 bp open reading frame that translates as a 14.3 kDa hydrophilic protein with a 19 amino acid signal peptide resulting in a secreted protein with a predicted molecular mass of 12.3 kDa (Fig. 1). Mobility in SDS PAGE gels is ~18 kDa for recombinant lacritin generated in *Escherichia coli* (Wang et al., 2006), and ~23–25 kDa with glycosylation in tears (Seifert et al., 2012). Such aberrant mobility may be attributable to lacritin's C-terminal amphipathic α-helix that supports lacritin cell surface targeting of the heparan sulfate proteoglycan syndecan-1 (Fig. 1), as confirmed by circular dichroism (Wang et al., 2006; Zhang et al., 2013) and point mutagenesis (Zhang et al., 2013). Lacking the C-terminal amphipathic α-helix and inactive (Wang et al., 2013) is splice variant lacritin-c (McKown et al., 2009). PSIPRED (v3.3) predicts three other C-terminal half α-helices (Fig. 1) in secreted lacritin. The

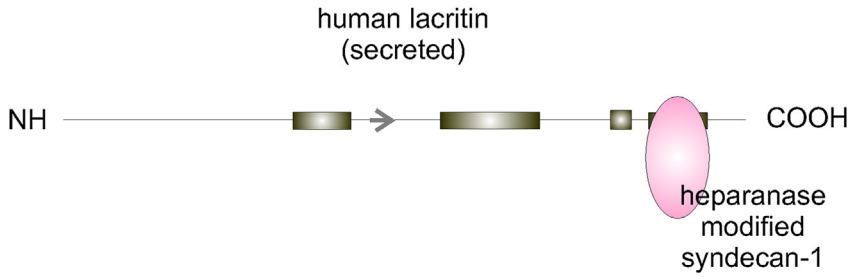

Fig. 1. Linear diagram of secreted lacritin. PSIPRED (v3.3) predicted α-helices are indicated by rectangles; the arrow indicates a short predicted β-strand. The most C-terminal α-helix is amphipathic and targets the cell surface proteoglycan syndecan-1 after heparanase modification (Wang et al., 2006). Both the hydrophobic face (L108/L109/F112) and the cationic face (Q103/K107 and K111) of lacritin are involved (Zhang et al., 2013). These target three syndecan-1 elements (Zhang et al., 2013): (i) the conserved hydrophobic sequence GAGAL, and (ii) chondroitin-4-sulfated and (iii) heparanase-cleaved 3-O-sulfated heparan sulfated chains on N-terminal serines 15, 23 and 25 (human syndecan-1 numbering excludes the signal peptide). α-Helicity of lacritin's C-terminal a-helix has been validated by circular dichroism (Wang et al., 2006; Zhang et al., 2013). Syndecan-1 has a short transmembrane domain known only for cytoskeletal signaling. Rapid lacritin signaling appears to be attributable to an associated G-protein coupled receptor, as first implied by pertussis toxin inhibitable mitogenic signaling (Wang et al., 2006).

C-terminal half is thus ordered, whereas the N-terminal half is largely disordered (Fig. 1; McKown et al., 2009). Thirteen sites of O-glycosylation (NetOGlyc 3.1) and one N-glycosylation site (NetNGlyc 1.0) are predicted (McKown et al., 2009), with O-glycosylation restricted to the disordered region predicted by PONDR ('Predictor of Naturally Disordered Regions') in the N-terminal half (McKown et al., 2009). The N-glycosylation site flanks the syndecan-1 binding domain, but is not generally conserved among orthologs. Patients with climatic droplet keratopathy display decreased N-linked glycosylation (Lei et al., 2009). However non-glycosylated lacritin is active in prosecretory, mitogenic, cytoprotective and syndecan-1 binding assays. It is possible that glycosylation enhances stability.

Lacritin mRNA and protein are highly expressed in human lacrimal gland as the sixth most common mRNA (Ozyildirim et al., 2005), with detection in lacrimal acinar cell secretory granules (Sanghi et al., 2001). Lacritin protein also appears to be expressed by the human meibomian gland (Tsai et al., 2006), and was recently detected in the gland of Wolfring (Ubels et al., 2012). In monkey, lacritin mRNA has been detected also in conjunctiva and corneal epithelium, and at low levels in other eye tissues including retinal and lens epithelia (Nakajima et al., 2007). A single retinal hit has been noted in Human Proteinpedia (HuPA_00710). In non-ocular tissues, lacritin is highly expressed in an apparent ductal-like cell in human submandibular and parotid glands – but not in acinar cells. Lacritin also appears to be produced at low levels in thyroid (Sanghi et al., 2001), and has been noted by RT-PCR in normal breast and invasive breast cancer tumors (Weigelt et al., 2003) and by proteomics in saliva (Human Proteinpedia [HuPA_00047]), lung lavage (HuPA_00022) and plasma (Schenk et al., 2008). No other expressing tissues were noted in a fifty tissue RNA dot blot and by tissue microarray of seventy-five different human organs (Sanghi et al., 2001). Release is apical from acinar cells into ducts that carry lacritin onto the surface of the eye (Morimoto-Tochigi et al., 2010), where lacritin is detected in tears (Sanghi et al., 2001; Nakajima et al., 2007; Seifert et al., 2012).

2.2. Predicted and demonstrated orthologs

We extracted LACRT aligned genomic sequences from over twenty-one species in Ensembl (release 70), guided by AceView defined exon boundaries for LACRT (Supplementary Fig. 1; see method in Laurie et al., 2012). Exon sequences were spliced (Supplementary Fig. 2) and then translated (Supplementary Fig. 3), with some displaying incomplete sequence. No complete mouse or rat ortholog was apparent although other rodents are represented, an observation possibly related to the predicted telomeric location or alternatively reflective of significant differences in lacrimal specific gene expression between mouse and human (Ozyildirim et al., 2005). Non-primate lacritins display on average 25% amino acid identity with human lacritin (vs 75% for primates), although bushbaby lacritin is only 41% identical (Table 2). Analysis of each by PSIPRED (version 3.3) displayed a predominance of predicted α-helices in the C-terminal half (Fig. 2).

Predicted identity of horse lacritin with human lacritin (45%) is greater than any other known non-primate ortholog (Table 2). Blotting of horse tears with anti-C vs anti-N terminal domain specific antibodies revealed that horse lacritin was mainly represented as a ~13 kDa C-terminal-half fragment with a predicted amphipathic α-helix at the C-terminus (Laurie et al., 2012).

2.3. Tools and manufacture

The 363 bp human coding region without signal peptide was subcloned into pTYB1 (New England Biolabs, Inc; Ipswich MA) as

Table 2
Human lacritin and orthologs (numbering includes signal peptide).

Species	Nucleotide#	Amino acid#	Nucleotide identity (%)	Amino acid identity (%)
Human [*H. sapiens*]	417	138		
Bushbaby [*O. garnettii*]	540	138	71	41
Cat [*Felis catus*]	356	119	72	38
Chimpanzee [*P. troglodytes*]	414	137	99	99
Cow [*B. Taurus*]	423	126	69	4
Dog [*Canis lupus familiaris*]	368	111	64	35
Elephant [*L. Africana*]	427	136	68	7
Gibbon [*N. leucogenys*]	331	108	93	67
Gorilla [*G. gorilla gorilla*]	417	137	99	97
Guinea pig [*C. porcellus*]	422	100	62	11
Horse [*E. caballus*]	336	111	75	45
Lesser hedgehog tenec [*E. telfairi*]	391	120	66	4
Macaque [*M. Mulatta*]	417	140	93	89
Marmoset [*C. jacchus*]	424	137	87	74
Microbat [*M. lucifugus*]	417	111	72	35
Mouse lemur [*M. murinus*]	417	135	70	*42
Orangutan [*P. abelii*]	417	138	97	94
Panda [*A. melanoleuca*]	433	126	68	37
Rabbit [*O. cuniculus*]	443	122	63	23
Shrew [*S. araneus*]	503	165	62	*32
Squirrel [*L. tridecemlineatus*]	514	143	61	26
Tree shrew [*T. belangen*]	437	139	64	*30

Values were determined by extraction of nucleotide sequence from Ensembl (release 70) genomic alignments with exon boundaries guided by AceView. *Identities differ slightly from those indicated by Ensembl.

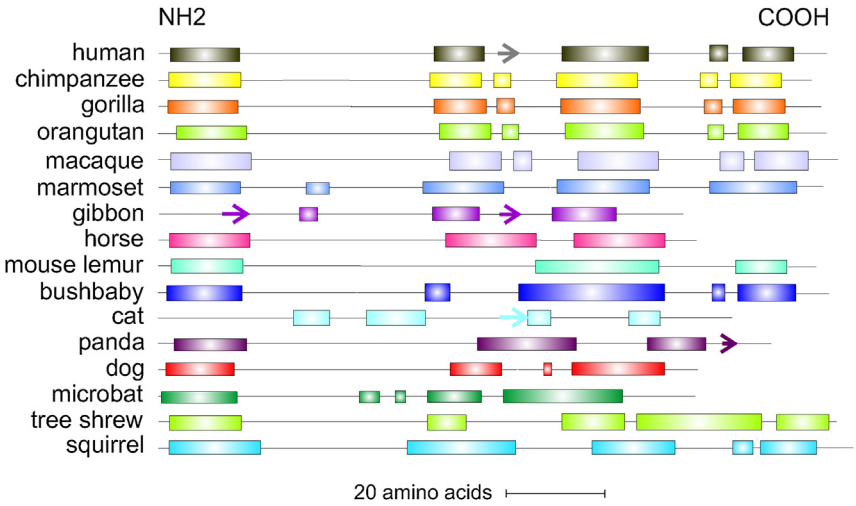

Fig. 2. Predicted and demonstrated lacritin orthologs. Shown are secondary structure predictions by PSIPRED (v3.3). Rectangles and arrow respectively indicate predicted α-helices and a β-strand. The N-terminal α-helix is the signal peptide. The most C-terminal α-helix in human lacritin regulates many of lacritin's activities via an amphipathic structure.

'pLAC' for expression of lacritin–intein fusion protein (Wang et al., 2006). Intein serves as a convenient affinity tag for purification that is retained on the chitin affinity column upon elution with β-mercaptoethanol. Eluted lacritin is concentrated, dialyzed versus PBS and then is further purified on DEAE (diethylaminoethanol) covalently linked to Sepharose. DEAE removes contaminating endotoxin. Also binding to DEAE is a C-terminal lacritin cleavage fragment, and the *E. coli* chaperone DnaK (McKown et al., 2013). DnaK is likely involved in lacritin synthesis.

Although lacritin's low nanomolar prosecretory (Sanghi et al., 2001), mitogenic (Wang et al., 2006) and cytoprotective (Wang et al., 2013) activities require only 1 or 10 nM lacritin, optimizing production from current ~0.1 g/l yields is desirable for cost efficiency – particularly for scale up. C-terminal hydrophobic residues might promote some misfolding. Misfolded molecules aggregate and become secluded in inclusion bodies. Single or double point mutagenesis to serine of I68, I68/I78, V69, I73, V91, I98, F104, L108, L109 or F112 enhanced yields two or three times (McKown et al., 2013). Some are inactivating, but lacritins V69S, I73S, L108S and L109S retain activity (Wang et al., 2013). Salt bridges can also promote misfolding, although many are stabilizing. Yield increases were noted for lacritins K66S/E70S, K66S/E70S/E103S/K107S and E103S/K107S (McKown et al., 2013), of which lacritin K66S/E70S retains activity. Another approach is to address codon usage. Some common human codons are rare or uncommon in *E. coli* such that tRNA's necessary for recombinant protein production are insufficient. Thirteen synonymous mutants were generated, however yield increases were low. It is possible that rare to common codon mutagenesis of lacritins V69S, I73S, L108S, L109S or K66S/E70S may increase yields (McKown et al., 2013).

2.4. Cell targeting

A deglycanated form of syndecan-1 was discovered to be the main cell surface binding protein for lacritin by mass spectrometric sequencing cell surface proteins bound to lacritin columns in buffer containing 100 mM NaCl. Validation was by affinity precipitation (Ma et al., 2006). Syndecan-1 is a widely expressed cell surface heparan sulfate proteoglycan with a carboxy terminal end anchored in the plasma membrane with short cytoplasmic tail. Its ectodomain is substituted proximally with chondroitin sulfate chain(s) at serines 184 and 194 (human syndecan-1; numbering excludes the signal peptide), and distally with up to three heparan sulfate chains (serines 15, 23, 25) without or with a short chondroitin sulfate chain (Kokenyesi and Bernfield, 1994). Lacritin's C-terminal α-helix (Wang et al., 2006) bound a domain within syndecan-1 amino acids 1–50, with binding dependent on prior heparanase cleavage of heparan sulfate (Ma et al., 2006). SiRNA knockdown of syndecan-1 abrogated lacritin dependent mitogenic activity, as did depletion of heparanase (but not heparanase-2), but could be rescued by addition of exogenous heparanase or with bacterial heparitinase (Ma et al., 2006). Recently, we narrowed the binding domain to hydrophobic amino acids 20–30 that when reduced to synthetic peptide GAGAL, enhanced lacritin C-terminal α-helicity (Zhang et al., 2013). Binding is equally dependent on substitution of S23 and S25 (and possibly S15) with both heparan sulfate and chondroitin sulfate as a novel hybrid domain of hydrophobic core protein, heparanase cleaved heparan sulfate and adjacent chondroitin sulfate (Zhang et al., 2013). Heparanase is not widely expressed, but is detected in tears by Western blotting (Ma, Wang and Laurie, unpublished), although at levels too low yet for proteomic detection.

With each modification essential for lacritin binding, cell targeting by lacritin is very selective. Contributing to cell selectivity is a signaling receptor(s). Signaling is initiated within seconds (Wang et al., 2006). A candidate G-protein coupled receptor has been identified (Zimmerman and Laurie, unpublished). SiRNA knockdown in cells abrogates lacritin dependent mitogenesis (Ma and Laurie, unpublished). Lacritin targets rat (Sanghi et al., 2001) and monkey (Fujii et al., 2013) primary lacrimal acinar cells in secretion assays, and primary human corneal epithelial (Wang et al., 2013) and HCE-T (Wang et al., 2006, 2013) human corneal epithelial (SV40-large T) cells in cytoprotection assays. Lacritin is mitogenic for HCE-T, HCE, human salivary HSG/HeLa and embryonic kidney (HEK293) cells, but not for human epidermal (A431), cervical (HeLa), foreskin fibroblast (HS68), fibrosarcoma (HT1080), erythroleukemia (K-562), breast carcinoma (MCF7), melanoma (SK-MEL, WM-164), Leydig (TM4), Sertoli (TM3), glioma (U-1242-MG, U251-MG) and mouse fibroblast (NIH3T3) (Wang et al., 2006). No fibrosis, angiogenesis, or inflammation has been observed in eyes of lacritin treated rabbits (Samudre et al., 2011), monkeys or rats (unpublished).

2.5. Prosecretory and protearing activities

Our original discovery screen employed primary cultures of rat lacrimal acinar cells (Sanghi et al., 2001) in a 96-well assay that monitored tear peroxidase release normalized to cellular DNA (Chen et al., 1998). 0.8–13 nM of recombinant human lacritin stimulated peroxidase secretion in a dose-dependent manner, without affecting carbachol/VIP stimulated secretion (Sanghi et al., 2001). Similarly, lacritin purified from monkey tears triggered protein secretion from monkey lacrimal acinar cells even under conditions of stress from the inflammatory cytokines interferon-γ and tumor necrosis factor that abrogated carbachol stimulated secretion (Fujii et al., 2013). Low level tear stimulation, induced in part by lacritin (Samudre et al., 2011), is responsible for continually wetting the ocular surface (Dartt, 2009) with basal tears.

3.2 nM recombinant human lacritin triggers calcium signaling by cultured human corneal epithelial cells (Sanghi et al., 2001). The corneal epithelium is intimately associated with sensory nerves that penetrate and form neuro/epithelial junctional complexes (Müller et al., 1996). Treating normal rabbits with 0.8–8 μM recombinant lacritin promoted tearing within 60 min – the earliest time point assayed, and lasted at least 240 min (Samudre et al., 2011). Tearing was measured via Schirmer strips 10 min after proparacaine anesthesia to minimize the inclusion of reflex tears. To test for toxicity, eyes were treated with 4 μM lacritin three times daily for two weeks, or alternatively with 1.1 μM lacritin truncation C-25 that lacks the syndecan-1 binding site. Lacritin steadily enhanced basal tearing without toxicity. One week after washout, basal tearing remained elevated, whereas C-25 had no effect (Samudre et al., 2011). These observations highlight the potential of lacritin as a tear secretagogue alone or in combination with other agonists.

2.6. Promitogenic activity and signaling

Early studies also noted that recombinant lacritin promoted HSG/HeLa cell proliferation over a 0.2–0.8 nM dose range that approximated the serum positive control (Sanghi et al., 2001), suggesting that lacritin was a pleiotropic tear factor that might contribute to regulation of epithelial renewal as it flowed downstream from lacrimal acinar cells onto the eye (Wang et al., 2006). Over a broader dose range, lacritin displayed a biphasic dose response with an optimum of 1 or 10 nM. Lacritin truncations lacking 15–49 amino acids from the C-terminus were inactive, whereas truncation of 5 or 10 C-terminal, or 24 N-terminal amino acids had no effect (Wang et al., 2006). Mitogenic signaling is initiated within seconds and proceeds through the G proteins $G\alpha_i$ or $G\alpha_o$ to the phosphatase PP2A (Karnati and Laurie, unpublished), leading to rapid dephosphorylation of PKCα. Dephosphorylated protein kinase C-α (PKCα) translocates to the perinuclear Golgi region where it activates phospholipase D1 (PLD1) and phospholipase Cγ2 (PLCγ2) to generate IP3. IP3 triggered release of calcium into the cytoplasm activates the phosphatase calcineurin to in turn dephosphorylate the transcription factor NFATC1 (nuclear factor of activated T cells, calcineurin dependent 1) that translocates into the nucleus (Wang et al., 2006) to co-regulate the transcription of genes involved in cell growth and secretion (Heit et al., 2006). Via a parallel pathway, $G\alpha_i$ or $G\alpha_o$/PKCα/PLC activates PLD1 and in turn mTOR that also promotes proliferation in a manner synergistic with NFATC1 (Wang et al., 2006).

2.7. Cytoprotective activity

Lacritin promotes the survival of human corneal epithelial cells stressed with interferon-γ and tumor necrosis factor (Wang et al., 2013). As a simple assay, we monitored the nuclear translocation of 'Forkhead box O3' (FOXO3). FOXO3 is nuclear in stressed or dying cells and cytoplasmic when cells are healthy. When interferon-γ/tumor necrosis factor stressed human corneal epithelial cells were treated 10 mM lacritin, FOXO was cytoplasmic – but remained nuclear with 10 nM C-25 (Wang et al., 2013). The same assay monitored manipulations with normal and dry eye tears. Normal, but not dry eye, tears are protective (cytoplasmic FOXO3), however protective activity is lost when lacritin is completely immunodepleted from tears (nuclear FOXO3). Similarly, spiking 10 nM lacritin, but not C-25, into dry eye tears restores protective activity (Wang et al., 2013).

To begin to discern how lacritin is prosurvival, we monitored the cleavage of caspases 3 and 9 in the absence or presence of lacritin. Lacritin had no effect on caspase cleavage and no DNA fragmentation was apparent, suggesting that interferon-γ/tumor necrosis factor treatment to induce stress did not trigger apoptosis and that the lacritin survival mechanism is not anti-apoptotic. However changes were observed in the lipidation of autophagy marker microtubule-associated protein 1 light chain 3 (LC3) (Wang et al., 2013). Autophagy captures stress damaged proteins and organelles by enclosure in lipidated LC3 covered autophagosomes. We monitored the process by transducing interferon-γ/tumor necrosis factor stressed human corneal epithelial cells with an LC3 construct double tagged with low pH sensitive green fluorescent protein and pH insensitive mCherry ('LC3RG') such that time dependent transition of LC3RG isolation membranes (double membrane that begins the enclosure of damaged proteins or organelles) to autophagosomes (enclosure completed and fused) and then fusion with lysosomes (low pH) could be followed. We discovered that lacritin, but not C-25, promoted the acceleration of autophagy for ~60 min, and then ceased. This was sufficient to restore oxidative phosphorylation, promote both mitochondrial fusion and cell survival, and trigger changes in 29 metabolites, including the suppression of kynurenine (Wang et al., 2013). Kynurenine is elevated in sera of patients with primary Sjögren's syndrome (Pertovaara et al., 2005), rheumatoid arthritis and other autoimmune diseases (Katz et al., 2008). When instead of interferon-γ/tumor necrosis factor stress, LC3RG cells were co-transduced with a cyan fluorescent protein-labeled huntingtin construct (Htt103Q) that is cell toxic, the same autophagic pulse was triggered by lacritin, but not C-25. No autophagic pulse was apparent when cells were co-transduced with a non-toxic huntingtin construct (Htt25Q) (Wang et al., 2013). Thus stress is a prerequisite for lacritin stimulated autophagy.

Unlike mitogenic signaling, survival signaling is mediated by FOXO3 and FOXO1. By 1 min, lacritin promotes the acetylation (and phosphorylation) of FOXO3 – a modification coincident with ligation of autophagy-related protein 101 and acceleration of autophagy (Wang et al., 2013). We also observed that stress promotes the immediate acetylation of FOXO1, as first reported by Zhao et al. (2010) in cancer cells, but unlike Zhao et al. (2010), stress was insufficient to promote ligation of acetylated FOXO1 with autophagy-related protein 7. Instead, lacritin is required and does so 5–15 min after administration (Wang et al., 2013). The autophagic pulse mechanism appears to be well-suited for the stressed ocular surface as each new bolus of lacritin is delivered to the eye, to then drain away into the nasolacrimal duct. Maintaining all elements of this system may be required for ocular surface health.

2.8. Molar levels in tears

Systematically discerning the bioactivity and molar variation of individual tear proteins in the entire tear proteome could open up a new era of ocular diagnosis and treatment. To gain information on

lacritin levels in tears, a 'lacritin' ELISA sold by USCN (#E2576Hu, L091229053) was tested. Unfortunately, it failed to detect recombinant human lacritin and the positive control migrated in SDS-PAGE as a broad smear (McKown and Laurie, unpublished). We therefore established an ELISA with N-terminal-specific anti-lacritin antibody 'anti-Pep Lac N-Term' that detects lacritin in tears without interference from other tear proteins (Seifert et al., 2012). Basal tear samples from 66 individuals aged 18–52 years were individually tested over six replicates revealing a mean of 4.2 ± 1.17 ng/100 ng of total tear protein with little difference by gender, although lacritin may be greater in female reflex tears (Ananthi et al., 2011). Assuming an estimated basal tear protein concentration of ~8 mg/ml (Sitaramamma et al., 1998), lacritin molar levels are 18–27 μM. In contrast, lysozyme is ~20 ng/100 ng total tear protein (Seifert et al., 2012), or ~100 μM – in agreement with others (Sen and Sarin, 1982 [80 μM], Velos et al., 1985 [86 μM]) with estimates to 300 μM (Eylan et al., 1977). To assess whether time of day influences lacritin levels, we collected tears from 34 additional individuals at 7:30–8:30 am (0 h), 11:30 am–12:30 pm (4 h), 4:30–5:30 pm (8 h) and 7:30–8:30 am the following day (24 h). No differences were observed.

18–27 μM lacritin is more than one-thousand fold greater than the accepted dose optimum of 1–10 nM (Wang et al., 2006), although 0.8–8 μM was effective when applied topically in rabbits (Samudre et al., 2011). Perhaps the availability of epithelial bioactive lacritin is restricted by an unknown ocular surface or secretory mechanism. Present in all anti-lacritin tear blots was the ~25 kDa lacritin band, and a ~13 kDa fragment. Also present, but overlooked were ~50 kDa, ~75 kDa and occasionally even higher molecular weight bands. Secondary antibodies showed no cross-reactivity with tears. With new anti-lacritin monoclonal antibody 1F5 displaying preference for the 75 kDa band, we explored the putatively larger species in more detail (Velez et al., 2013) and discovered that tissue transglutaminase in tears (Table 1) generates lacritin multimers. Multimers are largely inactive (Velez et al., 2013).

Exogenous tissue transglutaminase (1.5 μM) from guinea pig promoted crosslinking between deprotonated lacritin lysines 82 or 85 with acceptor glutamine 106 that was initiated within 1 min, and completed 40–90 min later. No crosslinking was observed in the presence of EDTA, or after prior denaturation of tissue transglutaminase by boiling, or when tissue transglutaminase was replaced by an inactive recombinant human tissue transglutaminase (Velez et al., 2013). We then immunodepleted all lacritin monomer, multimer and fragment from human tears. Recombinant lacritin spiked into immunodepleted tears formed dimers, trimers and tetramers after overnight incubation at 37 °C. In the negative control without tears, a small amount of dimer formed (Velez et al., 2013). Since glutamine 106 resides within the lacritin mitogenic domain (amino acids 100–109) that targets syndecan-1, we wondered whether lacritin activity was affected by cross-linking, and discovered that syndecan-1 binding was substantially decreased (Velez et al., 2013). Also suppressed was lacritin cytoprotective activity since tissue transglutaminase cross-linked lacritin was substantially less effective at rescuing interferon-γ/tumor necrosis factor stressed human corneal epithelial cells (Romano and Laurie, unpublished). Blotting suggests that normal human tears contain 0.6 μM tissue transglutaminase, that thus appears to act as a negative regulator of monomeric (epithelially active) lacritin. Primary human corneal epithelial cells express both transglutaminase 1 and tissue transglutaminase mRNAs. mRNA expression of both increases with hyperosmolar stress, particularly transglutaminase 1 (Chen et al., 2008), however transglutaminase 1 has not been detected in tears. Thus, lacritin may be subjected to enhanced cross-linking and deactivation in dry eye.

3. Lipocalin-1

3.1. Structure and expression

Tear lipocalin (LCN1; recently reviewed by Glasgow and Gasymov, 2011; Dartt, 2011) was originally noted as an unknown band in early electrophoretic separations of human tears and named tear pre-albumin (Erickson et al., 1956), based on its paper electrophoretic mobility near serum albumin proximal to the anode. Lack of immunological cross-reactivity in normal human tears suggested that the two were distinct (Josephson and Lockwood, 1964), in keeping with earlier studies by Fleming suggesting lack of serum immunoreactivity by rabbit anti-human tear antibodies (Fleming and Allison, 1925). Others confirmed this observation by gel electrophoresis, immunabsorption, gel fractionation and analytical ultracentrifugation, that also distinguished the substantially differing molecular weights of the two (Bonavida et al., 1969). In 1987, Pervaiz and Brew proposed the family name 'lipocalin' to describe a secondary or tertiary structurally homologous group of proteins with affinity for lipophilic ligands (Pervaiz and Brew, 1987). Subsequent cDNA cloning (Redl et al., 1992) from N-terminal tear albumin protein sequence out of a human lacrimal gland cDNA library identified a further member of the lipocalin family that was 58% identical to von Ebner's gland protein from rat, and identical to human von Ebner's gland protein, and was later designated as tear lipocalin with gene symbol LCN1. Tear lipocalin is a prominent component of tears with an estimated concentration of ~75 μM (Fullard and Kissner, 1991).

Tear lipocalin runs as a ~18 kDa band in SDS PAGE (Millar et al., 2009). O-linked glycosylation of C-terminal residues threonine 170 and serines 172 and 175 is weakly predicted by NetOGlyc 3.1 (numbering includes signal peptide). No N-linked glycosylation sites are predicted (NetNGlyc 1.0). Tear lipocalin forms an eight stranded antiparallel β barrel structure with C-terminal α-helix that creates a ~15 Å deep central cavity with positively charged bottom surface for ligand binding. Hydrophobic residues line the cavity (Gasymov et al., 2001; Breustedt et al., 2005), that best accommodates fatty acids of 18 (22.5 Å) to 24 carbons (Abduragimov et al., 2000). The disulphide bond between cysteines 79 and 171 restricts retinol binding in preference to native lipids (Glasgow et al., 1995). Tear lipocalin also binds lysozyme and lactoferrin through electrostatic interactions (Gasymov et al., 1999a,b), in keeping with the possibility that the three might be co-secreted as a complex.

Like LACRT, LCN1 is one of the most highly expressed human lacrimal gland genes and is equally eye specific (Ozyildirim et al., 2005). Western blotting has detected tear lipocalin in tears, saliva, sweat, and nasal mucus (Redl et al., 1992). Some expression in other tissues has been observed, including lung (Redl et al., 1998), pituitary gland (Wojnar et al., 2002), prostate (Holzfeind et al., 1996), plasma (Schenk et al., 2008) and semen (Pilch and Mann, 2006).

3.2. Orthologs

Twenty-eight tear non-overlapping orthologs are currently listed by Ensembl (release 70). Sequence identity of human tear lipocalin with primate and non-primate tear lipocalins is respectively 82 and 44%.

3.3. Tools and manufacture

Recombinant tear lipocalin has been largely produced in *E. coli*, although mammalian recombinant protein is commercially available. A large collection of point mutated tear lipocalins have been

generated by the Glasgow group for structural studies (for example, Gasymov et al., 2002).

3.4. Lipid binding activity

Tear lipocalin is the main lipid binding protein in tears (Glasgow et al., 1995). It copurifies out of human tears with stearic (5.9 μM), palmitic (4.4 μM) and lauric (0.4 μM) acids – levels in keeping with the relative affinities of each for tear lipocalin as determined by displacement of the fatty acid analog DAUDA (Gasymov et al., 1999a,b). By scavenging lipids, tear lipocalin is thought to help clear the ocular surface of sloughed cellular debris from epithelial turnover that might interfere with wetting. It also stabilizes the tear film (Schoenwald et al., 1998), and with ligand is itself stabilized (Tsukamoto et al., 2009).

3.5. Clearance of lipids coupled to tear lipocalin

Cell surface 'lipocalin-1 interacting membrane receptor' (LMBR1L) captures tear lipocalin for endocytosis. Endocytic internalization of FITC-labeled tear lipocalin (Wojnar et al., 2003) or β-lactoglobulin (Fluckinger et al., 2008) in NT2 neuronal cells was abrogated by antisense knockdown of LMBR1L. LMBR1L is a plasma membrane protein with nine predicted transmembrane domains (Wojnar et al., 2001), discovered in a phage display screen of tear lipocalin binding proteins (Wojnar et al., 2001). LMBR1L is widely expressed, but is not listed in corneal or lacrimal gland EST databases (NEIBank) suggesting either that it is absent, or more likely that expression is low (as is common for receptor proteins).

3.6. Cysteine protease inhibition activity

Recombinant tear lipocalin (5–10 μM) and tear lipocalin synthetic peptide (50–150 μM) inhibited papain activity with similar activity as cystatin C (CST3), indicating that tear lipocalin is a cysteine protease inhibitor (van't Hof et al., 1997). Tear lipocalin contains amino acid motifs similar to papain binding domains of family 2 cystatins. Leucine residues in the first cystatin like motif are necessary for protease inhibitor activity (Wojnar et al., 2001).

3.7. Bacterial growth inhibitory activity

Tear lipocalin (5 μM) inhibits the growth of *E. coli* in an $FeCl_3$ reversible manner by capturing secreted bacterial siderophores with an affinity similar to stearic acid – suggesting that it is physiologically relevant. Growth assays were performed in M9 minimal medium with a NaCl concentration of 10 mM (Fluckinger et al., 2004). Siderophores deliver extracellular iron necessary for bacterial growth in minimal medium.

3.8. Endonuclease activity

Homology of two tear lipocalin sequence motifs with an Mg^{2+} dependent endonuclease from gram negative *Serratia marcescens* was rationale for studies demonstrating that tear lipocalin displays endonuclease activity, although 1355 fold less active than DNase I (Yusifov et al., 2000). Human reflex tears contain 714 ng/ml DNA, and endonuclease activity. The activity largely co-fractionates with tear lipocalin, and is Mg^{2+} dependent and partially NaCl sensitive (Yusifov et al., 2008). Strands of DNA of increased length in dry eye tears have been detected on Schirmer strips, coincident with inflammation, and decreased tear nuclease activity – the latter thought to be contributed by tear lipocalin and DNase I (Sonawane et al., 2012).

4. New additions to the tear proteome

We previously assembled all tear proteomic data into a single table, restricting entry to proteins designated as 'extracellular' or 'plasma membrane' in their primary or alternative location (Laurie et al., 2008). Now updated with 139 new entries from Zhou et al. (2012), the additions supplement tears with proangiogenic, antiangiogenic, retinal survival, epithelial repair, cysteine protease inhibitor, immunosuppressive, and immunostimulatory activities (Table 1). Thirteen are highlighted below.

4.1. Angiogenesis

Exclusion of blood vessels from the cornea is essential for transparency (Ambati et al., 2006). Yet, tears contain both stimulators and inhibitors of angiogenesis. Now identified in tears are the stimulator angio-associated migratory cell protein (AAMP; 52 kDa) and the inhibitor fibulin 3 isoform 1 (EFEMP1; ~55 kDa). Antibody inhibition and antisense knockdown studies provide indirect evidence for the possibility that Angio-associated migratory cell protein is required for endothelial tube formation in co-culture with astrocytes (Beckner et al., 2002).

0.2–0.9 μM recombinant fibulin 3 isoform 1 inhibits sprouting of endothelial cells grown on collagen gels (Albig et al., 2006). Fibulin 3 isoform 1 binds the C-terminus of TIMP3 and, together with TIMP3, is a disease gene for macular degeneration (Klenotic et al., 2004).

4.2. Growth-like factors and epithelial biology

Tears contain growth factors. New tear growth factors (Zhou et al., 2012) are granulin (GRN; also known as epithelin), hepatoma derived growth factor (HDGF) and platelet derived growth factor C (PDGFC). Precursor progranulin (88 kDa) is processed to granulin (epithelin) 1 and 2. 0.8–3 nM granulin 1 enhances colony formation by normal rat kidney cells in agar in a manner that is opposed by 83 nM granulin 2 (Plowman et al., 1992).

Intraocular injection of 36 μM hepatoma derived growth factor (~28 kDa) after ocular nerve excision in rats increases the survival of retinal ganglion cells – in part via PI3K-Akt and MAP kinase signaling (Hollander et al., 2012).

Platelet derived growth factor C is secreted in inactive form in the vitreous, where it is activated by plasmin and thought to be involved in proliferative vitreoretinopathy (Lei et al., 2008). Platelet derived growth factor C is mitogenic for fibroblasts over a dose range of ~0.08–0.8 nM (Li et al., 2000).

Other interesting epithelial effectors now identified in tears include: trefoil factor 3 (TFF3; 7–12 kDa), cystatin-M (CST6; ~16.5 kDa) and growth arrest specific 6 (GAS6; ~75 kDa). Trefoil factor 3 plays an important role in epithelial repair. It is upregulated in injured cornea, and promotes the healing of NaOH wounded mouse corneas over a 7–400 μM dose range (Paulsen et al., 2008).

Cystatin-M is a cysteine protease inhibitor. Mice lacking cystatin-M develop metaplasia and keratitis of cornea (Zeeuwen et al., 2010).

Recombinant growth arrest specific 6 promotes photoreceptor outer segment phagocytosis with a dose optimum of 100 nM over a biphasic dose response (Hall et al., 2001). Anti-growth arrest specific 6 antibodies inhibit phagocytosis by retinal pigment epithelial cells (Karl et al., 2008).

4.3. Inflammation

Several new proteins are immunosuppressive (peptidylprolyl isomerase B [PPIB, also known as cyclophilin B], galectin 9 [LGALS9]), or immunostimulatory (pre B cell colony enhancing

factor [NAMPT; also known as visfatin], arginase [ARG1]). Peptidylprolyl isomerase B (~24 kDa) binds cyclosporine A with high affinity (Kd of 9.8 nM; Husi and Zurini, 1994) to together inhibit the phosphatase calcineurin within cells (Arber et al., 1992). Calcineurin is activated by calcium, as a downstream mediator of calcium signaling. Tear peptidylprolyl isomerase B would be expected to interact with topical cyclosporine A.

Galectin 9 (~40 kDa) is an S-type lectin with affinity for β-galactosides. Galectin 9 (025–0.75 μM) binds HAVCR2 (Tim-3) via carbohydrate recognition domain residues R64 and R238 to promote the death of T_H1-, but not T_H2-, $CD4^+$ and $CD8^+$ T cells, since HAVCR2 is a T_H1-specific cell surface protein. Galectin 9 is therefore involved in T cell suppression making it a potential therapeutic candidate for treatment of autoimmune diseases (Zhu et al., 2005).

Pre B cell colony enhancing factor (~56 kDa) is a proinflammatory adipokine. 9–45 nM pre B cell colony enhancing factor promotes the production of cytokines by CD14(+) monocytes (Moschen et al., 2007) and by rheumatoid arthritis synovial fibroblasts (Meier et al., 2012), including IL-6, TNF and IL1β.

Arginase (~35 kDa) is a manganese metalloenzyme that successfully competes for the substrate L-arginine with nitric oxide synthase, thereby reducing levels of immunoregulatory nitric oxide. Mice lacking arginase display little lipopolysaccharide induced uveitis (Zhang et al., 2009).

4.4. Innate defense

Tears contain the small leucine-rich keratan sulfate proteoglycan lumican (LUM; ~75 kDa), that is abundant in the cornea as a modulator of collagen fibril formation (Chakravarti et al., 1998), and deficient in macular corneal dystrophy in mature form (Hassell et al., 1980). *Pseudomonas aeruginosa* infection in lumican null versus wildtype mice suggests that lumican contributes to the innate response and clearance of bacteria (Shao et al., 2013).

5. Conclusions

Advantage should be taken of tear proteins as potential biomarkers, drug targets, and biotherapeutics. Tear-based biotherapeutics have considerable potential, particularly with the relatively small number of tear proteins that appear to be selectively downregulated in dry eye. Rather than simply alleviating symptoms, causes of ocular surface diseases may be addressable. Lacritin- and lipocalin-1-based therapeutics offer a platform to initiate this approach. Newly identified members of the tear proteome expand our appreciation of the functional capacity of the thin, but functionally dynamic tear film.

6. Notes added in proof

1. One tear proteomic article was overlooked. Aluru et al (PLoS One 7, e51979, 2012) compared tears from 73 normals to 129 individuals suffering from aqueous deficient dry eye. 2-D SDS PAGE with mass spectrometry identified seven downregulated proteins: AZGP1, CST_ [cystatin type not specified], IGJ, LACRT, LTF, PRR4 and SCGB2A2. As per Table 1, all except IGJ have been previously noted as downregulated in dry eye. Lacritin was downregulated in 95% of cases.
2. Low et al, 2013 (J. Proteomics [Epub ahead of print]) recently identified lacritin in the saliva of the vampire bat (*Desmodus rotundus*).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2013.05.020.

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