FUNCTIONAL PROFILE OF THE LIPOCALIN PROTEIN FAMILY

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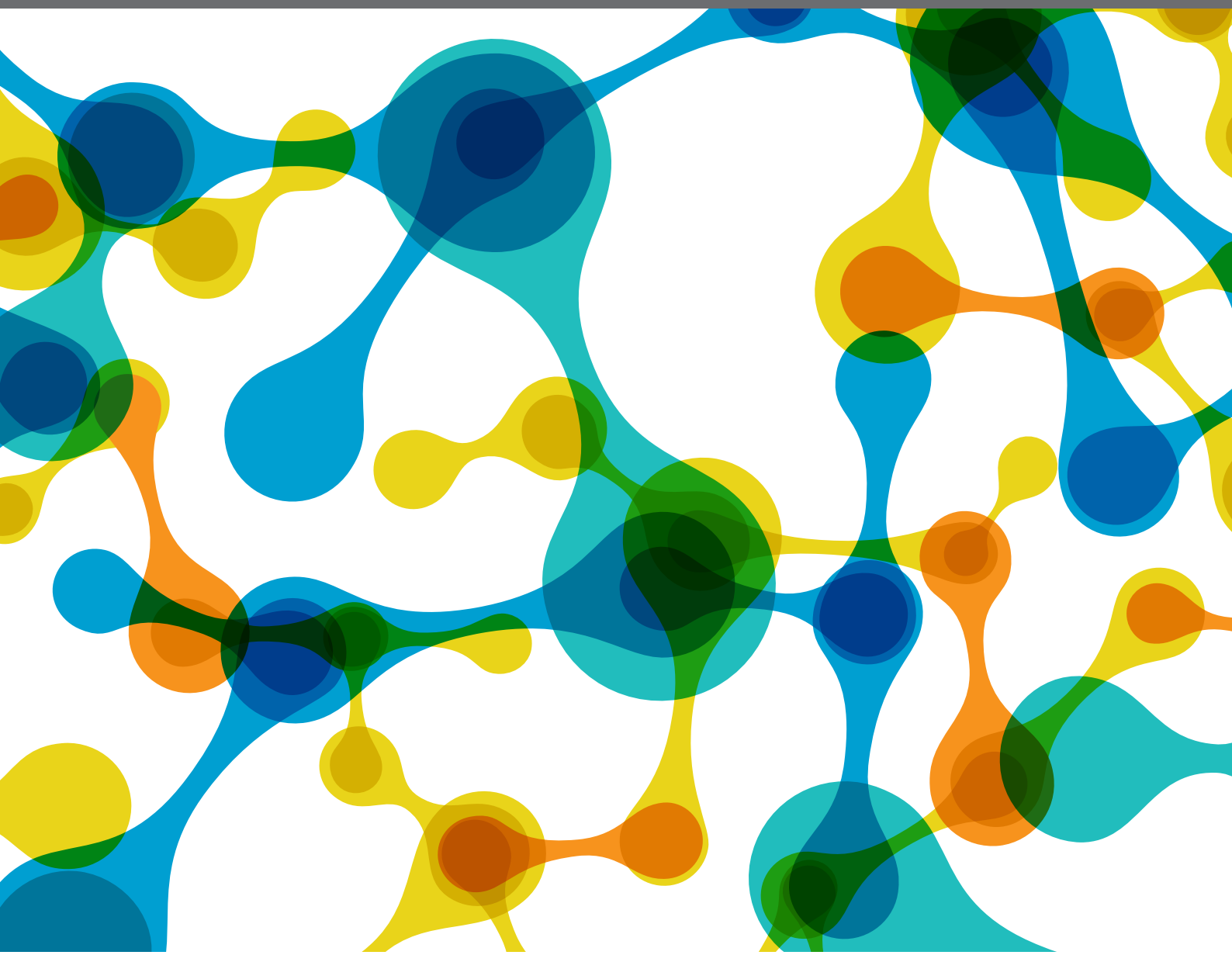

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FUNCTIONAL PROFILE OF THE LIPOCALIN PROTEIN FAMILY

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EDITORIAL
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Editorial: Functional Profile of the Lipocalin Protein Family

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Editorial on the Research Topic

Functional Profile of the Lipocalin Protein Family

INTRODUCTION

Lipocalins form an ancestral protein family found in all kingdoms of life, except for Archaea. From a small number of family members, Lipocalins rapidly evolved through duplication and divergence in the vertebrate genome, giving rise to nineteen different proteins in humans. Despite a high sequence diversity of homologous Lipocalins, their tertiary structure displays a resilient fold of eight beta-barrels delimiting a binding pocket where they can accommodate different ligands, mostly hydrophobic.

A close-to-linear accrual of publications marks the knowledge accumulation on Lipocalins since the family name was coined in 1985, but an inflection point appears in the literature after 2006, when the last comprehensive review of this protein family was collected. Since then, an explosion of association studies of Lipocalin expression with many human diseases has taken place, ranging from metabolic and endocrine syndromes to cancer, cardiovascular, neurodegenerative and psychiatric conditions. Regardless of this wealth of correlational data, with their understandable practical use as disease biomarkers, an analysis of publications devoted to Lipocalin biological function was needed.

In this Research Topic Issue, 28 authors have contributed a valuable collection of eleven reviews, focused on the function of most vertebrate Lipocalins. Their analyses, at every organizational level (molecular, cellular, tissues or organ systems), uncover an interesting pattern where the common Lipocalin structure provides a basic biochemical tool put to work in an amazingly varied set of physiological and pathological contexts. Specializations are combined with shared properties, and labour division with functional redundancy. The Research Topic starts with a novel view of Lipocalins evolution in chordates (Diez-Hermano et al.), where the use of unbiased selection of animal species, have yielded a phylogenetic tree with strong support of previously elusive relationships. On this evolutionary pattern, we can now map the Lipocalin functions (**Figure 1**), updated by all contributors to the Topic.

Two major categories can be established: homeostatic functions, regulating life processes like reproduction, development, or aging, and defensive functions triggered in response to damage or disease. Behind these physiological roles lie molecular functions that exploit the ligand binding pocket, the surface for intermolecular interactions, and a set of residues involved in enzymatic activities.

A rampant plethora of names has appeared for the individual Lipocalins, especially during the early years. Although several attempts to systematization of Lipocalin nomenclature have failed, a consensus in the name usage for each Lipocalin has slowly been reached during recent years. In this Editorial and

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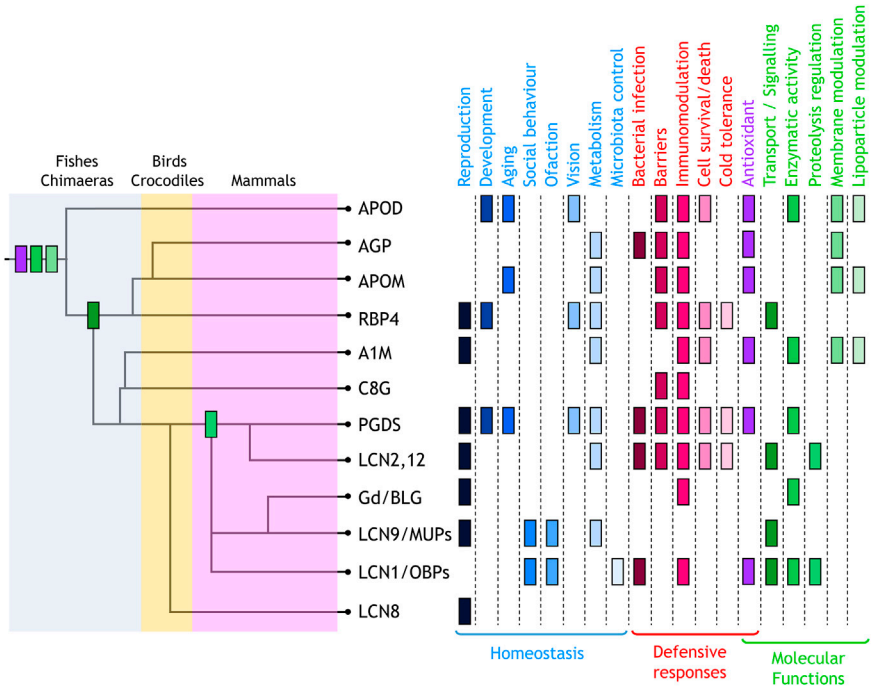

FIGURE 1 | Lipocalin functions mapped on the dendrogram of Lipocalin evolutionary history (Diez-Hermano et al.) where major monophyletic clades are shown. Physiological functions identified are classified into homeostatic (blue tones) and defensive (red tones). Functions defined at the molecular level (green tones) also include antioxidant (purple). The first appearance of these molecular functions in the evolutionary path of chordate Lipocalin are indicated in the tree using the same colour code. Antioxidant, enzymatic activity, and membrane modulation are ancestral functions. The current knowledge places transport/signalling at the divergence of RBP4 and PGDS-related radiation. In contrast, proteolysis regulation appeared later in evolution.

Figure we have settled upon the names, abbreviations and acronyms used in the separate contributions in the Research Topic Issue.

THE LIGAND PARTNERS OF LIPOCALINS

The family name was adequately chosen to highlight a prominent feature of Lipocalins: the pocket within the calix that can bind ligands. Ligand binding properties can be highly specific [e.g., retinol transport by RBP4 (Steinhoff et al.)], or rather "promiscuous" [AGP or Lcn1 being capable of binding many endogenous or exogenous molecules (Glasgow; Ruiz)].

Ligand binding can fulfil different purposes and have different spatial scales. Transport of ligands to target tissues to perform a function [RBP transport in the retina or Lcn2 as iron-provider (Bhusal et al.; Steinhoff et al.)] or the removal of toxic molecules [scavenging roles of Lcn1, OBPs or A1M (Bergwik et al.; Glasgow; Penn et al.)] are genuine transporter functions of Lipocalins. However, ligand-managing is not reduced to transport; Lipocalins can use the ligand to transform it [diverse enzymatic activities evolved in different family members like ApoD, A1M, PGDS or Lcn1 (Bergwik et al.; Glasgow; Sanchez and Ganfornina; Urade)], to prevent its oxidation [A1M heme quenching (Bergwik et al.)], or to retain a ligand in intra- or extracellular fluids [e.g., MUPs maintenance of pheromones in urinary scent marks guiding sexual and social behaviour (Penn et al.)]. Moreover, the ability of abundant Lipocalins to bind xenobiotics (including pharmacological agents) must be considered when analysing the pharmacokinetics and expected outcome of a treatment (Ruiz).

Biochemical moonlighting is also clear for some Lipocalins, where enzymatic activities (Bergwik et al.; Sanchez and Ganfornina; Urade) carried out by Met93 in ApoD, Cys34 in A1M or Cys65 in PGDS (all curiously located close to the binding pocket rim) can coexist with binding ligands deeper in the pocket, with physiological roles that can be experimentally dissociated.

As relevant as ligand-binding in the pocket can be for a Lipocalin, important functions have been demonstrated that employ interactions with high molecular weight partners, such as protein-protein interactions, or are unrelated to the ligand. Examples are the ligand-independent activation of TLR2/4 signalling in immune system cells by RBP4 (Steinhoff et al.), heparin binding by A1M (Bergwik et al.), or the nutritive and protective role of BLG [providing amino acids, and antioxidant and antibacterial peptides to the progeny (Sawyer)]. Indeed, surface residues potentially involved in ligand-independent functions are found as traces in the evolution of the different Lipocalin clades (Diez-Hermano et al.) paralleling their large functional divergence.

Finally, most Lipocalins are glycosylated and the oligosaccharides provide the basis of functional interactions of the Lipocalin, like the lectin-like binding of Gd to cell surfaces of endometrial decidua, oocyte and spermatozoa (Sawyer). Also, variation in glycosylation patterns are observed in various Lipocalins that can condition their

interactions [ApoD, AGP, Gd (Ruiz; Sanchez and Ganfornina; Sawyer)] and are worth further analysis.

LIPOCALINS IN THE BATTLEFIELD: IMMUNE AND DEFENSIVE RESPONSES

A prominent function of Lipocalins is their role as acute response proteins upon harmful stimuli (infection, inflammation, injury or disease) where up-regulation of Lipocalin expression is part of the general response. Lipocalins can synthesize immune system mediators [PGD_2 (Urade)] or control the availability of small immunomodulating molecules [like LPC by ApoD, or LPC and PAF by AGP (Ruiz; Sanchez and Ganfornina)]. They can either promote inflammation [PGDS or Lcn2 (Bhusal et al.; Urade)], restrain its extent or duration [ApoD, AGP (Ruiz; Sanchez and Ganfornina)], or have immunosuppressive functions [Gd, A1M (Bergwik et al.; Sawyer)]. They can control bacterial infections by scavenging precious iron-containing siderophores [AGP, Lcn2, C8G, Lcn1 and OBPs (Ruiz; Glasgow; Bhusal et al.; Stopková et al.)], transport lipids with antibacterial or antiviral activity or even degrade microbial DNA [Lcn1 (Glasgow)]. This general defensive function is part of the ancestral toolkit of Lipocalins (**Figure 1**) and lead to the concepts of Immunocalins or Siderocalins, applied to various members of the family [AGP, Lcn2, C8G, Lcn1, OBPs (Bhusal et al.; Glasgow; Ruiz; Stopková et al.)].

This Research Topic highlights yet another function also predicted to be ancestral in the family: regulation of barriers, so important for biological compartments protection [e.g., regulation of endothelial barriers to the passage of immune system cells by AGP (Ruiz), Gd or ApoM (Frances et al., 2021; Yao Mattisson and Christoffersen, 2021; Penn et al.) or the antagonistic roles of C8G and Lcn2 in preventing or promoting blood-brain-barrier disruption (Frances et al., 2021; Kim et al., 2021)]. Furthermore, many Lipocalins share functions dealing with the maintenance, composition and redox state of the most basic barriers of all: cellular membranes, with special attention to plasma and lysosomal membranes [ApoD, AGP, A1M, Gd (Bergwik et al.; Ruiz; Sanchez and Ganfornina; Sawyer)] vital for cell survival-death fates.

LIPOCALINS AS GUARDIANS OF OXIDATION STATE

It has become clear that antioxidant functions are also shared properties of Lipocalins (**Figure 1**) and they can be achieved by different molecular mechanisms: 1) by quenching oxidable molecules [PGDS, A1M (Bergwik et al.; Urade)]; 2) by scavenging oxidized molecules or radicals that propagate oxidation [OBPs, A1M (Bergwik et al.; Stopková et al.)]; 3) or by direct antioxidant activity, like Met-based lipid reduction by ApoD or Cys-based reduction by A1M (Bergwik et al.; Sanchez and Ganfornina).

Antioxidant effects of Lipocalins can be exerted on small free ligands (A1M), or on higher order lipidic structures by direct interaction with membranes (ApoD, AGP) or lipoprotein particles (ApoD, ApoM) (Bergwik et al.; Ruiz; Sanchez and Ganfornina; Christoffersen and Nielsen, 2013).

LIPOCALINS FOR HUNGRY ANIMALS IN A CHILLY WORLD

As for other lipid-binding proteins, metabolism regulation has been assigned to practically all Lipocalins, and correlations of metabolic diseases with Lipocalin expression are abundant. While the causal chain in some metabolic alterations observed upon modifying Lipocalin expression in model organisms still needs to be elucidated, examples of direct regulation of important metabolism-controlling signalling pathways have been demonstrated, like the regulation of insulin pathway by RBP or AGP (Ruiz; Steinhoff et al.) and the involvement in ER-stress, fat deposition and obesity of A1M (Bergwik et al., 2021b). Also, Lcn2 and AGP exert a direct control of food intake behaviours (Bhusal et al.; Ruiz). Metabolic response to cold stress is shared by RBP4, PGDS and Lcn2 (Bhusal et al.; Steinhoff et al.; Urade), a trait curiously present in the distant plant lipocalins (Frenette Charron et al., 2005). Indirect effects on metabolism are also accomplished by the modulation of lipoprotein composition and dynamics [ApoD (Sanchez and Ganfornina), ApoM (Frej et al., 2017)].

LIPOCALINS AND SEX. REPRODUCTION AND SOCIAL BEHAVIOUR

Lipocalins also play key roles in reproductive organ development and function, as well as in behaviours conditioning reproductive success. They influence various physiological processes like development or maintenance of reproductive organs [Lcn8 or PGDS (Stopková et al.; Urade)], prevention of oxidative stress in placenta [A1M (Bergwik et al.)], nutritive and protecting properties of milk [BLG (Sawyer)], fertilization, implantation and endometrial homeostasis [Gd (Sawyer)], or long-range sex-specific signal communication by MUPs and OBPs (Stopková et al.; Penn et al.). Indeed, the control of microbiota by OBPs not only influence host physiology, but also the perception of health state by putative mates (Stopková et al.).

LIPOCALINS AND LIFE. DEVELOPMENT AND AGING

The well-known roles of retinoic acid in embryonic development place RBP4 as a major player in developmental processes (Steinhoff et al.), but other Lipocalins cannot be discarded due to the shared ability of binding retinoids. Specific lipocalin-regulated developmental processes include development of organ-blood barriers in female and male reproductive organs [functionally linked to retinoic acid, and mediated by RBP receptor STRA6 (Steinhoff et al.)], but also unrelated mechanisms like angiogenesis or chondrogenesis [ApoD (Sanchez and Ganfornina)], or myelin development and maintenance [ApoD and PGDS (Sanchez and Ganfornina; Urade)]. Also, various Lipocalins have roles in the functional maintenance of specific organs upon aging [ApoD,

ApoM and PGDS, taking care of brain (Sanchez and Ganfornina), liver (Ding et al., 2020) or cartilage aging (Urade)].

WHERE DO LIPOCALINS FUNCTIONS TAKE PLACE?

All vertebrate Lipocalins have a signal peptide that, except for ApoM (Yao Mattisson and Christoffersen, 2021), is cleaved when the polypeptide is synthesized in the RER. There, they begin their path to the extracellular milieu through canonical secretion. Extracellular location is therefore shared by all Lipocalins. However, the picture is getting complicated since various Lipocalins are known to internalize in cells, and traffic to lysosomes either to be degraded [RBP or PGDS (Steinhoff et al.; Urade)] or to perform their function there [ApoD (Sanchez and Ganfornina)]. They can also bind to mitochondria in damaged cells [A1M (Bergwik et al.)].

Furthermore, extracellular Lipocalins appear in different formats: bound to protein partners [RBP4-transthyretin complex (Steinhoff et al.); A1M-IgA complex (Bergwik et al.)], lipoprotein particles [ApoD (Sanchez and Ganfornina), ApoM (Christoffersen and Nielsen, 2013)] or exosomes [ApoD (Sanchez and Ganfornina)]. Lipocalin dynamics within extracellular fluids or intracellular organelles have an important dependence not only on the format used, but also on their glycosylation "coat" that conditions their interactions, stability against proteases, or renal clearance (Ruiz; Sanchez and Ganfornina; Sawyer).

ARE LIPOCALINS ESSENTIAL FOR LIFE?

Not a single Lipocalin-KO animal model results in lethality, but no null-mutation for a Lipocalin is reported in humans. This paradox can be solved by three Lipocalins facts: 1) Functional redundancy: several Lipocalins can cooperate in the same physiological process [e.g., AGP-RBP4, Lcn2-AGP, RBP4-PGDS (Bhusal et al.; Ruiz; Steinhoff et al.; Urade)]. 2) Labour division: similar functions are performed by different lipocalins in different body compartments [e.g., siderophore scavenging by Lcn1 in tears and Lcn2 in plasma (Bhusal et al.; Glasgow)]. 3) Functional convergence: the enzymatic activity of PGDS is shared by the hematopoietic PGD_2-synthase (Urade).

LIPOCALINS AS OPTIMIZERS

A major conclusion we extract from the wide array of physiological processes reviewed in this Research Topic is that Lipocalins are key to organismal fitness, despite not being indispensable genes. Instead, they function as optimizers of several processes essential for life.

We need more experimental work to determine if loss of more than one Lipocalin is lethal (synthetic lethality) or if they are conditionally essential in particular environmental or internal stressful situations. Nevertheless, we can conclude that the proper response of an organism to disease or damage clearly depends on optimal performance of Lipocalins.

The functional map collaboratively built from this Research Topic can be a good starting point guiding future research on previously unpredicted functional properties of particular Lipocalins. It should help to increase our knowledge on important physiological processes and expand the applicability of Lipocalins to improve therapies for many pathological conditions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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BRIEF RESEARCH REPORT
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An Evolutionary Perspective of the Lipocalin Protein Family

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The protein family of Lipocalins is ubiquitously present throughout the tree of life, with the exception of the phylum Archaea. Phylogenetic relationships of chordate Lipocalins have been proposed in the past based on protein sequence similarities, but their highly divergent primary structures and a shortage of experimental annotations in genome projects have precluded a well-supported hypothesis for their evolution. In this work we propose a novel topology for the phylogenetic tree of chordate Lipocalins, inferred from multiple amino acid sequence alignments. Sixteen jawed vertebrates with fair coverage by genomic sequencing were compared. The selected species span an evolutionary range of ∼400 million years, allowing for a balanced representation of all major vertebrate clades. A consensus phylogenetic tree is proposed following a comparison of sequence-based maximum-likelihood trees and protein structure dendrograms. This new phylogeny suggests an APOD-like common ancestor in early chordates, which gave rise, via whole-genome or tandem duplications, to the six Lipocalins currently present in fish (APOD, RBP4, PTGDS, AMBP, C8G, and APOM). Further gene duplications of APOM and PTGDS resulted in the altogether 15 Lipocalins found in contemporary mammals. Insights into the functional impact of relevant amino acid residues in early diverging Lipocalins are also discussed. These results should foster the experimental exploration of novel functions alongside the identification of new members of the Lipocalin family.

Keywords: Lipocalin, Calycin, protein phylogeny, functional divergence, molecular evolution

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INTRODUCTION

Lipocalins form an ancestral protein family so far found in all kingdoms of life except for Archaea. The evolutionary paths followed by the Lipocalins have been explored using standard *in silico* molecular evolution methodologies based on protein sequence alignments, exon-intron architecture and protein tertiary structure comparisons (Ganfornina et al., 2000; Gutierrez et al., 2000; Sanchez et al., 2003, 2006; Lakshmi et al., 2015). These reports supported an evolutionary hypothesis where one (or a couple) of chordate Lipocalin genes found in separate chromosomes (e.g., APOD and RBP4 in human chromosomes 3 and 10 respectively) would repeatedly duplicate to achieve the remaining 15–20 Lipocalins that would cluster in a chromosome (e.g., human chromosome 9). See Charkoftaki et al. (2015) for a detailed chromosomal location of human and mouse genes.

Previous reports aimed at maximizing the number of protein sequences to be included, which might bias phylogenetic relationships in tree nodes overrepresented in databases due to the preferential sequencing of model organisms or to genome projects that have been prioritized. Some reports focused on pairwise comparisons of two organisms (Charkoftaki et al., 2015), which hinders a complete phylogenetic interpretation of the family. Other works (Lakshmi et al., 2015) studied a reduced number of Lipocalins, yet without a clear selection motive, in the context of other structurally related protein families.

In this work we focus on chordate Lipocalins, the central theme of this Topic Series. To identify the proteins to be included in the analysis, we selected comparable numbers of organisms belonging to representative vertebrate classes, whose genomes were sequenced with good coverage. We then aligned the recovered Lipocalin amino acid sequences and built phylogenetic trees following a maximum-likelihood reconstruction method. Furthermore, we compared the protein structure space of human Lipocalins and its dendrogram with the sequence-based phylogenetic tree.

Finally, we identified particular residues accounting for both the divergence and the specificities of the main Lipocalin clades that appeared early in chordate evolution. Marking such residues as important will be useful to reveal protein regions relevant for known or novel functions that can be further experimentally tested. With these results we propose an evolutionary history for Lipocalins in chordates.

METHODS

Sixteen jawed vertebrates (gnathostomata) were selected for analysis (**Figure 1A**). These species cover vertebrate clades that have evolved during the last ∼400 My. Their genome sequencing projects show fair coverage and annotation. A tunicate (*Ciona intestinalis*) was also used to find Lipocalins that could serve as outgroup for our phylogenetic trees. A Lipocalin protein sequence search was performed using PSI-BLAST (Altschul et al., 1997), starting with the set of eighteen human Lipocalin sequences used in our previous phylogenetic studies (Sanchez et al., 2006). We then searched each protein against the genome assemblies of the selected organisms (**Supplementary File 1** and **Supplementary Table 1**).

The recovered sequences were checked for full transcript coverage by comparison with the RefSeq_RNA database. The protein sequence of the Lipocalin Alpha-1-microglobulin was selected from the Ambp precursor protein, genetically encoded as a fusion protein with Bikunin (a non-Lipocalin trypsin inhibitor). The final list of proteins considered for our analysis is shown in **Supplementary File 1** (**Supplementary Table 2**). Sequences are named with an abbreviated species name and a Lipocalin label based on the human genome nomenclature.

Prediction of N-terminal signal peptides, present in most Lipocalins, was performed with SignalP-5.0 (Almagro Armenteros et al., 2019), and the predicted fragment was removed from sequences entering the analysis. Multiple sequence alignments (MSA) of Lipocalin mature protein sequences were then carried out using MAFFT v7.475 (Katoh and Standley, 2013) according to the following workflows: (1) To study the general phylogeny of chordate Lipocalins, we first performed a MSA with the iterative refinement G-INS-I method for the terrestrial early diverging (ED) Lipocalins. We then used the MAFFT-Add program to include the remaining terrestrial and fish Lipocalins with a progressive G-INS-I method keeping alignment length (Parameters: BLOSUM62; Gap 1.53; Offset 0.0). (2) To study within-clade phylogenies we used the MAFFT G-INS-I method with structural masks based on the human resolved tertiary structures. (3) For functional divergence analyses, we selected five ED-Lipocalins (APOD, RBP4, PTGDS, AMBP and C8G) and generated MSAs for clade pairs (each clade with the most related one in the general tree) using iterative G-INS-I MAFFT. For each MSA, we used a structural mask as above. All MSAs generated in our analyses appear in **Supplementary File 2**.

Phylogenetic trees were inferred following a maximum-likelihood (ML) method with IQ-TREE (Nguyen et al., 2015). The automatic ModelFinder selected JTT + F + R5 as the best model for the general tree, taking into account rate heterogeneity (Kalyaanamoorthy et al., 2017). An Ultrafast Bootstrap approximation with 1,000 replicas was used to estimate nodes support (Hoang et al., 2018). The same automatic approach of ModelFinder considering rate heterogeneity was used for individual clade phylogenies. The tree files generated in this work are available in **Supplementary File 2**. Tree visualization and drawing were performed with FigTree v 1.4.4[1] and iTOL[2].

Functional divergence of paralogous proteins and their correlated functional specificity, were studied with DIVERGE 3.0 (Gu et al., 2013) and JDet 1.4.9 (Muth et al., 2012) using the MSAs for clade pairs. This approach compares each Lipocalin with the expected evolved duplicate according to the ML general family tree. We followed the suggested DIVERGE protocol and selected residues with a false discovery rate (FDR) limit of 5% and supported by the Site-Specific Posterior Profile (SSPP) Probability. The Type-I divergence analysis files and the summary Table presenting the statistical results for the comparisons in each pair of Lipocalin clades are shown in **Supplementary File 3**. When using JDet to highlight specificity-determining positions (SDPs), we selected residues with a threshold of XDet:0.6, Entropy:2.5 and S3:10.0. The pairwise MSA used by JDet and its output are shown in **Supplementary File 4**. The 3D structures of relevant Lipocalins including selected residues were depicted with ViewerLite 4.2 or PyMOL 2.3.3.

RESULTS AND DISCUSSION

The phylogenetic relationship and evolutionary timescale of the sixteen jawed vertebrates used in our study were estimated with TimeTree (Kumar et al., 2017; **Figure 1A**). Red diamonds point at whole genome duplications (WGD) currently proposed to have occurred in chordates (Meyer and Van de Peer, 2005; Kasahara, 2007). **Figure 1B** shows the number of Lipocalins recovered for

[1]https://github.com/rambaut/figtree/releases

[2]https://itol.embl.de/

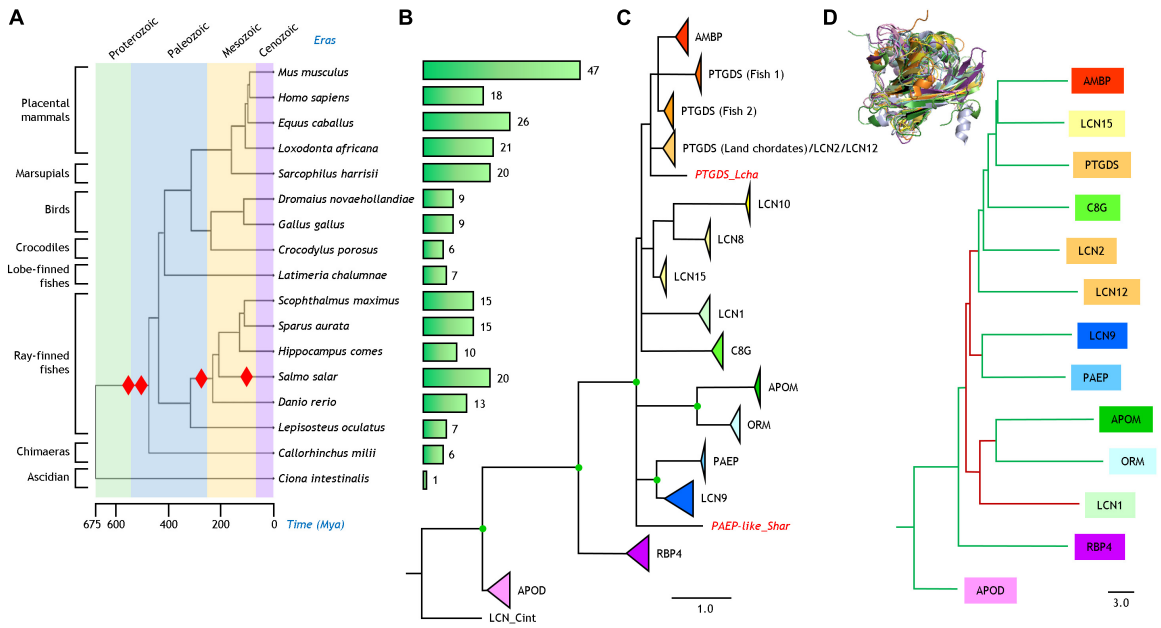

FIGURE 1 | Phylogenetic relationships of Lipocalins**. (A)** Phylogeny and evolution timescale of the organisms selected for our analysis. Red diamonds indicate whole genome duplications. **(B)** Bars depicting the number of *bona fide* Lipocalins found in each organism. **(C)** Phylogenetic tree of 250 Lipocalin protein sequences reconstructed from a structure-based multiple sequence alignment and a ML method. The tree was rooted with a tunicate Lipocalin (LCN_Cint) considered as outgroup. Unsupported nodes (bootstrap <65%) are excluded and branches joined as a polytomy. Lipocalin monophyletic clades (bootstrap >85%) are shown collapsed and color-coded. Scale branch length represents number of amino acid substitutions per site. Individual Lipocalins with unsupported groupings are shown in red and italics. Green dots point to full bootstrap support. **(D)** Dendrogram depicting the relationships of thirteen human Lipocalins structurally aligned (3D superimposition shown as an inset). Scale branch length represents distances obtained from the DALI similarity matrix. Branches in green and maroon point to relationships concordant or discordant, respectively, with the protein sequence-based phylogeny in **(C)**.

each species with our protein sequence similarity search in each genome. From the single Lipocalin detected in the urochordate *Ciona*, the graph helps to visualize the step-wise expansion of this protein family in early marine and land vertebrates due to WGD (leading to 6–7 Lipocalins), as well as new rounds of WGD in fish (resulting in sets of 10–20 Lipocalins). Further increases in the number of mammalian Lipocalins, some of them quite substantial, can be explained by tandem gene duplications, as it has been reported for other gene families (Gaunt, 2015).

Protein Sequence-Based Lipocalin Evolution

Our similarity search in the selected sixteen vertebrate genomes rendered 249 genes. After sequence processing, MSA and ML phylogenetic reconstruction, the resulting tree is shown in **Figure 1C** rooted with the *Ciona* Lipocalin. The nodes appearing in the tree are supported by bootstrap values >60%. Individual Lipocalin clades were supported by values >85% and are shown collapsed in the tree with a triangle whose area is proportional to the number of genes monophyletically related. Nodes with full 100% support are highlighted as green dots. Although we initially used the gene names present in the genome databases, we here propose to standardize Lipocalin names based on the strong support of our phylogenies, using the human genome nomenclature as a reference, combined with number-letter suffixes (**Supplementary Table 2** and **Supplementary File 1**). In this sense, the long-studied odorant-binding Lipocalins fully group together with LCN1 and are correspondingly renamed in our phylogeny as such for all species used in this work. Similarly, the rodent urinary proteins fall into the LCN9 monophyletic clade. An intriguing case is PTGDS, which is distributed over three independent and highly supported (>95%) clades. Two of these clades are composed of fish genes, while one combines in a single clade the terrestrial vertebrate PTGDS, LCN2, and LCN12.

The reconstructed phylogeny reinforces the hypothesis that APOD and RBP4 are the earliest diverging among ED Lipocalins. All other extant Lipocalins in chordates join in a monophyletic group. Within that group our organism-based phylogeny resolves several relationships that were not firmly supported in previous phylogenies. LCN9 relates to PAEP, and APOM relates to ORM. These links were previously suggested in a mouse-human Lipocalin comparison (Charkoftaki et al., 2015), but without evidence of phylogenetic support. Also, AMBP groups monophyletically with the two fish PTGDS clades. Finally, a different set of Lipocalins (LCN8, LCN10 and LCN15) forms another cluster with strong support.

Protein Structure-Based Lipocalin Relationships

Although the number of resolved tertiary structures for vertebrate Lipocalin is still insufficient, we used the DALI server (Holm, 2020) to structurally align thirteen human Lipocalins

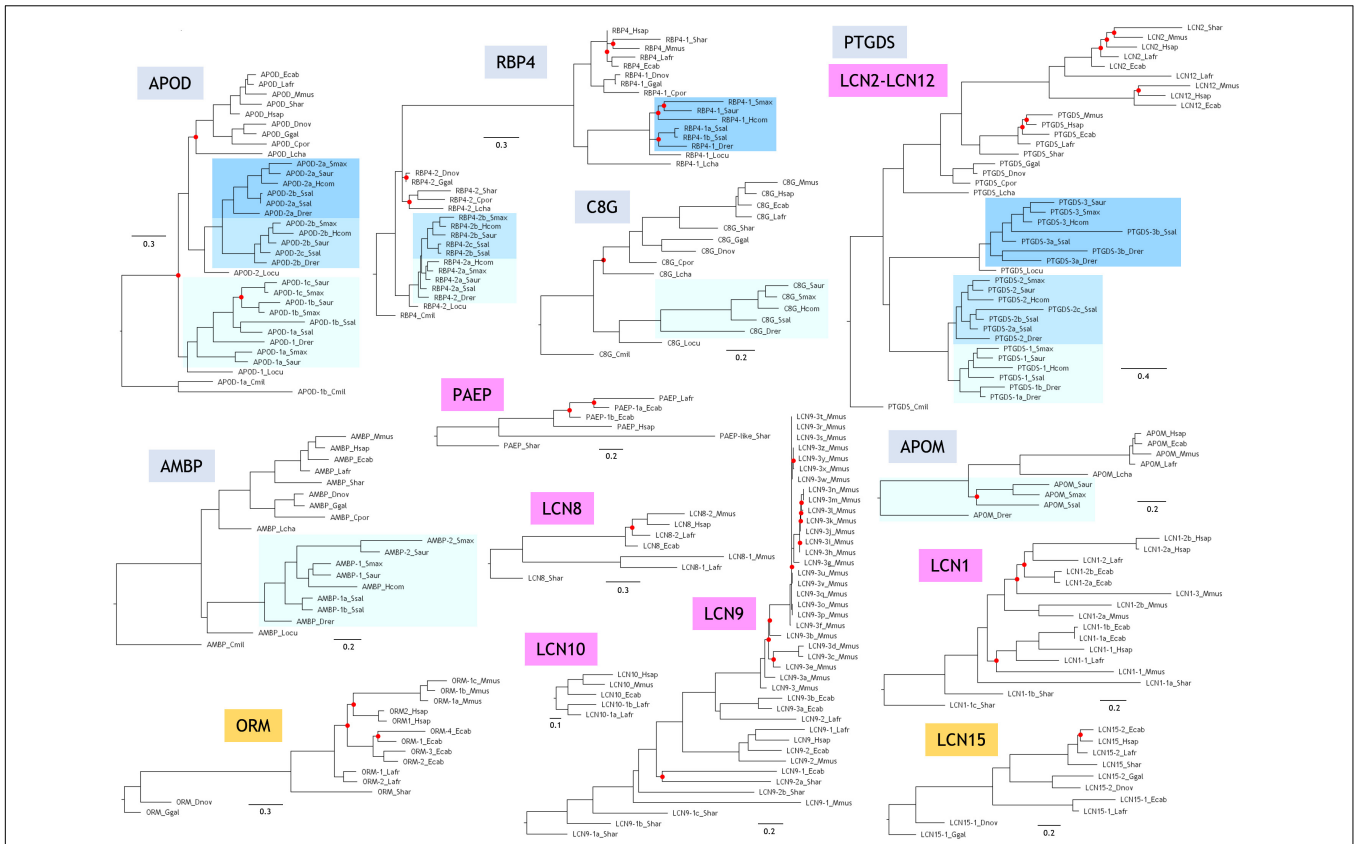

FIGURE 2 | Phylogenetic trees of Lipocalin clades. Each tree is based on structure-based multiple protein sequence alignments and a ML method. Trees were rooted with the Lipocalin(s) of the earliest diverging organism as shown in **Figure 1A**. Unsupported nodes (bootstrap <65%) are marked by a red dot. Scale branch lengths represent the number of amino acid substitutions per site.

(3D superimposition shown in **Figure 1D**). The dendrogram produced by DALI, based on average linkage clustering of the Z-scores of the structural similarity matrix, reasonably matches the topology of our amino acid sequence-based phylogeny (tree branches labeled in green in **Figure 1D**). Only LCN1 relates structurally to APOM and ORM in a different tree location (brown branches in **Figure 1D**). These structural relationships coming from a different comparison methodology, but lacking an evolutionary perspective, endorse the Lipocalin clade associations reported above.

Protein Sequence-Based Phylogenies and Organismal Representation of Lipocalin Clades

Phylogenetic relationships of independent Lipocalin clades are shown in **Figure 2**, each rooted with the protein(s) found in the organism showing the earliest time of divergence in **Figure 1A**. The six Lipocalin clades showing fish representatives (names boxed in light blue) are the ones defined as ED. Two clades debut in birds (names boxed in orange), while seven additional Lipocalins are latecomers, appearing during mammalian evolution (names boxed in purple). Except for APOM, all ED Lipocalins are present in cartilaginous fish, ancient ray-finned fish, and modern fish. Interestingly, APOD, RBP4, and PTGDS show groups of paralogous genes (boxed in shades of blue) reflecting the combination of regional tandem gene duplications with the different rounds of WGD that occurred in fish. However, AMBP, C8G, and APOM only show a single monophyletic fish group, suggesting processes of gene loss after genome duplication particularly prominent for APOM.

Two novel Lipocalins (ORM and LCN15) appear in our sample of terrestrial vertebrates. However, they are missing in the crocodile *C. porosus*, as it also happens with APOM, suggesting specific gene losses in this reptile representative.

Mammals display a great expansion of Lipocalins, mostly due to numerous and subsequent tandem gene duplications. As a result, five novel Lipocalins (PAEP, LCN1, LCN2, LCN8, and LCN9) are found in the marsupial *S. harrisii*, some of them showing evidence of recent gene duplications. Finally, our sample of placental mammals incorporate two novel Lipocalins (LCN10 and LCN12), configuring the final fifteen *bona fide* extant Lipocalins revealed by our species selection. This set is clearly the result of gene duplication and gene loss processes, with interesting selective losses resulting in Lipocalin pseudogenes still recognizable in late diverging (LD) Lipocalins (Schiefner et al., 2015). Our genome-wide strategy for Lipocalin identification does not retrieve some proteins considered Lipocalins in other

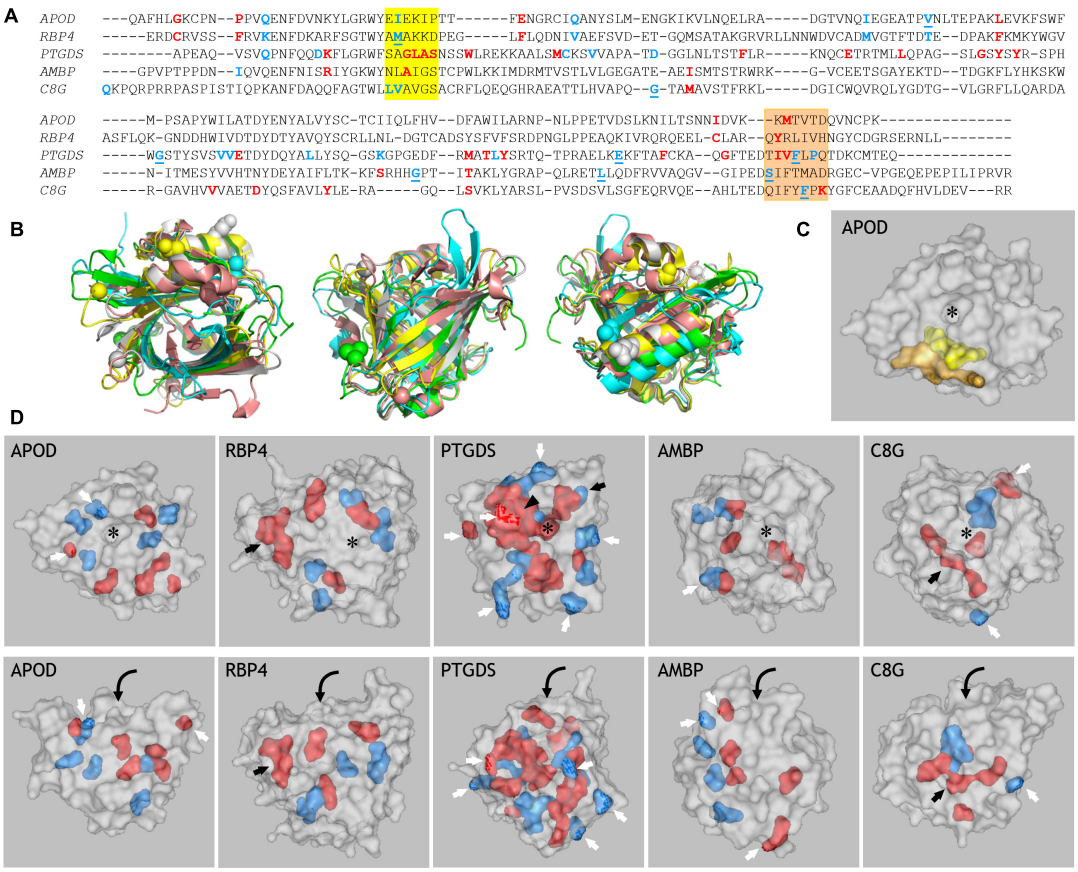

FIGURE 3 | Prediction of amino acid residues related to functional divergence in ED Lipocalins. **(A)** MSA of the five human representatives of the ED Lipocalins selected for this analysis. Residues related to Type-I divergence are labeled in blue, with underlined residues showing SSPP-probability support. Specificity-determining positions (SDPs) are labeled in red. Colored boxes mark two sequence regions enriched in functionally divergent residues. **(B)** Human Lipocalin structures superimposed, based on the structurally conserved beta-barrel, and colored (APOD, green; RBP4, cyan; PTGDS, gray; AMBP, yellow; C8G, salmon). Views into the ligand pocket, at the front side and the back side, revealing the alpha-helix attached to the beta-barrel, are shown. Colored spheres display SSPP-supported Type-I residues in all five Lipocalins. **(C)** Space-filled view of ApoD highlighting the residues marked by the two colored boxes in **(A)**. **(D)** Gray-colored space-filled views of the five human Lipocalin structures showing the color-coded residues identified in **(A)**. Panels in the upper row show views into the Lipocalin hydrophobic pocket (asterisk) while panels in the lower row show side views of the beta-barrel. Residues accessible at the protein surface (white arrows), or buried within the structure (black arrows) are pointed. Arrowhead points to a side wall of the binding pocket in PTGDS, and curved arrows suggest the binding pocket entrance on the side views of Lipocalins.

published reports. To cite just an example, a mouse ortholog of LCN15 is missing in our sequence list, while it is reported as a mouse Lipocalin by Charkoftaki et al. (2015). Our inclusion criteria do not include fatty acid-binding proteins as genuine Lipocalins, as it is the case for the alleged mouse Lcn15 homolog. They form a different protein family within the Calycin superfamily (Sanchez et al., 2006). We must therefore pay attention to these incorrect family assignments while further data help to curate genome annotations.

Functional Divergence Along the Evolution of Terrestrial ED Lipocalins

In an attempt to elucidate the functional traits and constraints potentially conditioning Lipocalin evolution in chordates, we have analyzed MSAs for clade pairs of the Lipocalins APOD, RBP4, PTGDS, AMBP, and C8G using the terrestrial vertebrate sequences. These pairs were explored following the predicted pattern of successive gene duplications (**Supplementary File 3**). The analysis identifies residues that experience altered functional constraints, as positions in the alignment that are variable in one clade and conserved in the other (Gu et al., 2013) or that define functional specificities as positions that are conserved but different in each clade (Muth et al., 2012). **Figure 3A** shows these predicted residues, colored in blue (Type-I) or red (SDPs), in the context of a MSA of the five human representatives of the Lipocalins mentioned above. To analyze the positions in the Lipocalins tertiary structure of residues showing correlated changes in evolutionary rates within a clade (Type-I), we focused on those best supported by SSPP-probability (underlined in **Figure 3A**) and displayed them as spheres in the superimposed Lipocalin structures (**Figure 3B**).

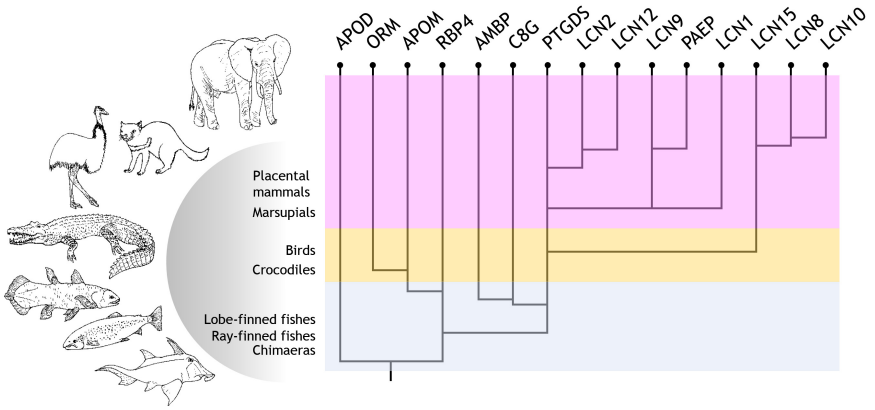

FIGURE 4 | Current proposal for the evolutionary history of Lipocalin in chordates. Evolutionary path suggested for Lipocalin duplication and diversification in chordates. Six Lipocalins evolved during fish evolution, while at least nine novel Lipocalins evolved in terrestrial vertebrates.

The highlighted residues tend to group in the three-dimensional structure, mostly on the surface of the Lipocalins and less in the ligand pockets. This result highlights the importance of residues potentially involved in protein-protein interactions in the radiation of Lipocalins, while those playing a role in ligand specificity might contribute less to the site-specific rate of change between Lipocalins.

When considering amino acids potentially involved in functional divergence (Type I and SDP), a group of them concentrate in two interesting areas in the MSA in all five Lipocalins (boxed in yellow and orange in **Figure 3A**). These regions therefore behave as hot-spots for generation of functional novelty (Ganfornina and Sánchez, 1999) along Lipocalin evolution. When located in a 3D structure (APOD shown as example in **Figure 3C**), the two regions pack together, with many of their residues lining the protein surface further highlighting intermolecular interactions as a relevant driving force.

We then explored the residues selected by DIVERGE (Type-I) and JDet (SDP) for each Lipocalin clade pair, and labeled them with different colors on the surface of each human Lipocalin structure (**Figure 3D**). A striking result of these studies is the large number of residues predicted to sustain the functional divergence of PTGDS from RBP4, with many residues located on the protein surface (white arrows) and a compact patch of residues (black arrowhead) lining a side wall of the binding pocket (asterisk). It is also interesting that the catalytic cysteine residue responsible for the unique enzymatic activity of PTGDS (black arrow) appears as a site-specific functional divergence, thus validating the methodology used. That PTGDS appears highly prone to shifted functional constraints might underlie its peculiar polyphyletic topology in the general ML tree discussed in section "Protein Sequence-Based Lipocalin Evolution" (**Figure 1B**), and could also represent the source of sequence variation that fueled the radiation of late-diverging Lipocalins from an ancestral PTGDS.

In the other clade pair analyses, a smaller number of residues are predicted to relate to functional divergence. These residues arrange around the binding pocket in APOD, with few of them accessible at the protein surface (white arrows). A similar pattern is seen for RBP4 in addition to two cysteine residues forming a peculiar third disulfide bond (a distinct feature of RBP4) that were correctly selected as SDPs (black arrow). AMBP shows an interesting polarization in the positioning of residues potentially contributing to functional divergence (easily seen in the side view of the molecule, bottom panel). Two of the selected amino acids of C8G contribute to the protein surface (white arrows), while three hydrophobic residues line up in the inner part of the protein (black arrow).

An Evolutionary Perspective of the Lipocalin Protein Family

Parsing our results, we propose an evolutionary path for Lipocalin duplication and diversification in chordates, which is schematically depicted in **Figure 4**. Starting from an APOD-like common ancestor, early chordates start a series of WGD and tandem duplications that soon gave rise to six Lipocalins present in extant fish (APOD, RBP4, PTGDS, AMBP, C8G, and APOM). Based on the topologies of our new phylogenetic trees, our previously studied Lipocalin gene structure similarities, their three-dimensional structures, their diverse posttranslational features and protein expression patterns, as well as their organismal representation (Ganfornina et al., 2000, 2006; Gutierrez et al., 2000; Sanchez et al., 2003, 2006; Schiefner and Skerra, 2015), we propose the following evolutionary scenario for the subsequent history leading to the extant vertebrate Lipocalin set. An ancestral RBP4 duplicated and diverged into PTGDS. PTGDS, with its tendency to sequence divergence, underwent an early tandem duplication that gave rise to C8G and AMBP, two Lipocalins that share a particular exon-intron gene structure unique in the family. Whether it is C8G or AMBP the first to arise early in chordates is debatable. However, AMBP appears as a fusion between the Lipocalin Alpha-1-microglobulin and the proteinase inhibitor Bikunin. This lead us to propose a more parsimonious hypothesis with C8G being the descendant

of PTGDS, and a subsequent gene duplication in conjunction with a gene fusion event generating AMBP. RBP4 also gave rise to APOM, with which it shares a unique pattern of three disulfide bridges (Schiefner and Skerra, 2015), and APOM in turn could have generated ORM in terrestrial vertebrates. Three independent PTGDS gene duplications in birds, early mammals and placental mammals, as well as local gene duplications in several clades, account for the catalog of Lipocalins present in contemporary mammals.

In summary, a protein sequence-based phylogeny of selected organisms supports a novel tree topology of chordate Lipocalins, which in turn enables us to propose an upgraded hypothesis for their evolutionary history. Moreover, we report a number of amino acid residues related to the functional divergence of early diverging Lipocalins. Needless to say that the hypothetical evolutionary path we offer for Lipocalins will need to be revisited whenever new vertebrate genomes become fully annotated. Inclusion of Lipocalins from other phyla as well as of sister families that together compose the Calycin superfamily, would further contribute to a comprehensive view of the evolution of these ancient and widespread genes. Finally, the *in silico* method used here to identify residues undergoing rapid divergence or functional specification in particular Lipocalins proved to be a helpful approach for the design of future experiments aiming at defining the origin of the diverse physiological roles of these essential proteins.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

GG, MG, and DS: conceptualization. SD-H, GG, AS, and DS: bioinformatics analyses. DS: writing—original draft. SD-H, MG, GG, AS, and DS: writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.718983/full#supplementary-material

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SYSTEMATIC REVIEW
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The Lipocalin Apolipoprotein D Functional Portrait: A Systematic Review

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Apolipoprotein D is a chordate gene early originated in the Lipocalin protein family. Among other features, regulation of its expression in a wide variety of disease conditions in humans, as apparently unrelated as neurodegeneration or breast cancer, have called for attention on this gene. Also, its presence in different tissues, from blood to brain, and different subcellular locations, from HDL lipoparticles to the interior of lysosomes or the surface of extracellular vesicles, poses an interesting challenge in deciphering its physiological function: Is ApoD a moonlighting protein, serving different roles in different cellular compartments, tissues, or organisms? Or does it have a unique biochemical mechanism of action that accounts for such apparently diverse roles in different physiological situations? To answer these questions, we have performed a systematic review of all primary publications where ApoD properties have been investigated in chordates. We conclude that ApoD ligand binding in the Lipocalin pocket, combined with an antioxidant activity performed at the rim of the pocket are properties sufficient to explain ApoD association with different lipid-based structures, where its physiological function is better described as lipid-management than by long-range lipid-transport. Controlling the redox state of these lipid structures in particular subcellular locations or extracellular structures, ApoD is able to modulate an enormous array of apparently diverse processes in the organism, both in health and disease. The new picture emerging from these data should help to put the physiological role of ApoD in new contexts and to inspire well-focused future research.

Keywords: protein physiology, lipid peroxidation, membrane management, oxidative stress, lipoprotein particles, extracellular vesicles, lysosome, ApoD

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INTRODUCTION

ApoD, identified and named almost 50 years ago, is a protein belonging to the Lipocalin family. Experimental research on ApoD has been accumulating, encouraged by numerous findings of ApoD relationship to many human diseases, from cancer to cardiovascular, metabolic or neurodegenerative conditions. This affluence of scientific reports has described many aspects of ApoD functional features, but a fundamental question remains to be responded: does ApoD moonlight, performing different biochemical functions in different biological contexts? or does it display a distinctive biochemical role that is being used in several physiological systems?

Along this half-a-century of ApoD research many reviews focused on this protein have been published. All of them are narrative in nature and many concentrate on specific details of ApoD

such as its relationship to disease, often underlining partial and not critically assessed views on many aspects of ApoD biology.

Aiming at answering the central questions posed above, we have performed a systematic review of all primary research published until January 2021 where ApoD properties have been investigated in the chordate phylum. Reports were tagged and classified according to their contributions to "molecular properties," "gene data," "regulation of expression," "disease-related," "cellular trafficking," "tissue and organ function," and "protein physiology." The level and quality of experimental evidence were critically evaluated to try to identify cause-effect relationships. The picture emerging from this approach should help to understand the physiological role of ApoD and to inspire well-focused future research.

METHODS

To assess the current knowledge on the physiology of ApoD, we performed a literature review of primary publications in a systematic manner, searching the National Library of Medicine database with the PubMed engine (published until January 31, 2021). Using the search query "Apolipoprotein D" OR Apo-D OR ApoD, we recovered 851 entries from which 39 narrative reviews were excluded. Following a Title/Keywords screen, we selected 787 entries for further assessment. Following exclusion criteria we finally selected 417 articles reporting research on the Lipocalin ApoD in chordates as the final review sources, and stored them in a Zotero (v5.0.88) collection. The complete collection is available in the **Supplementary Tables 1**–**8**. According to the abstract information, articles were tagged with the following terms: Disease-related (DR, $n = 216$), Regulation of Expression (RE, $n = 186$), Gene Data (GD, $n = 37$), Molecular Properties (MP, $n = 59$), Cellular Trafficking (CT, $n = 33$), Tissue and Organ Function (TOF, $n = 36$), and Protein Physiology (PP, $n = 35$). **Figure 1** summarizes the review process workflow and outcome.

Following full article reading, we classified each tagged report with subheading terms to guide the organization of the review. We then performed an evaluation of the conclusions statements of each report based on technical and argumentative consistency, according to existing state-of-the-art standards and required experimental controls. In cases of uncertainty, experts in each field were contacted and asked for their objective judgement.

The following databases and *in silico* prediction platforms and tools were used in this work: ProtParam (https://web.expasy.org/protparam/); DeepLoc-1.0 (http://www.cbs.dtu.dk/services/DeepLoc/); Gene Ontology database (http://geneontology.org/); Human Protein Atlas (https://www.proteinatlas.org); Mouse gene expression (http://www.informatics.jax.org/expression.shtml); miRNA database (mirdb.org). The ApoD multiple sequence alignment was generated with ClustalX2 (http://www.clustal.org), and the 3D structure of ApoD was visualized with ViewerLite 4.2 (https://chemweb.ir/accelrys-viewerlite/). A model of HApoD with sugars attached was built with GlyProt (http://glycosciences.de/modeling/glyprot/php/main.php).

Abbreviations: AA: arachidonic acid; BCF: breast cyst fluid; CSF: cerebrospinal fluid; E-3M2H: E-3-methyl-2-hexenoic acid; ECs: endothelial cells; EM: electron microscopy; EVs: extracellular vesicles; GuHCl: guanidine hydrochloride; HDX-MS: amide hydrogen-deuterium exchange mass spectrometry; LPC: lysophosphatidylcholine; LPS: bacterial lipopolysaccharide; MCs: blood vessel mural cells; MSCs: bone marrow stem cells; OS: oxidative stress; RER: rough endoplasmic reticulum; ROS: reactive oxygen species; SAXS: small-angle X-ray scattering; STR: short-tandem repeats; TG: triglycerides; UTRs: gene untranslated regions.

RESULTS AND DISCUSSION

ApoD is an early-diverging member of the Lipocalin family, with its phylogenetic origins traced back to the origin of chordates (Ganfornina et al., 2000; Diez-Hermano et al., 2021). Furthermore, ApoD is the chordate Lipocalin most similar to those in other phyla. ApoD primary structure is well-conserved in chordates, as deduced from a multiple sequence alignment of 22 chordate species (**Figure 2A**; **Table 1**), with an average 67% identity (range: 55–90%) in mature protein sequence. An intriguing aspect of this alignment is a favored residue conservation of the region encompassing the first three β-strands of the protein primary structure (**Figure 2A**).

Molecular Properties

ApoD is a monodomain globular glycoprotein with two intramolecular disulfide bonds, which are molecular properties suitable for working in extracellular non-reducing milieus. ApoD shows an N-terminal signal peptide in all chordates that lets the nascent protein to enter the endoplasmic reticulum. The protein can therefore follow a canonical secretion pathway, and is glycosylated along this path.

Protein Parameters

Since early characterization studies of ApoD, its apparent electrophoretic mobility, density of ApoD-positive fractions and behavior in size exclusion chromatography, suggested the existence of post-translational modifications (glycosylation), a potential for oligomerization, and an association with lipids. The predicted acidic isoelectric point (**Table 1**) implicates that ApoD polypeptide would have its lowest solubility in aqueous-salt solutions at the pH of acidic organelles in the cell, while at neutral pH the ApoD polypeptide would show a net negative charge. ApoD displays four conserved cysteine residues, while an additional cysteine (Cys116) is present only in humans (absent even in other primates) and allows for inter-molecular disulfide bond formation (**Figures 2A**, **3B,E**; **Table 1**).

References contributing to this section are listed in **Reference Collection 1**, **Supplementary Table 2**.

Protein Structure

The ApoD 3D crystal structure has been solved for the human protein after modification of several residues that rendered the protein prone to aggregation. The unique human Cys116 is close to one of the hydrophobic loops, and was also mutated to facilitate crystallization. The structure reveals a typical

Lipocalin fold (Skerra, 2000) composed of an eight-stranded β-barrel structure with an adjacent C-terminal α-helix. It has a closed end, and an open end with access to a pocket able to bind mostly hydrophobic ligands. Two intra-molecular disulfide bonds stabilize the structure. Three out of four loops at the barrel open-end are hydrophobic, making these regions candidate for interaction with hydrophobic surfaces, and contain residues relevant for ApoD antioxidant properties (see section Protein Physiology). Two N-glycosylation sites (**Figures 2A**, **3F**) are located on the side and bottom of the calyx, away from the ligand-binding pocket opening. **Figures 3A,B** show a surface representation of the ApoD monomer structure with charged or hydrophobic surface highlighted in color. Other relevant residues are shown in **Figures 3C–E**.

The presence of a ligand inside the pocket did not modify the general crystal structure of ApoD. When explored by amide hydrogen-deuterium exchange mass spectrometry (HDX-MS) or small-angle X-ray scattering (SAXS) in solution, interesting conformational changes elicited by ligand binding were detected, resulting in further ordering of the already stable Lipocalin fold. ApoD structure is also stable upon protein oxidation with H_2O_2. Dynamic information extrapolated from the crystal structure has allowed further modeling of ApoD binding to small ligands, lipoprotein particles or membranes. These studies help to understand a methionine-dependent lipid antioxidant mechanism (see below) and to study the influence of glycosylation on these functional properties. In addition, the ApoD monomer crystal structure, combined with modeled glycosylation conformations, was used to generate coherent models for the conformations of ApoD oligomers (**Figures 3G,H**) later confirmed experimentally (see below).

References contributing to this section are listed in **Reference Collection 2**, **Supplementary Table 2**.

Protein Glycosylation

As mentioned above, sugars were soon revealed to be linked to ApoD, with a relevant carbohydrate contribution (∼15–22%) to its apparent molecular weight. Two asparagine sites were experimentally demonstrated to be glycosylated, and *in silico* studies of human ApoD revealed no interference of sugars with binding pocket access. **Figure 3F** depicts a model of the N-linked oligosaccharides. The Asn45 glycosylation site is conserved in birds and mammals, but the second glycosylation site shows variations in position (**Figure 2A**). In ApoD of human plasma, Asn45 contains primarily trisialo-triantennary oligosaccharides, and Asn78 contains fucosylated disialo-biantennary oligosaccharides. The presence of negatively

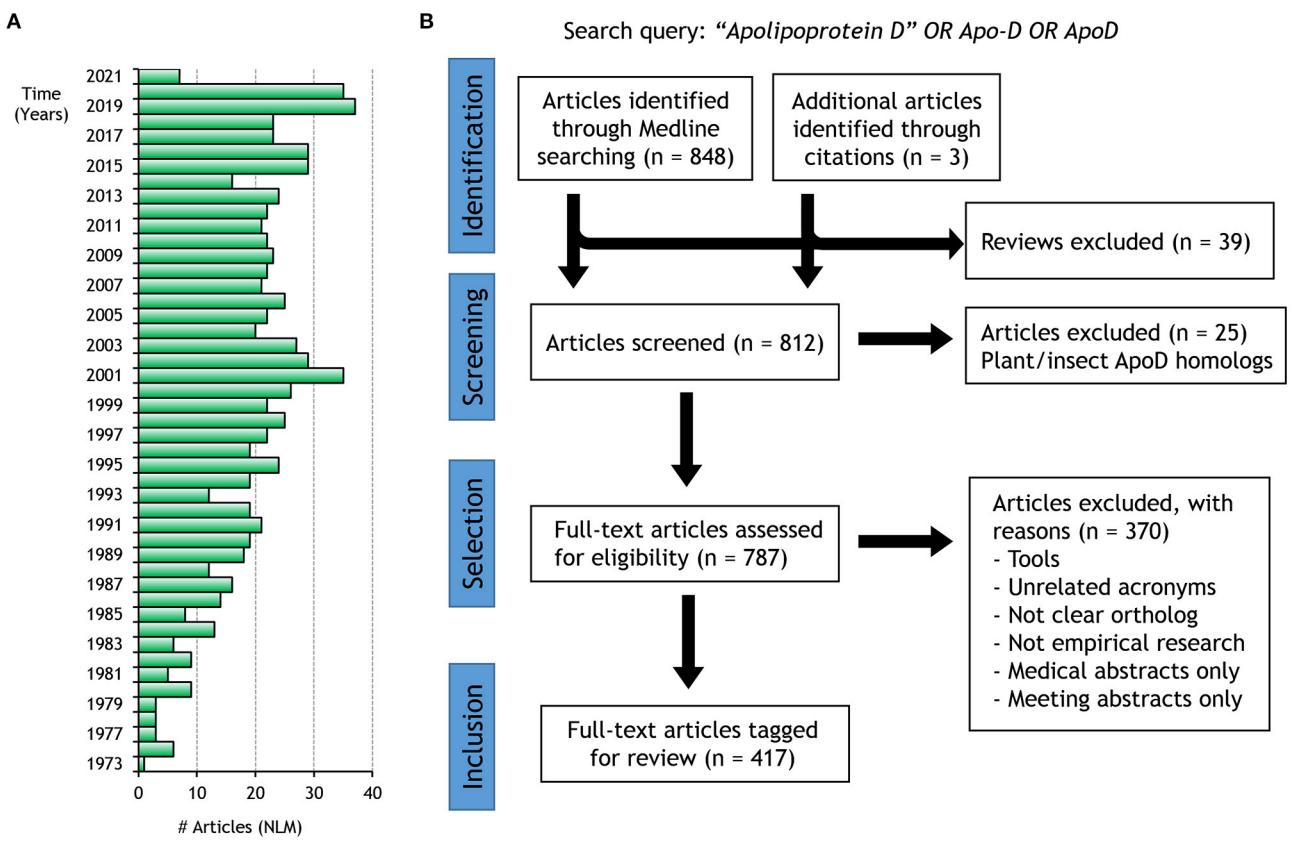

FIGURE 1 | ApoD literature search and inclusion criteria. **(A)** Yearly timeline of articles recovered by PubMed using the search query designed for our review. **(B)** PRISMA flow diagram for record inclusion in our review.

charged sialic acid in native ApoD sugar moiety contributes to its net negative charge in neutral pH environments.

Interesting variations of ApoD carbohydrate moiety have been reported between species (e.g., humans vs. other primates or rodents), between various tissues in a single species (brain tissue, cerebrospinal fluid, inner ear perilymph or plasma), within a tissue, or between health and disease conditions. Patterns of glycosylation have also been demonstrated to be sex-dependent (e.g., BCF in females, or axillary secretion in males). For example, less glycosylated forms of ApoD are present in mouse/human brain tissue compared to plasma, with differences in both terminal sialic acid and core N-linked oligosaccharides. A clear increase in α2-3 sialoglycosylation of plasma ApoD distinguishes, with high sensitivity, children with Autism Spectrum Disorder from healthy controls. Within a single tissue (cerebrospinal fluid; CSF) there is also variegation in the degree of ApoD sialylation. These variations generate size and charge heterogeneities with potential functional consequences worth exploring.

References contributing to this section are listed in **Reference Collection 3**, **Supplementary Table 2**.

Protein Oligomerization

Covalent and non-covalent homodimers and tetramers of ApoD have been detected in various experimental and biological systems. All studies of ApoD oligomerization have been focused so far on the human protein. Crystallization of bacterial recombinant human ApoD revealed that the protein tends to aggregate due to hydrophobic surface patches. This property

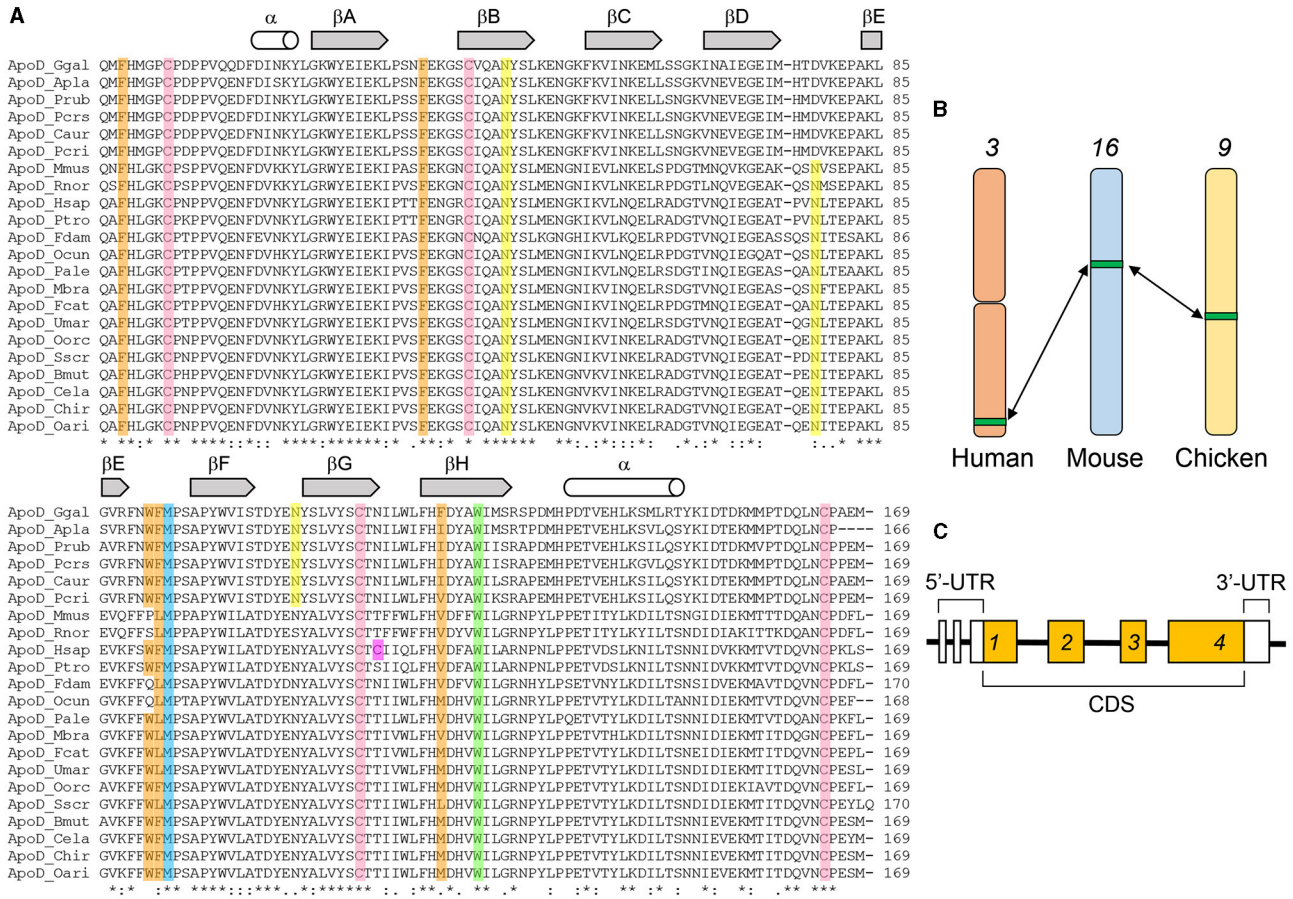

FIGURE 2 | ApoD protein sequence and gene features. **(A)** Multiple sequence analysis (MSA) of the mature amino acid sequence of selected vertebrate species recovered from GenBank (Apla, Anas platyrhynchos_EOB05196.1; Caur, Cathartes aura_KFP53002.1; Ggal, Gallus gallus_NP001011692.1; Pcri, Pelecanus crispus_KFQ60274.1; Pcrs, Podiceps cristatus_KFZ69168.1; Prub, Phoenicopterus ruber_KFQ85568.1; Bmut, Bos mutus_ELR54927.1; Cela, Cervus elaphus_ABB77207.1; Chir, Capra hircus_XP005675150.1; Oari, Ovis aries_XP004003075.1; Sscr, Sus scrofa_XP001926098.2; Fcat, Felis catus_XP006936237.1; Umar, Ursus marinus_XP008706566.1; Oorc, Orcinus orca_XP004278821.1; Mbra, Myotis brandtii_EPQ12038.1; Pale, Pteropus Alecto_XP006906222.1; Ocun, Oryctolagus cuniculus_ NP001075727.1; Hsap, Hoo sapiens_ NP001638.1; Ptro, Pan troglodites_XP516965.1; Fdam, Fukomys damarensis_KFO33128.1; Mmus, Mus musculus_CAA57974.1; Rnor, Rattus norvegicus_NP036909.1). Asterisks represent identical residues in all sequences, and dots/double dots point to similar residues. α-helices and β-strands are shown on top of the MSA, based on the solved tertiary structure of human ApoD. Colored residues are: four conserved cysteines involved in intramolecular disulfide bonds (pink), the human-specific unpaired cysteine (purple), conserved tryptophan residue in the ligand binding pocket (green), two glycosylated Asn residues (yellow), the antioxidant Met residue (blue), and residues in the hydrophobic surface patches at the rim of the binding pocket (orange). **(B)** Schematic representation of the chromosomal location of ApoD gene in human, mouse and chicken genomes. **(C)** Schematic representation of a consensus gene architecture for chordate ApoD, with four coding sequence (CDS)-containing exons and several 5′-UTR exons.

TABLE 1 | ApoD protein parameters.

Acc. number	Class/order	Common name	Species name	# Residues mature protein	Theor. Mw	Theor. pI	# Disulfide bonds	# Cys	N-linked sugars	Met-93	Mature protein sequence
EOB05196.1	Aves/Anseriformes	Mallard	*Anas platyrhynchos*	166	19,412	5.35	2	4	2	Yes	QMFHMGPCPDPPVQENFDISKYLGKWYEIEKLPSNFEKGSCIQANYSLKENGKFKVINKELLSSGKVNEVEGEIMHTDVKEPAKLSVRFNWFMPSAPYWVISTDYENYSLVYSCTNILWIFHIDYAWIMSRTPDMHPETVEHLKSVLQSYKIDTEKMMPTDQLNCP
KFP53002.1	Aves/Cathartiformes	Turkey vulture	*Cathartes aura*	169	19,750	5.23	2	4	2	Yes	QMFHMGPCPDPPVQEDFNINKYLGKWYEIEKLPSSFEKGSCIQANYSLKENGKFKVINKELLSNGKVNEVEGEIMHMDVKEPAKLGVRFNWFMPSAPYWVISTDYENYSLVYSCTNILWLFHIDYAWILSRAPEMHPETVEHLKSILQSYKIDTEKMMPTDQLNCPAEM
NP001011692.1	Aves/Galliformes	Chicken	*Gallus gallus*	169	19,780	5.51	2	4	2	Yes	QMFHMGPCPDPPVQQDFDINKYLGKWYEIEKLPSNFEKGSCVQANYSLKENGKFKVINKEMLSSGKINAIEGEIMHTDVKEPAKLGVRFNWFMPSAPYWVISTDYENYSLVYSCTNILWLFHFDYAWIMSRSPDMHPDTVEHLKSMLRTYKIDTDKMMPTDQLNCPAEM
KFQ60274.1	Aves/Pelecaniformes	Dalmatian pelican	*Pelecanus crispus*	169	19,778	5.23	2	4	2	Yes	QMFHMGPCPDPPVQEDFDINKYLGKWYEIEKLPSSFEKGSCIQANYSLKENGKFKVINKELLSNGKVNEVEGEIMHMDVKEPAKLGVRFNWFMPSAPYWVISTDYENYSLVYSCTNILWLFHVDYAWIKSRAPEMHPETVEHLKSILQSYKIDTEKMMPTDQLNCPPEM
KFZ69168.1	Aves/ Podicipediformes	Great crested grebe	*Podiceps cristatus*	169	19,719	5.09	2	4	2	Yes	QMFHMGPCPDPPVQEDFDINKYLGKWYEIEKLPSSFEKGSCIQANYSLKENGKFKVINKELLSNGKVNEVEGEIMHMDVKEPAKLGVRFNWFMPSAPYWVISTDYENYSLVYSCTNILWLFHIDYAWIISRAPEMHPETVEHLKGVLQSYKIDTDKMMPTDQLNCPPEM
KFQ85568.1	Aves/ Phoenicopteriformes	American flamingo	*Phoenicopterus ruber*	169	19,731	5.08	2	4	2	Yes	QMFHMGPCPDPPVQEDFDINKYLGKWYEIEKLPSSFEKGSCIQANYSLKENGKFKVINKELLSNGKVNEVEGEIMHMDVKEPAKLAVRFNWFMPSAPYWVISTDYENYSLVYSCTNILWLFHIDYAWIISRAPDMHPETVEHLKSILQSYKIDTDKMVPTDQLNCPPEM
ELR54927.1	Mammalia/ Artiodactyla	Wild yak	*Bos mutus*	169	19,466	5.07	2	4	2	Yes	QAFHLGKCPHPPVQENFDVNKYLGKWYEIEKIPVSFEKGSCIQANYSLKENGNVKVINKELRADGTVNQIEGEATPENITEPAKLAVKFFWFMPSAPYWVLATDYENYALVYSCTTIIWLFHMDHVWILGRNPYLPPETVTYLKDILTSNNIEVEKMTITDQVNCPESM
ABB77207.1	Mammalia/ Artiodactyla	Red deer	*Cervus elaphus*	169	19,564	4.96	2	4	2	Yes	QAFHLGKCPNPPVQENFDVNKYLGRWYEIEKIPVSFEKGSCIQANYSLKENGNVKVINKELRADGTVNQIEGEATQENITEPAKLGVKFFWFMPSAPYWVLATDYENYALVYSCTTIIWLFHMDHVWILGRNPYLPPETVTYLKDILTSNNIEVEKMTITDQVNCPEYM
XP005675150.1	Mammalia/ Artiodactyla	Goat	*Capra hircus*	169	19,488	4.96	2	4	2	Yes	QAFHLGKCPNPPVQENFDVNKYLGRWYEIEKIPVSFEKGSCIQANYSLKENGNVKVINKELRADGTVNQIEGEATQENITEPAKLGVKFFWFMPSAPYWVLATDYENYALVYSCTTIIWLFHMDHVWILGRNPYLPPETVTYLKDILTSNNIEVEKMTITDQVNCPESM
XP004003075.1	Mammalia/ Artiodactyla	Sheep	*Ovis aries*	169	19,488	4.96	2	4	2	Yes	QAFHLGKCPNPPVQENFDVNKYLGRWYEIEKIPVSFEKGSCIQANYSLKENGNVKVINKELRADGTVNQIEGEATQENITEPAKLGVKFFWFMPSAPYWVLATDYENYALVYSCTTIIWLFHMDHVWILGRNPYLPPETVTYLKDILTSNNIEVEKMTITDQVNCPESM
XP001926098.2	Mammalia/ Artiodactyla	Swine	*Sus scrofa*	170	19,592	4.83	2	4	2	Yes	QAFHLGKCPNPPVQENFDVNKYLGRWYEIEKIPVSFEKGSCIQANYSLKENGNIKVINKELRADGTVNQIEGEATPDNITEPAKLGVKFFWLMPSAPYWVLATDYENYALVYSCTTIIWLFHLDHVWILGRNPYLPPETVTYLKDILTSNDIDIEKMTITDQVNCPEYLQ
XP006936237.1	Mammalia/Carnivora	Domestic cat	*Felis catus*	169	19,474	4.82	2	4	2	Yes	QAFHLGKCPTPPVQENFDVHKYLGRWYEIEKIPVSFEKGSCIQANYSLMENGNIKVINQELRPDGTMNQIEGEATQANLTEPAKLGVKFFWLMPSAPYWVLATDYENYALVYSCTTIVWLFHMDHVWILGRNPYLPPETVTYLKDILTSNEIDIEKMTITDQVNCPEPL
XP008706566.1	Mammalia/Carnivora	Polar bear	*Ursus maritimus*	169	19,371	4.71	2	4	2	Yes	QAFHLGKCPTPPVQENFDVNKYLGRWYEIEKIPVSFEKGSCIQANYSLMENGNIKVINQELRSDGTVNQIEGEATQGNLTEPAKLGVKFFWLMPSAPYWVLATDYENYALVYSCTTIVWLFHMDHVWILGRNPYLPPETVTYLKDILTSNDIDIEKMTITDQVNCPESL

(Continued)

TABLE 1 | Continued

Acc. number	Class/order	Common name	Species name	# Residues mature protein	Theor. Mw	Theor. pI	# Disulfide bonds	# Cys	N-linked sugars	Met-93	Mature protein sequence
XP004278821.1	Mammalia/Cetacea	Killer whale	*Orcinus orca*	169	19,500	4.74	2	4	2	Yes	QAFHLGKCPNPPVQENFDVNKYLGRWYEIEKIPVSFEKGSCIQANYSLMENG NIKVINKELRADGTVNQIEGEATQENITEPAKLAVKFFWFMPSAPYWVLATDYE NYALVYSCTTIIWLFHMDHVWILGRNPYLPPETVTYLKDILTSNDIDIEKIAVTDQ VNCPEFL
EPQ12038.1	Mammalia/Chiroptera	Brandt's bat	*Myotis brandtii*	169	19,380	4.9	2	4	2	Yes	QAFHLGKCPTPPVQENFDVNKYLGRWYEIEKIPVSFEKGSCIQANYSLMENGN IKVINQELRSDGTVNQIEGEASQSNFTEPAKLGVKFFWLMPSAPYWVLATDYE NYALVYSCTTIVWLFHVDHVWILGRNPYLPPETVTHLKDILTSNNIDIEKMTITD QGNCPEFL
XP006906222.1	Mammalia/Chiroptera	Black flying fox	*Pteropus alecto*	169	19,359	5.35	2	4	2	Yes	QAFHLGKCPTPPVQENFDVNKYLGKWYEIEKIPVSFEKGSCIQANYSLMENGN IKVLNQELRSDGTINQIEGEASQANLTEAAKLGVKFFWLMPSAPYWVLATDYK NYALVYSCTTILWLFHVDHVWILGRNPYLPQETVTYLKDILTSNNIDIEKMTVTD QANCPKFL
NP001075727.1	Mammalia/ Lagomorpha	Rabbit	*Oryctolagus cuniculus*	168	19,433	5.15	2	4	2	Yes	QAFHLGRCPTPPVQENFDVHKYLGRWYEIEKIPVSFEKGNCIQANYSLMENG NIKVLNQELRPDGTVNQIEGQATQSNLTEPAKLGVKFFQLMPTAPYWVLATDY ENYALVYSCTTIIWLFHMDHVWILGRNRYLPPETVTYLKDILTANNIDIEKMTVT DQVNCPEF
NP001638.1	Mammalia/Primates	Human	*Homo sapiens*	169	19,303	5.2	2	5	2	Yes	QAFHLGKCPNPPVQENFDVNKYLGRWYEIEKIPTTFENGRCIQANYSLMENG KIKVLNQELRADGTVNQIEGEATPVNLTEPAKLEVKFSWFMPSAPYWILATDYE NYALVYSCTCIIQLFHVDFAWILARNPNLPPETVDSLKNILTSNNIDVKKMTVTD QVNCPKLS
XP516965.1	Mammalia/Primates	Chimpanzee	*Pan troglodytes*	169	19,301	5.43	2	4	2	Yes	QAFHLGKCPKPPVQENFDVNKYLGRWYEIEKIPTTFENGRCIQANYSLMENG KIKVLNQELRADGTVNQIEGEATPVNLTEPAKLEVKFSWFMPSAPYWILATDYE NYALVYSCTSIIQLFHVDFAWILARNPNLPPETVDSLKNILTSNNIDVKKMTVTD QVNCPKLS
KFO33128.1	Mammalia/Rodentia	Damaraland mole-rat	*Fukomys damarensis*	170	19,458	5.16	2	4	2	Yes	QAFHLGKCPTPPVQENFEVNKYLGRWYEIEKIPASFEKGNCNQANYSLKGNG HIKVLKQELRPDGTVNQIEGEASSQSNITESAKLEVKFFQLMPSAPYWVLATDY DNYALVYSCTNIIWLFHVDFVWILGRNHYLPSETVNYLKDILTSNSIDVEKMAVT DQVNCPDFL
CAA57974.1	Mammalia/Rodentia	House mouse	*Mus musculus*	169	19,478	4.71	2	4	2	Yes	QNFHLGKCPSPPVQENFDVKKYLGRWYEIEKIPASFEKGNCIQANYSLMENG NIEVLNKELSPDGTMNQVKGEAKQSNVSEPAKLEVQFFPLMPPAPYWILATDY ENYALVYSCTTFFWLFHVDFFWILGRNPYLPPETITYLKDILTSNGIDIEKMTTTD QANCPDFL
NP036909.1	Mammalia/Rodentia	Rat	*Rattus norvegicus*	169	19,584	5.04	2	4	2	Yes	QSFHLGKCPSPPVQENFDVKKYLGRWYEIEKIPVSFEKGNCIQANYSLMENG NIKVLNKELRPDGTLNQVEGEAKQSNMSEPAKLEVQFFSLMPPAPYWILATDY ESYALVYSCTTFFWFFHVDYVWILGRNPYLPPETITYLKYILTSNDIDIAKITTKDQ ANCPDFL
			Min	166	19,301	4.71					
			Max	170	19,780	5.51					
			Average	**169**	**19,519**	**5.07**					

In silico prediction (see section Methods) of molecular weight, pI and N-linked oligosaccharides, or experimentally tested (disulfide bonds and antioxidant Met-93 of human ApoD). ApoD from birds and mammals analyzed.

could promote self-association or association with lipid-based structures *in vivo* (**Figures 3J,K**).

Homodimers due to intermolecular disulfide bonds, evidenced by comparing electrophoretic mobility under reducing/non-reducing conditions, have been detected in urine and tear fluids. Experiments with sulfhydryl-trapping reagents during handling indicate that new disulfide bonds were not introduced along the experimental procedure. However, other forms of oligomerization are possible and compatible with these results, resulting from non-covalent stable binding between ApoD monomers. **Figures 3G,H** summarize the different forms of ApoD self-interaction.

Exposure of human ApoD to oxidized lipids promote dimerization and further oligomerization, in a way dependent on the oxidation state of particular methionine residues (Met93, see below), as demonstrated with recombinant ApoD, mutated at specific Met residues, and produced in a human cell line. Oxidation-dependent ApoD dimerization is resistant to guanidine hydrochloride (GuHCl) but not to urea, indicating that it is based on non-covalent intermolecular bonds. This property has allowed to detect ApoD dimers in specific brain regions of Alzheimer's disease patients (e.g., hippocampus, but not cerebellum) that also correlate with disease progression. In contrast, GuHCl extracts from healthy control brains show only monomeric forms of ApoD.

A tetrameric stable form of native ApoD in BCF, but not in plasma or CSF, has been demonstrated and characterized by multi-angle laser light scattering, analytical ultracentrifugation, HDX-MS and SAXS. Experimental data using progesterone as a ligand and the native ∼100 kDa ApoD tetramer from BCF, supports a particular tetramer conformation among those predicted by molecular modeling, where the binding pocket opening is accessible and the sugar moieties do not interfere in the inter-subunit interface (**Figure 3H**). Monomers interact with each other through the C-terminal α-helix and three β-sheets in close proximity, while glycosylated surfaces and Met93 are exposed in the tetramer. Oligomerization does not preclude ligand binding, and is not significantly altered upon binding of various ligands (biliverdin, palmitic acid, progesterone and sphingomyelin) or by *in vitro* protein oxidation with H_2O_2. The putative contribution of intermolecular disulfide bonds in the tetramer (involving human Cys116) has not been explored.

References contributing to this section are listed in **Reference Collection 4**, **Supplementary Table 2**.

Small Ligand Binding

The ability to bind progesterone was a defining feature of the most abundant protein in BCF, therefore named progesterone-binding cyst protein (PBCP) or gross cystic disease fluid protein 24 (GCDFP-24). Later on, this protein was demonstrated to be identical to ApoD purified from plasma HDL particles. Ligand-protein interaction at the ApoD binding pocket induces conformational changes leading to a more ordered structure, but does not result in major structural changes or altered oligomerization. These dynamic changes, though subtle, might have implications for ApoD interactions with other proteins or lipoprotein particles. Ligand binding reports are grouped in **Reference Collections 5, 6**, **Supplementary Table 2**.

Progesterone accommodation in the pocket involves a tryptophan residue heavily conserved in the Lipocalin family (Trp127 in human ApoD; **Figures 2A**, **3C**), whose fluorescence (Ex. $\lambda = 295$ nm) changes upon binding. This element in the pocket makes Trp-fluorescence titration a valid method to test a variety of ligands for ApoD (**Table 2**). All *in vitro* ligand-binding experiments have been performed with the human protein, using either recombinant ApoD (expressed by bacteria or eukaryotic cells) or native protein purified from BCF or plasma HDL. Arachidonic acid (AA) shows the highest affinity, while various AA derivatives (e.g., prostaglandins, 12-HETE or 5,15-diHETE) show no binding by Trp-fluorescence titration.

Cholesterol, a reasonable candidate because of its high presence in plasma lipoprotein particles, has been repeatedly tested, and reported to have no binding, or a very low affinity one (**Table 2**). A series of works (**Reference Collection 6**, **Supplementary Table 2**) demonstrate that ApoD has no cholesterol-transfer activity, a hypothesis originated by ApoD co-purification with lecithin-cholesterol acyltransferase (LCAT), whose activity is in fact modulated by ApoD (see section Protein Physiology) by a mechanism discarding ApoD as a cholesterol provider for LCAT.

Only one ligand has been identified bound to ApoD and extracted from the protein after purification from a natural source. E-3-methyl-2-hexenoic acid (E-3M2H), a male axillary precursor of odorants, was identified by gas chromatography-mass spectrometry (GC/MS) after temperature/pH switch and chloroform extraction from purified ApoD.

Interestingly, various ligands (e.g., bilirubin or E-3M2H) whose interaction with ApoD has been demonstrated by a different technique, do not alter Trp-fluorescence, raising the possibility of other sites of interaction. Molecular dynamics simulations infer flexible binding of oxidized derivatives of AA (5s-, 12s-, and 15s-HpETE) around the conserved Met93 at one of the hydrophobic patches at the entrance of the pocket. This particular form of lipoperoxide binding to ApoD is not expected to produce changes in fluorescence of Trp-127, located at the bottom of the binding pocket. A proof of interaction is experimentally supported by site-directed mutagenesis combined with HPLC-detection of reduced lipids (HETEs) after exposure to ApoD. This interaction underlies the antioxidant activity of ApoD (see below). **Figure 3I** summarizes in cartoon form this new view of small ligand-binding sites of ApoD, not restricted to the Lipocalin pocket.

Protein-Protein Interactions

Interactions of ApoD to higher-order lipid structures, like lipoprotein particles or cellular membranes, are particularly relevant since they determine the range of sites and biological contexts where ApoD function can be performed. They might depend on protein-protein or protein-lipid contacts.

As mentioned above, co-purification of ApoD with LCAT might indicate the potential for a protein-protein interaction

in nascent HDL particles, but a clear demonstration of ApoD-LCAT complex is not available. In contrast, a clear protein-protein interaction does account for human ApoD presence in HDL particles. An intermolecular disulfide link between ApoD Cys116 and ApoA-II Cys6 has been demonstrated by peptide digestion followed by sequencing and mass spectrometry (MS). This interaction, however, is an exclusive property of human ApoD due to its unique unpaired cysteine.

A putative disulfide-linked ApoD-ApoB100 complex was also proposed, but evidence is based on predictions from electrophoretic mobility in reducing/non-reducing conditions and immunoblot detection with anti-ApoD antibodies only, or with antibodies raised against LDL particles. An almost full characterization of 23 out of 25 cysteine residues in ApoB-100 by MS-analysis and peptide sequencing found no bonds with ApoD (Yang et al., 1990), strongly arguing against a disulfide-mediated interaction. Alternative mechanisms of ApoD interactions with plasma lipoprotein particles are therefore open to consideration.

Other potential interactions of ApoD have been explored with classic two-hybrid systems, where protein-protein contact takes place in the cell nuclei or cytoplasm, both requiring ectopic expression of ApoD in non-native biological compartments unsuitable for disulfide linked proteins (see sections Protein Structure and Cellular Trafficking). Alternatively, co-immunoprecipitation *in vitro* with or without crosslinking agents has been a method of choice. Using these approaches, ApoD has been proposed to interact with the extracellular glycoprotein Osteopontin (OPN), the intracellular domain of the Leptin Receptor (OB-Rb), the transmembrane glycoprotein Basigin (BSG), and the Scavenger receptor class B type 1 (SRB1).

The weak interaction reported between ApoD and the intracellular domain of OB-Rb, combined with its presumed topology within the cell, should discard this finding as a biologically relevant interaction for ApoD unless it is replicated. For membrane proteins such as BSG and SRB1, proposed as putative membrane receptors for ApoD, co-localization by

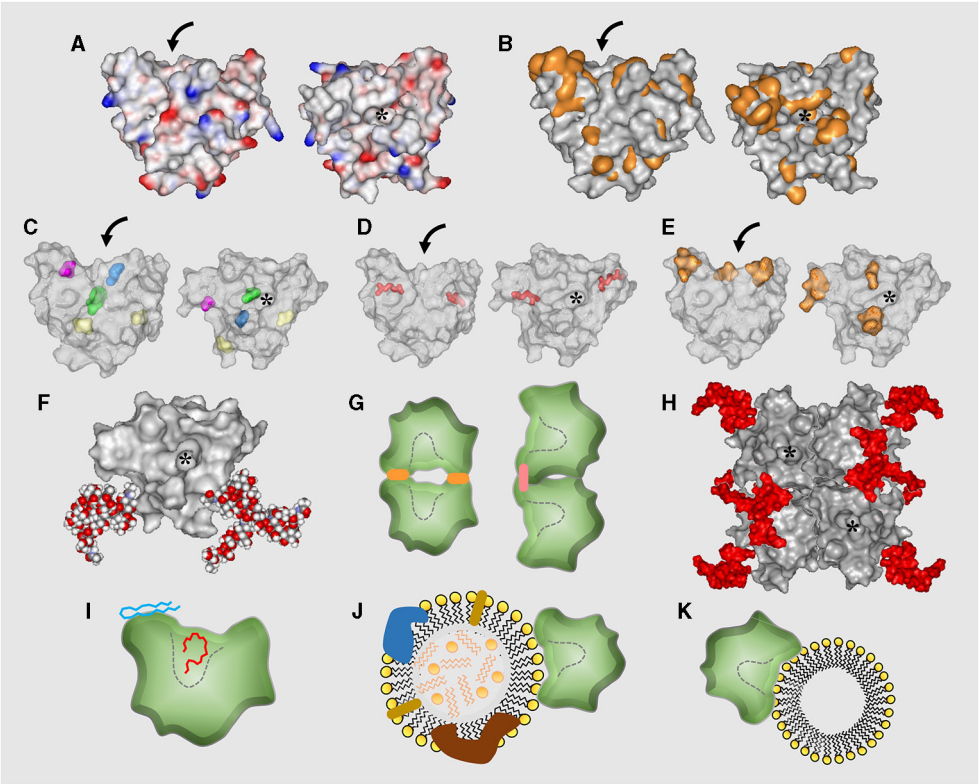

FIGURE 3 | Molecular features of ApoD. **(A,B)** Graycolored space-filled views of the human ApoD tertiary structure (modelled from PDB ID:2HZQ) showing charged residues in A (positive, red; negative, blue) and hydrophobic residues in B (orange). Side view of the β-barrel (left image; curved arrows point to the pocket entrance) and top view (right image) looking into the hydrophobic pocket (asterisk). **(C–E)** Human ApoD (PDB ID:2HZQ) side and top views with highlighted relevant residues. Colored residues in **(C)** are the antioxidant Met93 (blue); the human-specific unpaired Cys116 (purple); the conserved ligand binding pocket Trp127 (green); and the two glycosylated Asn45/Asn78 (yellow). Pink-colored residues in D are the four cysteines forming two intramolecular disulfide bonds. Orange-colored residues in E are those forming three hydrophobic loops around the pocket entrance. **(F)** Space-filled view of human ApoD with reported oligosaccharides linked to Asn45 and Asn78, as modelled by GlyProt (see Methods). **(G)** Cartoon representations of human ApoD dimers formed by hydrophobic patches (orange) or by intermolecular Cys116 disulfide bonds (pink). Variations of the particular configuration shown are possible. Dashed lines delineate the ligand pocket. **(H)** Representation of the best supported tetrameric structure of human ApoD found in BCF. Asterisks mark the ligand pocket accessible in all subunits (two facing back). Oligosaccharides shown in red. **(I)** Cartoon illustration of a side view of human ApoD with AA (red) and HpETE (blue) positioned into the hydrophobic pocket (marked by a dashed line) and interacting with the Met93-containing hydrophobic patch respectively. **(J,K)** Cartoon illustration of human ApoD interacting with higher-order lipid structures via the hydrophobic patches at rim of the pocket; **(J)** HDL particle; **(K)** Unilamellar vesicle (liposome).

TABLE 2 | ApoD ligand binding *in vitro* assays.

Ligand	Apparent Kd (μM)	Apparent Kd (μM)	Apparent Kd (μM)	Apparent Kd (μM)	Apparent Kd (μM)
References	Morais Cabral et al., 1995	Vogt and Skerra, 2001	Breustedt et al., 2006	Ruiz et al., 2013	García-Mateo et al., 2014
All-*trans*-retinoic acid			2.8	4.0 ± 2.6	
Retinol			0.08 ± 0.04	0.2 ± 0.1	
Arachidonic acid	0.006 ± 0.004	3.2 ± 0.2			
2-Arachidonyl-glycerol				n.d.	
12-HETE	n.d.				
5,15-diHETE	n.d.				
Prostaglandins (D2, E1, F2a)	n.d.				
Lysophosphatidylcholine					1.13 ± 0.05
Linoleic acid	n.d.				
Oleic acid	n.d.				
Palmitic acid	n.d.			3.3 ± 0.6	
Palmitoyl sphingomyelin				1.3 ± 0.5	
Cholesterol	n.d.			n.d.	
Pregnenolone		n.d.			
Progesterone	0.4 ± 0.1	1.7 ± 0.02			
Dihydrotestosterone		n.d.			
β-Estradiol				n.d.	
E-3M2H		n.d.			
Anandamide				1.6 ± 1.3	
Bilirubin	2.6 ± 0.5	n.d.			

Ligands tested by tryptophan fluorescence-based assays in vitro. Apparent Kd (μM) average ± SD are shown. "n.d." = no binding detected.

confocal imaging is often used as additional evidence. However, protein-complexes are below the resolution of standard co-labeling techniques, and methods relying on distance-dependent energy transfer, super-resolution or immunoelectron microscopy would be desirable as further evidence in relevant *in vivo* conditions. Other candidate ApoD receptors (LDLR and CXCR-4) are predicted from physiological contexts, where downstream consequences of ApoD exposure are modified by antagonists of these receptors. However, a direct interaction with these receptors has not been explored.

References contributing to this section are listed in **Reference Collection 7**, **Supplementary Table 2**.

Binding to Lipid-Rich Structures

The presence of ApoD in plasma lipoprotein particles lies at the base of its discovery in humans. ApoD was initially visualized as a "thin-line" polypeptide in immune-double diffusion analyses of plasma HDL particles, and was then identified as a low-abundance component of HDL_3 particles (defined as small-dense HDLs, d = 1.12–1.27 g/ml). Analysis of HDLs separated by electrophoretic mobility in non-denaturing PAGE followed by in-gel trypsinization, identified ApoD within the HDL-α2 type, in a 1:100 ratio with respect to ApoA-I. The presence of ApoD in HDLs has been confirmed also in human CSF and in baboon and mouse plasma. Additionally, plasma ApoB-100 positive LDL particles contain ApoD as well, but at lower concentrations (∼8 ng ApoD/μg LDL vs. ∼69 ng ApoD/μg HDL_3). The generalized interaction with different lipoparticles in several species suggests that ApoD-lipoparticle interactions must rely on a mechanism independent of ApoD-ApoA-II disulfide bond, a human HDL rarity. The fact that ApoD-LDL interaction is prevented by detergents, and do not take place with recombinant ApoD where hydrophobic surface residues have been mutated (to favor crystallization), suggests a hydrophobicity-dependent ApoD-lipoparticle binding mechanism (**Figure 3J**).

Also, direct binding of ApoD to unilamellar phospholipid vesicles (liposomes) further demonstrates its ability to bind to lipidic structures without requiring a protein-protein interaction. These unilamellar vesicles represent a simplified version of the outer phospholipid layer of HDLs, LDLs or a membrane bilayer (**Figure 3K**). In addition, ApoD has recently been identified in extracellular vesicles, characterized by the presence of CD81, CD63, and flotillin-1, and a density of d = 1.17–1.23 g/ml. The hydrophobic patches of ApoD at the entrance of the binding pocket are the likely site of interaction with liposomes or biological membranes, as indicated by experiments combining ApoD capacity to reduce oxidized liposomes with mutagenesis of Met residues that in fact contribute to the hydrophobicity of those patches.

The knowledge accrued on ApoD protein structure, its glycosylation and oligomerization properties, as well as its interactions with small ligands and other lipidic structures are relevant for its physiological roles in lipid management, and should help to get a global picture of how these molecular properties are put to work in various physiological contexts.

References contributing to this section are listed in **Reference Collection 8**, **Supplementary Table 2**.

Gene Data and Genomic Properties

Chromosomal Position and Gene Structure

The gene coding for ApoD locates in an autosomic chromosome that shows ample synteny in chordates (Sanchez et al., 2006), reflecting a strong evolutionary conservation of this genomic region (**Figure 2B**). The ApoD gene shows a standard metazoan exon-intron architecture, with a coding sequence interspersed in four exons that is conserved in chordates (Sánchez et al., 2003). Moreover, the gene upstream and downstream untranslated regions (UTRs) are also composed of several exons, mainly in the 5′-UTR, a property well-preserved in mammals (Mejias et al., 2019) (**Figure 2C**).

References contributing to this section are listed in **Reference Collection 9**, **Supplementary Table 3**.

Transcriptional Control of Gene Expression

The promoter region and elements controlling the expression of ApoD have being studied in detail for the human gene. The human promoter shows a canonic TATA-box upstream of the transcription start site. Several promoter elements and nuclear factors have been predicted to potentially regulate ApoD transcription in a number of organisms.

Experimental proof of a regulatory potential of human ApoD has been gathered for SRE1, AP-1, APR-3, NFκB, PARP1, HnRNP-U, and APEX-1 in cultured cells subjected to inflammation (LPS) and metabolic stress (serum deprivation). Also, the transactivator TAp73 mediates ApoD expression upon cell differentiation. The mouse ApoD promoter region has been recently assessed experimentally, and an alternative promoter region has been related to OS-induced ApoD expression.

DNA methylation, inferred from the CpG content of the gene promoter region, is also an important regulatory mechanism for ApoD transcription, with an inverse relationship between level of DNA methylation and ApoD gene transcription. This gene regulation mechanism has been shown in different physiological or pathological contexts: in esophageal, colorectal and astrocytic cancers, in the expression profile defining Th17 lymphocytes, and for the androgen receptor-response in male sexual development.

References contributing to this section are listed in **Reference Collection 10**, **Supplementary Table 3**.

Post-transcriptional and Translational Control of Gene Expression

The mRNA 3′-UTR is known to influence its stability and translation efficiency. ApoD 3′-UTRs show a high degree of conservation in mammals, and display shorter lengths and higher G+C content than those observed in average mammalian gene UTRs. These differences have been proposed to underlie a tight regulatory control of ApoD translation. In this context, a number of miRNAs have been predicted to control ApoD translation, possibly by binding to the 3′-UTR. Some of these miRNAs, like miR-229b-3p, miR-423-3p, and miR-490-3p, have been experimentally tested and implicated in the post-transcriptional downregulation of ApoD expression in rat male reproductive system upon metabolic dysfunction.

The 5′-UTR of ApoD also presents relevant properties for the regulation of ApoD expression. It is rich in short-tandem repeats (STR), specifically in primates. Long stretches of STRs are predicted to affect transcription and translation, which might have contributed to the neurodevelopmental changes that underlie primate evolution. Furthermore, mammalian ApoD genes show several alternative 5′-UTRs forms, possibly arising from alternative splicing. The alternative 5′-UTRs of the mouse ApoD gene have been experimentally tested and shown to underlie differential protein expression in several mouse tissues, with a particular 5′-UTR variant being strongly induced upon OS. Moreover, *in silico* analyses of these 5′-UTR variants in mouse and human ApoD show upstream initiation codons, upstream open reading frames, and predicted secondary structures that suggest a tight control on ApoD gene expression.

References contributing to this section are listed in **Reference Collection 11**, **Supplementary Table 3**.

Gene Polymorphisms

In terms of genetic variation for the ApoD gene, over 4,600 variants have been found in the GRCH38.p12 (annotation Release 109) assembly of the human genome, while 187 are reported in the short variants (dbSNP) and structural variants (dbVar) databases. Six variants that involve missense, intron insertions and 3′-UTR insertions, are predicted to involve molecular consequences. Some of these variants have been linked with variable support to human cancer, metabolic or neurological diseases (see **Supplementary Table 18**, and section ApoD-Disease Relationships), but a final proof of their clinical significance is currently missing.

References contributing to this section are listed in **Reference Collection 12**, **Supplementary Table 3**.

Regulation of Expression

A total of 186 primary publications (**Figure 4A**) were labeled with the *regulation of expression* (RE) tag for this systematic review (details recorded in **Supplementary Tables 9–17**). We combined our analysis with current data compiled in human and mouse expression atlases (see Methods section; **Supplementary Figures 1**, **2**).

ApoD in Body Fluids

Since its discovery in plasma HDL particles, ApoD protein and/or mRNA have been found in almost every organ, tissue or fluid. In addition to plasma, ApoD protein is present CSF, perilymph, urine, and secretions from exocrine glands (sweat, tears and mammary secretions) (**Supplementary Table 9**). The cellular origin of ApoD protein in each of these body fluids is not fully elucidated. With the exception of Th17 lymphocytes, blood cells in general do not express ApoD mRNA, and liver and intestine (major sites of HDL biogenesis) are among the ApoD low-expressing tissues both in humans and mice (**Supplementary Figures 1**, **2**). Plasma ApoD protein (∼128 mg/l) is approximately 25 times the concentration of CSF ApoD (∼5 mg/l) in healthy adult men, and they are uncorrelated, suggesting that a separate pool of ApoD protein is managed in these barrier-separated compartments.

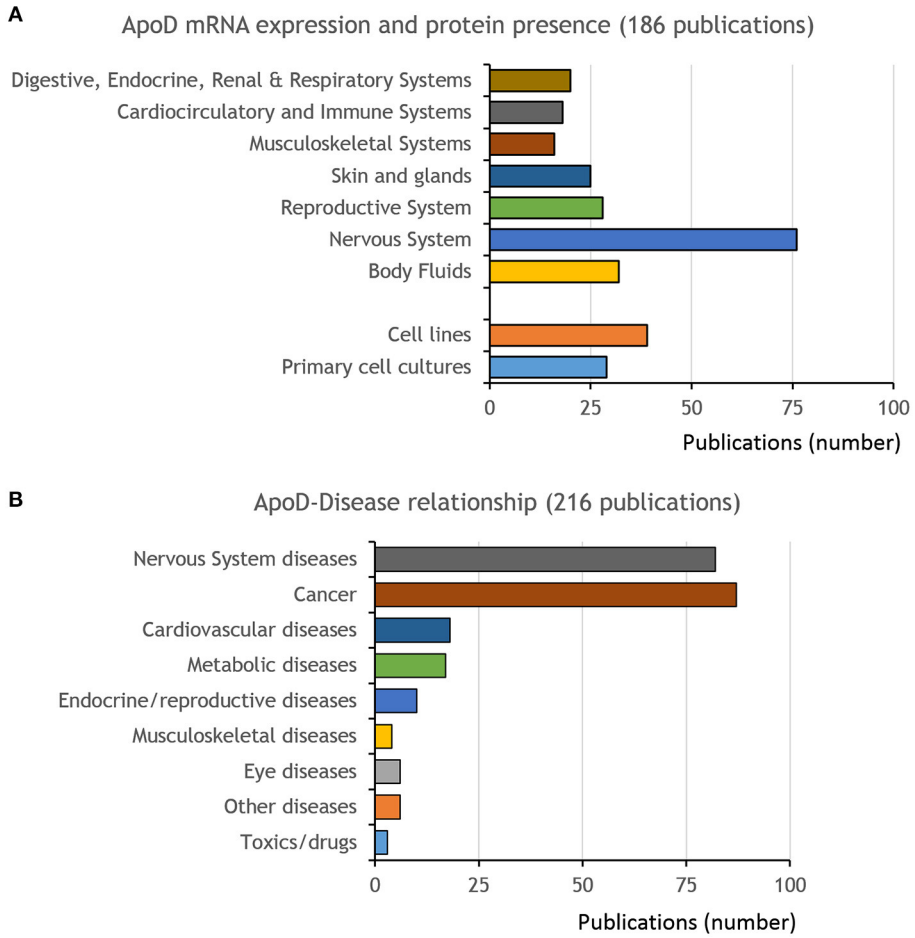

FIGURE 4 | Publications on ApoD expression and disease relationships. **(A)** Distribution of publications describing ApoD mRNA expression or protein presence *in vivo*, distributed by physiological systems and in cell cultures (primary cells or cell lines). **(B)** Publications with information on ApoD relationship to disease (expression changes triggered by disease or treatments, or association of ApoD gene variants with disease).

Avian egg fluids are also rich in ApoD, with the interesting property that egg white ApoD positively correlates with egg freshness.

References contributing to this section are listed in **Reference Collection 13**, **Supplementary Table 4**.

Tissue and Cellular Expression Patterns and Response to Stimuli

The analysis of tissue expression pattern leads to a general conclusion: in spite of its wide distribution, ApoD is never ubiquitously expressed, never in all cell types in a tissue, or at all times in a given cell type. ApoD is expressed in most tissues with a salt-and-pepper spatiotemporal pattern, suggesting a fine control that depends on particular physiological cell states. Furthermore, all tissues bear ApoD-expressing cells and cells able to endocytose ApoD protein from the extracellular milieu (see section Cellular Trafficking). These expression features, along with ApoD being a very stable protein, result in a high protein abundance when measured in high-throughput analyses, and in a lack of exact fit between mRNA and protein expression in a given tissue or cell (**Supplementary Figure 1**; **Reference Collections 14–16**; **Supplementary Table 4**). While tissues as the female breast present high levels of ApoD mRNA and protein, organs like the liver show high abundance of ApoD protein, but barely detectable ApoD mRNA both in human and mice. At the other end of the spectrum, blood cells and immune system-related organs are among those with low levels or no expression of ApoD, either mRNA or protein.

Organs and tissues involved in both male and female reproductive physiology express ApoD (**Supplementary Table 10**). The high expression of ApoD in breast has been located to the glandular epithelium (**Supplementary Figure 1**), and breast cysts accumulate high amounts of ApoD protein, making BCF a useful experimental source of native ApoD protein. ApoD mRNA is detected at all stages of the spermatogenesis process in testis and in ovarian theca cells. Along the female cycle, stromal and epithelial cells of the endometrium express ApoD mRNA and protein during the secretory phase. ApoD is also expressed during

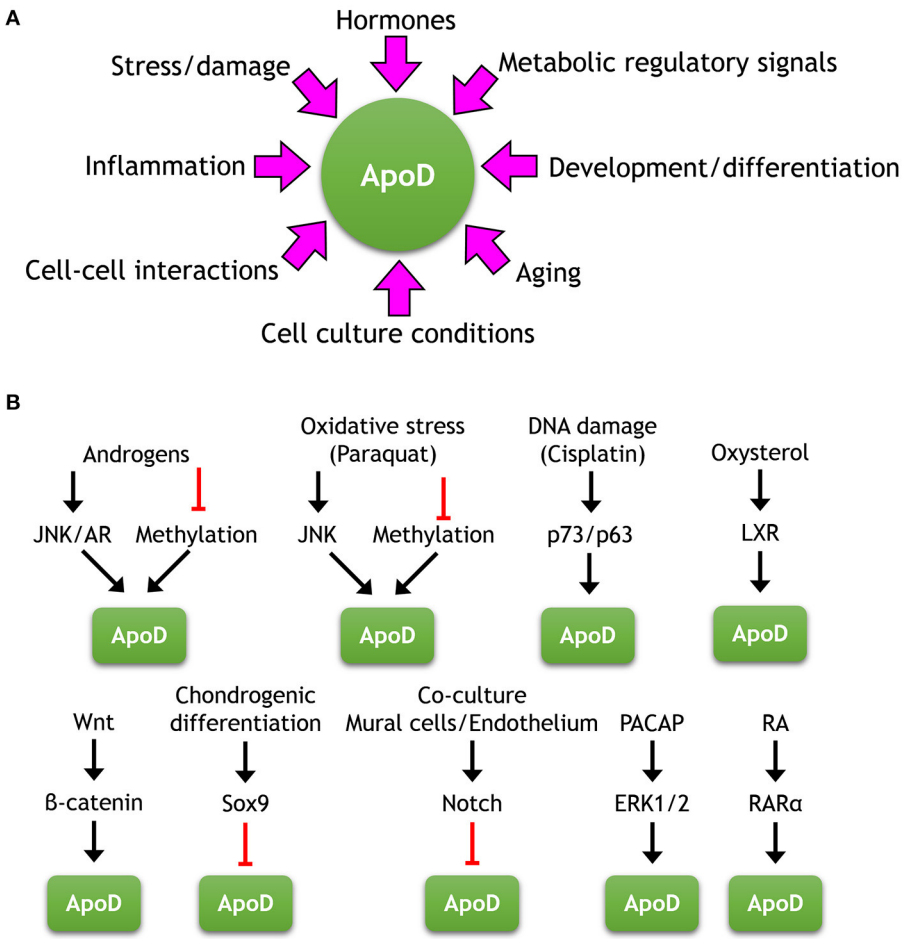

FIGURE 5 | Factors and pathways regulating the expression of ApoD. **(A)** Diverse stimuli regulate ApoD expression in a variety of cells and physiological conditions. **(B)** Summary of upstream regulatory pathways regulating ApoD expression where elements of the signaling cascade have been identified.

corpus luteum maturation in the ovary. Gestation alters ApoD abundance in plasma as well (**Supplementary Table 9**), with a decrease during a healthy pregnancy followed by a fast recovery if the mother breastfeeds her baby. ApoD expression in breast secretions and skin is also altered upon establishment of menopause.

These temporal patterns of expression are due to hormone regulation, as demonstrated by both *in vivo* and *in vitro* studies (**Supplementary Tables 10**, **16**, **17**; **Reference Collection 17**). Upregulation of ApoD by androgens is well documented in different preparations like breast explants, male genital fibroblasts or primary epithelial cells from male axillary apocrine glands. This regulation is mediated by nuclear androgen receptor (AR), and ApoD is being used as an AR activity assay (**Figure 5B**). Estrogens and progesterone, alone or in combination, also change ApoD expression in several experimental settings, with more variation in the final outcome depending on cell type (e.g., breast cancer cell lines up-regulate ApoD upon exposure to 17β-estradiol, while prostate cancer cell lines down-regulate it, **Supplementary Table 17**). Sex hormone-regulation of ApoD is also present in birds, in the context of oviposition cycles or egg fertilization, thus representing relevant biological stimuli for ApoD spatiotemporal regulation throughout evolution (**Figure 5**, **Supplementary Table 10**).

Expression in the nervous system (**Supplementary Table 11**) has been amply explored for ApoD, with primary publications doubling those devoted to other tissues or systems (**Figure 4**). All evidences support a prominent and consistent ApoD expression in the nervous system, where myelinating glial cells (oligodendrocytes and Schwann cells) constitute the main sites of expression in control conditions, followed by a more disseminated expression in astrocytes. ApoD protein abundance in the nervous system is accounted for by the fact that ApoD associates to myelin itself, a structure representing a large proportion of the vertebrate nervous system volume. As mentioned above, only subsets of cells express ApoD at a given time or location for each cell type (**Reference Collection 16**, **Supplementary Table 4**).

In addition to glial cells, ApoD has been found in meninges and the vascular system of the nervous system (**Supplementary Table 11**), particularly in pial and perivascular cells (mural cells or pericytes) associated to the capillary beds.

During mouse embryogenesis, ApoD has been detected in both pericytes and endothelial cells, and mRNA expression in the latter is under the control of Wnt/β-catenin signaling during the time interval of blood-brain-barrier formation (**Figure 5B**). ApoD-positive pericytes and other perivascular cells are also reported in the adult nervous system. RNAseq analyses of acutely isolated cortical cells show endothelial cells as second to myelinating oligodendrocytes in ApoD enrichment.

Although neuronal expression has been subject to debate (**Supplementary Table 11**), unambiguous detection of ApoD mRNA in neurons has been reported only in the developing brain. In contrast, detection of ApoD protein in some neurons has been reported at various ages in healthy control situations, while this finding is more abundant upon aging or disease. Neuronal uptake of ApoD upon disease has a certain degree of specificity. It is frequently found in the brain of Alzheimer's, but not in Parkinson's disease patients. Animal models of brain traumatic injury, stroke and Niemann-Pick type A disease do show neurons that have internalized ApoD protein, while they are not found in the Niemann-Pick type C mouse model. Transfer of ApoD from astroglial cells to neurons has been demonstrated in cell culture preparations, and shown to be mediated by extracellular vesicles (**Supplementary Table 17**; see section Cellular Trafficking).

A well-established fact with strong support from different studies is that ApoD expression increases throughout brain aging (**Reference Collection 18**, **Supplementary Tables 4**, **11**), a pattern conserved in several species analyzed with just one exception: a study documenting a decrease in ApoD mRNA in the aging avian hippocampus. A higher ApoD expression in cortex and brainstem in comparison with hippocampus or cerebellum are well-supported regional differences within the brain (**Supplementary Table 11**). In the highly-expressing prefrontal cortex, the increase of ApoD mRNA and protein throughout life positively correlates with proteins involved in antioxidant defense.

The expression data obtained from healthy individuals is coherent with an ApoD gene response to diverse experimental stress or injury paradigms (**Reference Collections 19–20**, **Supplementary Table 4**) that include oxidative stress (OS), peripheral nerve or traumatic brain injury, kainate excitotoxicity, damage by middle cerebral artery occlusion or by viral infection and experimental inflammation. All of the above results in increased ApoD expression *in vivo*. This ApoD stress response is mostly, but not exclusively, documented in the nervous system (e.g., OS-triggered upregulation is also observed in the cardiovascular system). These patterns of response can be extended to the many disease situations reviewed in section ApoD-Disease Relationships. In addition to the abundant correlative data from human diseases, experiments in animal models of disease analyzed *in vivo*, primary cell cultures and cell lines support a major conclusion: ApoD is a key player in the endogenous response to a variety of potentially harmful stimuli. The damage and stress responsive p73/p63 and JNK pathways have been demonstrated to up-regulate ApoD (**Supplementary Tables 16**, **17** and **Figure 5**), while the particular signaling cascades regulating ApoD upon other stress or inflammation inducers (e.g., H_2O_2, UV light or LPS) remains to be elucidated. Not all stressful conditions trigger ApoD expression (**Supplementary Table 17**), underscoring the specificity of pathways regulating ApoD (**Figure 5**). Moreover, a fine regulation of ApoD upon OS seems necessary, since it involves various non-exclusive mechanisms like DNA demethylation, the use of alternative promoters or 5′-UTR specific mRNA variants (see section Gene Data and Genomic Properties).

Nutritional and metabolic states also regulate ApoD expression (**Reference Collection 21**, **Supplementary Table 4**), and ApoD upregulation under caloric restriction or ADCY5 loss-of-function seems to be part of a common signature leading to lifespan extension. Curiously, these results derived from *in vivo* studies agree with ApoD upregulation upon serum starvation in cell culture systems (**Reference Collection 22**, **Supplementary Table 4**). New studies on how metabolic switches can modulate ApoD in different contexts, and searching for the specific signaling pathways that trigger ApoD expression are therefore valuable. A particular lipid-managing pathway is known to control ApoD expression: ApoD is a target gene for LXR in liver, skeletal muscle, adipocytes and endothelial cells, thus becoming part of the response to oxysterol stimulation.

Pathways involved in development and cell differentiation are also known to regulate ApoD expression (**Reference Collection 23**; **Supplementary Tables 4, 16, 17**). In addition to its regulation by the Wnt/β-catenin pathway mentioned above, ApoD is downstream of Sox9 during chondrogenic differentiation, and of PACAP/Erk signaling during adipocyte differentiation. Also, particular cell-cell interactions regulate ApoD expression in one of the cellular partners, like endothelial-mural cell interactions relevant during the angiogenesis process. In this scenario, ApoD is downregulated in mural cells by contact-dependent (Notch-3) and contact-independent mechanisms.

Finally, confluency and senescence in cell cultures also trigger ApoD expression (**Reference Collection 22**, **Supplementary Table 4**). These culture conditions parallel steady-state situations of cells in their physiological tissue environment and the *in vivo* upregulation by aging, respectively. Both conditions concur with a halt in cell division, as it is also the case for serum starvation conditions. The good prognosis of some types of cancers where ApoD increases, also relates its expression to low cell-division rate (see section ApoD-Disease Relationships). Retinoic acid induction of ApoD expression, mediated specifically by RARα in breast cancer cells, correlates with the anti-proliferative action of this signaling pathway. However, the potential role of ApoD in regulating cell division (see section Protein Physiology) must be dependent on the physiological/pathological context. For example, in the model of pericyte-endothelium interactions mentioned above, mural cells decrease ApoD expression upon interaction with endothelial cells, when they would stop dividing to generate mature capillary structures.

Figure 5 summarizes stimuli regulating ApoD expression and the particular upstream signaling pathways known to date.

ApoD-Disease Relationships

The reports in this section either study the expression of ApoD in response to disease and therapies, or evaluate association of ApoD gene variants with disease. Information was accrued from a total of 216 primary publications (**Figure 4B**; details in **Supplementary Table 18**).

That ApoD is part to the endogenous response to a wide range of diseases, with diverse primary causes, is uncontentious. Data support the existence of common factors underlying diverse disease situations that cause ApoD expression changes, and OS is the strongest candidate. The ApoD upregulation upon experimental stress or injury reviewed above is coherent with prominent examples concurring in the nervous system (**Reference Collection 27**, **Supplementary Table 5**), where 85% of 66 reports on degenerative/psychiatric diseases or naturally occurring injury identify an over-expression of ApoD. Exceptions are the down-regulation observed in neurotransmission-centered diseases, like depression and a DOPA-decarboxylase deficiency.

Cancer is the other major disease where changes in ApoD expression have been analyzed (82 reports). A clear negative correlation between ApoD expression and malignancy has been found in nervous system tumors, fibrosarcomas, breast, colorectal, hepatic, renal and cervical cancers. The general association of a good prognosis with high ApoD expression strongly suggests a protective anti-tumoral function for this Lipocalin. While studies of prostate cancer have not evidenced unambiguously such a pattern, some studies show regional ApoD expression differences (high in juxta-tumoral tissue) that are still compatible with a defensive tissue response to neoplastic transformation. ApoD tumor-suppressing activity has been experimentally tested and an inverse relationship between ApoD promoter methylation, ApoD expression and outcome is supported by various reports (**Supplementary Table 18**). Whether a common mechanism of ApoD function can promote survival of damaged postmitotic cells in neurodegenerative diseases, and also prevents proliferation of cancerous cells deserves further analysis.

Cardiovascular and metabolic diseases (particularly diabetes) as well as infection or injury, are also accompanied by ApoD upregulation. Again, OS might be a common link to ApoD response to these diseases, for instance in atherosclerotic plaques depending on disease progression, or upon oxidative degradation of glycated proteins in diabetes.

In contrast to the many diseases where ApoD expression changes have been reported, few genetic variations of ApoD have been widely or robustly linked to disease risk or prognosis (see section Gene Polymorphisms and **Supplementary Table 18**). Among the few cases reported, it is striking that most of them occur in non-coding sequences (introns or UTRs) revealing that pathogenic variations in ApoD protein sequence must be too deleterious to survive in extant populations.

Cellular Trafficking

The consistent finding of ApoD in body fluids and the signal sequence present in the translated polypeptide indicate that ApoD is exocytosed from cells expressing the protein. A consistent set of experimental work supports the association

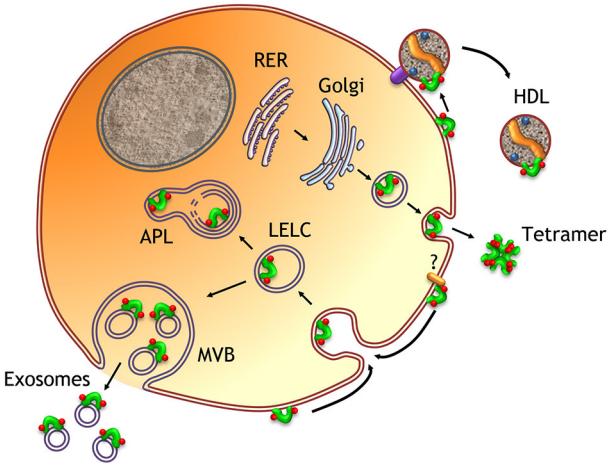

FIGURE 6 | Schematic representation of ApoD subcellular traffic. A model of an ApoD-expressing cell is represented. Canonical exocytosis through the RER-Golgi pathway generates the mature, glycosylated (red dots) protein. The tetrameric form identified in the breast cyst fluid is represented as the format detected in extracellular fluids. Once at the plasma membrane, ApoD can be endocytosed (by non-expressing cells as well) and targeted to lysosomes and autophagolysosomes. When endolysosomes develop into multivesicular bodies, ApoD would be carried on the outer surface of exosomes. Finally, ApoD can be transferred to HDL during their biogenesis, or during their lipid-efflux activity (upon HDL-receptor interaction).

of ApoD to the rough endoplasmic reticulum (ER), the signal peptide removal in the protein sorting process, the N-linked oligosaccharide modification carried out in RER-Golgi, and a secretion of the mature glycoprotein to the extracellular environment in several tissues and cultured cells. All these data make ApoD a typical soluble extracellular protein undergoing a canonical secretory pathway, a consensus attained by subcellular localization prediction algorithms and data present in gene ontology databases (see Methods). Additionally, subcellular traffic of ApoD can also lead to its exportation out of the cell in different formats that include ApoD tetramers, HDL-associated ApoD and extracellular vesicle-associated ApoD (**Figure 6**).

However, several reports have interpreted their findings about ApoD biological roles on the basis of protein partitioning in cytoplasm and/or nuclear compartments. Aside of technical issues questioning those results, some reports use overexpression of fusion-tagged proteins, which are known to undergo unnatural compartmentalization or degradation. Also, strategies based on *in vitro* interaction assays that were designed for proteins naturally occurring in the cytoplasmic or nuclear compartments (like the classic two-hybrid assays) preclude the finding of functionally relevant interactions for ApoD. An alleged cytosolic ApoD would likely be non-glycosylated and improperly folded in the absence of its intramolecular disulfide bonds.

Intracellular Traffic

A number of studies have shown the presence of ApoD in RER and vesicular compartments of different eukaryotic cells. Immunoelectron microscopy (EM) of nervous system cell types has unambiguously identified ApoD in the outer

nuclear membrane-RER membrane complex and in lysosomes. Lysosomes isolated from placental cells, monitored with biochemical techniques, contain ApoD. Likewise, clathrin-coated vesicles isolated from hen's ovaries also contain ApoD.

Recently, the subcellular location of ApoD has been studied in detail in basal conditions and under experimental stimuli, either by immunogold-EM microscopy, or by fluorescence immunocytochemistry combined with established cell compartment markers and monitored by well-documented standardized confocal microscopy. These studies have detected the presence of ApoD in RER, the Golgi apparatus, endosomes, lysosomes, autophagosomes, multivesicular bodies, as well as in the outer side of plasma membrane, clathrin-coated vesicles and caveolae. The protein does not localize to mitochondria or peroxisomes, and has not been immunolocalized inside cell nuclei. Lysosomal ApoD localization has been demonstrated in astrocytes, oligodendrocytes, Schwann cells, fibroblasts, and neurons.

Although those experiments were performed at a fixed time point, serial-time experiments demonstrated that the presence of ApoD in the endosome-lysosome compartment is stable and dynamically enriched upon OS exposure. Long-lasting lysosomal location of ApoD depends on its glycosylation and hydrophobicity, as mutated bacterial recombinant ApoD is maintained in lysosomes only transiently. Targeting of ApoD to the lysosomal compartment occurs not only in ApoD-expressing cells, but also in non-expressing neurons upon exposure to exogenous ApoD or when co-cultured with astrocytes. The fact that ApoD is a stable component of subsets of lysosomes at a given time, connects many of the apparently diverse physiological roles of ApoD (see section Protein Physiology).

References contributing to this section are listed in **Reference Collection 33**, **Supplementary Table 6**.

Exocytosis

It is undoubtedly established, as discussed above, that ApoD is being secreted to the extracellular environment following a canonical secretory pathway (**Figure 6**). However, whether this pathway leads to actual secretion of ApoD in monomeric form has not been established. In addition, extracellular vesicles (EVs) constitute an alternative exocytotic path for ApoD (see section Binding to Lipid-Rich Structures). Proteomic analyses have identified ApoD in EVs from human plasma and CSF. Experimental characterization of EVs produced by a human astroglial cell line and by mouse primary astrocytes, identified the ApoD-positive vesicles as exosomes originated from multivesicular bodies, according to their size (∼100 nm), density (1.17–1.23 g/ml) and molecular markers. When exported by glial cells in exosomes, ApoD must be located on the external surface of these EVs (**Figure 6**).

A third mechanism by which ApoD becomes extracellular is by traveling in HDL particles. ApoD-HDL association can take place during HDL biogenesis, or ApoD can associate to HDLs while the lipoparticles bind to cell membranes and perform their lipid efflux activity. However, these mechanistic details and the particular subcellular origin of the HDL-associated ApoD detected in body fluids need to be investigated. The plasma membrane location of both ApoA-I dependent HDL biogenesis (Denis et al., 2008) and ABCA1-dependent cholesterol efflux activity (Phillips, 2018), makes it a likely location for the origin of ApoD-positive HDL particles (**Figure 6**).

References contributing to this section are listed in **Reference Collection 34**, **Supplementary Table 6**.

Endocytosis

The immunolocalization of ApoD in cells not expressing the gene (see section Tissue and Cellular Expression Patterns and Response to Stimuli), as well as the internalization of ApoD by cells cultured in the presence of its native or recombinant forms, are the experimental basis supporting the endocytosis of this Lipocalin. It takes place both under control conditions and in response to specific biological stimuli. ApoD endocytosis appears as a general property of this protein, as it has been reported in birds and mammals. Particularly, in glia-neuron co-cultures ApoD is found to be exclusively transported in EVs from astrocytes to neurons, where it gets internalized. The current view of several extracellular formats of ApoD (HDL, EVs or tetramers in solution) makes it worth to study whether different membrane interaction mechanisms or endocytosis paths are used for ApoD internalization.

ApoD association to the extracellular side of the plasma membrane is coherent with both, its traffic from RER to plasma membrane by the canonical exocytotic path and with its cell contact before internalization. ApoD-plasma membrane interaction has been experimentally demonstrated and is currently considered an established localization for ApoD in human cells (https://www.proteinatlas.org/ENSG00000189058-APOD/cell). Whether ApoD-membrane association is mediated by protein-protein or protein-lipid interactions requires further research (see sections Protein-Protein Interactions and Binding to Lipid-Rich Structures). **Figure 6** summarizes ApoD intra and extracellular traffic as currently known.

References contributing to this section are listed in **Reference Collection 35**, **Supplementary Table 6**.

Tissue and Organ Function

The reports tagged in this section were selected because they study the function of ApoD by experimentally altering ApoD natural expression levels, or by subjecting cells or tissues to defined concentrations of the protein in a controlled experimental situation. A critical review of these reports aims at uncovering common and distinct roles for ApoD in different physiological organ and cellular systems.

ApoD Functions in Cardiovascular System

The process of angiogenesis has been a focus of interest to study the role of ApoD, given its reported expression by blood vessel mural cells (MCs: smooth muscle cells and pericytes). Both in embryonic development and during the remodeling process of wound healing, ApoD increased expression is causally linked to undifferentiated mural cells migration, though is not consistently related to cell proliferation (as it is often found in cancer cells; see section ApoD-Disease Relationships). A crosstalk between endothelial cells (ECs) and MCs governs the switch of

the angiogenic cellular process from a proliferative/migratory state to a differentiation state, characterized by quiescence and adhesion. This switch is essential for vessel morphogenesis. Blood vessel angiogenesis involves reactive oxygen species (ROS), EC-derived PDGF-BB, JAGGED1 and NO, as well as MC-expressed NOTCH3. These secreted and cell contact-mediated signaling downregulates ApoD in MCs, a process linked to blood vessel maturation. Experimental manipulation of ApoD levels demonstrates that ApoD regulates the adhesion of MCs to the extracellular matrix, and low levels of ApoD promote Zyxin- and Vinculin-positive focal adhesion contacts. Concordant effects have been reported in retinal choroid vessels: ApoD-KO mice show neovascularization with increased permeability.

Myocardial cells show slight expression of ApoD under normal circumstances, but the tissue surrounding an injured/infarcted area promptly upregulates ApoD. The study of this process in ApoD-KO mice indicates that ApoD is associated to protection from cell death in the injured tissue. This ApoD protective role on cardiomyocytes is dependent on a proper protein fold and strongly correlates with its antioxidant activity (see sections Binding to Lipid-Rich Structures and Protein Physiology). In this experimental paradigm, the protective activity is attained by increasing ApoD in plasma, though it is unclear whether ApoD levels are also elevated in the infarcted tissue. We thus propose that ApoD function in myocardial tissue protection and remodeling might be based on: (1) A modulation of cell viability in cardiomyocytes and vessel ECs, possibly due to internalization of plasma-derived ApoD, and/or (2) a regulation of cell differentiation related to the angiogenic response described above, organized by ECs and MCs.

References contributing to this section are listed in **Reference Collection 36**, **Supplementary Table 7**.

Roles of ApoD in Metabolism Regulation

The role of ApoD in metabolism has been analyzed *in vivo* by using two different ApoD-KO mouse lines and a transgenic mouse (hApoD-Tg) driving the expression of human ApoD under the control of the human THY1 gene. This hApoD-Tg mouse ectopically expresses hApoD mostly in neurons, but the protein is present in plasma and other organs physiologically relevant to metabolism. Also, adenovirus-driven liver production of mouse ApoD has been used as a paradigm of acute overexpression, leading to elevated protein levels in plasma.

The metabolic consequences of altering ApoD levels have been evaluated mostly in plasma and liver, although also in retina, and measured in a variety of experimental settings: fasting or non-fasting conditions, different feeding diets, and different sex or age of animals. No clear pattern can be extracted for the role of ApoD on carbohydrate metabolism, where reports describe varied outcomes on glucose tolerance or insulin resistance depending on experimental conditions. Some consistency is observed in the effects on triglycerides (TG): Loss of ApoD leads to decreased hepatic TG content and increased plasma TG, while overexpression leads to elevated TG levels in liver and unaltered or decreased triglyceridemia (depending on the strategy used for ApoD overexpression). On the other hand, variations in plasma cholesterol levels are also reported, with various outcomes upon ApoD loss or overexpression. Also, association of ApoD polymorphisms have been found with both increased and decreased HDL-cholesterol species. The finding of ApoD being able to mediate binding of HDL to LDL, and of HDL particles to actively dividing carcinoma cells, suggests that it can regulate lipid traffic indirectly by influencing lipoparticle dynamics. Variations in local physiological contexts of this traffic mechanism might contribute in very different ways to the final systemic outputs measured in the experimental settings studied *in vivo*. However, more work is needed to derive definitive evidence for understanding the role of ApoD in lipid and carbohydrate metabolism. So far, the relevant results indicate that the functional relationship of ApoD with various metabolic parameters is, at most, indirect and dependent on other physiological conditions.

References contributing to this section are listed in **Reference Collection 37**, **Supplementary Table 7**.

ApoD Functions in Skeletal System

Bone cells, from bone marrow stem cells (MSCs) to osteoblasts, are reported to express ApoD in cell culture systems (**Supplementary Tables 16**, **17**), and two reports have focused on testing the effects of experimental manipulations of ApoD levels on bone formation and remodeling. Relevant sex and hormone-related patterns have been found using ApoD-KO or hApoD-Tg mice and cell culture systems. With both approaches ApoD appears as an osteogenic factor. Lack of ApoD in mice reduces bone volume and thickness. These effects are observed in trabecular and cortical bone in females, but only in cortical bone in males. Enhanced bone turnover in female ApoD-KO mice is indicated by increased osteoblast surface and osteoclast numbers. Primary MSCs from ApoD-KO mice have lower survival and proliferation, and increased osteoclastogenesis, but an uptake of exogenous hApoD partially reverts their osteogenic potential. When osteoporosis is modeled by glucocorticoid (dexamethasone) treatment after osteogenic induction of MSCs, overexpression of ApoD reverts the effects of dexamethasone, as measured by PI3K/Akt pathway activity and downstream osteogenic gene expression, thus promoting the osteogenic process. Osteogenesis is accompanied by SOD and catalase upregulation, and oxidative damage is associated with glucocorticoid-induced osteoporosis, thus linking ApoD function in this context to its antioxidant activity.

References contributing to this section are listed in **Reference Collection 38**, **Supplementary Table 7**.

ApoD Functions in the Nervous System

As presented above, the current evidence supports a general view in which non-neuronal cells become the source of ApoD in response to different stimuli, and neurons count on the Lipocalin for its cellular functions by internalizing ApoD. Neurotransmission is one of those functions modulated by ApoD. Analysis of downstream effects in gene expression in the brain of ApoD-KO or hApoD-Tg mice, reveal an enrichment of genes related to synaptic transmission. Particularly, changes in glutamate, somatostatin,

dopamine and acetylcholine neurotransmission have been independently documented by receptor binding assays, HPLC determination of neurotransmitters or their catabolites, or receptor immunodetection. These effects might underlie the behavioral phenotypes related to locomotor function, motor and spatial learning, and retinal function observed in mice with altered expression levels of ApoD.

ApoD addition to cultured neurons results in neuritogenesis and synaptogenesis, which are crucial during neuronal development and underlie neuronal plasticity of established circuits. Neuritogenesis is promoted in immature neurons in culture by the combined addition of ApoD and retinoic acid in the absence of serum. Experiments combining ApoD addition with receptor antagonists indicate that ApoD-dependent neurite extension can be mediated by LDLR, and possibly also by CXCR4 activation, pathways known to be involved in neuronal differentiation.

Another general function of ApoD, extensively analyzed in loss-of-function and transgenic mice, is its role in the glial response to dyshomeostatic changes in the nervous system due to oxidative, metabolic or traumatic stresses. Many studies have reported an acute regulation of ApoD expression under these insults, either experimental or triggered by disease (see sections Regulation of Expression and ApoD-Disease Relationships), supporting an overall neuroprotective role now widely accepted as a functional label for this Lipocalin. Both astrocytes and oligodendrocytes express and secrete ApoD in response to stress. The protein exerts an autocrine and paracrine neural tissue protection, which results in functional preservation of OS-challenged dopaminergic systems, of neurons affected by kainate excitotoxicity or suffering from Aβ-related degeneration. Astrocytes, although not an abundant source of ApoD in basal conditions, quickly respond to OS with a JNK-dependent expression of ApoD, which is secreted to the extracellular milieu as cargo on the surface of extracellular vesicles (**Figure 6**). The protein is internalized by glial and neuronal cells, improving their viability thanks to a control by ApoD of OS-dependent lipid peroxide accumulation. Moreover, a surge of ApoD in a stressed neural tissue behaves as an off-signal limiting the dimension and duration of gliosis and inflammation. The inflammatory response is linked to OS due to increased PLA2 expression and AA production, among other factors. Quenching of AA is proposed as part of this inflammation control by ApoD (see section Protein Physiology).

A long-lasting homeostasis maintenance role for ApoD has been also proposed in the process of physiological aging of the nervous system, where this protein has been shown as the most consistently overexpressed in primates and rodents. Also, life-expanding strategies in model organisms, like caloric restriction, promote ApoD expression not only in the nervous system but also in cardiac and skeletal muscle (**Supplementary Table 11**). The homeostatic role predicted by the expression pattern is supported by the phenotypes exhibited by aged ApoD-KO mice, which do not display altered lifespan but do present signs of early neurodegeneration at 3 months of age, with oxidative damage and proteostasis defects in cortex and hippocampus. These alterations underlie cognitive defects and a hyperkinetic phenotype evident in old (21 months) ApoD-KO mice.

The predominant expression of ApoD in myelinating cells under control conditions (oligodendrocytes in CNS and Schwann cells in PNS; see section Regulation of Expression) has prompted experimental studies, using cultured primary cells and ApoD-KO and hApoD-Tg mice, that clearly support the implication of ApoD in the myelination process during development, in the lifelong maintenance of the myelin sheath, and in the remyelination that occurs in response to environmental insults. These processes have been analyzed in the mouse brain corpus callosum as well as in the peripheral sciatic nerve. ApoD is required for a proper and timely response to a crush injury in PNS nerves, helping to recover locomotor function. ApoD promotes myelin clearance and regulates angiogenesis and macrophages recruitment to the wound site, processes that are essential for subsequent axonal regeneration and remyelination. ApoD contributes to optimize myelin clearance, carried out by transdifferentiated Schwann cells and infiltrating macrophages, through two complementary actions: control of lipid-mediated inflammatory signaling and optimization of the phagocytosis process itself. Data indicates that ApoD regulates and control the tissue levels of AA and lysophosphatidylcholine (both *in vitro*-demonstrated ApoD ligands, **Table 2**). They are needed for an adequate cytokine inflammatory response and recruitment of bone marrow-derived macrophages. Although macrophages do not express ApoD, the levels of this Lipocalin in the injured nerve environment influence their phagocytic activity, since myelin-associated ApoD is phagocytosed as well. Flow cytometry experiments with primary macrophages demonstrated that ApoD affects the initiation and efficacy of phagocytosis.

A dynamic spatiotemporal regulation of ApoD expression is apparent in myelinating cells, with a prominent increase at the height of postnatal myelination followed by continuous rise throughout life. The absence of ApoD results in a defective and irreversible compaction, mostly in the extracellular leaflet of both CNS and PNS myelin. This altered myelin structure results in a decreased conduction velocity, reported for the sciatic nerve, and compromises motor learning tasks. As downstream effects, both the mTORC1-dependent lipogenic switch and the ERK-mediated growth pathways are altered in the absence of ApoD. A lack of myelin compaction is due to inadequate removal of myelin glycocalyx, mostly affecting gangliosides GM1–2b, GD1b, and GT1b content and distribution. This role of ApoD on glycocalyx physiology was demonstrated to be linked to the adequate subcellular localization of lysosomal and plasma membrane sialidase (Neu1 and Neu3) and of the regulatory Fyn kinase. This mechanism requires preservation of lysosomal membrane integrity (see section Protein Physiology).

References contributing to this section are listed in **Reference Collection 39**, **Supplementary Table 7**.

Protein Physiology

In this final section we aim at discussing the available knowledge, derived from state-of-the-art research critically assessed in this

review, to give a plausible answer to the central question posed in the Introduction: In order to achieve its pleiotropic roles, does ApoD moonlight between different biochemical functions when expressed in different contexts, or instead ApoD displays a distinctive biochemical role that works on varied physiological situations?

The presence of ApoD in extracellular formats such as lipoprotein particles and exosomes evidences its ability to associate to higher-order lipid structures. According to our systematic search no unambiguous evidence exists for the secretion of protein monomers in native conditions. Non-denaturing electrophoresis analysis of CSF revealed only high-molecular weight ApoD oligomers, while in plasma it has been repeatedly identified in lipoprotein particles preparations. In the particular case of BCF, where an extremely high concentration of ApoD is produced, the protein assembles in tetramers through protein-protein interactions. Finally, ApoD secreted by cultured astrocytes is internalized by neurons only if the conditioned extracellular media has not been depleted of extracellular vesicles. A protein region encompassing the first three β-strands, highly conserved in chordates (**Figure 2A**), and several hydrophobic patches located at the protein pocket entrance (**Figure 3E**) are proposed to underlie ApoD self-association and ApoD-lipid structure interactions, respectively.

These results shed doubts on a view of ApoD widely cited (a functional tag in most databases for this Lipocalin) as a "lipid transporter," a task that a secreted globular monomer could easily achieve.

Unquestionably though, it is the ability of ApoD to bind small hydrophobic ligands of varied shapes inside its β-barrel pocket. However, when free ligands (e.g., AA) have been mechanistically related to ApoD function, binding data are compatible with a buffering or quenching function, or a very local shuttling of the ligand at the most, rather than to a generalized long-range ligand transport between cells. A curious case is the expression of ApoD in feather follicles of pheasants, only in skin areas with specific plumage colors, suggestive of a pigment-retention function. Similarly, the ligand bound to ApoD in sweat from human axilla could be the source of slowly released volatile odor molecules. These ligand-retention functions compare well with that of crustacean ApoD homologs, also linked to their carapace coloration (Wade et al., 2009).

In a different context, ligand shuttling has been repeatedly proposed for ApoD in the cholesterol transfer to LCAT. However, ApoD has been demonstrated not to bind cholesterol, not to contribute to LCAT-cholesterol transfer and not to show a direct interaction with LCAT. On the contrary, experimental data suggests that ApoD exerts "stabilizing effects" on LCAT activity. After reviewing the relevant information on this issue, we propose a different view that can guide new testable hypotheses: ApoD binds lysophosphatidylcholine (LPC), a LCAT reaction product that exerts a negative feedback on LCAT activity. By quenching LPC, ApoD would maintain LCAT activity over a wide range of LPC product concentration. This specific LPC quenching function is compatible with the small amounts of ApoD recovered from HDLs, since only a transitory presence of ApoD might be needed when LCAT is adding cholesterol to the lipoparticle. As for the HDL-LDL interaction (see section Roles of ApoD in Metabolism Regulation), the putative consequences of ApoD presence in HDLs on cholesterol management in the organism would therefore be of an indirect nature, and could explain the lack of correlation between ApoD and cholesterol content in many physiological or pathological situations.

A fundamental advance in defining ApoD molecular function was its role in organismal protection against OS, achieved by a control of the magnitude of lipid peroxidation, measured at tissue or cellular levels. This role has received strong experimental support from *in vitro* biochemical assays, cellular experimental systems, and *in vivo* experiments with animal models where ApoD expression was manipulated. Moreover, further validation for this role comes from experimental approaches testing the expression of human ApoD in evolutionary distant organisms. Overexpression of human ApoD in Drosophila increases lifespan in both normal and pro-oxidative experimental conditions. Also, replacement in plants of the native chloroplast Lipocalin (LCNP) by human ApoD, targeted to thylakoids, rescues drought and OS sensitivity of the mutant. Lipid peroxidation control is evidenced in both reports as the mechanism mediating the organism response.

An ApoD antioxidant mechanism has been demonstrated using oxidized AA-derivatives in solution or auto-oxidized liposomes. ApoD is able to reduce free radical-generating lipid hydroperoxides to inert lipid hydroxides. In this reaction, the residue Met93 exposed on one of the surface hydrophobic patches of the protein (**Figure 3C**) is converted to Met93-sulfoxide. This residue is preserved in ApoD chordate orthologs (**Figure 2A**) and contributes to the functional differentiation of ApoD from its closest Lipocalin relative, RBP4, where that position is occupied by charged (Lys or Arg) residues (Diez-Hermano et al., 2021). To maintain ApoD antioxidant activity, the action of a methionine sulfoxide reductase (MRS) would be required. However, oxidized ApoD tends to self-associate. Interesting data from Alzheimer's disease brain samples reveal that hippocampal (but not cerebellar) MRS levels decrease with disease progression, while ApoD oligomerization increases. This suggests that the ApoD redox cycling might be blocked if Met93 does not return to its native form and the protein self-associates. This effect sets an upper limit to ApoD antioxidant activity, since it would result in the consumption of ApoD-Met93. Whether this depletion triggers a feedback regulatory loop promoting ApoD gene expression under OS situations in different physiological and pathological contexts would be an interesting aspect to explore. In this context, we must keep in mind that ApoD structure is stable under pro-oxidative situations, making it suitable for the biological contexts where ApoD function is beneficial (from neurodegenerative conditions to cancer). Also, ApoD ligand binding ability is preserved at low pH and its glycosylation prevents a rapid degradation, both good assets to perform its ligand binding and antioxidant functions inside the endolysosomal compartment.

The direct antioxidant activity of ApoD and its demonstrated stable location in the lysosomal compartment put forward a new

view of ApoD protein physiology that holds high explanatory power in the understanding of a number of apparently varied ApoD roles.

ApoD control of redox state can be performed directly on both, cell membranes and lipoprotein particles. The unilamellar vesicles where ApoD-reducing activity has been demonstrated are a good experimental model for both types of lipid-based structures. Lipid peroxide products are mainly derived from cellular membranes, which are a major target for cell-generated ROS. The ability of ApoD to keep low levels of membrane-originated lipid peroxides, together with the positive correlation of ApoD content in HDLs with their antioxidant capacity, and the promotion of HDL-LDL interaction by exogenously-added native ApoD, support the protective action of ApoD in both types of lipidic structures (membranes and lipoparticles). Additional evidence comes from the existence of ApoD insect homologs stably anchored to cell membranes (Ganfornina et al., 1995; Ruiz García, 2013), which suggests that membrane interaction is part of an ancestral ApoD property.

In addition to the immediate effects on the redox state of membranes and other lipid structures, ApoD can give rise to indirect effects when performing its antioxidant function in the lysosome. The lysosome is considered a "lipid-controlling" cellular hub. ApoD maintenance of lysosomal membrane redox balance and integrity results in the control of plasma membrane composition. This is for example the case for plasma membrane glycolipids, with important consequences for membrane-membrane interactions like those required in the process of myelin compaction. Lysosomal membrane stability can, by extension, influence the lipid export/import balance in cells, another way of ApoD indirectly conditioning the organism lipid metabolism. Altered ApoD expression in response to mutations of the lysosomal cholesterol transporters (as in Niemann-Pick type C disease) supports this notion. Plasma membrane modulation is also coherent with the observed correlation of ApoD content in HDLs and their ABCA1-dependent cholesterol efflux capability in macrophages, or the subtle changes in lipid content in lipoprotein particles of subjects with ApoD polymorphisms. Through its influence on membranes and lipoparticle dynamics, without a need of binding cholesterol, ApoD can modulate its flux within and between cells.

Additionally, the lysosome is a "cell death/survival controller" by its fundamental recycling, detoxifying and proteostatic functions. Lysosomal ApoD would condition whether a failure in the lysosomal compartment takes place upon a wide array of disease/injury situations, thus contributing to the final cell fate. This ApoD-dependent cell fate decision can be extended to developmental processes as well.

Finally, a role of ApoD in innate immunity has been frequently reported, while no mechanistic link to the protein physiology was proposed. We suggest that ApoD, with its lysosomal optimization mechanism, can modulate the efficiency of phagocytic cells, like it has been demonstrated in injury-recruited macrophages, therefore influencing many of the maintenance and immune responses of the organism.

This view makes us to propose that ApoD lipid-binding properties are more related to management of lipid-based structures composition (membranes or lipoparticles) and a control of their redox state, than to lipid transport. Whether similar membrane-stabilizing properties endow ApoD-positive exosomes with resistance properties to be efficient cargo transporters in disease or tissue damage situations, would be worth studying.

A different aspect of ApoD physiology scarcely studied is the role of its demonstrated N-linked glycosylation, which has been proven to be tissue and species specific, and to be essential for both, ApoD interaction with lipoparticles and for its cellular localization in the endolysosomal compartment. This is particularly important because of the association between redox signaling and glycan profiles, which in turn could affect several signaling pathways (Khoder-Agha and Kietzmann, 2021). In relation to this, modulation of signaling pathways by ApoD has been confirmed in endothelial cells and osteoblasts (PI3K-Akt pathway) and nervous tissue (pERK). How ApoD controls signaling cascades is open to discussion. Although several protein candidates have been proposed as ApoD membrane receptor, no clear demonstration is available for a receptor-mediated signaling transduction. Alternatively, ApoD might not require a protein receptor and trigger a unique signaling cascade. Instead, it could be working as a quencher of lipid modulators (e.g., AA), or conditioning the membrane partitioning of signaling complexes that are known to be dependent on membrane lipids distribution.

In summary, the available information supports a parsimonious hypothesis for the biological function of ApoD, with a unique biochemical role related to the management and redox state of lipid cellular and extracellular structures. This proposition is compatible with the wealth of experimental results showing that multiple stimuli in varied cellular contexts trigger ApoD expression with a tight spatiotemporal regulatory control. The protein can then become associated with the challenged membranes or being exported to the extracellular milieu to act in a paracrine fashion. Both direct and indirect downstream effects, depending on the cell type affected, would explain pleiotropy at the organismal level with a single biochemical function.

The proposed unique molecular mechanism also explains ApoD biological role in response to tissue/organ damage and disease, where homeostatic maintenance is disturbed and ApoD will contribute to restore the equilibrium through tissue repair/reconstruction. Under this paradigm, we can also explain ApoD roles in organismal developmental processes implying building-deconstruction cycles. **Figure 7** summarizes the new view on ApoD physiology. References contributing to this section are listed in **Reference collection 40**, **Supplementary Table 8**.

Future Goals for ApoD Biology

In spite of the explanatory power of our proposed biological role for ApoD, many questions keep been unresolved and many others are likely to arise, which can spur and guide new research programs. A few of them follow:

(1) To explore the functional relationship between the protein antioxidant capacity and the pocket ligand binding. In this

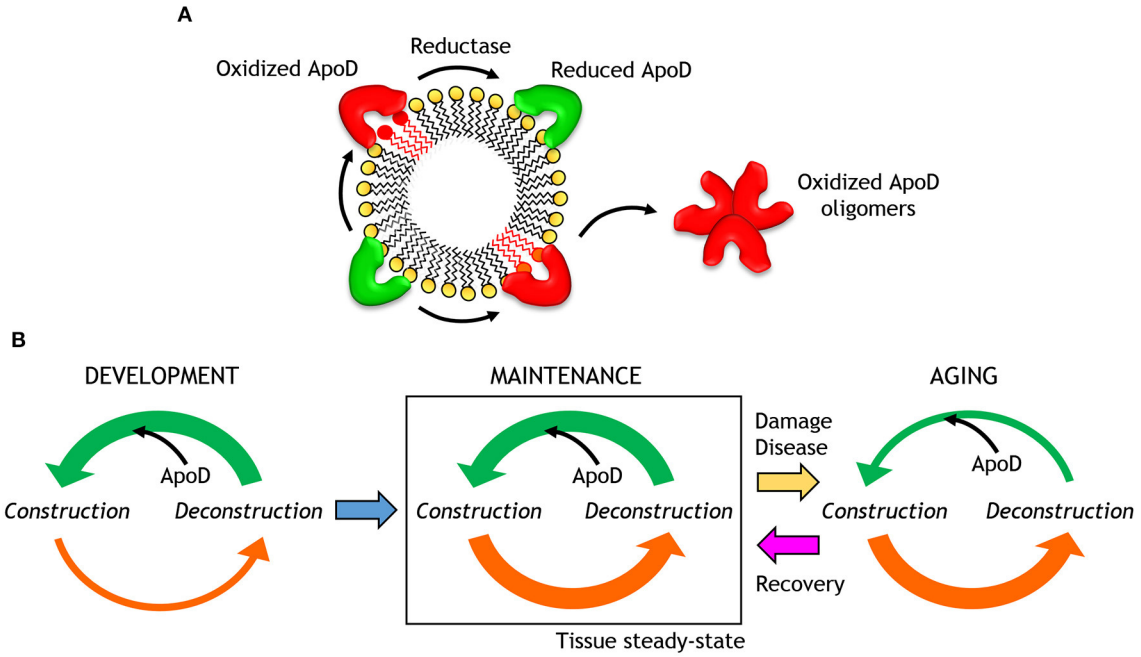

FIGURE 7 | ApoD physiology summary. **(A)** Schematic view of the lipid-managing biochemical function of ApoD. The lipid structure depicted can equally represent the surface of a lipoprotein particle, extracellular vesicle or cellular membrane. ApoD antioxidant activity can be maintained by redox cycling, requiring a reductase activity, or the cycle can terminate by oligomerization of oxidized ApoD. **(B)** Summary of global tissue function of ApoD, where it contributes to the turnover and maintenance of tissues and organs. This equilibrium is reached after developmental processes in which ApoD is also involved, and switches to a different state upon disease, injury or physiological aging.

respect, the hypothesis of ApoD working on oxidized lipid "whiskers" (Greenberg et al., 2008; Del Caño-Espinel, 2014) on cell membrane bilayers or lipoparticles is appealing and worth contrasting.

(2) To test whether ApoD downstream effects on signaling pathways rely on a canonical receptor-mediated transduction, or alternatively they depend on the modulation of the lipid context of signaling elements (e.g., PI3K). Findings in Drosophila reveal that loss of an ApoD homolog alters PI3K association to the plasma membrane (Hull-Thompson et al., 2009).

(3) To test whether oligomeric *vs.* monomeric forms of ApoD underlie its managing function on cell membranes or lipoparticles.

(4) To characterize the extent of ApoD redox cycle, maintaining antioxidant ApoD activity thanks to the intervention of reductases, and the implications of a potential upper limit to this mechanism due to ApoD oligomerization. This aspect can be key to fully understand ApoD function in aging and disease.

(5) To analyze the effects of differential glycosylation on ApoD interactions and functions.

(6) Recent studies on a Drosophila homolog (Yin et al., 2021) point to lipid droplets as another higher-order lipid structure susceptible to be modulated by ApoD. Searching for lipid droplet-managing functions of vertebrate ApoD is therefore pertinent.

(1) At a more general tissue/organ level, several functions are relevant to be studied in more depth, such as the ApoD role on feather and skin physiology, neuronal synaptic function, and metabolism.

(8) Finally, it is worth to analyze the potential exchange between the nervous system and systemic pools of extracellular ApoD in its different formats, not only to fully understand its roles in the organism, but also for a potential therapeutic use of ApoD in nervous system diseases.

ApoD SYSTEMATIC REVIEW. REFERENCE COLLECTIONS

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MG and DS: conceptualization, writing, review, and editing. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.738991/full#supplementary-material

Supplementary Figure 1 | ApoD mRNA and protein expression summary of the Human Protein Atlas.

Supplementary Figure 2 | ApoD mRNA and protein expression summary of the Mouse Gene Expression Database.

Supplementary Table 1 | Complete ApoD Reference Library.

Supplementary Tables 2–8 | References organized by subheadings within each tag (MP, GD, RE, DR, CT, TOF, and PP).

Supplementary Tables 9–15 | Analysis of primary papers describing ApoD mRNA expression or protein detection *in vivo* in different physiological systems: body fluids, reproductive system, nervous system, skin and glands, musculoskeletal system, cardiovascular and immune systems, and other functional systems (digestive system, liver, pancreas, adipose tissue, kidney).

Supplementary Tables 16, 17 | Analysis of primary papers describing ApoD mRNA expression or protein detection in primary cultures and cell lines.

Supplementary Table 18 | Analysis of primary papers describing ApoD relationships to disease (expression regulation by disease or treatment, genetic variations associated to disease).

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REVIEW
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Biological Functions of RBP4 and Its Relevance for Human Diseases

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Retinol binding protein 4 (RBP4) is a member of the lipocalin family and the major transport protein of the hydrophobic molecule retinol, also known as vitamin A, in the circulation. Expression of RBP4 is highest in the liver, where most of the body's vitamin A reserves are stored as retinyl esters. For the mobilization of vitamin A from the liver, retinyl esters are hydrolyzed to retinol, which then binds to RBP4 in the hepatocyte. After associating with transthyretin (TTR), the retinol/RBP4/TTR complex is released into the bloodstream and delivers retinol to tissues via binding to specific membrane receptors. So far, two distinct RBP4 receptors have been identified that mediate the uptake of retinol across the cell membrane and, under specific conditions, bi-directional retinol transport. Although most of RBP4's actions depend on its role in retinoid homeostasis, functions independent of retinol transport have been described. In this review, we summarize and discuss the recent findings on the structure, regulation, and functions of RBP4 and lay out the biological relevance of this lipocalin for human diseases.

Keywords: RBP4, retinol transport, liver, retinoids, vitamin A, lipocalin, metabolism

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GENERAL INTRODUCTION

Vitamin A, which comprises retinol and its biologically relevant metabolites, belongs to the group of fat-soluble vitamins and is essential for humans (McCollum and Davis, 1913). It is famously involved in the physiology of vision, but moreover, it is also known to play roles in immune cell function, reproduction, embryonic development, and the regulation of cell proliferation and differentiation (Morriss-Kay and Sokolova, 1996; Napoli, 1996; Blaner et al., 1999; Blaner, 2007).

Most of vitamin A's actions are mediated by its active metabolite all-*trans* retinoic acid (atRA) acting as the high affinity agonist ligand for the three known isoforms of retinoic acid receptors (RAR) α, β, and γ (Petkovich et al., 1987). A 9-*cis* configurated RA derivative, whose exact nature and physiological context is still under investigation, activates retinoid X receptors (RXR) (Heyman et al., 1992; Levin et al., 1992; Rühl et al., 2015). Both RAR and RXR belong to the family of nuclear receptors that serve as ligand-activated transcription factors. In contrast to regulating gene expression, 11-*cis* retinaldehyde in the eye is the light-sensitive chromophore of the rhodopsin complex to allow for the visual cycle in the retina (Palczewski et al., 2000). A deficit of vitamin A and as a consequence 11-*cis* retinaldehyde is evident by impaired vision, leading to night blindness or even full blindness (Blegvad, 1924). Vitamin A deficiency due to malnutrition during pregnancy is the leading cause for visual defects in newborns in developing countries (Pirie, 1983). Thus, vitamin A is required for both development (RA- and nuclear receptor-mediated) and functionality (11-*cis*

retinaldehyde-mediated) of the eye, underlining its extraordinary dependence on vitamin A.

Lipocalins are a family of proteins with the ability to bind and transport small lipophilic proteins (Pervaiz and Brew, 1987). A prominent member of this family is retinol binding protein 4 (RBP4, also known as RBP) (Kanai et al., 1968; Quadro et al., 1999), which is central to this review. As suggested by its name, RBP4 transports retinol and is considered the only specific binding protein in the circulation (Kanai et al., 1968). As such and most likely also via non-canonical functions, RBP4 is implicated in a variety of human conditions that include impaired vision and ocular diseases (Li et al., 2010), disorders of glucose and lipid homeostasis (Yang et al., 2005), and cardiovascular diseases (Sun et al., 2013). This review will summarize the current knowledge of the biological functions of RBP4 and how the lipocalin is linked to these pathologies.

DISCOVERY AND PROTEIN STRUCTURE OF RBP4

In 1968 Kanai et al. were first to describe RBP4 as a human plasma protein that is bound specifically to retinol and functioning as its transporter in the circulation. In their study, they analyzed plasma of subjects that had been injected intravenously with radio-labeled retinol. They were able to purify the protein that had bound labeled retinol and named it RBP (Kanai et al., 1968). Besides binding retinol, they found it to be circulating in complex with another, larger protein with prealbumin mobility on electrophoresis. Since then, there have been numerous studies on the biology of RBP4, dissecting its structure, function, and its role in the context of human diseases.

The primary structure of the 21 kDa protein was characterized as a single polypeptide chain, containing 201 amino acids and three disulfide bridges in humans (Rask et al., 1980, 1987). An N-terminal secretory signal peptide of 18 amino acid is cleaved upon protein processing. The complete 3D structure of RBP4 was reported by Newcomer et al. (1984). Using x-ray crystallography, they found that its structure is built not only of an N-terminal coil, a C-terminal α-helix followed by a coil region, but also a characteristic β-barrel core that they described as an eight-stranded up-and-down β-barrel. This β-barrel core is the structural part of the RBP4 molecule that is able to specifically host one molecule of retinol, which keeps this hydrophobic vitamin soluble in an aqueous milieu, and therefore capable of transporting it through the bloodstream.

TRANSCRIPTIONAL AND POST-TRANSLATIONAL REGULATION OF RBP4

RBP4 is highest expressed in the liver followed by robust expression in all adipose tissue depots (Tsutsumi et al., 1992; Wu C. et al., 2009, 2013). Nevertheless, its mRNA can be detected in several other tissues and anatomical structures, such as kidney, retinal pigment epithelium, testes, brain, lung, and the choroid plexus (Soprano et al., 1986; Blaner, 1989; MacDonald et al., 1990; Duan and Schreiber, 1992; Soprano and Blaner, 1994; O'Byrne and Blaner, 2013). Highest expression in liver coincides with the highest retinoid stores of any organ, corresponding to about 80% of all retinoids in the body (O'Byrne and Blaner, 2013).

Several genomic elements in the 5' flanking region of the human *RBP4* gene confer its high expression in hepatocytes, although the occupancy with specific transcriptional regulators has not been characterized thoroughly (D'Onofrio et al., 1985; Colantuoni et al., 1987; Blaner, 1989). However, a multiprotein complex including high mobility group A1, protein-associated splicing factor, steroidogenic factor 1, and other proteins was shown to be recruited to its promoter, especially upon stimulation by cyclic AMP (Bianconcini et al., 2009; Chiefari et al., 2009), a known inducer of *Rbp4* mRNA expression in murine hepatocytes (Jessen and Satre, 1998). In mice, liver *Rbp4* mRNA is elevated by injecting glucagon (Bianconcini et al., 2009). Consistently, *Rbp4* mRNA expression in liver is induced by fasting. Interestingly, this fasting induction was also observed in mice that lack peroxisome proliferator-activated receptor α (PPARα) (Smati et al., 2020), the master regulator of the transcriptional response to fasting in liver (Kersten et al., 1999), which is in accordance with a cAMP-dependent and PPARα-independent mechanism.

Rats with either normal, retinol-depleted, or retinol-repleted status did not show any alterations in the expression or synthesis of *Rbp4* mRNA and protein in liver, respectively (Soprano et al., 1982), suggesting that hepatic gene expression of *Rbp4* is independent of the overall vitamin A status. On the other hand, both atRA and 9-*cis* RA induced *Rbp4* mRNA expression in murine Hepa 1-6 cells and in mouse liver *in vivo* in a dose- and time-dependent manner (Jessen and Satre, 2000), showing that at least at higher concentrations, these retinoids are able to induce *Rbp4* mRNA. Another study showed that atRA treatment of mice reduced mRNA expression of *Rbp4* in adipose tissues but not liver, whereas protein levels of RBP4 were reduced in liver and increased in serum (Mercader et al., 2008).

Very little is known about the translational control of *Rbp4* mRNA. Welles et al. showed that in response to re-feeding of fasted mice, translation of *Rbp4* mRNA in liver was enhanced, most likely in a 'mechanistic target of rapamycin in complex 1' (mTORC1)-dependent manner. In support of this was the finding that rapamycin, an inhibitor of mTORC1, prevented the nutrient-induced translation of *Rbp4* mRNA (Welles et al., 2020). Major regulators of *Rbp4* transcription and translation are depicted in **Figure 1**.

In humans, RBP4 circulates as the native full-length protein of 183 amino acids. Interestingly, in patients with chronic renal failure, two additional forms of RBP were identified, lacking one or both of the C-terminal amino acids leucine (Jaconi et al., 1995). The authors suggested that these C-terminally truncated RBP4 proteins are generated in hepatocytes and, after its release, are rapidly cleared by the kidney in healthy individuals, but not in patients with chronic renal failure (Jaconi et al., 1995). Moreover, chronic diseases of the kidney but not of the liver in human patients associated with higher levels of truncated proteins in the circulation (Frey et al., 2008). The physiologic role of these

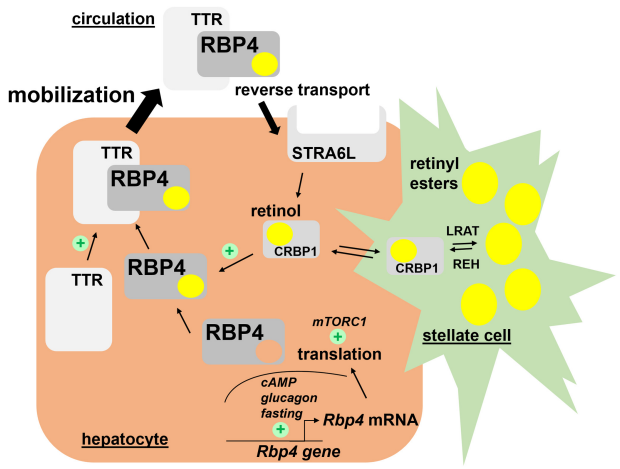

FIGURE 1 | Hepatic retinol mobilization by RBP4. Most of liver retinoids are stored as retinyl esters in hepatic stellate cells in a LRAT-dependent manner. Upon hydrolysis by REH, retinol binds CRBP1 and becomes available to associate with RBP4 in hepatocytes. After complex formation with TTR, retinol/RBP4/TTR is secreted into the circulation. Both steps enhance secretion of the complex since depletion of retinol or TTR induces RBP4 accumulation in hepatocytes. Hepatocyte-expressed STRA6L is thought to confer reverse retinol transport from circulating holo-RBP4. Expression of *Rbp4* mRNA is induced by cAMP/glucagon signaling and RBP4 protein translation enhanced by mTORC1 activation. Please note that pathways for uptake and storage of dietary retinoids from circulating lipoproteins, which are thought to be RBP4-independent, are not included in the figure. cAMP, cyclic adenosine monophosphate; CRBP1, cellular retinol binding protein 1; LRAT, lecithin retinol acyltransferase; mTORC1, mechanistic target of rapamycin in complex 1; RBP4, retinol binding protein 4; REH, retinyl ester hydrolases; STRA6L, stimulated by retinoic acid 6-like; TTR, transthyretin.

truncated RBP4 proteins, which are still able to bind retinol (Jaconi et al., 1996), is currently unknown.

TISSUE-SPECIFIC CONTRIBUTIONS TO CIRCULATING RBP4

The concentration of RBP4 in blood, and therefore also retinol, is rather tightly regulated and kept normally at about 2–3 μM in humans and around 1 μM in mice, despite changes in the daily uptake of retinoids with the diet (Shirakami et al., 2012). Since RBP4 is expressed in many tissues and cell types, the specific contribution of each of these sites to circulating RBP4 is of great interest for understanding its biological function. In particular its aforementioned robust expression in adipose tissue and the observed correlation to serum levels in a variety of metabolic conditions (Yang et al., 2005) sparked the idea that adipose tissue may significantly contribute to blood RBP4 (Muoio and Newgard, 2005; Tamori et al., 2006).

The site of origin was elegantly addressed by a recent study, reporting that circulating RBP4 derives exclusively from hepatocytes. This was concluded from the observation that it was undetectable in blood of mice with a hepatocyte-specific RBP4 knockout (Thompson et al., 2017). Thus, RBP4 should be considered primarily a hepatokine rather than an adipokine, which is further supported by the finding that a modest liver-specific overexpression of RBP4 readily translates in increased serum levels (Muenzner et al., 2013; Fedders et al., 2018) and that chronic liver diseases in humans that interfere with the hepatic biosynthetic capacity lead to lower RBP4 in serum (Yagmur et al., 2007). Consistently, adiponectin promoter-driven overexpression of human RBP4 increased its protein levels in adipose tissue without leading to a major elevation of circulating RBP4 when mice were fed standard chow (Lee et al., 2016). Thus, in mice and most likely also in humans, probably all circulating RBP4 is liver-derived. However, it has been hypothesized that there are disease states or certain conditions that may allow extra-hepatic RBP4 to reach the circulation. The reasons for why hepatocyte-derived RBP4 reaches the circulation but not adipocyte-expressed RBP4 are currently unknown. This is even more puzzling since cultured adipocytes or fat explants readily secrete RBP4 into the culture media (Tsutsumi et al., 1992; Wei et al., 1997; Thompson et al., 2017). Notably, muscle-specific overexpression of human RBP4 rescued RBP4 levels in the circulation of RBP4-deficient mice, suggesting that also *in vivo*, non-hepatocyte expressed RBP4 can *per se* contribute to blood RBP4 (Quadro et al., 2002).

Besides the circulation, RBP4 is also found in other compartments such as the cerebrospinal fluid (CSF), most likely by its secretion from choroid plexus cells of the blood brain barrier (MacDonald et al., 1990; Duan and Schreiber, 1992).

It should be noted that not all commercially available kits quantify RBP4 reproducibly. When comparing different methods, including sandwich enzyme-linked immunosorbent assays and competitive enzyme-linked immunoassays, quantitative western blotting standardized to full-length RBP4 came out as the superior method for measuring RBP4 in serum (Graham et al., 2007).

HEPATIC RETINOL MOBILIZATION BY RBP4

Upon dietary uptake, retinoids, in form of retinyl esters, are together with other lipids packed into chylomicrons, and then released from the intestine into the lymphatic system (Goodman et al., 1965; Vogel et al., 1999). By the action of membrane-bound lipoprotein lipase (LPL), cells hydrolyze triglycerides and retinyl esters from these lipoproteins to meet their specific demands. Especially when RBP4's function is compromised, LPL-mediated uptake of lipoprotein-derived retinyl esters can be a significant alternative source for cellular retinoids (Quadro et al., 2004). Chylomicron remnants that are formed are then taken up by the liver which serves as the main site of retinoid storage. An alternative dietary source of retinoids are provitamin A carotenoids, whose transport, cellular uptake, and metabolism differs from these pathways and which are not the focus of this article. The reader is kindly referred to other excellent reviews on this topic (von Lintig, 2012; von Lintig et al., 2020).

RBP4's primary function is to mobilize retinol from liver (**Figure 1**). The liver hosts the bulk of retinoids of dietary origin as retinyl esters (Kane et al., 2008) in hepatic stellate

cells, morphologically distinct and lipid droplets containing cells that are specialized in storing high concentrations of retinyl esters (Yamada et al., 1987). Stellate cells express lecithin retinol acyltransferase (LRAT), very likely the only enzyme to esterify retinol in these cells (Batten et al., 2004; Liu and Gudas, 2005; O'Byrne et al., 2005), and a number of potential lipases for retinyl ester hydrolysis to provide retinol for mobilization (Haemmerle and Lass, 2019; Wagner et al., 2020). Although it is not completely understood how retinol travels from stellate cells to hepatocytes and *vice versa*, it was shown that an absence of RBP4 did not prevent retinyl ester storage in stellate cells, implying RBP4-independent mechanisms at play (Quadro et al., 1999). For retinol mobilization into the circulation, however, RBP4 expression in hepatocytes is indeed required (Shirakami et al., 2012). Mice that lack RBP4 globally showed increased amounts of hepatic retinol and retinyl esters, whereas circulating retinol levels were decreased by almost 90% (Quadro et al., 1999). Reciprocally, an acute overexpression of RBP4 specifically in liver increased serum retinol levels with a simultaneous reduction in liver retinyl esters (Muenzner et al., 2013), underlining the pivotal role of RBP4 expression in hepatocytes for mobilizing retinol into the circulation.

Notably, residual levels of retinol in the circulation of mice lacking RBP4 show that alternative carrier proteins are present, including albumin that can readily bind retinol (Muenzner et al., 2013). Moreover, retinyl esters are also found in liver-derived very low density lipoprotein (VLDL) and LDL (Krasinski et al., 1990), suggesting that either RBP4-independent pathways for mobilization from liver exist or that retinyl esters from dietary chylomicrons can be transferred by the action of cholesteryl ester transfer protein (CETP). Collectively, these pathways seem, at least partially, to compensate when RBP4 is dysfunctional or absent (Quadro et al., 1999, 2005) and maintaining a basal degree of retinoid delivery to target cells.

Hepatocytes release RBP4 bound to retinol (holo-RBP4) and the availability of retinol facilitates its secretion (Bellovino et al., 1999). Consistently, vitamin A-deficiency in rats decreases serum-RBP4 levels while inducing its accumulation in liver (Smith et al., 1975) and adding retinol to retinol-depleted hepatocytes increases RBP4 secretion (Dixon and Goodman, 1987). Before exiting the cell, RBP4 associates with the earlier mentioned prealbumin, also known as transthyretin (TTR), a tetrameric protein of ∼55 kDa (Monaco et al., 1995; Naylor and Newcomer, 1999), forming a complex within the endoplasmic reticulum (ER) (Bellovino et al., 1996). Total lack of TTR or an acute, liver-specific depletion induced an accumulation of RBP4 in liver, suggesting that TTR enhances RBP4 secretion (Wei et al., 1995; Fedders et al., 2018). However, TTR seems not absolutely required since some RBP4 was still detectable in the circulation of TTR knockout mice and isolated hepatocytes with or without TTR expression secreted comparable amounts of RBP4 (van Bennekum et al., 2001). Moreover, despite strongly reduced serum RBP4 and retinol concentrations, TTR-deficient mice did not show any major alterations in tissue retinoid levels, including the liver and the eye (Wei et al., 1995), arguing against a limiting function of TTR in hepatic retinol mobilization and systemic homeostasis. Thus, TTR's primary effect on RBP4 is in regard to increasing its serum half-life by formation of a higher molecular weight complex that prevents renal filtration (van Bennekum et al., 2001). Intriguingly, TTR functions also as one of the specific transport proteins for thyroid hormone (Ingbar, 1963). The potential biological interplay of retinol and thyroid hormone transport via the liver-secreted RBP/TTR complex have not been fully dissected yet.

RECEPTORS FOR RBP4

The exact nature of an earlier postulated cell-surface receptor for RBP4 remained enigmatic until 2007, when Sun et al. identified 'stimulated by retinoic acid 6' (STRA6) as a cell surface receptor that transports retinol provided by holo-RBP4 across the cell membrane (Kawaguchi et al., 2007). Prior to this new function, it was already known that STRA6 contains several transmembrane domains and that its expression is inducible by atRA (Taneja et al., 1995). Strikingly, STRA6 mediates bi-directional retinol transport as well as exchange between intracellular binding proteins and extracellular RBP4, thereby balancing circulating RBP4 with import and export, metabolism, and storage of intracellular retinoids and counteracting states of both retinoid insufficiency or oversupply (Kawaguchi et al., 2012). TTR partially blocks the STRA6-mediated release of retinol from holo-RBP4 and may need to dissociate from the complex before RBP4 can bind STRA6 (Kawaguchi et al., 2011). Retinol uptake by STRA6 is coupled to intracellular binding and/or storage via cellular retinol binding protein 1 (CRBP1) and LRAT activity, respectively (Isken et al., 2008; Kawaguchi et al., 2011). Accordingly, free retinol present in the membrane blocks STRA6-catalyzed retinol release (Kawaguchi et al., 2011). A site-directed mutagenesis screen identified a characteristic loop in the structure of STRA6 that is responsible for the release of retinol from its binding pocket in the β-barrel core of RBP4 (Kawaguchi et al., 2008).

STRA6 is strongly expressed during embryonic development, in structures of blood-organ barriers, and organs that required high levels of retinoids, including the retinal pigment endothelium (RPE) and the female reproductive organs, testis, brain, and the kidney (Bouillet et al., 1997; Wu C. et al., 2009). In mice, differential promotor usage of the *Stra6* gene gives rise to different mRNA transcripts that show distinct regulations by retinol deficiency, potentially encoding also a shorter, N-terminally truncated STRA6 protein (Laursen et al., 2015). Mice that lack STRA6 were viable but had a dramatic reduction of retinoids in the RPE as well as retina and suffered from impaired vision and irregular ocular morphology (Ruiz et al., 2012; Amengual et al., 2014). More recently, the structure of zebrafish STRA6 was solved by cryo-electron microscopy, revealing one intramembrane and nine transmembrane helices in an intricate dimeric assembly that forms a deep lipophilic cleft for retinol internalization into the membrane lipid bilayer (Chen et al., 2016). Unexpectedly, STRA6 was tightly bound by calmodulin, which, in conjunction with Ca^{2+}, was shown to favor binding of apo-RBP4 and retinol export (Zhong et al., 2020; **Figure 2A**). Dissecting the biological significance of

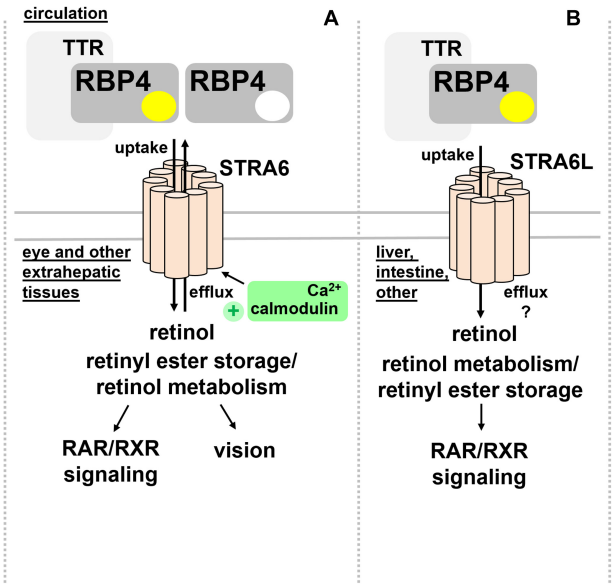

FIGURE 2 | RBP4 membrane receptors for retinol transport. **(A)** Extrahepatic tissues including the eye take up retinol via binding of circulating holo-RBP4 to STRA6. Uptake is coupled to intracellular binding, storage and metabolism of retinol. STRA6 can also mediate efflux of cellular retinol to apo-RBP4, which was shown to be further stimulated by binding of calmodulin/Ca^{2+} to an intracellular domain of STRA6. **(B)** Liver, intestine, and several other organs but not the eye express STRA6L, predicted to have a similar transmembrane domain structure like STRA6. STRA6L catalyzes retinol uptake from holo-RBP4. Whether or not it can mediate bidirectional retinol transport, and how it couples to intracellular binding, storage and metabolism of retinol is currently unknown. RAR, retinoic acid receptor; RBP4, retinol binding protein 4; RXR retinoid X receptor; STRA6, stimulated by retinoic acid 6; STRA6L, stimulated by retinoic acid 6-like; TTR, transthyretin.

calmodulin/Ca^{2+} on retinol transport by RBP4 and STRA6 may reveal an additional level of understanding of how this process is coordinated.

In addition to STRA6, a structurally related protein of about 20% overall homology acting as RBP4 receptor was identified in 2013 and designated first RBP4 receptor 2 (RBPR2) and later as 'stimulated by retinoic acid 6-like' (STRA6L) (Alapatt et al., 2013). Since it was known that retinol cycles between the circulation and liver and that expression of STRA6 is almost undetectable in this organ (Bouillet et al., 1997), the existence of another receptor was assumed. Indeed, STRA6L's expression is highest in liver, and somewhat lower in intestine and colon (Alapatt et al., 2013). In contrast to STRA6, STRA6L expression is suppressed by retinol and atRA. Furthermore, it is still unknown whether STRA6L can also mediate bidirectional retinol transport (Alapatt et al., 2013; **Figure 2B**). Functionally, STRA6L is required for development and function of the eye in zebrafish (Shi et al., 2017; Lobo et al., 2018; Solanki et al., 2020). Also in zebrafish, STRA6L is expressed in tissues involved in retinol uptake and storage, like intestinal enterocytes, hepatocytes, and pancreatic cells but not the eye itself. However, STRA6L mutant fish exhibit visual defects that are likely due to systemic vitamin A deficiency that was observed in these fish (Shi et al., 2017).

At this point, STRA6 and STRA6L are the only known specific receptors for RBP4. It should be pointed out that retinol uptake from circulating holo-RBP4 may also involve receptor-independent membrane passage/diffusion, in particular when receptor-mediated uptake is compromised (Berry et al., 2013).

RBP4 CATABOLISM

After its dissociation from TTR and retinol release, retinol-free apo-RBP4 in the circulation is filtrated by the kidney. More than 99% of that is reabsorbed by the proximal renal tubule, which renders urinary RBP4 a highly sensitive marker for tubular dysfunction (Bonventre et al., 2010). Tubular reabsorption of RBP4 is mediated by the megalin-cubilin receptor complex (Christensen et al., 1999; Raila et al., 2005). Besides its endocytic uptake, RBP4 staining in lysosomes, endoplasmic reticulum, Golgi, and basal vesicles suggest basolateral RBP4 secretion by these cells via a degradation-synthesis cycle (Christensen et al., 1999). Strikingly, kidney-specific megalin deletion in mice, besides inducing loss of RBP4 in the urine, led also to urinary retinol excretion and reduced hepatic retinol and retinyl esters, suggesting a more complex and rather unexplored role of the kidney in retinoid homeostasis (Raila et al., 2005).

RBP4 LOSS-AND GAIN-OF-FUNCTION INDUCED PATHOLOGIES IN MICE

Embryonic development. Mice with genetic deletion of RBP4 exhibit very low retinol concentrations in their circulation (Quadro et al., 1999). However, they give birth to viable embryos that show rather mild and transient developmental abnormalities of the heart (Wendler et al., 2003), a much less severe phenotype as one would have predicted from the relevance of vitamin A for the developing organism. However, this is only observed when mice were fed diets with sufficient vitamin A and implying RBP4-independent pathways for retinoid delivery already discussed above. Reducing the dietary intake of vitamin A in females lacking RBP4 before and during pregnancy has fatal consequences on embryonic development (Quadro et al., 2005). Feeding a vitamin A-deficient diet (<0.22 IU/g), severe embryonic malformations were observed, including smaller embryo size, undetectable or abnormal midfacial regions and forelimbs, and exencephaly (Quadro et al., 2005). These characteristics are in line with the severe fetal vitamin A deficiency syndrome and overlap with the phenotypes of mice with retinoid receptor knockouts (Mark et al., 2009).

Vision. Mice on a mixed genetic background (129xC57BL/6J) that lack RBP4 show impaired retinal function and visual acuity during the first couple of months but develop normal vision by 4–5 months of age when fed standard chow, in this particular study 22 IU of retinol/g (Quadro et al., 1999). However, when maintained on a vitamin A-deficient diet after weaning (<0.22 IU/g), vision of RBP4-deficient mice (129xC57BL/6J) further deteriorated whereas that of control mice was not compromised. A more recent study suggests that visual defects upon loss

of RBP4 are even more pronounced and become chronic on a pure C57BL/6 genetic background even when feeding a vitamin A sufficient diet with ∼15 IU/g (Shen et al., 2016). Transgenic expression of human RBP4 in muscle (Quadro et al., 2002) or from the murine *Rbp4* gene locus (Liu et al., 2017) rescued serum retinol levels and suppressed visual defects due to loss of endogenous RBP4. These results demonstrate that visual performance depends on RBP4-mediated retinol transport but also highlights the aforementioned existence of alternative, although less efficient, pathways for the eye to acquire retinoids (Vogel et al., 2002). It also underlines that most other tissues in the body are less dependent on RBP4-mediated retinol delivery, especially when dietary retinoid supply is ample. This notion is further supported by the phenotype of mice lacking the RBP4 receptor STRA6, in which rod photoreceptor outer and inner segment length as well as cone cell numbers were reduced, as were scotopic and photopic responses (Ruiz et al., 2012; Amengual et al., 2014). Besides visual defects, these mice showed no other major vitamin A-related abnormalities (Berry et al., 2013), somewhat similar to RBP4-deficient mice. Notably, also transgenic overexpression of human RBP4 in muscle of mice can lead to progressive retinal degeneration, although this appears to be independent of alterations in retinoids and more likely to be mediated by retinal neuroinflammation (Du et al., 2015, 2017).

Insulin sensitivity and glucose tolerance. Elevated RBP4 in the circulation of type 2 diabetic patients was reported many years ago (Basualdo et al., 1997; Abahusain et al., 1999) but it took until 2005, when a causal link between circulating RBP4 and insulin resistance was presented: Yang et al. showed, besides increased RBP4 in blood of multiple insulin resistant mouse models, that RBP4-deficient mice were less prone to develop insulin resistance (Yang et al., 2005). Moreover, transgenic overexpression of human RBP4 or injection of recombinant human RBP4 in wild-type mice caused glucose intolerance and insulin resistance. Since RBP4 expression was increased in adipose tissue but not liver, these findings led to the hypothesis that RBP4 acts as an adipokine, linking obesity with insulin resistance. Intriguingly, serum RBP4 correlated positively with adipose *RBP4* mRNA and intra-abdominal fat mass and inversely with insulin sensitivity also in humans (Klöting et al., 2007) and was lowered by exercise (Graham et al., 2006). However, at least in mice and as already pointed out above, circulating RBP4 is derived primarily from liver and not adipose tissue. Mechanistically, RBP4 was shown to induce expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in liver and to impair insulin signaling in muscle (Yang et al., 2005). However, follow-up studies by the same laboratory implicated the immune system, in particular antigen-presenting cells, such as dendritic cells, macrophages, and also CD4 T cells as the drivers of an inflammatory response that is induced by RBP4 within adipose tissue (Norseen et al., 2012; Moraes-Vieira et al., 2014, 2016, 2020). Strikingly, this inflammatory reaction was independent of RBP4's association with retinol (Norseen et al., 2012) and mediated by an activation of toll-like receptors 2/4 (TLR2/4) and proinflammatory cytokine secretion from macrophages, involving nuclear factor κ-B (NFκB), c-Jun N-terminal kinases (JNK), and interleukin 1β (Moraes-Vieira et al., 2014, 2020; **Figure 3A**).

An alternative functional model was suggested by Berry et al. (2011), where holo-RBP4's binding to STRA6 triggers receptor phosphorylation close to its C-terminus and the recruitment and activation of Janus kinase 2 (JAK2) and the signal transducer and activator of transcription 5 (STAT5) (**Figure 3B**). Interestingly, neither retinol nor retinol free apo-RBP4 were able to induce STAT5 phosphorylation. Activated STAT5 then leads to the upregulation of genes which are known to inhibit insulin signaling, such as suppressor of cytokine signaling 3 (SOCS3) (Berry et al., 2011). Follow-up studies provided evidence that activation of JAK/STAT by STRA6 requires transfer of retinol from holo-RBP to an intracellular acceptor, such as CRBP1 or LRAT (Berry et al., 2012b). TTR's association with holo-RBP4 prevented STRA6 binding and, subsequently, induction of JAK/STAT signaling (Berry et al., 2012a). These findings were corroborated by the observation that injected recombinant holo-RBP4 failed to induce JAK/STAT or impair insulin signaling in mice lacking STRA6 (Berry et al., 2013).

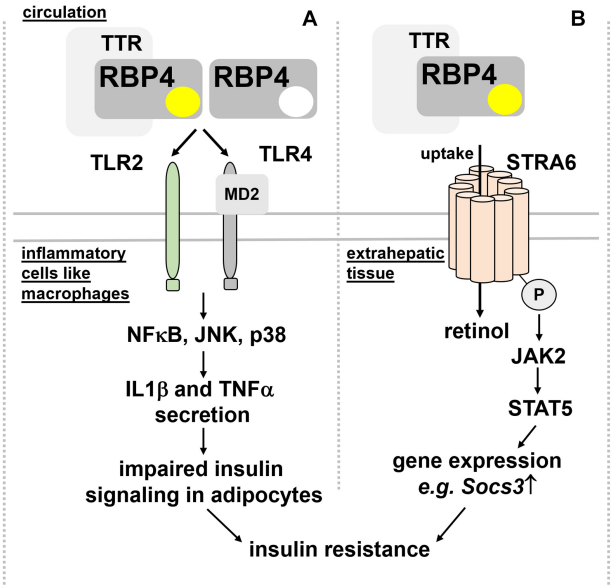

FIGURE 3 | Proposed mechanisms for RBP4's detrimental effects on insulin sensitivity. **(A)** RBP4, irrespective of its association with retinol and thus independent of retinol transport, was shown to activate TLR2/4 on inflammatory cells like macrophages. A downstream signaling cascade involving NFκB, JNK, and p38 leads to the secretion of IL1β and TNFα, which, in turn, impairs insulin signaling in adipocytes and leading to insulin resistance. **(B)** Binding of holo-RBP4 to STRA6 was shown to trigger tyrosine phosphorylation of the membrane receptor, resulting in recruitment and activation of JAK2 and the transcription factor STAT5. As a consequence, the induction of STAT5 target genes like *Socs3* impairs insulin signaling. IL1β, interleukin 1β; JAK2, Janus kinase 2; JNK, c-Jun N-terminal kinases; NFκB, nuclear factor κ-B; MD2, myeloid differentiation protein-2; RBP4, retinol binding protein 4; *Socs3*, suppressor of cytokine signaling 3; STAT5, signal transducer and activator of transcription 5; STRA6, stimulated by retinoic acid 6; TNFα, tumor necrosis factor α; TLR2/4, toll-like receptors 2/4; TTR, transthyretin.

Hence, RBP4's causal role in inducing insulin resistance is still vividly debated within the field (Fedders et al., 2015). Although elevated levels of circulating RBP4 in states of insulin resistance and type 2 diabetes were reproduced by the majority of studies and by many independent laboratories, the underlying reasons for this elevation, and whether or not RBP4 is indeed actively contributing to insulin resistance is still under investigation. A few examples of unanswered issues for the reader's consideration are described in the following sentences. For instance, not all studies identified elevated expression of RBP4 in adipose tissue of obese patients (Janke et al., 2006), and instead of adipose tissue expression, kidney function was proposed as major determinant of its serum levels, which is known to deteriorate upon the onset of type 2 diabetes and thus may lead to an accumulation of RBP4 in the blood (Raila et al., 2007; Henze et al., 2008). Other studies failed to reproduce improved insulin sensitivity and glucose tolerance in RBP4-deficient mice when fed a high-fat diet (Motani et al., 2009). Also mouse models that increase circulating RBP4 levels by an extent that is comparable to insulin resistant states by an acute or long-term liver-specific overexpression of murine RBP4 instead of the human protein did not observe an impairment in insulin responses and glucose tolerance (Muenzner et al., 2013; Fedders et al., 2018). From a physiological perspective, how can the lipocalin RBP4 that is so strongly expressed in liver and circulating at rather high levels in the blood become an inflammatory stressor upon a 2–3 fold elevation that is observed in these states? Are there yet unidentified post-translational modifications of RBP4 that occur upon extended serum half-life that could mediate this? Moreover, is retinol transport just a bystander or an active participant in these presumed RBP4 effects? Why are some of the metabolic effects of transgenically elevated RBP4 in mice seen with human but not the murine protein? Further research is needed to address these questions for building a consensus on this important, but continuously controversial topic.

Adipose tissue lipolysis and hepatic fat content. Adipose tissue-specific overexpression of human RBP4 in mice induces hepatic steatosis, despite an absence of changes in total RBP4 levels in the circulation or alterations in retinoid concentrations (Lee et al., 2016). Hepatic steatosis was accompanied by glucose intolerance and elevated concentrations of non-esterified fatty acids in plasma, which were shown to contribute to hepatic triglyceride accumulation. Feeding a high-fat diet aggravated the RBP4-induced metabolic disturbances in these mice. These findings indicate that locally expressed RBP4 stimulates adipose tissue lipolysis and fatty acid release, which subsequently triggers hepatic steatosis. Whether this is due to direct effects of RBP4 on fatty acid handling or indirect, via an induction of pro-inflammatory cytokines like tumor necrosis factor α (TNFα) or changes in local retinoid homeostasis, will be interesting to dissect (Lee et al., 2016). Additional insights come from another study showing that treatment of human adipocytes with RBP4 directly stimulated lipolysis and that, when co-cultured with macrophages, pro-inflammatory cytokines contribute to this by interfering with the insulin-dependent suppression of lipolysis (Kilicarslan et al., 2020). In agreement with RBP4's stimulating effect on lipolysis are the reported lower levels of non-esterified fatty acids in serum of RBP4-deficient mice (Yang et al., 2005).

Cold tolerance. Exposing mice or humans to low temperatures increases plasma concentrations of RBP4 and retinol (Fenzl et al., 2020). In mice, this coincides with an elevation of *Rbp4* mRNA expression in liver (Fenzl et al., 2020), which may indicate increased retinol mobilization from liver into the blood stream for supporting the adaptation to cold. Indeed, mice lacking RBP4 failed to induce thermogenic reprogramming of their subcutaneous adipose tissue, rendering these mice more sensitive to cold and dropping of their body temperature. Retinol induced thermogenic gene expression also in human white adipocytes and increased mitochondrial respiration (Fenzl et al., 2020). Thus, elevated retinol mobilization by RBP4 from liver upon the exposure to cold may be required for an adequate thermogenic adaptation of specific white adipose tissues depots in order to maintain body core temperature, which so far has not been formally tested. Moreover, loss of RBP4 in mice was associated with reduced phosphorylation of hormone sensitive lipase during the exposure to cold (Fenzl et al., 2020), a lipolytic enzyme that is activated by phosphorylation and essential for adequate fatty acid release from white adipose tissue (Stralfors and Belfrage, 1983). Providing fatty acids is required for uncoupling protein-induced thermogenesis (Schreiber et al., 2017). Thus, RBP4's function during cold exposure may be linked to the above described stimulating effects on lipolysis, thereby ensuring fatty acid supply to brown adipose tissue as fuel for functional uncoupling protein-mediated non-shivering thermogenesis.

Cardiovascular system and blood pressure. Circulating RBP4 levels also correlate with blood pressure and cardiovascular disease (Solini et al., 2009; Meisinger et al., 2011; Sun et al., 2013; Li et al., 2019), potentially also secondary to decreased renal clearance due to hypertensive nephropathy (Majerczyk et al., 2017). Strikingly, systolic and diastolic blood pressure were lower in the RBP4-knockout mice and higher in the RBP4-overexpressing mice compared with the corresponding wild-type littermates (Kraus et al., 2015), suggesting indeed a functional role of RBP4 in the control of blood pressure. RBP4-deficient mice were partially protected from angiotensin 2-induced hypertension and cardiac hypertrophy. Further studies are needed to interrogate whether these effects are dependent on retinol transport and to delineate the underlying mechanisms in more detail. In this regard, effects of RBP4 on endothelial cells are likely to be involved since carbachol-induced, and therefore endothelium-dependent, vasodilation of carotid arteries *ex vivo* was slightly enhanced or reduced in RBP4 knockout and overexpressing mice, respectively (Kraus et al., 2015).

Behavior and neuropathology. Besides its expression in various regions of the central nervous system and associated structures (MacDonald et al., 1990; Duan and Schreiber, 1992; Wu C. et al., 2009), very little is known about RBP4's role in the brain. Interestingly, RBP4-deficient mice have decreased locomotor activity and increased anxiety-like behavior. At a

structural level these mice show neuronal loss and gliosis in the cortex and hippocampus and a reduction in proliferating neuroblasts in the subventricular zone (Buxbaum et al., 2014). It thus appears that altered vitamin A transport affects brain development and neuronal function, thereby altering behavior. Moreover, these neuropathological alterations might complicate the interpretations of other phenotypes observed in whole body RBP4-deficient mice. Of note, Buxbaum et al. investigated TTR-deficient mice in parallel and found some, but not complete overlap in the above described phenotypes, which is in accordance with the interdependent transport characteristics of the RBP/TTR complex.

PATHOLOGIES ASSOCIATED WITH RBP4 MUTATIONS IN HUMANS

So far, null mutations of RBP4 have not been characterized in humans (Zhong et al., 2014), which may suggest that in contrast to mice, complete absence of RBP4 is incompatible with embryonic survival, regardless of maternal vitamin A intake. It is interesting to note that compared to rodents, humans seem to be much more sensitive to dysregulated retinoid homeostasis and retinoid toxicity associated with random retinoid diffusion (Nau, 2001), which could be a likely consequence of dysfunctional RBP4 variants.

Vision. Two mutations p.I59N and p.G93D, corresponding to I41N and G75D of RBP4 after cleavage of the signal peptide of the mature protein, were associated with night blindness and modest retinal dystrophy without apparent effects on growth in compound heterozygous sisters (Biesalski et al., 1999; Seeliger et al., 1999). Both mutations were associated with reduced plasma RBP4 and retinol concentrations most likely due to reduced stability of the retinol-RBP4 complex (Folli et al., 2005). Another two missense mutations, p.A73T and p.A75T, were identified to cause autosomal dominant congenital eye malformations, including microphthalmia, anophthalmia, and coloboma (MAC) disease and with a maternal penetrance significantly greater than paternal penetrance (Chou et al., 2015). Similar to p.I59N and p.G93D, both mutant proteins bound retinol rather poorly, but strikingly, the unliganded mutant proteins were found to occupy STRA6 with much higher affinity than wild-type RBP4, and consequently are likely to disrupt delivery of vitamin A to target cells in accordance with a dominant-negative effect (Chou et al., 2015). Rare bi-allelic mutations (c.248 + 1G > A) of *RBP4* were identified in a patient with retinal dystrophy and ocular coloboma (Khan et al., 2017). A homozygous splice site variant in *RBP4* (c.111 + 1G > A) was found to cause retinal dystrophy and developmental abnormalities (Cukras et al., 2012). Both mutations were associated with low or undetectable RBP4 levels in the circulation. Another homozygous mutation (c.67 C > T) of *RBP4*, predicted to encode an early stop codon (p.Arg23*) and resulting a premature termination of translation, associated with retinitis pigmentosa (Cehajic-Kapetanovic et al., 2020). Although assumed to yielding no functional RBP4, whether or not the latter mutation represents a null mutation in homozygosity is currently unclear.

It is interesting to note that mutations in the RBP4 receptor *STRA6* in humans cause a wide array of malformations including anophthalmia, congenital heart defects, diaphragmatic hernia, alveolar capillary dysplasia, lung hypoplasia, and intellectual disability, also referred to as Matthew-Wood syndrome (Golzio et al., 2007; Pasutto et al., 2007; Chassaing et al., 2009). Why the human phenotype is much more severe than STRA6 loss-of-function in mice is still under investigation but may be linked to the aforementioned differences in sensitivities to retinoid toxicity between humans and rodents.

Other conditions. Although also restricted to very few case studies, some of the above described RBP4 mutations were associated with acne vulgaris, osteoarthritis, and hypercholesterolemia, conditions that can be linked to dysregulated retinoid homeostasis (Seeliger et al., 1999; Cukras et al., 2012; Khan et al., 2017; Cehajic-Kapetanovic et al., 2020).

RBP4 POLYMORPHISMS IN HUMANS AND DISEASE ASSOCIATION

Not unexpectedly, single-nucleotide polymorphisms of *RBP4* exist that associate with circulating retinol levels (Mondul et al., 2011). In regard to disease states, polymorphisms of *RBP4* were shown to associate with the risk for coronary artery disease (Wan et al., 2014), childhood obesity and cardiovascular risk factors (Codoñer-Franch et al., 2016), with plasma RBP4 levels and hypertriglyceridemia risk in Chinese Hans (Wu Y. et al., 2009), serum HDL (Shea et al., 2010), the risk for gestational diabetes (Hu et al., 2019), body mass index (Munkhtulga et al., 2010), insulin resistance (Kovacs et al., 2007), and type 2 diabetes (Craig et al., 2007; Munkhtulga et al., 2007; van Hoek et al., 2008). As delineated above, genetic mouse models seem to support a causative role for RBP4 in some of these conditions.

PHARMACOLOGICAL APPROACHES TO LOWER CIRCULATING RBP4

The notion that elevated RBP4 in the circulation may contribute to certain pathologies sparked renewed interest in RBP4-lowering therapies. The retinoid fenretinide, first produced in the United States in the 1960s, is a synthetic amide derivative of atRA and has antiproliferative and apoptotic effect in certain tumor cells (Ulukaya and Wood, 1999). Fenretinide was also shown to bind RBP4, to affect its hepatic secretion, and to sterically disrupt complex formation with TTR (Malpeli et al., 1996; Holven et al., 1997). As a consequence of the latter, renal clearance of RBP4 is increased and its serum concentration and also that of retinol decline (Formelli et al., 1993; Schaffer et al., 1993). This may be why in humans, fenretinide administration can lead to impaired visual adaptation to darkness (Decensi et al., 1994).

Treating high-fat diet fed mice with fenretinide for 6 weeks normalized circulating RBP4 levels to that of mice fed normal chow, at the same time improving insulin action and glucose tolerance (Yang et al., 2005), which is in support of RBP4's detrimental effect on glucose homeostasis. Moreover,

TABLE 1 | Major findings from mouse models and human mutations of RBP4.

Species	Mouse model or human mutation	Findings
Mouse	Global RBP4 knockout	circulating retinol levels decreased by ~90% (Quadro et al., 1999) increased contents of retinol and retinyl esters in liver at an age of 5 months (Quadro et al., 1999) impaired retinal function and visual acuity during first months of life that normalize after 4–5 months when fed a vitamin A-sufficient diet (Quadro et al., 1999), analyzed on mixed 129xC57BL/6J genetic background viable embryos with mild and transient developmental abnormalities of the heart (Wendler et al., 2003) increased utilization of lipoprotein-derived retinyl esters (Quadro et al., 2004) increased insulin sensitivity (Yang et al., 2005) lower circulating levels of non-esterified fatty acids (Yang et al., 2005) feeding a vitamin A-reduced diet before and/or during pregnancy leads to severe embryonic malformations (smaller size, undetectable or abnormal midfacial regions and forelimbs, and exencephaly) (Quadro et al., 2005) no effect on insulin sensitivity and glucose tolerance on normal chow and after feeding a high-fat diet (Motani et al., 2009) decreased locomotor activity (Buxbaum et al., 2014) increases anxiety-like behavior (Buxbaum et al., 2014) neuronal loss (Buxbaum et al., 2014) gliosis in the cortex and hippocampus (Buxbaum et al., 2014) reduction in proliferating neuroblasts in the subventricular zone (Buxbaum et al., 2014) lower blood pressure (Kraus et al., 2015) partially protected from angiotensin 2-induced hypertension (Kraus et al., 2015) reduced cardiac hypertrophy (Kraus et al., 2015) serum retinol levels below detection threshold (Shen et al., 2016), analyzed on pure C57BL/6 genetic background more severe and persistent visual impairments (Shen et al., 2016), analyzed on pure C57BL/6 genetic background improved insulin responses and lower adipose tissue inflammation and CD4 T-cell activation when fed normal chow or high-fat diet (Moraes-Vieira et al., 2016), analyzed after feeding a low vitamin A diet (4 IU/g) for 4–5 generations prior to characterization lower body core temperature when exposed to cold (Fenzl et al., 2020) reduced thermogenic activation and hormone sensitive lipase activity in subcutaneous white adipose tissue upon cold exposure (Fenzl et al., 2020)
Mouse	Overexpression of human RBP4 under the control of the mouse muscle creatine kinase promoter	rescue of RBP4 and retinol levels in circulation when crossed into RBP4-deficient mice (Quadro et al., 2002) suppression of visual defects when crossed into RBP4-deficient mice (Quadro et al., 2002) insulin resistant at 12 weeks of age (Yang et al., 2005) progressive retinal degeneration (Du et al., 2015) no effect on serum insulin levels or insulin sensitivity (Du et al., 2015) higher blood pressure (Kraus et al., 2015) impaired glucose tolerance and insulin sensitivity and increased adipose tissue inflammation (Moraes-Vieira et al., 2016, 2020)
Mouse	Acute liver-specific overexpression of murine RBP4	increased serum RBP4 and retinol levels and RAR activation in epididymal white adipose tissue (Muenzner et al., 2013) decreased liver retinyl ester levels (Muenzner et al., 2013) glucose tolerance not impaired (Muenzner et al., 2013)
Mouse	Adipocyte-specific overexpression of human RBP4	increased RBP4 protein levels in adipose tissue (Lee et al., 2016) circulating RBP4 and retinol levels unaltered when fed normal chow, increased when fed high-fat diet (Lee et al., 2016) glucose intolerance and hepatic steatosis (Lee et al., 2016) elevated concentrations of non-esterified fatty acids in plasma (Lee et al., 2016) increased weight gain when feeding a high-fat diet (Lee et al., 2016) elevated non-esterified fatty acid uptake and increased gluconeogenic gene expression in liver (Lee et al., 2016) signs of increased visceral adipose tissue inflammation and altered retinoid homeostasis in liver (Lee et al., 2016)
Mouse	Hepatocyte-specific knockout of RBP4	RBP4 undetectable and retinol reduced by more than 93% in serum (Thompson et al., 2017) hepatic retinol and retinyl ester levels unchanged (Thompson et al., 2017) moderately increased body weights, weight gain, and fat mass when fed a high-fat/high-sucrose diet (Thompson et al., 2017) no alterations in insulin sensitivity or glucose tolerance on control or high-fat/high-sucrose diet (Thompson et al., 2017)
Mouse	Human *RBP4* open reading frame in the mouse *Rbp4* locus	rescue of plasma RBP4 levels, of retinol levels and retinal function when crossed into RBP4-deficient mice (Liu et al., 2017), analyzed after backcrossing to C57BL/6 genetic background
Mouse	Long-term liver-specific overexpression of murine RBP4	increased serum RBP4 and retinol levels (Fedders et al., 2018) liver retinyl ester levels unchanged (Fedders et al., 2018) insulin response and glucose tolerance not impaired on either normal chow or high-fat diet (Fedders et al., 2018)

(Continued)

TABLE 1 | Continued

Species	Mouse model or human mutation	Findings
Human	Compound heterozygous p.I59N and p.G93D	Night blindness (Biesalski et al., 1999; Seeliger et al., 1999) modest retinal dystrophy (Biesalski et al., 1999; Seeliger et al., 1999) undetectable RBP4 and reduced retinol concentrations in serum (Seeliger et al., 1999)
Human	Homozygous c.111 + 1G > A	Retinal dystrophy (Cukras et al., 2012) developmental abnormalities (Cukras et al., 2012) undetectable RBP4 and reduced retinol concentrations in serum (Cukras et al., 2012)
Human	Heterozygous p.A73T and p.A75T	Causing autosomal dominant congenital eye malformations (incl. microphthalmia, anophthalmia, and coloboma disease) (Chou et al., 2015) poor binding of mutated RBP4 to retinol but higher affinity to STRA6 (Chou et al., 2015)
Human	Bi-allelic c.248 + 1G > A	Retinal dystrophy (Khan et al., 2017) ocular coloboma (Khan et al., 2017) undetectable RBP4 levels in serum (Khan et al., 2017)
Human	Homozygous c.67 C > T	Retinitis pigmentosa and childhood acne vulgaris (Cehajic-Kapetanovic et al., 2020)

long-term treatment with fenretinide for several months prevented high-fat diet induced obesity, insulin resistance, and hepatic steatosis, whereas, unexpectedly, some of these beneficial effects were also observed in mice lacking RBP4 (Preitner et al., 2009), proposing also RBP4-independent mechanisms at play. Indeed, follow-up studies implied direct regulation of retinoid homeostasis genes by fenretinide as a likely mechanism for its metabolic benefits (McIlroy et al., 2013). Moreover, lowering RBP4 by fenretinide was dependent on the presence of LRAT, which hints toward even more complex effects of this compound (Amengual et al., 2012). Fenretinide has also been used in human trials for age-related macular degeneration with some positive outcome but limited efficacy (Mata et al., 2013). RBP4 lowering is thought to limit the accumulation of lipofuscin bisretinoids in the RPE, a process known to contribute to a variety of degenerative retinal diseases (Radu et al., 2005). However, as a retinoid, fenretinide is accompanied by a problematic safety profile including teratogenicity.

Another small molecule RBP4 ligand is A1120, which, in contrast to fenretinide, is a non-retinoid compound, bearing no similarities with atRA. A1120 disrupts the RBP4 and TTR complex by inducing a conformational change of the interaction interface and potently lowers serum RBP4 and retinol (Motani et al., 2009). In high-fat diet fed mice, both fenretinide and A1120 lowered serum RBP4 but only fenretinide improved insulin responses and glucose tolerance, which let the authors argue that fenretinide's effects are independent of lowering RBP4 (Motani et al., 2009). By lowering retinol levels in serum and its non-retinoid nature, which may confer a more favorable safety profile compared to fenretinide, also A1120 is an investigational therapy for Stargardt macular dystrophy (Dobri et al., 2013; Hussain et al., 2018). Moreover, human RBP4 was shown to interact with engineered RBP4 protein scaffolds in an A1120-dependent manner, allowing for a synthetic and conformation-specific ON-switch system with a broad potential for pharmacological applications (Zajc et al., 2020).

BPN-14136 is another non-retinoid compound that disrupts the RBP4 and TTR complex with the potential to treat atrophic age-related macular degeneration and Stargardt disease (Cioffi et al., 2014). Interestingly, a BPN-14136 derivative was able to lower RBP4 and partially prevent high-fat diet induced obesity and hepatic steatosis in mice with adipose tissue-specific transgenic overexpression of human RBP4 described above (Cioffi et al., 2019).

INTERACTION OF RBP4 WITH NON-RETINOID LIGANDS

Whether endogenously expressed RBP4 transports non-retinoid ligands is currently unknown. Clues come from Nanao et al., who presented RBP4 crystal structures that contain expression host-derived oleic and linoleic acid in its ligand binding pocket, suggesting that RBP4 can indeed bind fatty acids [Protein data bank ID (Berman et al., 2000): 2WQ9 (Nanao et al., 2010), 2WR6 (Huang et al., 2010)]. Another study analyzed crystal structures of retinol-free RBP4 purified from human plasma, urine, or amniotic fluid and confirmed its binding to certain fatty acids. This included palmitic acid, which could be purified from RBP4 in human urine (Perduca et al., 2018). Moreover, palmitic and also linoleic acid, among others, were able to replace ^{3}H-retinol from human RBP4 (Cioffi et al., 2019). Thus, RBP4 may be a physiologically relevant binding and transport protein for fatty acids. One could hypothesize that this may actually be involved in some of the aforementioned effects on glucose and fatty acid homeostasis and should be tested in future research.

CONCLUDING REMARKS

Much has been learned about RBP4's function in health and disease since its discovery more than 50 years ago. Extraordinary progress was made by generating and analyzing transgenic mouse models, in particular mice that lack RBP4 (Quadro et al., 1999), and by studying human mutations. Major findings from these studies are summarized in **Table 1**. What becomes obvious is that transport of retinol by RBP4 is the single most important route for its distribution in the body although, especially in mice, evolutionary pressure has led to the alternative delivery

pathways to compensate for RBP4 dysfunction as a backup plan for the organism. This is also in accordance with the fundamental role of vitamin A for cell homeostasis. As pointed out in the introductory section, development of the eye and visual function show the highest dependence on the supply of retinol by RBP4, which is mirrored by the phenotypes of transgenic mouse models and the consequences of mutations in humans, and also therapeutic strategies that target this lipocalin. Besides mobilizing retinol from liver, its role within other tissues is much less well explored. Tissue-specific knockout mouse models of RBP4 may help to decipher new biological aspects. Together with potential functions beyond transporting retinol, as discussed in this review, the next decades will warrant many more exciting findings for a more complete picture of RBP4's biology.

AUTHOR CONTRIBUTIONS

JSS and MS wrote the manuscript. AL gave intellectual input and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Biochemical and Structural Characteristics, Gene Regulation, Physiological, Pathological and Clinical Features of Lipocalin-Type Prostaglandin D_2 Synthase as a Multifunctional Lipocalin

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Lipocalin-type prostaglandin (PG) D_2 synthase (L-PGDS) catalyzes the isomerization of PGH_2, a common precursor of the two series of PGs, to produce PGD_2. PGD_2 stimulates three distinct types of G protein-coupled receptors: (1) D type of prostanoid (DP) receptors involved in the regulation of sleep, pain, food intake, and others; (2) chemoattractant receptor-homologous molecule expressed on T helper type 2 cells (CRTH2) receptors, in myelination of peripheral nervous system, adipocyte differentiation, inhibition of hair follicle neogenesis, and others; and (3) F type of prostanoid (FP) receptors, in dexamethasone-induced cardioprotection. L-PGDS is the same protein as β-trace, a major protein in human cerebrospinal fluid (CSF). L-PGDS exists in the central nervous system and male genital organs of various mammals, and human heart; and is secreted into the CSF, seminal plasma, and plasma, respectively. L-PGDS binds retinoic acids and retinal with high affinities (Kd < 100 nM) and diverse small lipophilic substances, such as thyroids, gangliosides, bilirubin and biliverdin, heme, NAD(P)H, and PGD_2, acting as an extracellular carrier of these substances. L-PGDS also binds amyloid β peptides, prevents their fibril formation, and disaggregates amyloid β fibrils, acting as a major amyloid β chaperone in human CSF. Here, I summarize the recent progress of the research on PGD_2 and L-PGDS, in terms of its "molecular properties," "cell culture studies," "animal experiments," and "clinical studies," all of which should help to understand the pathophysiological role of L-PGDS and inspire the future research of this multifunctional lipocalin.

Keywords: sleep, food intake, adipogenesis, innate immunity, inflammation, beta-trace protein, amyloid-beta chaperon, reproduction

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INTRODUCTION

In 1985, I purified lipocalin-type prostaglandin (PG) D_2 synthase (L-PGDS) from rat brain as a prostaglandin H_2 (PGH_2) D-isomerase (EC:5.3.99.2), that catalyzes the isomerization of a 9,11-endoperoxide group of PGH_2, a common intermediate of the two series of prostanoids, to produce prostaglandin D_2 (PGD_2) with 9-hydroxy and 11-keto groups (Urade et al., 1985). The cDNA cloning of L-PGDS demonstrated that the amino acid sequence of L-PGDS has the homology with

members of the lipocalin family which is composed of a variety of secretory proteins that bind and transport lipophilic small substances, such as β-lactoglobulin, α2-urinary globulin, placental protein 14, and α1-microglobulin (Urade and Hayaishi, 2000a). L-PGDS possesses a typical lipocalin fold of β-barrel with a central hydrophobic cavity, the retinoid-binding activity similar to other lipocalins, and the chromosomal gene structure with the same numbers and sizes of exons and phase of splicing of introns as those of other lipocalins (Urade and Hayaishi, 2000a).

Those studies open a new field of the lipocalin research, because that L-PGDS is the first lipocalin characterized as an enzyme among members of the lipocalin family and that L-PGDS produces an important bioactive lipid mediator PGD_2. PGD_2 plays important roles in the regulation of a variety of pathophysiological functions, such as sleep, pain, food intake in the central nervous system (CNS), inflammation and innate immunity, diabetes, cardiovascular functions and also in the reproduction systems.

I published several reviews of L-PGDS and PGD_2 (Urade and Hayaishi, 2000a,b, 2011; Urade and Eguchi, 2002; Smith et al., 2011). In this article, I classify the reports concerning with L-PGDS mainly after publication of those reviews and summarize the new finding of each section as follows:

1) Biological function of prostaglandin D_2 produced by lipocalin-type prostaglandin D_2 synthase,
2) Ligand binding properties of lipocalin-type prostaglandin D_2 synthase as an extracellular transporter,
3) Structural characterization of lipocalin-type prostaglandin D_2 synthase by nuclear magnetic resonance (NMR) and X-ray crystallography,
4) Cell culture studies of lipocalin-type prostaglandin D_2 synthase,
5) Mammalian experiments for the study of lipocalin-type prostaglandin D_2 synthase,
6) Pharmacokinetic analyses and functionalization with recombinant lipocalin-type prostaglandin D_2 synthase,
7) Studies of nonmammalian orthologs of lipocalin-type prostaglandin D_2 synthase,
8) Clinical studies of pathophysiological function of lipocalin-type prostaglandin D_2 synthase,
9) Future subjects.

BIOLOGICAL FUNCTION OF PROSTAGLANDIN D_2 PRODUCED BY LIPOCALIN-TYPE PROSTAGLANDIN D_2 SYNTHASE

Supplementary Table 1 summarizes the research history of L-PGDS and PGD_2. PGD_2 was originally discovered as a by-product of biosynthesis of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Both PGE_2 and $PGF_{2\alpha}$ exhibit potent activities on the smooth muscle contraction, whereas PGD_2 does not show the strong smooth muscle contractile activity. Therefore, the physiological function of PGD_2 was not extensively investigated until the discovery of potent action of PGD_2 on the regulation of inflammation and sleep.

In the early 1980s, PGD_2 was found to be a major prostaglandin produced in the brain of various mammals (Narumiya et al., 1982) including humans (Ogorochi et al., 1984) and to induce sleep after administration into the brain of freely moving rats (Ueno et al., 1982) and monkeys (Onoe et al., 1988). From those findings, the biochemical and neurological studies of PGD_2 in the CNS were accelerated. In 1979, other type of PGD_2 synthase, hematopoietic PGD_2 synthase (H-PGDS), was purified from rat spleen (Christ-Hazelhof and Nugteren, 1979). Comparisons of L-PGDS with H-PGDS revealed that these two enzymes are quite different proteins from each other (Urade et al., 1987), i.e., L-PGDS is a member of the lipocalin family (Nagata et al., 1991) and H-PGDS is the first identified invertebrate ortholog of sigma class of glutathione *S*-transferase (Kanaoka et al., 1997; Kanaoka and Urade, 2003). L-PGDS and H-PGDS have been evolved from different origins to acquire the same catalytic ability, being new examples of functional convergence (Urade and Eguchi, 2002; Smith et al., 2011).

Supplementary Table 2 summarizes the catalytic, molecular and genetic properties of human L-PGDS and H-PGDS. We isolated cDNAs and chromosomal genes of L-PGDS (*L-Pgds* or *Ptgds*; Urade et al., 1989; Igarashi et al., 1992) and H-PGDS (*Hpgds*; Kanaoka et al., 1997, 2000), and then determined X-ray crystallographic structures of the recombinant proteins of L-PGDS and H-PGDS expressed in *E. coli* (Kanaoka et al., 1997; Kumasaka et al., 2009), respectively. The inhibitors selective to L-PGDS, $SeCl_4$ and AT56 (Irikura et al., 2009), and to H-PGDS, HQL79 (Aritake et al., 2006), TFC007 (Nabea et al., 2011) and TAS204 (Urade, 2016) were found. We also generated KO mice of *L-Pgds* and *Hpgds* genes (Eguchi et al., 1999; Park et al., 2007), respectively, human enzyme-overexpressing transgenic (TG) mice (Fujitani et al., 2002, 2010), respectively, and flox mice used for conditional KO mice (Kaneko et al., 2012; Nakamura et al., 2017), respectively. In double KO mice of *L-Pgds* and *Hpgds* genes, the production of PGD_2 in the brain and other tissues is almost undetectable, indicating that these two enzymes are major components responsible for the biosynthesis of PGD_2 in our body (Kaushik et al., 2014).

Figure 1 shows the biosynthesis of PGD_2. PGD_2 is produced from arachidonic acid (C20:4), a major polyunsaturated fatty acid in our body, integrated in C2 position of phospholipids. Once cells are stimulated by various hormones, cytokines, and other signals, arachidonic acid is released from phospholipids by the action of cytosolic phospholipase A_2 ($cPLA_2$) or group III phospholipase A_2 (PLA2G3). A part of the released arachidonic acid is oxygenated by cyclooxygenase (Cox)-1 or 2 (PGH_2 synthase-1 or -2, these genes are *ptgs* or *ptgs2*, respectively) to produce PGH_2, a common intermediate of the two series of PGs, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and thromboxane (TX) A_2, in which 2 indicates the number of unsaturated C=C bond. PGH_2 is converted by L-PGDS or H-PGDS to PGD_2, in the presence of exogenous sulfhydryl compounds, most likely a reduced form of glutathione within the cells. Both Cox-1 and -2 are microsomal membrane-binding enzymes and produce PGH_2 within the cells. PGH_2 is chemically unstable

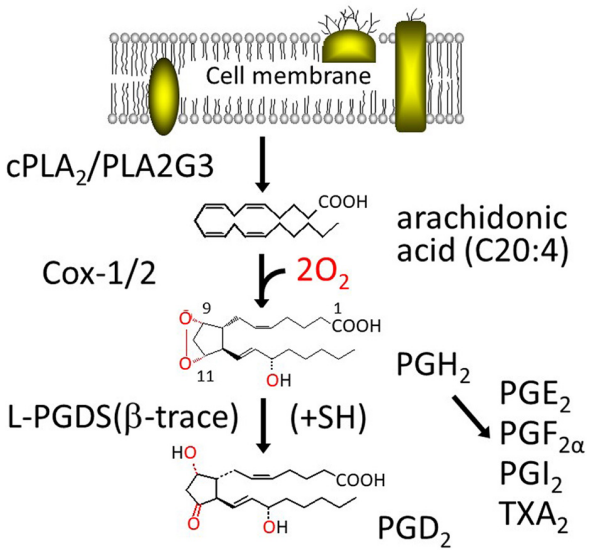

FIGURE 1 | Biosynthesis pathway of PGD_2.

in aqueous solution with a $t_{1/2}$ of several min to degrade a mixture of PGE_2 and PGD_2 at a ratio of 2:1. Therefore, it is unlikely that L-PGDS in the extracellular space interacts with PGH_2 to produce PGD_2 selectively. As L-PGDS binds a variety of hydrophobic substances, such as retinoids and thyroids, L-PGDS in the extracellular space may act as an extracellular transporter for these hydrophobic ligands.

Prostaglandin D_2 stimulates three distinct types of G-protein coupled receptors (**Supplementary Table 3**). One is D type of prostanoid (DP) receptors (also abbreviated as DP_1) coupled with Gs-protein to increase intracellular cAMP levels (Hirata et al., 1994). DP receptors are involved in the regulation of sleep (Qu et al., 2006), pain (Eguchi et al., 1999), food intake (Ohinata et al., 2008) and others. Two is chemoattractant receptor-homologous molecule expressed on T helper type 2 cells (CRTH2) receptors (also abbreviated as DP_2, previously known as GPR44 of an orphan receptor, or CD294) coupled with Gi-protein to decrease intracellular cAMP levels (Nagata et al., 1999; Hirai et al., 2001). CRTH2 receptors are involved in myelination of peripheral nervous system (Trimarco et al., 2014), adipocyte differentiation (Wakai et al., 2017), inhibition of hair follicle neogenesis (Nelson et al., 2013) and others. Three is F type of prostanoid (FP) receptors (Abramovitz et al., 1994) coupled with Gq-protein to increase intracellular Ca^{2+} concentrations. FP receptors are activated by either PGD_2 or $PGF_{2\alpha}$ at almost the same binding affinities and involved in protection of the heart against ischemia-reperfusion injury by activating Nrf2 (Katsumata et al., 2014). Human genes, stable agonists and antagonists, and physiological functions mediated by DP, CRTH2, and FP receptors are also shown in **Supplementary Table 3**. The KO mice of *Ptgdr* (for DP, Matsuoka et al., 2000), *Ptgdr2* (for CRTH2, Satoh et al., 2006) and *Ptgfr* (FP, Sugimoto et al., 1997) genes and the flox mice of *Ptgdr* (Kong et al., 2016) and *Ptgfr* (Wang et al., 2018) genes are already generated. The flox mice of *Ptgdr2* gene are not yet available.

The pathophysiological function of PGD_2 was extensively studied in the last two decades by pharmacological analyses with selective inhibitors for L-PGDS and H-PGDS, agonists and antagonists for DP, CRTH2 and FP receptors, as well as by *in vivo* analyses with various gene-manipulated mice of *L-Pgds*, *HPgds*, *Ptgdr*, *Ptgdr2* and *Ptgfr* genes, as described in the later sections.

The cDNA and genome cloning of *L-Pgds* (Nagata et al., 1991; Igarashi et al., 1992) revealed that L-PGDS is a member of the lipocalin family, as judged by the homology of amino acid sequence and gene structure. The gene structure of *L-Pgds* (Igarashi et al., 1992) is remarkably analogous to those of other lipocalins, such as β-lactoglobulin, α2-urinary globulin, placental protein 14, and α1-microglobulin. All those proteins have the same numbers and sizes of exons and phase of splicing of introns. Positions of exon/intron junction of the *L-Pgds* gene are highly conserved and located around the same positions of those other lipocalins, in a multiple alignment of amino acid sequences despite a weak homology (Urade and Hayaishi, 2000a; Urade et al., 2006).

In 1993, the amino acid sequence of β-trace protein purified from human cerebrospinal fluid (CSF, Hoffmann et al., 1993) was found to be exactly identical to that of human L-PGDS (Nagata et al., 1991) after cleavage of its N-terminal hydrophobic signal sequence. β-Trace is a major protein of human CSF which was originally found in human CSF in 1961 (Clausen, 1961), but its molecular properties and physiological functions remained unidentified. In 1994, we purified L-PGDS from human CSF and confirmed that CSF L-PGDS is enzymatically and immunologically the same as β-trace (Watanabe et al., 1994).

Lipocalin-type prostaglandin D_2 synthase is distributed in the CNS (Urade et al., 1989, 1993), male genital organs (Urade et al., 1989; Tokugawa et al., 1998) and human heart (Eguchi et al., 1997), and secreted into CSF (Hoffmann et al., 1993; Watanabe et al., 1994), seminal plasma (Tokugawa et al., 1998) and plasma during coronary circulation (Eguchi et al., 1997), respectively. L-PGDS maintains the binding activity of lipophilic ligands similar to other members of lipocalin superfamily (Urade et al., 2006), suggesting that L-PGDS acts as an extracellular transporter of lipophilic substances, as described below.

LIGAND BINDING PROPERTIES OF LIPOCALIN-TYPE PROSTAGLANDIN D_2 SYNTHASE AS AN EXTRACELLULAR TRANSPORTER

Supplementary Table 4 summarizes various types of ligands bound to L-PGDS and their binding affinities and kinetics. L-PGDS binds retinoids (Tanaka et al., 1997), thyroids (Beuckmann et al., 1999), and various types of lipophilic ligands. L-PGDS binds also amyloid β (Aβ) peptides (Kanekiyo et al., 2007), prevents the formation of amyloid fibrils (Kanekiyo et al., 2007; Kannaian et al., 2019) and disaggregates the fibrils (Kannaian et al., 2019), acting as a major Aβ chaperone in human CSF. L-PGDS binds PGD_2 at a molar ratio of 1:2 with high and low affinities, suggesting that L-PGDS may function

as an extracellular PGD_2-transporter in the absence of substrate PGH_2 (Shimamoto et al., 2021). All those ligands exhibit binding affinities to L-PGDS much higher than the Km value of PGH_2 but do not potently inhibit the L-PGDS activity. L-PGDS has a large central hydrophobic cavity within a molecule, in which two to three molecules of those small ligands are captured. Docking analyses suggest that those ligands bind to the hydrophobic pocket at the bottom of a large central cavity of L-PGDS, which is different from the PGH_2-binding catalytic pocket at the upper entrance of L-PGDS, as described later.

Binding of Lipophilic Hormones Including Retinoids and Thyroids

Lipocalin-type prostaglandin D_2 synthase binds *all-trans*- and 9-*cis*-retinoic acids and *all-trans*- and 13-*cis*-retinal, but not retinol, with high affinities of Kd = 70–80 nM at a 1:1 molar ratio (Tanaka et al., 1997; Shimamoto et al., 2007; Inoue et al., 2009), similar to several other lipocalins. L-PGDS/β-trace is secreted into various body fluids, such as CSF of the brain (Hoffmann et al., 1993; Watanabe et al., 1994), interphotoreceptor matrix of the retina (Beuckmann et al., 1996), plasma (Eguchi et al., 1997), and seminal plasma (Tokugawa et al., 1998), suggesting that L-PGDS acts as an extracellular transporter of those retinoids within these compartments. Retinoid transporting function by L-PGDS is demonstrated in the study of glial migration (Lee et al., 2012) and the placode formation in *Xenopus* embryo (Jaurena et al., 2015) by using the Cys65Ala mutant without the PGD_2 synthase activity (Urade et al., 1995).

Thyroid hormones, such as thyroxine (T4), 3-3′,5′-triiodo-L-thyronine (T3) and 3-3′,5-triiodo-L-thyronine (reverse T3), bind to L-PGDS with affinities of Kd = 0.7 to 3 μM (Beuckmann et al., 1999). L-PGDS expression is upregulated in rat brain through activation of a thyroid hormone/retinoic acid-responsive element in the promoter region of the rat *L-Pgds* gene (García-Fernández et al., 1998). Thyroid hormones upregulate *L-Pgds* gene expression in the male genital organs, the seminal vesicle and testis, of cat fish (Sreenivasulu et al., 2013). These results suggest that L-PGDS gene expression is controlled by its own ligands by an autonomic positive regulation mechanism. Fish L-PGDS ortholog does not contain an active thiol of Cys65 in mammalian L-PGDSs so that the fish L-PGDS does not show the PGD_2 synthase activity but maintains the retinoid/thyroid-binding activity (Fujimori et al., 2006). Therefore, the fish L-PGDS is also predicted to function as a non-enzymic transporter of lipophilic hormones (Sreenivasulu et al., 2013).

Binding of Heme-Degradation Products and Heme

Lipocalin-type prostaglandin D_2 synthase binds bilirubin and biliverdin (Beuckmann et al., 1999; Inoue et al., 2009; Miyamoto et al., 2009; Sreenivasulu et al., 2013), harmful degradation products of heme with high affinities (Kd of 20–40 nM) (Tanaka et al., 1997). This binding is also confirmed in the CSF of patients after subarachnoid hemorrhage, in which a part of biliverdin is covalently bound to the Cys65 residue, an active thiol of L-PGDS, as demonstrated by NMR (Inui et al., 2014). Therefore, L-PGDS scavenges those harmful heme-metabolites from CSF. L-PGDS also binds heme itself, as examined by NMR (Phillips et al., 2020). The heme-binding L-PGDS is associated with the pseudo-peroxidase activity, which is proposed to contribute to the anti-apoptotic activity of L-PGDS against the H_2O_2-induced cytotoxicity (Phillips et al., 2020).

Binding of Amyloid β Peptides

Lipocalin-type prostaglandin D_2 synthase binds to Aβ peptides 1–40 and 1–42, and their fibrils with high affinities (Kd = 18–50 nM). The aggregation of Aβ peptides is crucial in the pathogenesis of Alzheimer's disease. L-PGDS recognizes a region of amino acid residues of 25–28 in Aβ peptides, the key region for conformational change to β-sheet structures (Kanekiyo et al., 2007). L-PGDS inhibits the spontaneous aggregation of Aβ (1–40) and Aβ (1–42) in a physiological concentration range from 1 to 5 μM in human CSF, and also prevents the seed-dependent aggregation of 50 μM Aβ (1–40) with Ki of 0.75 μM. When L-PGDS is removed from the human CSF by immunoaffinity chromatography with anti-(L-PGDS) IgG, the inhibitory activity toward Aβ (1–40) aggregation in human CSF decreases by 60%. Recombinant human L-PGDS disaggregates Aβ fibrils and dissolves many insoluble proteins existed in amyloid plaques in the brain of patients with Alzheimer's disease (Kannaian et al., 2019). These results indicate that L-PGDS is a major endogenous Aβ-chaperone in the human brain.

Binding of Lipids, Gangliosides, and Lipophilic Drugs

Lipocalin-type prostaglandin D_2 synthase also binds gangliosides, such as GM_1 and GM_2, with a high affinity with Kd = 65–210 nM (Mohri et al., 2006a). L-PGDS is upregulated in oligodendrocytes and a few neurons in the brain of murine models of various lysosomal storage diseases, such as Krabbe's disease (Taniike et al., 1999; Mohri et al., 2006b), Tay–Sachs disease, Sandhoff disease, GM_1 gangliosidosis and Niemann–Pick type C1 disease (Mohri et al., 2006a). These results suggest that L-PGDS plays a protective role on oligodendrocytes in scavenging harmful lipophilic substrates accumulated by malfunction of myelin metabolism in lysosomal storage diseases, as demonstrated by using double mutant mice with *L-Pgds* gene KO mice as shown later (Taniike et al., 2002).

Lipocalin-type prostaglandin D_2 synthase also binds fatty acids (Zhou et al., 2010; Elmes et al., 2018) and various water insoluble drugs, including a sleeping pill Diazepam (Fukuhara et al., 2012a), a drug used for high blood pressure Telmisartan (Mizoguchi et al., 2015), anti-cancer drugs (Nakatsuji et al., 2015; Teraoka et al., 2017), cannabinoid receptor antagonists (Yeh et al., 2019), cannabinoid metabolites (Elmes et al., 2018), synthetic cannabinoids (Elmes et al., 2018), and anti-cholinergic drugs (Low et al., 2020), as listed in **Supplementary Table 4**. The binding affinities for those compounds are lower than those for retinoids, thyroids and Aβ. The drug-binding ability of L-PGDS is used for the drug delivery system and is recognize to induce off-target effects of anti-cholinergic drugs, such as Chlorpheniramine and Trazodone, to modulate the cytotoxicity of Aβ fibrils (Low et al., 2020).

Binding of Nicotinamide Coenzymes

Lipocalin-type prostaglandin D_2 synthase binds NADPH, $NADP^+$, and NADH, as examined by thermodynamic and NMR analyses. These hydrophilic ligands, especially NADPH, interact with the upper pocket of a ligand-binding cavity of L-PGDS with an unusual bifurcated shape (Qin et al., 2015). The binding affinity of L-PGDS for NADPH is comparable to that of NADPH oxidases. Therefore, L-PGDS may attenuate the NADPH oxidase activities through interaction with NADPH, being involved in anti-oxidative stress function of L-PGDS.

Binding of Substrate Analog and Product

Most recently, we demonstrated by isothermal titration assay and NMR analyses that L-PGDS binds a stable PGH_2 analog U-46619 at two binding sites of high and low affinities with Kd values of 0.53 and 7.91 μM, respectively, and also its product PGD_2 with 0.3 and 44 μM, respectively, in the hydrophilic catalytic pocket at the upper entrance of L-PGDS molecule (Shimamoto et al., 2021). The high affinity binding is lost in the Cys65Ala mutant of L-PGDS, indicating that the thiol group of Cys65 is important for high affinity binding of the substrate PGH_2 and the product PGD_2 to L-PGDS. On the other hand, the low affinity binding site has a wide binding spectrum for other PGs including PGE_2 and $PGF_{2\alpha}$ with comparable affinities. These results indicate the substrate-induced catalytic mechanism for L-PGDS. The sleep-inducing activity by an intracerebroventricular infusion of PGD_2 is significantly reduced in *L-Pgds* gene KO mice than wild-type mice, suggesting that L-PGDS binds and transport PGD_2 in the brain to stimulate effectively DP receptors in the sleep-promoting system (Shimamoto et al., 2021).

STRUCTURAL CHARACTERIZATION OF LIPOCALIN-TYPE PROSTAGLANDIN D_2 SYNTHASE BY NUCLEAR MAGNETIC RESONANCE AND X-RAY CRYSTALLOGRAPHY

Figure 2 shows NMR (**Figure 2A**) and X-ray crystallographic structures (**Figures 2B,C**) of recombinant L-PGDS expressed in *E. coli*. We first determined in 2007 the solution structure of mouse L-PGDS by NMR (Shimamoto et al., 2007). L-PGDS possesses a typical lipocalin fold of β-barrel with two sets of β-sheet composed of each four strands of anti-parallel β-sheet and a 3-turn α-helix associated with the outer surface of the barrel. L-PGDS possesses a large central cavity with a flexible rid of a wide entrance opening to the upper end of the barrel. The central cavity of L-PGDS is larger than those of other lipocalins and contains two pockets (**Figure 3A**). NMR titration analyses demonstrate that *all-trans*-retinoic acid occupies the hydrophobic pocket 2 with amino acid residues important for the retinoid binding well conserved in other lipocalins (**Figures 3B,C**) and that PGH_2 occupies the hydrophilic pocket 1 containing Cys65 and a hydrogen network of Ser45, Thr67, and Ser81 (**Figures 3D,E**).

We determined in 2009 the crystal structures of mouse L-PGDS Cys65Ala mutant (Kumasaka et al., 2009) and revealed that L-PGDS exhibits two different conformers due to the movement of the flexible EF-loop (**Figures 2A,C**). One conformer of L-PGDS has an open cavity of the β-barrel and the other, a closed cavity (**Figure 2C**). The upper hydrophilic pocket 1 of the central cavity contains the catalytically essential Cys65 residue with a hydrogen bond network with Ser45, Thr67, and Ser81 (**Figures 3E**, **4**). The SH titration analyses combined with site-directed mutagenesis (Kumasaka et al., 2009) demonstrate that Cys65 residue is activated by its interaction with Ser45 and Thr67. The crystal structure of L-PGDS confirmed that the lower compartment of the central cavity is composed of hydrophobic amino acid residues highly conserved among other lipocalins (**Figure 3C**).

X-ray crystallographic structures of human L-PGDS was also reported, in which fatty acids (Zhou et al., 2010) and polyethylene glycol used as precipitants (Perduca et al., 2014) are identified to be inserted into the central cavity of the molecule. The NMR structures of L-PGDS complexed with an L-PGDS-selective inhibitor AT56 (Irikura et al., 2009) or a variety of lipophilic (Qin et al., 2015) or hydrophobic (Shimamoto et al., 2007; Sreenivasulu et al., 2013; Inui et al., 2014; Kannaian et al., 2019; Low et al., 2020; Phillips et al., 2020) ligands were also already reported (**Supplementary Table 4**). Those structural information is useful for designing inhibitors specific for L-PGDS.

Figure 5 shows a substrate-induced product release mechanism for L-PGDS (Shimamoto et al., 2021). L-PGDS has two binding sites for the substrate PGH_2 and the product PGD_2 in the pocket 1 of the upper part of the cavity. Site 1 is the catalytic site containing Cys65 and site 2, the non-catalytic site. Apo-form of L-PGDS has a wide open entrance, through which PGH_2 enters to the pocket 1. PGH_2 binds to the site 1 at step (i). H2-helix, CD-loop, and EF-loop of L-PGDS interact with PGH_2 so that the cavity is closed at step (ii). The closed conformation of L-PGDS holds the 9,11-endoperoxide group of PGH_2 to interact with the thiol group of the catalytic Cys65. The catalytic reaction occurs to produce PGD_2 at step (iii). After the catalytic reaction, PGD_2 still binds to site 1 at the high affinity with Kd of 0.3 μM (**Supplementary Table 4**). In the absence of PGH_2, L-PGDS acts as a carrier of PGD_2 as shown at step (iv). In the presence of excess concentrations of PGH_2, the next molecule of PGH_2 binds to site 2, inducing movement of CD-loop to open site 1 and release PGD_2 at step (v). PGH_2 then move from site 2 to site 1 and the catalytic cycle starts again (Shimamoto et al., 2021).

Post transcriptional modification of L-PGDS/β-trace in human CSF is identified by a proteoform analysis (Zhang et al., 2014) to be *N*-glycans at Asn51 and Asn78 with different *N*-glycan compositional variants, a core-1 HexHexNAc-O-glycan at Ser29, acetylation at Lys38 and Lys160, sulfonation at Ser63 and Thr164, and dioxidation at Cys65 and Cys167 after cleavage of an N-terminal signal sequence to Ala22. Two N-truncated forms of L-PGDS from Gln31 and Phe34 exist in human urine (Nagata et al., 2009).

A loop structure of residues 55–63 between β-strand A and B of L-PGDS is proposed to be homologous to a sequence domain in apolipoprotein E responsible for binding to low-density lipoprotein (LDL) receptor (Portioli et al., 2017). Two potential GTPase Rab4-binding sites are proposed to locate

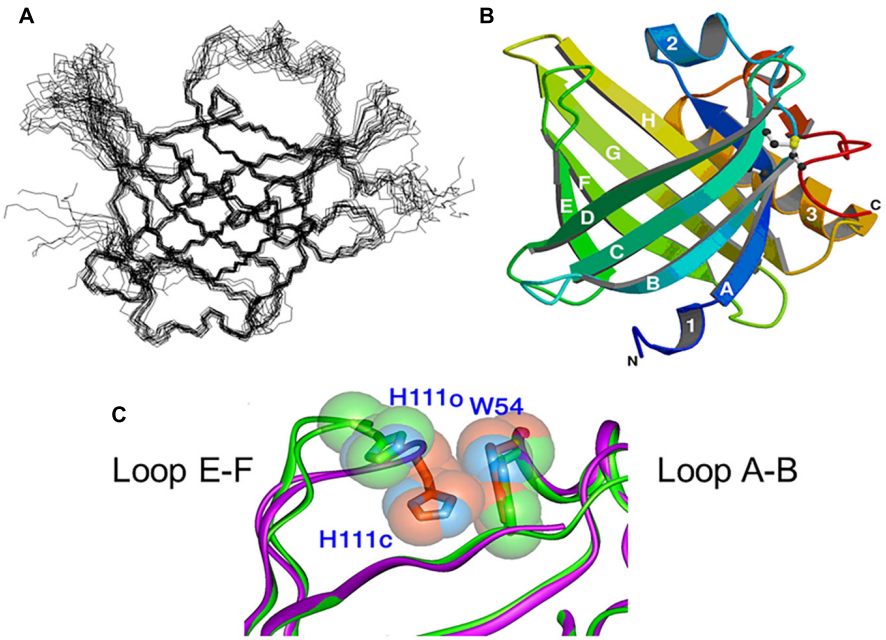

FIGURE 2 | NMR **(A)** and X-ray crystallographic **(B)** structures of L-PGDS (Shimamoto et al., 2007; Kumasaka et al., 2009, respectively). **(C)** Overlapping views of the entrance of the catalytic pocket of the open (*green*) and closed (*purple*) conformers of L-PGDS (Kumasaka et al., 2009). Positions of EF-loop and H2-helix are indicated.

residues 75–98 and 85–92 within β-strand B and C of human L-PGDS (Binda et al., 2019).

CELL CULTURE STUDIES OF LIPOCALIN-TYPE PROSTAGLANDIN D_2 SYNTHASE

Lipocalin-type prostaglandin D_2 synthase is constitutively expressed in several cell lines including human brain-derived TE671 cells, human neuroblastoma SH-SY5Y cells, mouse adipocytic 3T3-L1 cells, and others. Several types of primary cultured cells, such as leptomeningeal cells, vascular endothelial cells, bone marrow-derived macrophages or mast cells are also used to study the regulation mechanism of *L-Pgds* gene and the functional analyses of L-PGDS, as summarized in **Supplementary Table 5**.

L-Pgds Gene Regulation in the Central Neuronal Cells

In mouse neuronal GT1-7 cells, L-PGDS is induced by dexamethasone (García-Fernández et al., 2000). The L-PGDS induction is suppressed by 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), whereas TPA induces the synthesis of PGs in many tissues. The L-PGDS induction by glucocorticoids is also found in mouse adipocytic 3T3-L1 cells (Yeh et al., 2019). Dexamethasone and glucocorticoids are known to suppress inflammation by inhibition of PG production. However, PGD_2 produced by L-PGDS may, in part, be also involved anti-inflammatory effects by those hormones.

In rat leptomeningeal cells, the Notch-Hes signal represses *L-Pgds* gene expression by interaction with an atypical E-box (aE-box) in the promoter region. IL-1β upregulates *L-Pgds* gene expression through the NF-kB pathway at two NF-kB sites of the *L-Pgds* gene (Fujimori et al., 2003) and by contact with astrocytes (Fujimori et al., 2007b).

In human TE671 cells, the Notch-Hes signal represses *L-Pgds* gene expression by interaction with an N-box in the promoter region and the AP-2β binding to the AP-2 element in the promoter region is involved in maintenance of *L-Pgds* gene expression (Fujimori et al., 2005). TPA induces L-PGDS in TE671 cells. Protein kinase C phosphorylates Hes-1, inhibits DNA binding of Hes-1 to the N-box, and induce L-PGDS. Activation of the AP-2β function is involved in up-regulation of *L-Pgds* gene expression. The aE-box is critical for transactivation of the *L-Pgds* gene in TE671 cells. Upstream stimulatory factor (USF)-1 binds to the aE-box in intron 4 of the human *L-Pgds* gene and activates *L-Pgds* gene expression. Binding of AP-2β in the promoter also cooperatively contributes to the transactivation of *L-Pgds* gene (Fujimori and Urade, 2007). Serum starvation induces PGD_2 production in TE671 cells through transcriptional activation of *Pghs-2* and *L-Pgds* genes. The serum starvation up-regulates *L-Pgds* gene expression by binding of USF-1 to aE-box within intron 4. The USF1 expression is enhanced through activation of p38 mitogen-activated protein kinase in TE671 cells (Fujimori et al., 2008). **Figure 6** summarizes the transcriptional regulation of the human *L-Pgds* gene in TE671 cells.

In human neuroblastoma SH-SY5Y cells, L-PGDS prevents neuronal cell death caused by oxidative stress (Fukuhara et al., 2012b). An NF-kB element in the proximal promoter region of

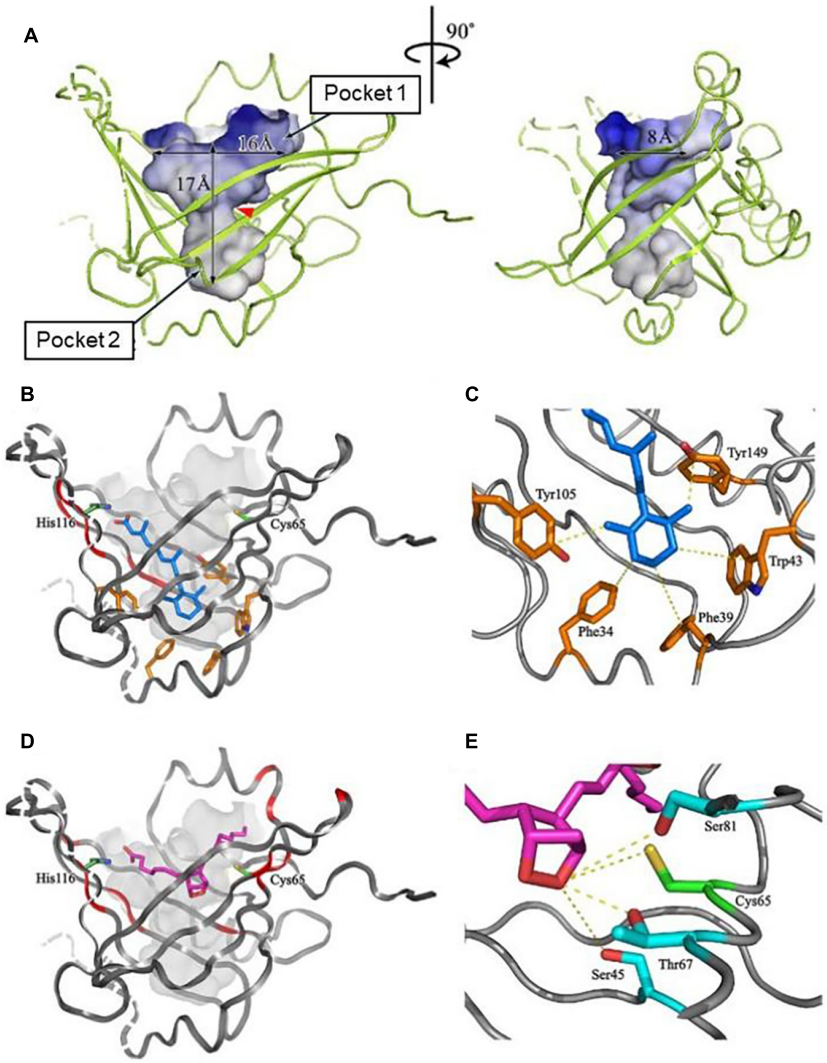

FIGURE 3 | The structure of a large central cavity of L-PGDS **(A)** and docking models of the complexes with *all-trans*-retinoic acid **(B,C)** or PGH_2 **(D,E)** determined by NMR (Shimamoto et al., 2007). The molecule of *all-trans*-retinoic acid are shown in *light blue* **(B,C)**. The carbon chain of PGH_2 is shown in *purple* and a 9,11-endoperoxide group, in *orange* **(D,E)**. Amino acid residues important for the ligand binding and the catalytic activity are shown in panels **(C,E)**, respectively.

L-Pgds gene mediates paraquat-induced apoptosis of these cells (Fujimori et al., 2012a).

In U251 glioma cell line expressing estrogen receptor (ER) α, estradiol (10^{-11} M) increases the promoter activity of *L-Pgds* gene. Conditioned media from estradiol-treated neurons increases the *L-Pgds* gene promoter activity in glial cells, suggesting that a paracrine factor released from the neighboring neurons after stimulation of estrogen induces L-PGDS in glial cells (Devidze et al., 2010).

In primary cultured astrocytes, microglial cells, and fibroblasts (Lee et al., 2012), L-PGDS accelerates the migration of these cells and changes the morphology to the characteristic phenotype in reactive gliosis. Activation of AKT, RhoA, and JNK pathways mediates L-PGDS-induced cell migration. L-PGDS interacts with myristoylated alanine-rich protein kinase C substrate (MARCKS) and promotes the cell migration in a PGD_2-independent manner, because that the inactive Cys65Ala mutant without the PGD_2 synthase activity shows the same effect.

L-Pgds Gene Regulation in Vascular Cells

Fluid shear stress induces L-PGDS in human vascular endothelial cells (Taba et al., 2000). c-Fos and c-Jun bind to the AP-1 binding site of the 5′-promoter region of the *L-Pgds* gene to induce L-PGDS. Shear stress elevates the c-Jun phosphorylation level in a time-dependent manner, similar to that of *L-Pgds* gene expression. A c-Jun N-terminal kinase inhibitor decreases the c-Jun phosphorylation, DNA binding of AP-1, and shear stress-induced *L-Pgds* gene expression (Miyagi et al., 2005). Homozygosity for the C variant of the T-786C single-nucleotide polymorphism of the human endothelial NO synthase gene

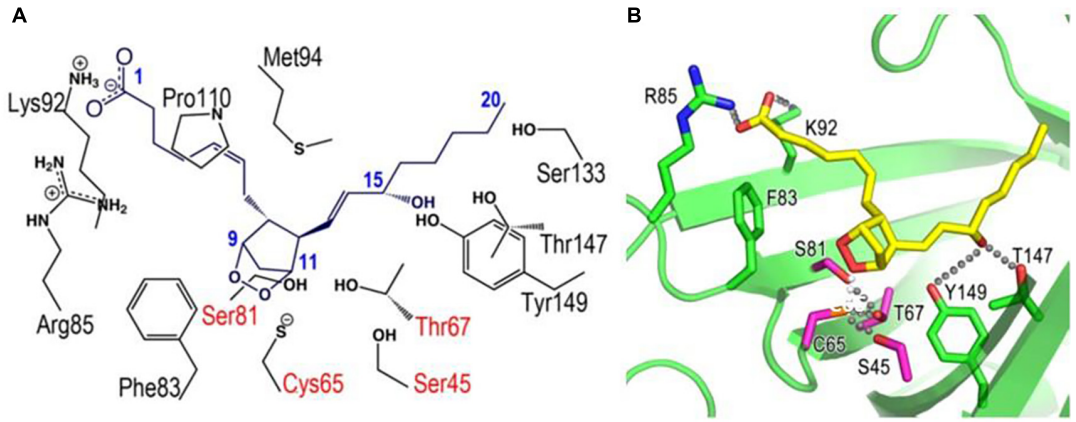

FIGURE 4 | Docking models of L-PGDS complexes with PGH_2 based on X-ray crystallography (Kumasaka et al., 2009). Amino acid residues important for interaction with PGH_2 are shown in panel **(A)**. R85, K92, F83, S82, C65, S45, T67, Y149, and T147 are shown in stick-model form in panel **(B)**. Hydrogen bonding network around Cys65 and 15-hydroxy group of PGH_2 are also indicated in panel **(B)**.

exhibits a reduced endothelial cell capacity to generate NO and is less sensitive to fluid shear stress. In the CC-genotype of endothelial cells, fluid shear stress elicits a marked rise in *Pghs-2* and *L-Pgds* gene expression (Urban et al., 2019). These results suggest that the increased production of PGD_2 is the reason why CC-genotype endothelial cells maintain the robust anti-inflammatory characteristics, despite a reduced capacity to produce NO.

Exogenously added L-PGDS induces apoptosis of epithelial, neuronal and vascular smooth muscle cells. L-PGDS-induced apoptosis is inhibited by mutations in a glycosylation site Asn51, a putative protein kinase C phosphorylation site Ser106, and the enzymatic active site Cys65, although the action mechanism remains unclear (Ragolia et al., 2007).

L-Pgds Gene Regulation in Adipocytes

Lipocalin-type prostaglandin D_2 synthase is involved in adipocyte differentiation of mouse 3T3-L1 cells (Fujimori et al., 2007a). A responsive element for liver receptor homolog-1 (LRH-1) in the promoter region of the *L-Pgds* gene plays a critical role in *L-Pgds* gene expression in pre-adipocytes of 3T3-L1 cells. Two sterol regulatory elements (SREs) in the promoter region act as *cis*-elements for activation of *L-Pgds* gene. Synthetic liver X receptor agonist T0901317 activates the expression of SRE-binding protein-1c (SREBP-1c) and upregulates *L-Pgds* gene expression in these cells. LRH-1 and SREBP-1c bind to their respective binding elements in the promoter of *L-Pgds* gene and increase *L-Pgds* gene expression in pre-adipocytes and adipocytes, respectively, of 3T3-L1 cells. **Figure 7** summarizes the transcriptional regulation of the mouse *L-Pgds* gene in preadipocytes and adipocytes of mouse 3T3-L1 cells.

Gene knockdown of *L-Pgds* by antisense *L-Pgds* gene in pre-adipocytes stimulates fat storage during the maturation stage of these cells (Chowdhury et al., 2011). In these cells, Δ^{12}- prostaglandin J_2 (Δ^{12}- PGJ_2), a dehydrated product of PGD_2 produced by L-PGDS, activates adipogenesis through both PPARγ-dependent and -independent pathways (Fujimori et al., 2012b), although the J series of PGD_2 is not physiologically produced *in vivo* as described below.

Prostaglandin D_2 is relatively unstable in water, as compared with PGE_2 and $PGF_{2\alpha}$, both of which are chemically very stable for several months to years. PGD_2 is spontaneously dehydrated in water to prostaglandin J_2 (PGJ_2), Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2). These cyclopentene PGs with a O=C-C=C- bond are chemically reactive with various SH and amino groups to make conjugates. As d15-PGJ_2 acts as a ligand of PPARγ *in vitro*, several reports emphasize the importance of d15-PGD_2 *in vivo*. However, those cyclopentene PGs are not produced physiologically *in vivo* and are artificially generated from PGD_2 during extraction and purification for measurement. Those cyclopentene PGs are cytotoxic and induce apoptosis of cultured cells because of their massive reactivities. We have to be careful about the artificial cytotoxic effect of those dehydrated PGD_2 products in the cell culture system. In cases that the target receptor of PGD_2 is predicted, it is desirable to use chemically stable agonists, rather than PGD_2 itself.

Adipocytes dominantly express CRTH2 receptors (Wakai et al., 2017). CRTH2 antagonist, but not DP antagonist, suppresses the PGD_2-elevated intracellular triglyceride level. CRTH2 agonist 15R-15-methyl PGD_2 increases the mRNA levels of the adipogenic and lipogenic genes, decreases the glycerol release level, and represses the forskolin-mediated increase of cAMP-dependent protein kinase A activity (PKA) and phosphorylation of hormone-sensitive lipase (HSL). The lipolysis is enhanced in the adipocytes differentiated from embryonic fibroblasts of CRTH2 KO mice. These results indicate that PGD_2 produced by L-PGDS suppresses the lipolysis by repression of the cAMP-PKA-HSL axis through CRTH2 receptors in adipocytes in an autocrine manner.

Glucocorticoid induces *L-Pgds* gene expression and leptin production in differentiated primary preadipocytes (Yeh et al., 2019). *L-Pgds* siRNA and L-PGDS inhibitor AT56 suppress glucocorticoid-induced leptin production, whereas overexpression of *L-Pgds* gene enhances leptin production. The

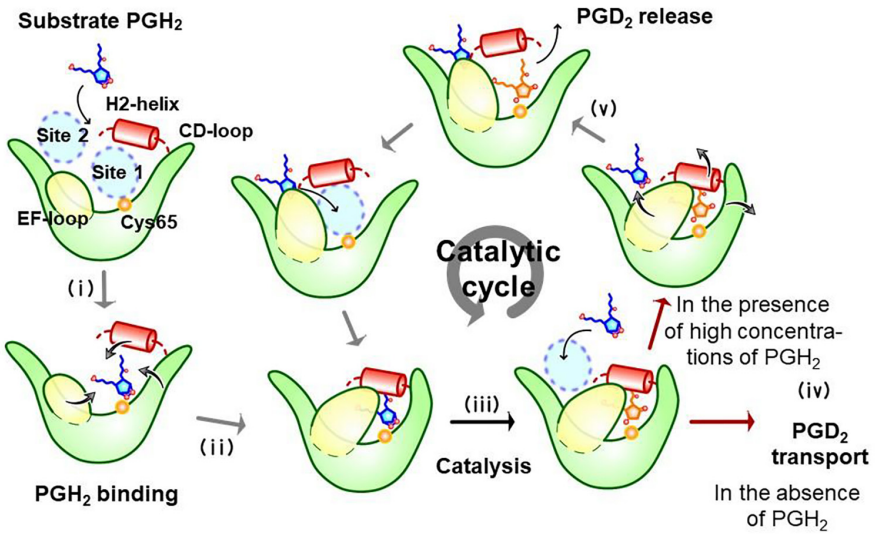

FIGURE 5 | Substrate-induced product-release mechanism for L-PGDS (Shimamoto et al., 2021).

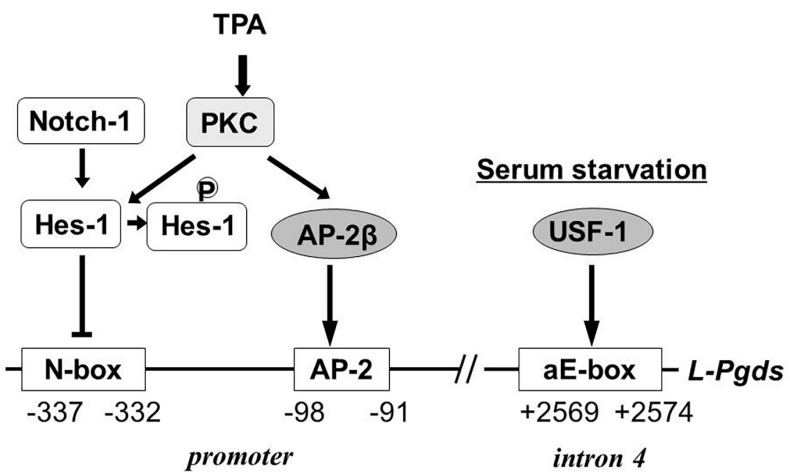

FIGURE 6 | Regulatory mechanism of human *L-Pgds* gene expression in brain-derived TE671 cells (Fujimori et al., 2005, 2008; Fujimori and Urade, 2007). *L-Pgds* gene expression is lowered by the Notch-Hes signal via an N-box, but enhanced by AP-2β through the AP-2 element. TPA activates PKC, followed by phosphorylation of Hes-1 to cancel the binding of Hes-1 to the N-box. Upstream stimulatory factor-1 (USF-1) activates *L-Pgds* gene expression through the atypical E-box (aE-box) in the intron 4. Serum starvation induces further *L-Pgds* gene expression.

L-PGDS-leptin pathway may be involved in undesired effects of clinical used glucocorticoid including obesity. In human mesenchymal stroma cells used in cellular therapies, L-PGDS is involved in differentiation of adipocytes, suggesting that *L-Pgds* gene expression is a potential quality marker for these cells, as it might predict unwanted adipogenic differentiation after the transplantation (Lange et al., 2012).

Lipocalin-Type Prostaglandin D_2 Synthase Studies in Skeletal Muscle Cells

Lipocalin-type prostaglandin D_2 synthase stimulates glucose transport of the insulin-sensitive rat skeletal muscle cell line L6 at the basal level and after insulin-stimulation. L-PGDS increases translocation of glucose transporter 4 to the plasma membrane, suggesting that L-PGDS, via production of PGD_2, is an important mediator of muscle and adipose glucose transport, which plays a significant role in the glucose intolerance associated with type 2 diabetes (Ragolia et al., 2008).

L-Pgds Gene Regulation in Peripheral Nervous Cells

L-Pgds gene is the most upregulated gene in the primary culture of rat dorsal root ganglion (DRG) neurons infected with the intracellular domain of neuregulin 1 type III, a member of the neuregulin family of growth factors. DRG neurons

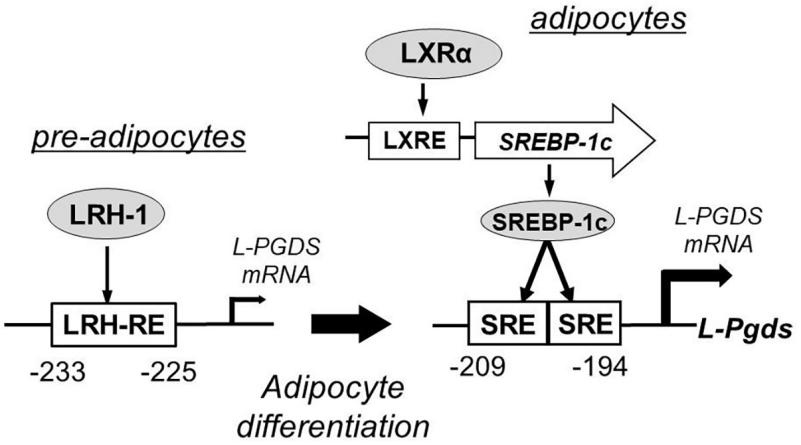

FIGURE 7 | Transcriptional regulation of mouse *L-Pgds* gene in adipocyte 3T3-L1 cells (Fujimori et al., 2007a). *L-Pgds* gene expression is activated by liver receptor homolog-1 (LRH-1), one of orphan nuclear receptors, through the LRH-responsive element (LRH-RE) in pre-adipocytes. In the differentiated adipocytes, activation of liver X receptor (LXR) elevates the expression of sterol regulatory element-binding protein-1 (SREBP-1c) through the LXR-responsive element (LXRE), and then SREBP-1c strongly enhances the *L-Pgds* gene expression via two sterol regulatory elements (SREs) in the promoter region of *L-Pgds* gene.

secrete L-PGDS and PGD_2, the latter of which stimulates CRTH2 receptors and enhances myelination of Schwann cells (Trimarco et al., 2014).

Lipopolysaccharide (LPS) stimulation increases *L-Pgds* gene expression in intestinal neurons and glial cells in the primary culture of rat enteric nervous system. L-PGDS inhibitor AT56 inhibits PGD_2 production in the primary culture treated with LPS. As *Pghs-2* and *L-Pgds* gene expression increase in the inflamed colonic mucosa of patients with active Crohn's disease, the L-PGDS pathway may be a new therapeutic target in this disease (Le Loupp et al., 2015).

L-Pgds Gene Regulation in Macrophages

Incubation with LPS or *Pseudomonas* upregulates *L-Pgds* gene expression in mouse bone marrow-derived macrophages and macrophage cell line RAW 264.7 cells, in which AP-1 and p53 regulate the *L-Pgds* gene expression positively and negatively, respectively (Joo et al., 2007). Binding of PU.1, a transcription factor essential for macrophage development and inflammatory gene expression, to the cognate site in the *L-Pgds* gene promoter mediates the L-PGDS induction. LPS stimulation triggers TLR4 signaling and activates casein kinase II (CKII). CKII phosphorylates PU.1 at Ser148. The activated phosphorylated PU.1 binds to its cognate site in the promoter. The TLR4 signaling also activates JNK and p38 kinase that phosphorylate and activate cJun. Activated phosphorylated cJun binds to both PU.1 and the AP-1 site of the promoter. PU.1 and cJun form a transcriptionally active complex in the *L-Pgds* gene promoter, leading to *L-Pgds* gene expression (Joo et al., 2009). Therefore, L-PGDS is also important for the innate immunity.

L-Pgds Gene Regulation in Chondrocytes

L-Pgds gene expression is upregulated in human primary cultured chondrocytes by treatment with IL-1β. Inhibitors of MAPK p38, c-jun N-terminal kinase (JNK), and the NF-κB/Notch signaling pathways suppress the *L-Pgds* gene upregulation (Zayed et al., 2008).

Lipocalin-Type Prostaglandin D_2 Synthase Studies in Mast Cell Maturation

Lipocalin-type prostaglandin D_2 synthase in fibroblasts is important for maturation of mouse and human mast cells (Taketomi et al., 2013). Mast cells secrete a group III phospholipase A_2 (PLA2G3), a mammalian ortholog of anaphylactic bee venom phospholipase A_2. The secreted PLA2G3 couples with L-PGDS in neighboring fibroblasts to provide PGD_2. The PGD_2 produced by fibroblasts stimulates DP receptors on mast cells to facilitate maturation of mast cells. Mast cells maturation and anaphylaxis are impaired in KO mice of PLA2G3, L-PGDS or DP, mast cell–deficient mice reconstituted with PLA2G3-null or DP-null mast cells, or mast cells cultured with *L-Pgds* gene–ablated fibroblasts. The PLA2G3-L-PGDS-DP paracrine axis is important for the innate immunity and inflammation by the mast cell-fibroblast interaction.

Lipocalin-Type Prostaglandin D_2 Synthase Studies in Cells of the Skin

Follicular melanocytes in the mouse skin express L-PGDS. B16 mouse melanoma cells express L-PGDS under the control of microphthalmia-associated transcription factor (MITF) responsible for differentiation of melanocytes (Takeda et al., 2006). In human epidermal keratinocytes, antimycotics induce PGD_2 release and suppress the expression of thymic stromal lymphopoietin, the NF-kB activity, and IkBα degradation induced by poly I:C plus IL-4. L-PGDS inhibitor AT-56 counteracts the antimycotic-induced suppression of thymic stromal lymphopoietin production and the NF-kB activity (Hau et al., 2013).

Lipocalin-Type Prostaglandin D_2 Synthase Studies in Cancer Cells

Overexpression of Yes-associated protein 1 (YAP) suppresses *L-Pgds* and *Ptgdr2* gene expression in gastric cancer cells. Overexpression of *L-Pgds* and *Ptgdr2* genes decreases proliferation and self-renewal of gastric cancer cells by YAP. These results indicates that YAP inhibits *L-Pgds* and *Ptgdr2* gene expression to promote self-renewal of gastric cancer cells (Bie et al., 2020). Chemotherapeutics induce Cox-2 and lead apoptosis of human cervical carcinoma cells. *L-Pgds* gene knockdown by siRNA prevents the chemotherapeutics-induced apoptosis, suggesting that the apoptosis occurs through PGD_2 production by L-PGDS (Eichele et al., 2008).

Lipocalin-Type Prostaglandin D_2 Synthase Studies in Cells of the Prostate Gland

Bisphenol A, a synthetic plasticizer widely used to package daily necessities, induces *Pghs2* and *L-Pgds* gene expression in human prostate fibroblasts and epithelial cells. Cox-2 inhibitor NS398 and L-PGDS inhibitor AT56 suppress the cell proliferation enhanced by bisphenol A and increase apoptosis of those cells. Thus, Cox-2 and L-PGDS mediate low-dose bisphenol A-induced prostatic hyperplasia through pathways involved in cell proliferation and apoptosis (Wu et al., 2020).

Lipocalin-Type Prostaglandin D_2 Synthase Studies in Seminal Plasma and Oviduct Fluid

The seminal plasma and oviduct fluid contain L-PGDS. Pretreatment of bovine sperm and/or oocytes with anti-L-PGDS antibody inhibits *in vitro* fertilization and increases sperm-oocyte binding (Gonçalves et al., 2008a). Anti-L-PGDS antibody reacts with cow oocytes incubated with oviduct fluid. Pretreatment of oocytes with anti-L-PGDS antibody inhibits sperm binding, fertilization and embryonic development *in vitro* (Gonçalves et al., 2008b).

Amphibian Ortholog of Lipocalin-Type Prostaglandin D_2 Synthase and Its Function During Development of *Xenopus* Embryo

Amphibian ortholog of *L-Pgds* gene is identified and its protein is expressed in *Xenopus* A6 cells. Amphibian ortholog of L-PGDS is associated with both PGD_2 synthase activity and *all-trans*-retinoic acid-binding activity (Irikura et al., 2007). During development of *Xenopus* embryo, amphibian *L-Pgds* gene expression is induced by zinc-finger transcription factor Zic 1 specifically at the anterior neural plate and allows for the localized production and transport of retinoic acid, which in turn activates a cranial placode developmental program in neighboring cells (Jaurena et al., 2015). This effect is reproduced by the Cys65Ala mutant of amphibian L-PGDS, indicating that amphibian L-PGDS functions independently of its enzymatic activity yet as an extracellular retinoic acid transporter.

Intracellular Interaction of Lipocalin-Type Prostaglandin D_2 Synthase With D Type of Prostanoid Receptors in HEK293 or HeLa Cells

Lipocalin-type prostaglandin D_2 synthase interacts intracellularly with DP receptors co-expressed in HEK293 or HeLa cells. L-PGDS or its Cys65Ala mutant promotes cell surface expression of DP receptors, but not of other G-protein coupled receptors including CRTH2 receptors. Interaction of L-PGDS with the C-terminal MEEVD residues of Hsp90 within cells is crucial for export of DP receptors to the cell surface. Co-expression of L-PGDS with DP receptors and Hsp90 promotes PGD_2 synthesis. Depletion of *L-Pgds* gene decreases DP receptor-mediated ERK1/2 activation. L-PGDS inhibitor AT-56 or DP antagonist BWA868C inhibit L-PGDS-induced ERK1/2 activation. These results indicate that L-PGDS increases the DP receptor-ERK1/2 complex formation and increases DP receptor-mediated ERK1/2 signaling as an intracrine/autocrine signaling mechanism (Binda et al., 2014).

Depletion of endogenous *L-Pgds* gene in HeLa cells decreases recycling of endogenous DP receptors to the cell surface after agonist-induced internalization. *L-Pgds* gene overexpression increases the recycling of DP receptors. Depletion of endogenous GTPase Rab4 prevents L-PGDS-mediated recycling of DP receptors. *L-Pgds* gene depletion inhibits Rab4-dependent recycling of DP receptors. These results indicate that L-PGDS and Rab4 are involved in the recycling of DP receptors. DP receptor stimulation promotes interaction between the intracellular C terminus of DP receptors with Rab4 to form the L-PGDS/Rab4/DP complexes. L-PGDS interacts preferentially with the inactive, GDP-locked Rab4S22N variant rather than with wild-type Rab4 or with constitutively active Rab4Q67L proteins. L-PGDS is involved in Rab4 activation after DP stimulation by enhancing GDP-GTP exchange on Rab4. The region of amino acid residues between 85 and 92 in L-PGDS is proposed to be involved in the interaction with Rab4 and DP recycling, as assessed by deletion mutants and using synthetic peptides (Binda et al., 2019). These mechanisms may amplify the autocrine L-PGDS/PGD_2/DP receptor function.

MAMMALIAN EXPERIMENTS FOR THE STUDY OF LIPOCALIN-TYPE PROSTAGLANDIN D_2 SYNTHASE

Lipocalin-type prostaglandin D_2 synthase is enriched in the CNS, male genital organs, skin of various mammals, and the human heart; and involved in a variety of pathophysiological function. Recent studies using various gene-manipulated mice including systemic or cell/tissue-selective KO mice of *L-Pgds* and *Ptgdr*,

Ptgdr2, *Ptgfr* genes explore the understanding of the function of L-PGDS, as summarized in **Supplementary Table 6**.

Lipocalin-Type Prostaglandin D_2 Synthase-Mediated Functions in Central Nervous System

In the CNS, L-PGDS is predominantly localized in leptomeningeal cells (arachnoid trabecular cells, arachnoid barrier cells and arachnoid border cells), choroid plexus epithelial cells and oligodendrocytes (Urade et al., 1993; Beuckmann et al., 2000), and is secreted into the CSF to be β-trace (Hoffmann et al., 1993; Watanabe et al., 1994). PGD_2 is the most potent endogenous sleep-inducing substance (Urade and Hayaishi, 2011) as well as a potent inflammatory mediator (Urade and Hayaishi, 2000b). *L-Pgds* gene KO and TG mice show normal sleep pattern, suggesting that sleep of these mice is normalized by unknown compensation mechanism against gene-manipulation. PGD_2-induced sleep is mediated by both adenosine A_{2A} receptor-dependent and independent systems (Zhang et al., 2017a). Several cytokines, such as IL-1β and TNFα induce sleep in a PGD_2-independent manner (Zhang et al., 2017b), Therefore, adenosine and those cytokine systems may be involved in sleep maintenance by compensation for the *L-Pgds* gene deletion. RNA-Seq analyses of the brain of KO mice of *L-Pgds* and/or *ptgdr* genes will provide the information to understand the compensation mechanism.

However, a variety of abnormality was detected in *L-Pgds* gene-manipulated mice after any stimulation or pathological conditions. *L-Pgds* gene KO mice exhibit many abnormalities in the CNS function including regulation of sleep, pain, neural protection, food intake, and others as follows:

Pain Regulation

L-Pgds gene KO mice do not show allodynia (touch-evoked pain) after an intrathecal administration of PGE_2- or bicuculline, a $GABA_A$-antagonist (Eguchi et al., 1999). The PGE_2- or bicuculline-induced allodynia is reproduced in *L-Pgds* gene KO mice by administration of subfemtomole amount of PGD_2 (Eguchi et al., 1999). In a rat lumbar disk herniation model with thermomechanical allodynia and degeneration of DRG, overexpression and knockdown of *L-Pgds* gene, respectively, attenuates and worsens the allodynia and tissue degradation (Xu et al., 2021).

Sleep Regulation

Human *L-Pgds* gene-overexpressing TG mice show sleep attack with a transient increase in PGD_2 content in the brain after pain stimulation for tail cutting (Pinzar et al., 2000). *L-Pgds* or *Ptgdr* gene KO mice do not show the rebound sleep after sleep deprivation (Hayaishi et al., 2004). Thus, the L-PGDS/DP system is crucial for the homeostatic regulation of sleep.

An intraperitoneal administration of an L-PGDS inhibitor $SeCl_4$ decreases the PGD_2 concentration in the brain without changing the PGE_2 and $PGF_{2\alpha}$ concentrations and induces complete insomnia during 1hr after the administration. *L-Pgds*, *L-Pgds*/*Hpgds* double or *Ptgdr* gene KO mice do not show the $SeCl_4$-induced insomnia, whereas *Hpgds* or *Ptgdr2* gene KO mice show the $SeCl_4$-induced insomnia, indicating that the insomnia depends on PGD_2 produced by L-PGDS and recognizes by DP receptors (Qu et al., 2006). Pentylenetetrazole induces seizure in wild-type mice with a remarkable increase in the PGD_2 concentration in the brain and induces excess sleep after seizure. The postictal sleep is not induced in *L-Pgds*, *L-Pgds*/*Hpgds* double or *Ptgdr* KO mice but observed in *Hpgds* and *Ptgdr2* KO mice, indicating that the sleep depends on the L-PGDS/PGD_2/DP receptors system (Kaushik et al., 2014).

The $SeCl_4$-induced insomnia is disappeared in leptomeninges-selective *L-Pgds* gene KO mice, which is generated by conditional gene depletion after injection of adeno-associated virus (AAV)-Cre vectors into the subarachnoidal space of newborn *L-Pgds* gene flox mice, but found in oligodendrocytes- or choroid plexus-specific conditional KO mice (Cherasse et al., 2018). These results indicate that the leptomeningeal L-PGDS is important for maintenance of physiological sleep.

Estradiol differentially regulates *L-Pgds* gene expression in the female mouse brain. Estradiol increases *L-Pgds* gene expression in the arcuate and ventromedial nucleus of the medial basal hypothalamus, a center of neuroendocrine secretions, and reduced in the ventrolateral preoptic area, a sleep center (Mong et al., 2003). Estradiol benzoate reduces *L-Pgds* gene expression in the sleep center and induces high motor activity in ovarectomized female mice (Ribeiro et al., 2009).

Protection of Neurons and Glial Cells

Lipocalin-type prostaglandin D_2 synthase plays important roles for protection of neurons and glial cells under various pathological conditions. *L-Pgds* gene expression is upregulated in oligodendrocytes in *twitcher* mice as a model of human globoid cell leukodystrophy (Krabbe disease) caused by the mutation of galactosylceramidase (GALC). In double mutant of $GALC^{twi/twi}$ *L-Pgds* gene KO mice, many neurons and oligodendrocytes exhibit apoptosis, indicating that L-PGDS protect neurons and glial cells against apoptotic loss cause by accumulation of cytotoxic glycosylsphingoid psychosine produced by the lack of GALC (Taniike et al., 2002). *L-Pgds* gene expression is upregulated in oligodendrocytes in mouse models of a variety of lysosomal storage disorders, such as Tay–Sachs disease, Sandhoff disease, GM_1 gangliosidosis, and Niemann–Pick type C1 disease (Mohri et al., 2006a), suggesting that L-PGDS is involved in pathology of these diseases or may protect neurons and glial cells also in these diseases.

In a model of the hypoxic-ischemic encephalopathy of neonates, L-PGDS is induced as an early stress protein and protects neurons in neonatal mice (Taniguchi et al., 2007). *L-Pgds* gene KO mice exhibit greater infarct volume and brain edema after cerebral ischemia than wild type mice (Saleem et al., 2009). In neonatal rats, dexamethasone upregulates *L-Pgds* gene expression in the brain and protects hypoxic-ischemic brain injury. L-PGDS inhibitor SeCl4 or DP antagonist MK-0524 suppress the neuroprotective effect (Gonzalez-Rodriguez et al., 2014). In chronic intermittent hypoxia of adult rats, *L-Pgds* gene expression is increased in the brain from the second week (Shan et al., 2017). *L-Pgds* gene expression is upregulated in COX-2-overexpressing APP/PS1 mice, which exhibit more

severe amyloid fibril formation than APP/PS-1 mice (Guan et al., 2019). These results suggest that L-PGDS is involved in the neuroprotection against the stress condition or the recovery from brain damage.

Amyloid β Clearance

Lipocalin-type prostaglandin D_2 synthase binds Aβ (1–40) and Aβ (1–42) peptides at high affinities (**Supplementary Table 4**) and prevents their fibril formation *in vitro* (Kanekiyo et al., 2007; Kannaian et al., 2019). L-PGDS is a major CSF component responsible for prevention of Aβ fibril formation in human CSF *in vitro* (Kanekiyo et al., 2007). Infusion of Aβ (1–42) peptide into the brain attenuates and worsens, respectively, the Aβ fibril precipitation in the brain in *L-Pgds* gene KO and human *L-Pgds* gene-overexpressing TG mice, as compared with each wild-type mice (Kanekiyo et al., 2007). These results indicate that L-PGDS prevents Aβ fibril formation in the brain *in vivo*.

Depression-Related Behavior

Chronic stress via corticosterone treatment increases mRNA levels of COX-2 and L-PGDS in the brain. *Ptgdr2* gene-KO mice show antidepressant-like activity in a chronic corticosterone treatment-induced depression. The pharmacological inhibition of CRTH2 receptors in wild-type mice with a dual antagonist for CRTH2/TXA receptors, ramatroban, rescues depression-related behavior in chronic corticosterone-, LPS-, and tumor-induced pathologically relevant depression models. These results indicate that the L-PGDS/PGD_2/CRTH2 axis is involved in progression of chronic stress-induced depression (Onaka et al., 2015).

Light-Induced Phase Advance

L-Pgds gene KO mice exhibit impaired light-induced phase advance, while they show normal phase delay and nonvisual light responses. *Ptgdr2* gene KO mice or CRTH2 antagonist CAY10471-administered wild-type mice also show impaired light-induced phase advance. These results indicate that L-PGDS is involved in a mechanism of light-induced phase advance via CRTH2 signaling (Kawaguchi et al., 2020).

Food-Intake

Several reports demonstrate the involvement of L-PGDS in food intake. Fasting upregulates *Ptgs2* and *L-Pgds* gene expression in the hypothalamus of mice, in which the orexigenic center exists. Intracerebroventricular administration of PGD_2 or DP agonist stimulates food intake. DP antagonist, antisense oligonucleotide of DP receptors or an antagonist of neuropeptide Y (NPY) receptors suppress the orexigenic effects. Thus, the L-PGDS/DP/NPY axis regulates the food intake (Ohinata et al., 2008). Oral administration of a δ opioid peptide rubiscolin-6 stimulates food intake in wild-type and *Hpgds* gene KO mice, but not *L-Pgds* or *Ptgdr* gene KO mice. The orexigenic activity is found in *L-Pgds*^{flox}/Nes-Cre mice, which lack *L-Pgds* gene in neurons and glial cells within the brain parenchyma but maintain *L-Pgds* gene in leptomeninges, choroid plexus and cerebroventricular ependymal cells. Thus, PGD_2 produced by L-PGDS in leptomeningeal cells or cerebroventricular ependymal cells mediates the orexigenic effect (Kaneko et al., 2012). The activation of central δ-opioid receptor stimulates normal diet intake mediated by the orexigenic L-PGDS/PGD_2/DP system but conversely suppresses high-fat diet intake through α-MSH/CRF pathway in a PGD_2-independent manner (Kaneko et al., 2014).

Lipocalin-Type Prostaglandin D_2 Synthase in Peripheral Nervous System

Lipocalin-type prostaglandin D_2 synthase and H-PGDS exist in neurons and glial cells, respectively, of chicken DRG (Vesin et al., 1995) and play an important role for maintenance of peripheral nervous system (PNS). *L-Pgds* gene KO mice exhibit hypomyelination of Schwann cells in PNS (Trimarco et al., 2014). In the primary culture, DRG neurons produce L-PGDS and PGD_2, secrete these substances into the extracellular space, and stimulates myelination of Schwann cells. The L-PGDS inhibitor AT56 or gene knockdown of CRTH2 by shRNA suppresses the myelination of Schwann cells. Therefore, the L-PGDS/PGD_2/CRTH2 axis plays important roles as a paracrine signal for development and maintenance of myelination of PNS (Trimarco et al., 2014). L-PGDS is necessary for macrophage activity and myelin debris clearance in a non-cell autonomous way during the resolution of PNS injury. In late phases of Wallerian degeneration, L-PGDS regulates the blood–nerve barrier permeability and SOX2 expression in Schwann cells, prevents macrophage accumulation, and exerts an anti-inflammatory role (Forese et al., 2020). Thus, L-PGDS has a different role during development and after injury in the PNS.

Lipocalin-Type Prostaglandin D_2 Synthase in Lung Inflammation

Prostaglandin D_2 is an important inflammatory mediator involved in allergic asthma and L-PGDS is involved in the pro- and anti-inflammatory functions. Eosinophilic lung inflammation and Th2 cytokine release in ovalbumin-induced asthma model are enhanced in human *L-Pgds* gene-overexpressing TG mice (Fujitani et al., 2010). LPS or *Pseudomonas* treatment upregulates *L-Pgds* gene expression in the lung and alveolar macrophages. Removal of *Pseudomonas* from the lung is accelerated in the TG mice, or by intratracheal instillation of PGD_2 to wild type mice, but impaired in *L-Pgds* gene KO mice. Thus, L-PGDS plays a protective role against the bacterial infection (Joo et al., 2007). L-PGDS inhibitor AT56 suppresses accumulation of eosinophils and monocytes in the broncho-alveolar lavage fluid in an antigen-induced asthma model of *Hpgds* gene KO mice. PGD_2-produced by L-PGDS is involved in inflammatory cell accumulation in the alveolar lavage fluid (Irikura et al., 2009). Intratracheal administration of HCl results in lung inflammation accompanies by tissue edema and neutrophil accumulation. The deficiency of both *L-Pgds* and *Hpgds* genes exacerbates HCl-induced lung dysfunction to a similar extent. In this model, inflamed endothelial/epithelial cells express L-PGDS, while macrophages and neutrophils express H-PGDS. Vascular hyperpermeability in the inflamed lung is accelerated in *L-Pgds* gene KO mice and is suppressed by DP agonist. Thus, PGD_2 is produced locally by L-PGDS in inflamed endothelial and epithelial cells

and enhances the endothelial barrier through DP receptors (Horikami et al., 2019).

Lipocalin-Type Prostaglandin D_2 Synthase in Cardiovascular Function

L-Pgds gene is the most extensively expressed in the human heart (Eguchi et al., 1997) and L-PGDS plays cardioprotective function in several models. Estrogen induces *L-Pgds* gene expression in the heart of female mice by stimulating estrogen receptor (ER) β. An estrogen-responsive element in the *L-Pgds* gene promoter region is activated by ERβ, but not by ERα (Otsuki et al., 2003). Chronic hypoxia (10% O2) upregulates *L-Pgds* gene expression in the mouse heart. L-PGDS increases in the myocardium of auricles and ventricles and the pulmonary venous myocardium at 28 days of hypoxia. *L-Pgds* gene expression in the heart is two-fold more higher in heme oxygenase-2 KO mice, a model of chronic hypoxemia, than that of wild-type mice. Hypoxemia increases *L-Pgds* gene expression in the myocardium to adapt to the hemodynamic stress (Han et al., 2009).

Glucocorticoid stimulation with corticosterone or cortivazol induces calcium-dependent $cPLA_2$, Cox-2 and L-PGDS in neonatal mouse cardiomyocytes. Glucocorticoids upregulate the expression of $cPLA_2$, *Ptgs2* and *L-Pgds* genes and stimulate PGD_2 synthesis in adult mouse heart. In isolated Langendorff-perfused mouse hearts, dexamethasone protects ischemia/reperfusion injury. Cox-2 inhibitor or depletion of *L-Pgds* gene completely suppresses the cardioprotective effect of dexamethasone. Dexamethasone reduces the infarct size by *in vivo* ischemia/reperfusion experiments of wild-type mice. The cardioprotective effect is markedly reduced in *L-Pgds* gene KO mice (Tokudome et al., 2009). Dexamethasone upregulates the expression of target genes characteristic erythroid-derived 2–like 2 (Nrf2) in wild-type but not *L-Pgds* gene-KO mice. Dexamethasone increases Nrf2 expression in an L-PGDS-dependent manner. Nrf2 KO mice do not show L-PGDS-mediated, dexamethasone-induced cardioprotection. Dexamethasone induces FP receptors in the mouse, rat and human heart. *Ptgfr* gene KO mice do not show the dexamethasone-induced cardioprotection. FP receptors bind PGD_2 and $PGF_{2\alpha}$ with almost identical affinities. These results indicate that the dexamethasone-induced cardioprotective effect is mediated by the L-PGDS/PGD_2/FP receptors axis through the Nrf2 pathway (Katsumata et al., 2014).

In perfused beating rat atria, hypoxia increases hypoxia-inducible factor (HIF) 1α, stimulates atrial natriuretic peptide (ANP) secretion, and upregulates *Ptgs2* and *L-Pgds* gene expression. HIF-1α antagonist, 2-methoxyestradiol, downregulates HIF-1α, *Ptgs2* and *L-Pgds* gene expression, and decreases hypoxia-induced ANP secretion. L-PGDS inhibitor AT-56 downregulates L-PGDS protein levels. The hypoxia-induced ANP secretion increases PPARγ protein levels and PPARγ antagonist GW9662 attenuates it. 2-Methoxyestradiol and AT-56 inhibit hypoxia-induced increase in atrial PPARγ protein. Thus, hypoxia activates the HIF-1α-L-PGDS-PPARγ signaling to promote ANP secretion in beating rat atria (Li et al., 2018). In the same model, acute hypoxia stimulates endothelin (ET)-1 release and expression of ET_A and ET_B receptors. ET-1 upregulates *Ptgs2* and *L-Pgds* gene expression and increases PGD_2 production through activation of ET_A and ET_B receptors. L-PGDS-derived PGD_2 promotes hypoxia-induced ANP secretion and in turn regulates *L-Pgds* gene expression by an Nrf2-mediated feedback mechanism. ET-1 induced by hypoxia activates the Cox-2/L-PGDS/PGD_2 signaling and promotes ANP secretion. The positive feedback loop between L-PGDS-derived PGD_2 and hypoxia-induced *L-Pgds* gene expression is a part of the mechanism of hypoxia-induced ANP secretion by ET-1 (Li et al., 2019).

Lipocalin-Type Prostaglandin D_2 Synthase in Obesity and Adipocyte Differentiation

A high-fat diet (HFD) upregulates *L-Pgds* gene expression in adipose tissues and differentiates adipocytes (Fujimori et al., 2007a, 2012b; Chowdhury et al., 2011) so that *L-Pgds* gene KO mice show several abnormalities in the regulation of energy homeostasis. *L-Pgds* gene KO mice exhibit glucose-intolerant and insulin-resistant at an accelerated rate as compared with wild-type mice. Adipocytes are larger in *L-Pgds* gene KO mice than those of control mice with the same diet. *L-Pgds* gene KO mice develop nephropathy and an aortic thickening reminiscent to the early stages of atherosclerosis when fed HFD (Ragolia et al., 2005). Adipocytes of *L-Pgds* gene KO mice are less sensitive to insulin-stimulated glucose transport than those of wild-type mice (Ragolia et al., 2008).

L-Pgds gene KO mice exhibit body weight gain more than WT mice when fed HFD, and increase subcutaneous and visceral fat tissues. HFD-fed *L-Pgds* gene KO mice exhibit increased fat deposition in the aortic wall, atherosclerotic plaque in the aortic root, macrophage cellularity and the expression of pro-inflammatory cytokines such as IL-1β and monocyte chemoattractant protein-1. Thus, *L-Pgds* gene deficiency induces obesity and facilitates atherosclerosis through the regulation of inflammatory responses (Tanaka et al., 2008).

L-Pgds gene KO mice display features of the metabolic syndrome in the absence of HFD as well as with HFD feeding and this correlates with hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis, i.e., increases in plasma ACTH and corticosterone concentrations, at 20-week-old. C57BL/6 mice exhibit age-related increases in HPA activity, whereas *L-Pgds* gene KO mice are resistant to changes in HPA activity with age and long-term HFD feeding. Thus, these events depend on *L-Pgds* gene expression (Evans et al., 2013).

Vertical sleeve gastrectomy (VSG) is used to improve metabolic complications in patients with obese and diabetes. VSG improves glycemic parameters 10 weeks after operation in WT and *L-Pgds* gene knock-in (KI) mice but not in *L-Pgds* gene KO mice, as compared with the sham-operated

group. *L-Pgds* gene KO mice develop glucose intolerance and insulin resistance even after VSG similar to or greater than the sham group. *L-Pgds* gene KO mice exhibit post-VSG peptide YY levels slightly increased but significantly less than other groups and the leptin sensitivity in response to VSG less than KI mice. Total cholesterol level is unchanged in all groups irrespective of sham or VSG surgery. However, *L-Pgds* gene KO mice show higher cholesterol levels and increase the adipocyte size in post-VSG. Thus, L-PGDS plays an important role in the beneficial metabolic effects by VSG (Kumar et al., 2016).

L-Pgds gene KO mice show hypertension and acceleration of thrombogenesis but *Hpgds* gene KO mice do not change these functions (Song et al., 2018). Therefore, L-PGDS is also important for the control of blood pressure and thrombosis.

There are two distinct types of adipose-specific *L-Pgds* gene KO mice: one is fatty acid binding protein 4 (fabp4, aP2)-Cre/*L-Pgds*$^{flox/flox}$ mice and the other is adiponectin (AdipoQ)-Cre/*L-Pgds*$^{flox/flox}$ mice. The former strain lacks the *L-Pgds* gene in adipocytes even in the premature stage and the latter strain, only after maturation. The former strain decreases *L-Pgds* gene expression and PGD_2 production levels in white adipose tissue under HFD conditions, whereas the latter strain does not change the L-PGDS and PGD_2 levels. When fed an HFD, the former strain aP2-Cre/*L-Pgds*$^{flox/flox}$ mice reduce body weight gain, adipocyte size, and serum cholesterol and triglyceride levels. In white adipose tissue of HFD-fed aP2-Cre/*L-Pgds*$^{flox/flox}$ mice, the expression levels of adipogenic, lipogenic, and M1 macrophage marker genes are decreased, whereas the lipolytic and M2 macrophage marker genes are enhanced or unchanged. Insulin sensitivity is improved in HFD-fed aP2-Cre/*L-Pgds*$^{flox/flox}$ mice. Therefore, PGD_2 produced by L-PGDS in premature adipocytes is involved in the regulation of body weight gain and insulin resistance under nutrient-dense conditions (Fujimori et al., 2019).

In PPARγ-KO mice, *L-Pgds* gene expression is increased in brown adipose tissue (BAT) and subcutaneous white adipose tissue but reduced in the liver and epididymal fat (Virtue et al., 2012b). Double KO mice of PPARγ and *L-Pgds* genes exhibit reduced expression of thermogenic genes, the *de novo* lipogenic program and the lipases in subcutaneous white adipose tissue but elevated expression of lipolysis genes in epididymal fat. These results indicate that PPARγ and L-PGDS coordinate to regulate carbohydrate and lipid metabolism (Virtue et al., 2012b). In BAT, *L-Pgds* gene expression increases after HFD feeding or cold exposure at 4C (Virtue et al., 2012a). The L-PGDS induction depends on PGC1α and 1β, positive regulators of BAT activation, and is reduced by RIP140, a negative regulator of BAT activation. Under cold–acclimated conditions, *L-Pgds* gene KO mice exhibit elevated reliance on carbohydrate used for thermogenesis and increase expression of genes regulating glycolysis and *de novo* lipogenesis in BAT.

In *ob/ob* mice, *L-Pgds* gene expression is decreased in white adipose tissue whereas *Hpgds* gene expression is markedly increased. In white adipose tissue, H-PGDS exists in macrophages and is involved in polarization of macrophages toward to M2, anti-inflammatory, state in both mice and human (Virtue et al., 2015).

Lipocalin-Type Prostaglandin D_2 Synthase in Bone and Cartilage Metabolism

In the mouse model of collagen-induced arthritis (CIA), PGD_2 is produced in the joint during the early phase. Serum PGD_2 levels increase progressively throughout the arthritic process and reach to a maximum during the late stages. The expression of *L-Pgds*, *Hpgds*, *Ptgdr*, and *Ptgdr2* genes increases in the articular tissues during arthritic process. DP antagonist MK0524 increases the incidence and severity of CIA, and the local levels of IL-1β, CXCL-1, and PGE_2 but reduces the IL-10 levels. CRTH2 antagonist CAY10595 does not modify the severity of arthritis. The administration of PGD_2 or a DP agonist BW245C reduces the incidence of CIA, the inflammatory response, and joint damage. In the articular tissue during development of CIA, PGD_2 plays an anti-inflammatory role through DP receptors (Maicas et al., 2012). In the spontaneous Hartley guinea pig and experimental dog arthritis models, *L-Pgds* gene expression also increases over the course of osteoarthritis. In the guinea pig model, L-PGDS levels are correlated positively with the histological score of osteoarthritis (Nebbaki et al., 2013).

In an experimental osteoarthritis model induced by destabilization of the medial meniscus, *L-Pgds* gene KO mice exhibit exacerbated cartilage degradation and enhanced expression of matrix metalloproteinase 13 (MMP-13) and a disintegrin and metalloprotein-ase with thrombospondin motifs 5 (ADAMTS-5), and display increased synovitis and subchondral bone changes (Najar et al., 2020). Cartilage explants from *L-Pgds* gene KO mice show enhanced proteoglycan degradation after treatment with IL-1α. Intra-articular injection of AAV2/5 encoding *L-Pgds* gene attenuates the severity of osteoarthritis in wild-type mice, by an increase in L-PGDS level in the osteoarthritis tissue. *L-Pgds* gene KO mice show the accelerated development of naturally occurring age-related osteoarthritis (Ouhaddi et al., 2020). *L-Pgds* gene deletion promotes cartilage degradation during aging and enhances expression of extracellular matrix degrading enzymes, MMP-13 and ADAMTS-5, and their breakdown products. Moreover, *L-Pgds* gene deletion enhances subchondral bone changes without effect on its angiogenesis, increases mechanical sensitivity, and reduces spontaneous locomotor activity. *L-Pgds* gene expression increases in aged mice, suggesting that L-PGDS plays an important role to protect against naturally occurring age-related osteoarthritis. L-PGDS may be a new efficient therapeutic target in osteoarthritis.

Lipocalin-Type Prostaglandin D_2 Synthase in Keratinocytes and Hair Follicle Neogenesis

In a wound-induced hair follicle neogenesis model, L-PGDS and PGD_2 levels of the skin are negatively correlated with the hair follicle neogenesis among C57Bl/6J, FVB/N, and mixed strain

mice (Nelson et al., 2013). The hair follicle regeneration increases in mice with an alternatively spliced transcript variant of *L-Pgds* gene without exon 3 and *Ptgdr2* gene KO mice, but not in *Ptgdr* gene KO mice. Keratinocytes produce L-PGDS in the skin. PGD_2 produced by L-PGDS in keratinocytes inhibits wound-induced hair follicle neogenesis through CRTH2 receptors.

Lipocalin-Type Prostaglandin D_2 Synthase in Mast Cell Differentiation and Anaphylaxis

Mast cells play important roles in anaphylaxis. Phenotypes of mast cells are changed by microenvironment. Mast cells dominantly express H-PGDS (Urade et al., 1990) and release PGD_2 after anti-IgE stimulation. For maturation of bone-marrow derived mast cells *in vitro*, it is necessary for immature mast cells to interact with fibroblasts, suggesting that fibroblasts release some mediators for maturation of mast cells. The mediator is PGD_2 produced by L-PGDS in fibroblasts1. Mast cells secrete phospholipase A_2 (PLA2G3), which couples to the Cox/L-PGDS system in fibroblasts to produce PGD_2. PGD_2 released from fibroblasts then stimulates DP receptors on immature mast cells to promote their maturation (Taketomi et al., 2013).

Lipocalin-Type Prostaglandin D_2 Synthase in Colon

In experimental colitis model with dextran sodium sulfate in the drinking water, the disease activity reduces in *L-Pgds* gene KO mice than WT mice (Hokari et al., 2011). PGD_2 derived from L-PGDS plays pro-inflammatory roles in the dextran-induced colitis.

Lipocalin-Type Prostaglandin D_2 Synthase in Adenoma

In tumor generation in $Apc^{Min/+}$ mice mated with various mutations of *L-Pgds*, *Hpgds* and *Ptgdr* genes, adenoma production is enhanced in KO mice of *Hpgds* or *Ptgdr* gene but slightly reduced in *L-Pgds* gene KO mice. $Apc^{Min/+}$ mice overexpressing human *Hpgds* or *L-Pgds* gene suppress the tumor generation (Tippin et al., 2014).

Lipocalin-Type Prostaglandin D_2 Synthase in Melanoma

Lipocalin-type prostaglandin D_2 synthase is expressed in endothelial cells of human melanoma and oral squamous cell carcinoma (Omori et al., 2018). Human endothelial cells produce L-PGDS and PGD_2 after stimulation with IL-1 and TNFα derived from tumor cells. Melanoma growth is accelerated in *L-Pgds* gene KO mice or endothelial cell-specific *L-Pgds* gene KO mice and is attenuated by administration of a DP agonist BW245C. *L-Pgds* gene deficiency in endothelial cells accelerates vascular hyperpermeability, angiogenesis, and endothelial-mesenchymal transition in tumors to reduce tumor cell apoptosis. Tumor cell-derived inflammatory cytokines increase *L-Pgds* gene expression and PGD_2 production in tumor endothelial cells. PGD_2 is a negative regulator of the tumorigenic changes in tumor endothelial cells.

Lipocalin-Type Prostaglandin D_2 Synthase in Renal Function

In a mouse model of Adriamycin-induced nephropathy, L-PGDS is induced in tubules including proximal, Henle's loop and distal compartments of the kidney (Tsuchida et al., 2004). Urinary L-PGDS excretion increases from day 1 onward, and apparently precedes the increase in urinary albumin excretions. Neither serum L-PGDS nor creatinine levels are changed by administration of Adriamycin. However, serum creatinine levels are inversely correlated to urinary L-PGDS excretions. The urinary L-PGDS is a useful marker of renal permeability dysfunction.

Otsuka Long-Evans Tokushima Fatty (OLETF) rats develop diabetes associated with hypertension and exhibit higher urinary L-PGDS excretion than non-diabetic Long-Evans Tokushima Otsuka rats (Ogawa et al., 2006). The urinary L-PGDS excretion in OLETF rats increases in an age-dependent manner and is due to increased glomerular permeability to L-PGDS. Renal tissue contains L-PGDS mRNA and immunoreactivity. However, glomerular filtration of L-PGDS contributes to urinary L-PGDS excretion much more than the *de novo* synthesis. Multiple regression analysis shows that urinary L-PGDS is determined by urinary protein excretions and not by high blood pressure *per se*. Conversely, the urinary L-PGDS excretion in the early stage of diabetes predicts the urinary proteinuria in the established diabetic nephropathy.

Crude extracts of monkey kidney and human urine contain L-PGDS isoforms with its original N-terminal sequence starting from Ala23 after the signal sequence, and from Gln31 and Phe34 with its N-terminal-truncation (Nagata et al., 2009). The mRNA and the intact form of L-PGDS exist in the cells of Henle's loop and the glomeruli of the kidney, as examined by *in situ* hybridization and immunostaining with monoclonal antibody 5C11, which recognizes the amino-terminal loop from Ala23 to Val28 of L-PGDS. Those cells and tissues produce L-PGDS *de novo* within the kidney. Truncated forms of L-PGDS exist in the lysosomes of tubular cells, as visualized by immunostaining with monoclonal antibody 10A5, which recognizes the 3-turn α-helix between Arg156 and Thr173 of L-PGDS. Therefore, tubular cells uptake L-PGDS and degrade within lysosomes to produce the truncated form.

Lipocalin-type prostaglandin D_2 synthase contributes to the progression of renal fibrosis via CRTH2-mediated activation of Th2 lymphocytes (Ito et al., 2012). In a mouse model of renal fibrosis caused by unilateral ureteral obstruction, the tubular epithelium produces L-PGDS *de novo*. *L-Pgds* or *Ptgdr2* gene KO mice exhibit less renal fibrosis, reduce infiltration of Th2 lymphocytes to the cortex, and decrease production of Th2 cytokines IL-4 and IL-13. Administration of a CRTH2 antagonist Cay10471 at 3 days after the obstruction suppresses the progression of renal fibrosis. IL-4- or IL-13 KO mice also ameliorate the kidney fibrosis in the unilateral obstruction model. Blocking of CRTH2 receptors may be useful to slowdown the progression of renal fibrosis in chronic kidney diseases.

Lipocalin-Type Prostaglandin D_2 Synthase in Preterm Birth

LPS-induced preterm birth occurs 89% and 100%, respectively, in C57BL/6 mice and *L-Pgds* gene-overexpressing TG mice and significantly reduced to 40% in *L-Pgds* gene KO mice (Kumar et al., 2015). Administration of DP or CRTH2 antagonists to C57BL/6 mice increases the number of viable pups 3.3-fold, indicating that PGD_2-mediated inflammation is involved in the preterm birth and the dead birth.

Lipocalin-Type Prostaglandin D_2 Synthase in Uterus

Double KO mice of both *L-Pgds* and *Hpgds* genes develop adenomyotic lesions in the uterus at 6-month-old (Philibert et al., 2021). The disease severity increases with age, suggesting that the PGD_2 signaling has major roles in the uterus by protecting the endometrium against development of adenomyosis.

Lipocalin-Type Prostaglandin D_2 Synthase in Testis and Epididymis

Male genital organs highly express L-PGDS in various animals including humans (Tokugawa et al., 1998; Gerena et al., 2000). The regional distribution and regulation of L-PGDS expression are examined in rat testis and epididymis by *in situ* hybridization and immunohistochemistry under the conditions of sexual maturation, castration, and ethylene dimethane sulfonate treatments, which eliminates Leydig cells in the testicular interstitium (Zhu et al., 2004). In sexually mature rats, testicular peritubular cells weakly expressed L-PGDS but Leydig cells in the testis highly expressed L-PGDS by day 70 postpartum. L-PGDS is highly detected in the caput, corpus, and cauda of the epididymis during sexual maturation 70 days after birth. Castration and ethylene dimethane sulfonate treatments significantly decrease L-PGDS expression in the epididymis. Testosterone propionate treatment increases L-PGDS expression in the epididymis of both castrated and ethylene dimethane sulfonate-treated rats. Testosterone up-regulates *L-Pgds* gene expression in rat epididymis.

Both heterozygous and homozygous *L-Pgds* gene KO mice present unilateral cryptorchidism affecting the second phase of testicular descent in 16% and 24% of cases, respectively (Philibert et al., 2013). The adult cryptorchid testes increases the spermatogonia apoptosis and decreases the global tubule size parameters. The gubernaculum of newborn mutants shows some histological abnormalities. In 29 children with cryptorchidism, none of the investigated cases presented mutations in the *L-Pgds* gene. L-PGDS is a novel component in the cryptorchidism (Philibert et al., 2013).

Lipocalin-Type Prostaglandin D_2 Synthase in Prostate Gland

Administration of a low dose of bisphenol A to male SD rats induces prostatic hyperplasia and upregulates *Ptgs2* and *L-Pgds* gene expression in the prostate. The prostatic hyperplasia is suppressed by administration of inhibitors for Cox-2 and L-PGDS with increased apoptosis levels, indicating that Cox-2 and L-PGDS mediate low-dose bisphenol A-induced prostatic hyperplasia (Wu et al., 2020).

Lipocalin-Type Prostaglandin D_2 Synthase in Male Germ Cell Differentiation in the Fetal Testis

The male/female ratio and the number of infant are unchanged among WT, KO mice of *L-Pgds*, *Hpgds*, *ptgdr* and *ptgdr2* genes, and double KO mice of *L-Pgds* and *Hpgds* genes. However, the delayed development of fetal mouse testis occurs in several KO lines, indicating that PGD_2 produced by L-PGDS and H-PGDS is involved in differentiation of somatic and germ cells in testis by stimulating DP and CRTH2 receptors, respectively. The role of the PGD_2 signaling pathway in reproduction was reviewed (Rossitto et al., 2015). L-PGDS is one of the most male-enriched gene transcripts expressed at 12.5 days post coitum (dpc) in an early stage of mouse embryonic gonads (Adams and McLaren, 2002). The time course and place of L-PGDS expression in mouse embryo are similar to those of the sex-determining factor Sox9. In embryonic gonads, L-PGDS exists only in male XY gonads but not in female XX gonads. L-PGDS is expressed downstream of Sox9. PGD_2 is an autocrine factor inducing Sox9 nuclear translocation after DP receptor-mediated cAMP-PKA phosphorylation, which induces subsequent Sertoli cell differentiation (Malki et al., 2005). Sox9 dimer binds to a paired Sox/Sry binding site in a promoter region of the L-PGDS gene and trans-activates the male-specific L-PGDS gene expression (Wilhelm et al., 2007) to make a positive feed-back loop of the L-PGDS-Sox9 signaling. In Sox9 KO mice, L-PGDS disappears in embryonic Sertoli cells of the XY gonads. In L-PGDS KO mice, the Sox9 transcript decreases in Sertoli cells of the XY embryonic gonad and the subcellular localization of Sox9 protein is perturbed (Moniot et al., 2009). H-PGDS exists in somatic and germ cells of the embryonic gonad of both sexes at embryonic day 10.5, before the onset of L-PGDS expression. Administration of H-PGDS inhibitor HQL79 to WT mice suppresses the nuclear translocation of Sox9 protein in embryonic Sertoli cells. In *Hpgds* gene KO mice, Sox 9 protein remains mainly within the cytoplasm of embryonic Sertoli cells at the early stage of mouse testicular differentiation. H-PGDS-produced PGD_2 is involved in the initial nuclear translocation of Sox9 during the early stage of testicular differentiation (Moniot et al., 2011). In double KO mice of *L-Pgds* and *Hpgds* genes, i.e., depleted for PGD_2, and *ptgdr2* gene KO mice, a significant proportion of the germ cells are not mitotically arrested and still engaged in the cell cycle at a time which should be quiescent (Moniot et al., 2014). Germ cells of those KO mice downregulate cell cycle inhibitors $p21^{Clip1}$ and $p57^{Kip2}$ and upregulate cell cycle activators CyclinE1 and E2. Therefore, the PGD_2 signaling is involved in the control of cell cycle genes in fetal testis to arrest mitotic process. At late embryonic stages of those KO mice, the ectopic expression of pluripotency markers Pou5f1 (Oct4), Sox2 and Nanog is detected in the testis and the male germ cell marker Nanos2 is downregulated. The PGD_2 system is also involved in germ cell differentiation in the embryonic testis. Somatic factor Cyp26B1 is a retinoic acid–metabolizing enzyme of P450 family produced by

Sertoli cells. Cyp26B1 protects germ cells from retinoic acid. The mutant gonads reduce Cyp26B1. Thus, PGD_2 produced by Sertoli cells influences the differentiation of the embryonic germ cells.

The L/H-PGDS/PGD_2 system is important for sex organ development. Therefore, the *in utero* exposure to NSAIDs, such as acetaminophen and ibuprofen, during the sex determination period results in malfunction of both male and female genital organs and also intergenerational subfertility (Rossitto et al., 2019a,b).

PHARMACOKINETIC ANALYSES AND FUNCTIONALIZATION WITH RECOMBINANT LIPOCALIN-TYPE PROSTAGLANDIN D_2 SYNTHASE

Pharmacokinetic analyses with recombinant L-PGDS after an intravenous injection to dogs (Li et al., 2008) reveal that the serum concentration of L-PGDS decreases with t1/2 of 0.77 h, which is shorter than that of other proteins with the same molecular mass as that of L-PGDS. Only about 10% of administered L-PGDS is recovered into the urine. Thus, majority of L-PGDS seems to be taken up by the tissues and/or degraded within the body. After the intrathecal injection, about one third of the administered L-PGDS is excreted to the blood, suggesting that L-PGDS is degraded or taken up by neural cells within the CNS or excreted through the lymphatic fluid.

Lipocalin-type prostaglandin D_2 synthase is useful for functionalization of nanoparticles to increase the brain-blood barrier permeability and intracellular uptake efficiencies (Portioli et al., 2017). L-PGDS-conjugated nanoparticles are taken up by neurons and glial cells mediated by an LDL receptor-mediated mechanism after administration from the tail vein of mice. This report also suggest that other cells uptake L-PGDS through LDL receptors as an intercellular transporter of various lipophilic substances. The intercellular transport of L-PGDS may be involved in the finding that L-PGDS protein injected into the mouse brain promotes migration and accumulation of astrocytes *in vivo* (Lee et al., 2012).

STUDIES OF NONMAMMALIAN ORTHOLOGS OF LIPOCALIN-TYPE PROSTAGLANDIN D_2 SYNTHASE

There are many studies of L-PGDS in non-mammals, including birds, amphibians and fishes.

In chicken DRG, the L-PGDS-immunoreactivity is detected in neurons and the H-PGDS-immunoreactivity, in glial cells (Vesin et al., 1995). cDNA for the chicken ortholog of L-PGDS is identified (Fujimori et al., 2006). The recombinant chicken L-PGDS is associated with the weak PGD_2 synthase activity of about 3% of that of the mouse enzyme and possesses the binding activities of retinoic acid and thyroxine with almost comparable affinities (Kd = 0.6–0.7 μM) to those of the mouse enzyme (Fujimori et al., 2006). The chicken L-PGDS is also expressed in a male specific mechanism regulated by Sox9 (Moniot et al., 2008), similar to mouse L-PGDS.

The amphibian ortholog of L-PGDS is identified in three different species of frogs, *Xenopus laevis*, Cane Todd (*Bufo marinus*) and Japanese treefrog (*Hyla japonica*) (Irikura et al., 2007). The recombinant protein of toad L-PGDS is associated with the weak PGD_2 synthase activity of about 4% of that of the rat enzyme and possesses the binding activities of bilirubin, biliverdin and retinoic acid with a weak affinity (Kd = 2 μM) and of thyroid hormones with almost comparable affinities (Kd = 0.9–1.6 μM) to those of the rat enzyme (Irikura et al., 2007). The toad L-PGDS exists in choroid plexus and is secreted into the CSF. *Xenopus* L-PGDS is one of the genes activated by Zic1, a transcription factor expressed at the anterior neural plate to promote the placode fate of embryo, and act as a retinoic acid-transporter directly participate in the establishment of the pre-placodal region (Jaurena et al., 2015).

The fish ortholog of L-PGDS is found in Zebrafish (Fujimori et al., 2006). The fish L-PGDS ortholog is not associated with the PGD_2 synthase activity due to the loss of the active site Cys65 residue but maintains the binding affinities for thyroxine and *all-trans*-retinoic acid (Kd = 0.4–1.0 μM), like mammalian L-PGDSs. In Catfish, the L-PGDS ortholog is expressed in seminal vesicle, in which the *L-Pgds* gene expression is decreased by L-thyroxin overdose and increased by depletion of thyroid hormone, suggesting that the L-PGDS protein is involved in the thyroid hormone pathway even in fish (Sreenivasulu et al., 2013).

CLINICAL STUDIES OF PATHOPHYSIOLOGICAL FUNCTION OF LIPOCALIN-TYPE PROSTAGLANDIN D_2 SYNTHASE

In human tissues, L-PGDS mRNA expression is most intense in the heart following the brain, and widely and weakly detected in many other organs, such as the placenta, lung, liver, skeletal muscle, kidney, pancreas, and others (Eguchi et al., 1997). Clinical studies of patients with various types of diseases reveal a variety of pathophysiological function of L-PGDS, as summarized in **Supplementary Table 7**.

Central Nervous System Function

Sleep Regulation

In the CNS, L-PGDS is involved in sleep regulation (Urade and Hayaishi, 2011). Sleep attack or coma caused by overproduction of PGD_2 is reported in patients with mastocytosis (Roberts et al., 1980) and African sleeping sickness (Pentreath et al., 1990). Under normal physiological condition, serum L-PGDS levels increase in evening and decrease by total sleep deprivation but not by partial sleep deprivation selective to rapid eye movement sleep (Jordan et al., 2004). CSF L-PGDS levels in day time (10:00 AM – 1:00 PM) are lower in patients with excess daytime sleepiness (EDS) than control healthy volunteers (Bassetti et al., 2006). Narcolepsy is a sleep disorder associated with EDS in daytime and caused by degeneration of hypothalamic

neurons which produce a neuropeptide hypocretin/orexin. CSF and serum levels of L-PGDS are higher in patients with narcolepsy than control patients (Jordan et al., 2005; Wang et al., 2020). CSF and serum levels of L-PGDS are also higher in patients with ideopathic hypersomnia without degeneration of hypocretin/orexin-producing neurons than control patients (Wang et al., 2020). In patients with obstructive sleep apnea (OSA), serum L-PGDS levels are higher in patients with EDS than those without EDS or controls (Barceló et al., 2007). Urinary L-PGDS levels are higher in patients with severe OSA than those in control or moderate OSA. The urinary excretion of severe OSA patients decreases to the control level after continuous positive airway pressure treatment to maintain the respiration during sleep (Chihara et al., 2013). The L-PGDS/β-trace concentrations in CSF, serum and urine are positively related with sleepiness in many sleep disorders.

Food Intake

Cerebrospinal fluid L-PGDS levels are positively related with food intake in a weight loss study with a 3-week dietary lead-in followed by 12-weeks of leptin or placebo treatment (Elias et al., 2011). CSF L-PGDS levels at the base line are related positively with NPY, galanin, visceral adipose tissue volume, corticotropin-releasing hormone and beta-endorphin, and inversely with CSF leptin. Leptin treatment does not affect CSF L-PGDS and NPY levels. Serum L-PGDS levels are not related with any of the measured variables either at baseline or after leptin treatment.

Cerebrospinal Fluid Circulation

Cerebrospinal fluid levels of L-PGDS are altered in malfunction of CSF circulation. Normal pressure hydrocephalus exhibits an abnormal CSF flow in the ventricles or cavities within the brain. This disease is caused by blocking CSF flow throughout the brain and spinal cord. Patients exhibit progressive mental impairment and dementia, problems with walking, and impaired bladder control. CSF L-PGDS levels decrease in patients with this disease (Mase et al., 2003) in disproportionately enlarged subarachnoid-space (Nishida et al., 2014) and show a trend of increase in the cognitive-improved patients after lumboperitoneal shunting but not in the poor cognitive-improved patients (Nakajima et al., 2015).

Spontaneous intracranial hypotension is a secondary headache etiology caused by CSF leakage, in which CSF L-PGDS levels are higher than control (Murakami et al., 2018).

Lipocalin-type prostaglandin D_2 synthase is useful to monitor the CSF drainage from human brain (Tsutsumi et al., 2015). The diploic vein/peripheral vein ratio of L-PGDS concentrations increases in the frontal, temporal, parietal and skull base. The diploic vein/peripheral vein ratio decreases in the frontal region for patients older than 45 years. The diploic veins constitute CSF drainage pathways with heterogeneous function intensity at different cranial locations.

Neurodegenerative Diseases

Induction of L-PGDS in neurons and glial cells is found in several neurodegenerative diseases. CSF L-PGDS levels are unchanged in patients with multiple sclerosis (Kagitani-Shimono et al., 2006). However, as examined with autopsy samples from patients with multiple sclerosis, L-PGDS immunoreactivity increases in oligodendrocytes within the shadow plaques and in hypertrophied astrocytes within the chronic plaques (Kagitani-Shimono et al., 2006). In neonatal hypoxic-ischemic encephalopathy, the surviving neurons in the infarcted lesions express L-PGDS immunoreactivity (Taniguchi et al., 2007).

After subarachnoid hemorrhage, CSF L-PGDS levels increase ∼two-fold at days 3 and 5, and return to the basal level at day 17 (Mase et al., 1999). L-PGDS in CSF from those patients covalently binds biliverdin, a by-product of heme breakdown, at Cys65 residue. L-PGDS scavenges harmful heme-degradation products in CSF after subarachnoid hemorrhage (Inui et al., 2014). Therefore, L-PGSDS acts as an extracellular scavenger of lipophilic toxic substance in CSF.

Cardiovascular Diseases

In human heart, L-PGDS exists in myocardiocytes, atrial endocardiocytes and a synthetic phenotype of smooth muscle cells in the arteriosclerotic intima, as examined by immunostaining of autopsy specimens (Eguchi et al., 1997). L-PGDS also exists in the atherosclerotic plaque of coronary arteries with severe stenosis. In patients with stable angina, the plasma level of L-PGDS is higher in the great cardiac vein than in the coronary artery. Therefore, the stenotic site produces and secrets L-PGDS to the coronary circulation (Eguchi et al., 1997). In patients undergoing percutaneous transluminal coronary angioplasty, L-PGDS levels in coronary sinus blood at 48 h after the treatment reduce to the baseline level in patients with restenosis but increase in those without restenosis (Inoue et al., 2001).

Serum L-PGDS levels are altered by many vascular diseases. Hypertensive patients show the increased serum L-PGDS levels with the renal function worsened. The urinary excretion is higher in hypertensive patients than normotensive patients (Hirawa et al., 2002). There is a common SNP of 4111 A>C in 3′-untranslated region of the L-PGDS gene in Japanese. Serum levels of HDL cholesterol are higher in hypertensive subjects with the A/A genotype than those with the A/C and C/C genotypes. The subjects with the A/A genotype exhibit the maximum intima-media thickness in the common carotid artery smaller than those with the A/C and C/C genotypes (Miwa et al., 2004). Serum L-PGDS levels increase in associated with the progression of atherosclerosis in non-treated asymptomatic subjects of atherosclerosis (Miwa et al., 2008). Serum L-PGDS levels are powerful biomarkers of severity of stable coronary artery disease in patients with coronary angiography (Inoue et al., 2008). Serum L-PGDS levels are higher in patients with vasospastic angina, and negatively correlated with the degree of the left anterior descending coronary artery vasomotion in response to acetylcholine (Matsumoto et al., 2011). Serum L-PGDS levels are positively correlated with cardiovascular diseases in Japanese.

On the other hand, in a study with gender- and age-matched Scandinavian individuals, serum L-PGDS is unchanged between patients with or without cardiovascular disease, while osteoprotegerin concentrations increase in patients with acute coronary syndrome (Hosbond et al., 2014). Pathological changes in serum L-PGDS levels may be different among populations with different genetics and nutrient circumstances.

Serum L-PGDS levels are higher in the pulmonary venous blood in patients with pulmonary embolism than control (Mutlu et al., 2020).

Metabolic Syndrome

Serum L-PGDS levels are associated with hypertriglyceridemia but not diabetes in patients with metabolic syndrome (Cheung et al., 2013).

Renal Function

Urinary L-PGDS levels are useful marker of renal function. Urinary L-PGDS excretion increases in the early stage of kidney injury in patients with type-2 diabetes mellitus (Hirawa et al., 2001) and is reversed by blood sugar control (Hamano et al., 2002). L-PGDS appears in the renal tubules in diabetes patients but does not exist in nondiabetic patients (Hamano et al., 2002). Urinary L-PGDS levels in type 2 diabetes patients are related with cardiovascular disease and useful as a supplemental or additional marker to the criteria of metabolic syndrome (Yoshikawa et al., 2007). A prospective study for ∼2 years with 121 patients of type-2 diabetes with <30 mg/g Cr albuminuria reveals that urinary L-PGDS levels are useful to predict the future status of renal injury in those patients (Uehara et al., 2009).

Urinary L-PGDS levels increase by the renal injury after long-term administration of gentamycin (Nakayama et al., 2009). In patients with systemic lupus erythematosus, urinary L-PGDS levels are significantly higher with active vs. inactive lupus nephritis or in patients without lupus nephritis. Urinary L-PGDS excretion increases as 3 months before a clinical diagnosis of worsening lupus nephritis (Suzuki et al., 2009) and decreases in patients with mucopolysaccharidosis type II disease (Hunter disease) as compared with age- and gender-matched healthy controls (Yuan et al., 2019).

Cancers

Lipocalin-type prostaglandin D_2 synthase expression is different among the types of cancers. L-PGDS exists in tumor cells of all various types of ovarian cancers (Su et al., 2001) but not in lung tumors (Ragolia et al., 2010). L-PGDS expression increases in malignant melanomas (Shimanuki et al., 2012), and is negatively correlated with Yes-associated protein 1 (YAP) in gastric cancers (Bie et al., 2020).

Bone and Cartilage

Lipocalin-type prostaglandin D_2 synthase is induced in cartilages of patients with osteoarthritis, suggesting that L-PGDS has an important role in the pathophysiology of OA (Zayed et al., 2008).

Digestive Tract

Lipocalin-type prostaglandin D_2 synthase is involved in inflammation of digestive tract. L-PGDS is induced in fibroblasts close to infiltrating cells in *Helicobacter pylori*-infected gastric mucosa (Hokari et al., 2009), and lamina proprial infiltrating cells and muscularis mucosa in patients with ulcerative colitis in parallel with the disease activity (Hokari et al., 2011). Patients with Crohn's disease show L-PGDS and Cox-2 mRNA expressions and increased PGD_2 levels in inflamed colonic mucosa at the active stage (Le Loupp et al., 2015). L-PGDS appears in neurons of both myenteric and submucosal plexi of the patients with Crohn's disease (Le Loupp et al., 2015).

Inflammation

In clinically healthy 58-year-old 100 Swedish men, serum L-PGDS levels positively correlate with soluble TNF-receptors 1 and 2 and negatively with alcohol consumption and serum HDL but not with insulin sensitivity (Wallenius et al., 2011). These results suggest that serum L-PGDS acts as an inflammatory marker.

Reproduction

Lipocalin-type prostaglandin D_2 synthase is involved in reproduction in both female and male genital organs. Serum L-PGDS levels are similar between pregnant and non-pregnant women (Shiki et al., 2004). L-PGDS levels are higher in the umbilical cord blood and amniotic fluid newborn urine than the maternal blood. L-PGDS levels in the cervicovaginal fluid are higher in patients with rupture of membrane than that without rupture of membrane (Shiki et al., 2004). In the normal pregnant women, urinary L-PGDS levels are higher in the third trimester than earlier pregnancy, while the plasma levels remain unchanged. Urinary L-PGDS levels are higher in early onset of preeclampsia as compared with late onset and in the severe compared to mild preeclampsia (Kinoshita et al., 2014). Urinary L-PGDS levels are a potential diagnostic marker for preeclampsia. L-PGDS levels in cervicovaginal secretion are two-fold higher in preterm births than full term births and inversely correlate against the days to expected delivery (Kumar et al., 2015). DP and CRTH2 antagonists may represent novel tocolytic agents for the treatment of preterm birth.

Patients with azoospermia show seminal plasma L-PGDS levels lower in oligozoospermic group than those in normozoospermic group (Tokugawa et al., 1998). L-PGDS levels in seminal plasma significantly reduce in severe oligozoospermic subfertile patients (Leone et al., 2001), and positively correlate with the alpha-glucosidase activity (Chen et al., 2005). Seminal plasma levels of L-PGDS are lower in patients with obstructive azoospermia than those with normal semen parameters, after vasectomy, or with nonobstructive azoospermia (Heshmat et al., 2008).

FUTURE SUBJECTS

In the last two decades, more than 200 papers reported the multifunctional properties of L-PGDS. New reports are publishing almost every month in a variety of pathological and physiological function. To further accelerate understanding the function of the L-PGDS/PGD_2 system, we still need several research probes as follows:

1) CRTH2-flox mice to make cell- or tissue-selective conditional KO mice.
2) L-PGDS-selective inhibitors with high affinities to study the L-PGDS-selective function *in vivo* pharmacologically.
   To understand the production of PGD_2 within the cell, it remains unclear
3) How Cox and L-PGDS couple to each other topologically to achieve the efficient conversion from arachidonic acid, via unstable intermediate PGH_2, to produce PGD_2.
   To demonstrate the intercellular transport of L-PGDS and its ligands, we need the data of
4) LDL receptor-mediated internalization of L-PGDS in any types of cells.
5) L-PGDS-selective receptors to mediate the internalization of L-PGDS.
   Moreover, novel promising animal models are proposed as follows:
6) APP multiple mutant mice without L-PGDS may be a new animal model of Alzheimer disease with early accumulation of Aβ plaques.
7) Double KO mice of *L-Pgds* and *Hpgds* genes are new model animals of aging, because they show progressive age-related cartilage degradation (Ouhaddi et al., 2020) and adenomyosis (Philibert et al., 2021).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.718002/full#supplementary-material

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REVIEW
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Structure, Functions, and Physiological Roles of the Lipocalin α_1-Microglobulin (A1M)

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α_1-microglobulin (A1M) is found in all vertebrates including humans. A1M was, together with retinol-binding protein and β-lactoglobulin, one of the three original lipocalins when the family first was proposed in 1985. A1M is described as an antioxidant and tissue cleaning protein with reductase, heme- and radical-binding activities. These biochemical properties are driven by a strongly electronegative surface-exposed thiol group, C34, on loop 1 of the open end of the lipocalin barrel. A1M has been shown to have protective effects *in vitro* and *in vivo* in cell-, organ-, and animal models of oxidative stress-related medical conditions. The gene coding for A1M is unique among lipocalins since it is flanked downstream by four exons coding for another non-lipocalin protein, bikunin, and is consequently named α_1-microglobulin-bikunin precursor gene (*AMBP*). The precursor is cleaved in the Golgi, and A1M and bikunin are secreted from the cell separately. Recent publications have suggested novel physiological roles of A1M in regulation of endoplasmic reticulum activities and erythrocyte homeostasis. This review summarizes the present knowledge of the structure and functions of the lipocalin A1M and presents a current model of its biological role(s).

Keywords: antioxidant, reduction, heme, radicals, thiol, preeclampsia, acute kidney injury, radioprotection

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INTRODUCTION

Proteins of the lipocalin family are found in all but one branch of life, and 15–20 different proteins of this family have been confirmed and studied in the human body. The lipocalins share a common fold and are characterized by their ability to bind small, most commonly hydrophobic, molecules (Schiefner and Skerra, 2015). A multitude of different biochemical and enzymatic properties have been described for the lipocalins and they are involved in a large variety of biological functions, including immunoregulation, signal transduction, smell reception, tissue development, storage and transportation of molecules, and cell homeostasis. One of the members of the lipocalin family is α_1-microglobulin (A1M), a protein that has been described as a tissue housekeeping protein responsible for removal of and protection against harmful oxidants and reparation of macromolecules. A1M was discovered over 45 years ago as a yellow-brown protein purified from human urine (Ekström et al., 1975), and it has been shown to be conserved in all studied vertebrate species. Following synthesis in the liver, it is secreted into the circulation and it is subsequently

equilibrated over the vessel wall between the intra- and extravascular compartments of all organs. Early reports proposed an immunoregulatory role of A1M (Babiker-Mohamed et al., 1990a; Åkerström et al., 2000), but more recent reports have presented data of enzymatic reductase activity as well as heme- and radical binding functions, which reconceptualized the biological role to a housekeeping protein responsible for cleaning of tissues and antioxidant protection (Åkerström and Gram, 2014). The aim of this review is to give an overview of the structure, mechanistic functions and life cycle of A1M, and to put these into a physiological context.

STRUCTURE

The proteins in the lipocalin family share a similar tertiary structure, which resembles a bucket. The bucket is formed by eight antiparallel β-strands shaped into a β-barrel with one open and one closed end. The lipocalin polypeptides contain 150–190 amino acids and are composed of a single polypeptide chain. Commonly, the lipocalins contain a binding site within the β-barrel where small hydrophobic compounds are bound (Flower et al., 2000). The crystal structure of A1M displays the characteristic lipocalin fold with a β-barrel and four loops at the open end of the bucket (Meining and Skerra, 2012). A1M has a disulfide bridge between C72 and C169, and a free thiol on the unpaired C34. C34 is located on one of the loops, loop 1, close to the open end of the bucket. It has been shown that C34 participates in one-electron oxidation and reduction reactions (Allhorn et al., 2005) and is involved in binding and neutralization of target compounds (Åkerström et al., 2007). Human A1M has a molecular weight of 26 kDa and is composed of a single polypeptide with 183 amino acids (Takagi et al., 1981; López Otin et al., 1984; Kaumeyer et al., 1986). A1M is a glycoprotein, which carries three different oligosaccharides (Amoresano et al., 2000) that can be found in different glycoforms. Two N-linked sialylated glycans are present on A1M, a biantennary glycan attached to N17 and a biantennary or triantennary glycan attached to N96. Additionally, O-linked structures, consisting of HexHexNAc glycans, are attached to T5 (Amoresano et al., 2000). A1M isolated from urine has been shown to be covalently modified with "chromophores," suggested to be the result of binding of free radicals and/or heme degradation products. Covalent modifications have been shown to be located on C34, K69, K92, K118, and K130, and these chromophores contribute to the heterogeneous charge and yellow-brown color of urinary A1M (Escribano et al., 1991; Åkerström et al., 1995; Berggård et al., 1999a). Additionally, *in vitro* experiments using the synthetical radical 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) have identified radical adducts on Y22 and Y132 (Åkerström et al., 2007). Finally, A1M has been described to contain two heme binding sites with different affinities for heme (Karnaukhova et al., 2014). Using molecular simulation, one heme binding site was found to be located buried in the lipocalin pocket in the vicinity of K92, K118 and K130, and the other binding site was located closer to the surface involving C34 and H123 (Rutardottir et al., 2016). The 3D structure of A1M, with important residues highlighted, is shown in **Figure 1**.

GENE

The gene encoding A1M is denoted the α_1-microglobulin-bikunin precursor gene (*AMBP*), and besides A1M it encodes a second protein, bikunin (Kaumeyer et al., 1986; Vetr and Gebhard, 1990). Bikunin is a structural component of the extracellular matrix and a Kunitz-type plasma proteinase inhibitor (Chen et al., 1992; Shigetomi et al., 2010). The *AMBP* gene is composed of 10 exons, where A1M is encoded by exons 1–6 and bikunin by exons 7–10. Additionally, exon 1 encodes a signaling peptide (Vetr and Gebhard, 1990). Transcription of the *AMBP* gene produces the AMBP protein, which is constituted by A1M and bikunin linked together by the tripeptide VRR (Lindqvist et al., 1992). Folding of the AMBP protein occurs in the endoplasmic reticulum (ER), from where it is further transported to the Golgi apparatus and post-translationally modified through the attachment of a chondroitin sulfate chain to the N-terminal part of bikunin. Subsequently, after leaving the Golgi apparatus, heavy chains (HC) are covalently bound to the chondroitin sulfate chain (Enghild et al., 1991). Three different kinds of HC exist in humans: HC1, HC2, and HC3. Attachment of HC1 and HC2 results in the formation of inter-α-trypsin inhibitor (IαI), whereas attachment of HC3 generates pre-α-inhibitor (PαI) (Fries and Blom, 2000). Before being secreted from the cell, the AMBP protein is proteolytically cleaved between A1M and bikunin. After secretion, no physical or functional association between A1M and bikunin, or their complexes, has so far been described. Consequently, the reason for co-synthesis of the two proteins is largely unknown, although recent evidence suggests that the presence of A1M is important for correct synthesis and post-translational modification of bikunin (Bergwik et al., 2020).

Transcription of the *AMBP* gene in the liver is regulated by hepatocyte nuclear factors (HNF 1–4) (Rouet et al., 1992, 1995, 1998). The expression of A1M is, similar to other antioxidation proteins, also regulated by the Keap1/Nrf2 system (Chorley et al., 2012; Campbell et al., 2013). Upregulation of the A1M expression has been found during oxidative stress related conditions induced by heme, hydrogen peroxide (H_2O_2), hemoglobin (Hb) and hydroxyl radicals ($OH^{\bullet}$) in primary skin keratinocytes, human cell lines and in skin and retinal explants (Olsson et al., 2007, 2011; Åkerström et al., 2017; Kristiansson et al., 2020b). Increased A1M concentrations in plasma samples have also been described in clinical conditions associated with oxidative stress. Women with preeclampsia (PE), a pregnancy related condition, displayed increased plasma A1M concentrations, as well as a correlation between plasma A1M and Hb concentrations, and between A1M and markers of oxidative stress, i.e., plasma peroxidation capacity and the amount of protein carbonyl groups (Olsson et al., 2010a). In that study, an upregulated A1M expression, i.e., increased A1M mRNA levels, was also found in placentas from the preeclamptic women.

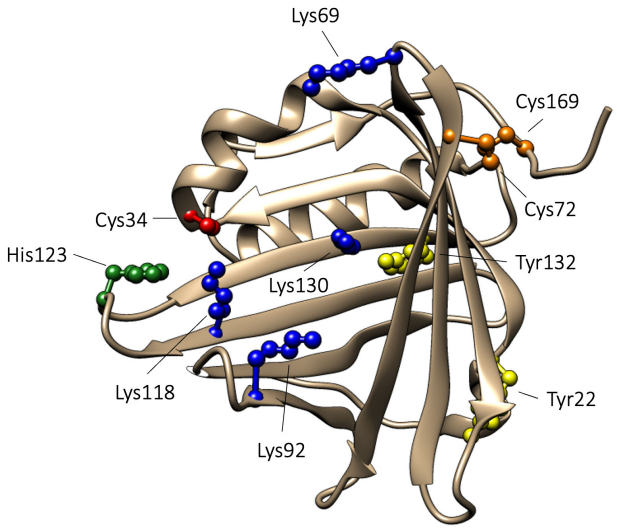

FIGURE 1 | 3D-structure of A1M. The 3D-structure of A1M is based on the published crystal structure (Meining and Skerra, 2012). Functionally important amino acids are highlighted: C34, described to be important for reduction, radical scavenging, and heme binding, is marked with red. Lysine residues (K69, K92, K118, and K130), described to be related to the yellow-brown modifications, are marked with blue. H123, suggested to take part in coordination of heme-iron, is marked with green. Tyrosine residues (Y22 and Y132), shown to be modified in radical scavenging reactions, are marked in yellow. The disulfide bridge, formed between C72 and C169, is marked in orange.

The *AMBP* gene has been mapped to the lipocalin gene cluster in the 9q-32-33 region in man (Diarra-Mehrpour et al., 1989) and on chromosome 4 in mice (Salier et al., 1992). Intron number 6, located between exon 6 and 7, is very large and contains A/T-rich regions and retroposon elements. This suggests that intron 6 is an unstable region and a recombinatorial hotspot, supporting the theory that the A1M and bikunin genes are assembled from two ancestral genes (Lindqvist et al., 1999). The genetic construction of the *AMBP* gene is conserved in all species studied, which is covered in more detail below.

α_1-MICROGLOBULIN IN DIFFERENT SPECIES

α_1-microglobulin has been found in a wide range of vertebrate species. The A1M protein has been detected, identified and purified from mammals (Åkerström and Berggård, 1979; Vincent et al., 1983; Åkerström, 1985; Åkerström et al., 1987; Tavakkol, 1991; Chan and Salier, 1993; Ide et al., 1994; Lindqvist and Åkerström, 1996; Nakata et al., 2011) amphibians (Kawahara et al., 1997), fish (Hanley and Powell, 1994; Lindqvist and Åkerström, 1999) and birds (Åkerström, 1985). The amino acid sequences from more than 60 species are available in public databases[1,2]. Interestingly, the *AMBP* gene construction, i.e., the co-expression with bikunin, is preserved in all species where the *AMBP* gene has been described. Most of the published A1M-sequences (>50) contain several of the A1M-specific functional groups described above, i.e., the free thiol group of C34, the targets for covalent modifications K92, K118, K130, Y22, and Y132, the suggested heme binding residue H123 and the disulfide bridge C72-C169 (**Figure 2**). The A1M homolog sequences also contain the lipocalin motifs (SCR1, 2, 3), proposed to be essential for the lipocalin structural folding (Flower, 1996), whereas the predicted carbohydrate binding sites at position T5, N17, and N96 in human A1M (Escribano et al., 1990) are less conserved.

SYNTHESIS, BIODISTRIBUTION, AND DEGRADATION

Figure 3 illustrates the life cycle of A1M, from its synthesis in liver, transport via blood to the extravascular space of all organs, and degradation in kidneys. The major site of synthesis is the liver (Tejler et al., 1978; Åkerström and Landin, 1985; Åkerström et al., 1995; Olsson et al., 2007). The protein is secreted from the liver to the blood, where the total concentration of A1M is approximately 2 μM (DeMars et al., 1989). In humans, about 50% of A1M in plasma is complex-bound to IgA via a reduction-resistant disulfide bond, 7% to albumin and 1% to prothrombin (Grubb et al., 1983; Berggård et al., 1997). Complex formation with other plasma proteins has been demonstrated in several other species. In rat, A1M was found to be covalently linked to fibronectin (Falkenberg et al., 1994) and α_1-inhibitor-3 (Falkenberg et al., 1990), a homolog to the human protease inhibitor α_2-macroglobulin. In plaice, high-molecular weight A1M-complexes have also been described (Lindqvist and Åkerström, 1999). Thus, the complex-forming propensity seems to be conserved from fish to mammals. Both free A1M and the complexed forms are rapidly equilibrated between the intra- and extravascular compartments and their half-lives in blood were determined to approximately 2–3 min in rats and mice (Wester et al., 2000; Larsson et al., 2001).

Due to its small size, free A1M is almost freely filtrated through the glomerular membranes out to the primary urine, where most of it is reabsorbed by the proximal tubular cells and catabolized (Nordberg et al., 2007). Some A1M, however, is excreted in the urine and the A1M-concentration in urine is a clinically used indicator of tubular renal damage (Ekström and Berggård, 1977; Nordberg et al., 2007).

A1M mRNA has been detected in most other human cell types besides the liver, *c.f.* kidney (Kastern et al., 1986; Leaver et al., 1994), placenta (Olsson et al., 2010a), stomach (Tavakkol, 1991), pancreas (Itoh et al., 1996; Berggård et al., 1998), skin (Olsson et al., 2011), retina (Cederlund et al., 2013) and blood cells (Leaver et al., 1994; Olsson et al., 2007). The A1M protein has been identified in the extravascular space of most organs, where it is associated to perivascular basal membranes and connective tissue (Ødum and Nielsen, 1994; Berggård et al., 1998), and especially abundant in the epidermis of skin (Bouic et al., 1985; Allhorn et al., 2003; Olsson et al., 2011) and intestinal/colon epithelium (Bouic et al., 1984; Larsson et al., 2001). It is often co-localized

[1]www.ncbi.nlm.nih.gov/gene
[2]www.uniprot.org

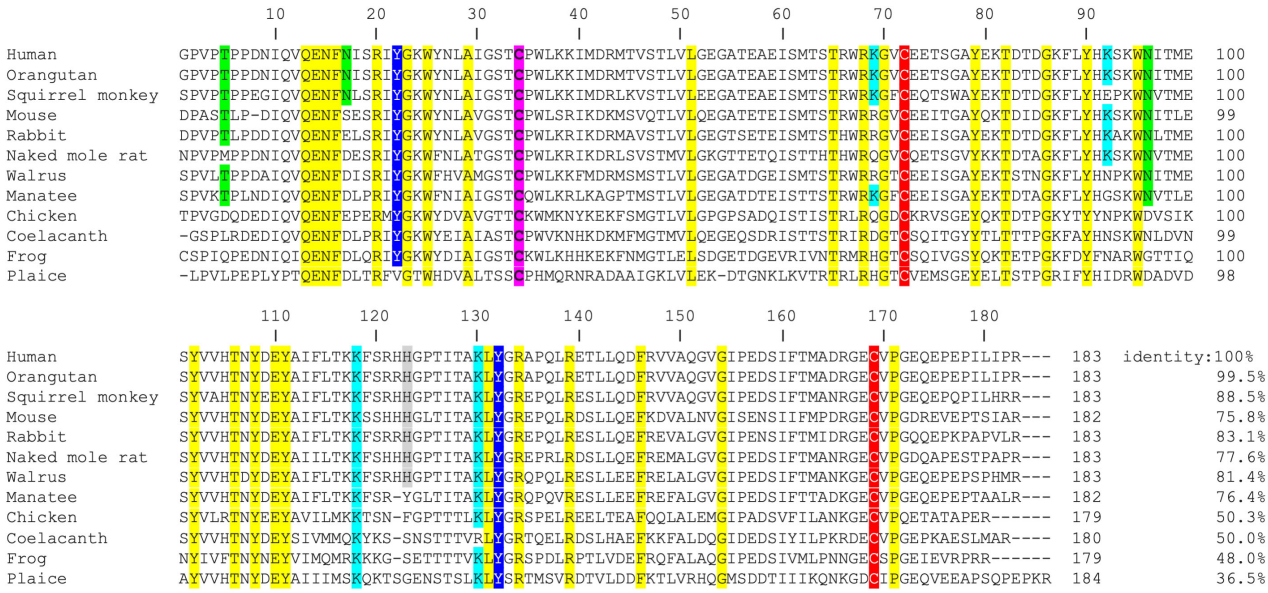

FIGURE 2 | Amino acid sequence alignment of A1M from 12 different species. The amino acid sequence of human wild-type (wt) A1M and 11 additional species were retrieved from public data bases (www.ncbi.nlm.nih.gov and www.uniprot.org) and aligned using the http://www.ebi.ac.uk/Tools/msa/clustalw2/ software. The degree of identity of the different sequences to the human sequence is presented as percent relative to human A1M. Amino acids with reported functional impact in human A1M are marked: the C34, described to be important for reduction and antioxidant properties as well as heme binding, is marked with pink. Glycosylated positions (T5, N17, and N96) are marked with green. Lysine residues (K69, K92, K118, and K130), described to be related to the yellow-brown modifications, are marked with light blue. The disulfide bridge, formed between C72 and C169, is marked in red. H123, suggested to take part in coordination of heme-iron, is marked with gray. Tyrosines (Y22 and Y132), shown to be modified in radical scavenging reactions are marked in dark blue. Of these functionally important amino acids, five are completely conserved in all 12 species (C34, C72, K118, Y132, and C169). Additional 28 amino acids, which are identical between all species in the set, are marked with yellow. Human, *Homo sapiens*; Orangutan, *Pongo abelii*; Squirrel monkey, *Saimiri boliviensis boliviensis*; Mouse, *Mus musculus*; Rabbit, *Oryctolagus cuniculus*; Naked mole rat, *Heterocephalus glaber*; Walrus, *Odobenus rosmarus divergens*; Manatee, *Trichechus manatus latirostris*; Chicken, *Gallus gallus*; Coelacanth, *Latimeria chalumnae*; Frog, *Xenopus laevis*; and Plaice, *Pleuronectes platessa*.

with elastin and collagen (Bouic et al., 1985; Ødum and Nielsen, 1994; Olsson et al., 2011) and has been shown to bind to collagen *in vitro* (Santin and Cannas, 1999; Olsson et al., 2011). Such a distribution of A1M in the extravascular space and extracellular matrix allows the protein to execute its protective functions (see below) mainly outside the blood circulation, at interfaces between the cells and the ambient environment (blood/tissue, air/tissue, intestinal lumen/villi), as well as at the interface between maternal blood and fetal tissues in placenta (Berggård et al., 1999b; May et al., 2011).

CELL BINDING, UPTAKE AND INTERACTION WITH MITOCHONDRIA

Cell surface binding of A1M has been reported for a variety of cells, including peripheral lymphocytes (Fernandez-Luna et al., 1988; Babiker-Mohamed et al., 1990b; Wester et al., 1998, 2000), neutrophils (Méndez et al., 1986), erythrocytes (Kristiansson et al., 2020a), blood cell lines (Olsson et al., 2007, 2008), and keratinocytes (Olsson et al., 2011). The cell surface binding of A1M is specific and saturable, suggesting the presence of an A1M-receptor on the cells. The dissociation constant for binding of A1M on human T cells and mouse peripheral lymphocytes was estimated to approximately 10^{-5} M (Babiker-Mohamed et al., 1990b; Wester et al., 1998), but was slightly higher on the U937 histiocyte cell line, 10^{-7} M (Fernandez-Luna et al., 1988). However, up to date, a specific A1M-receptor has not yet been identified.

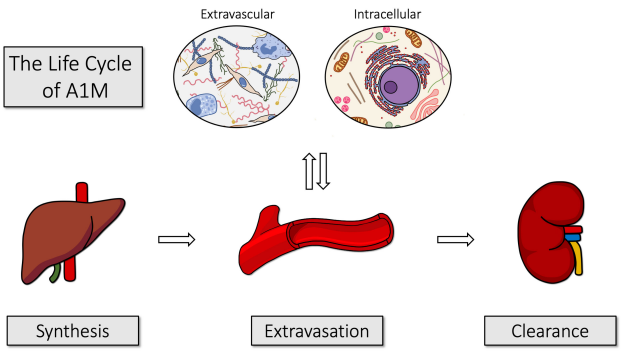

FIGURE 3 | The life cycle of A1M. A1M is primarily synthesized in the liver, from where it is secreted into the blood. It is rapidly equilibrated over the vessel walls of all organs and can be taken up intracellularly. After exerting its functions, it is transported to the kidneys and filtrated in the glomeruli. Finally, it is reabsorbed by the proximal tubular cells where it is degraded together with any bound heme groups and/or radicals.

The erythroid cell line K562 (Olsson et al., 2008), erythrocytes (Kristiansson et al., 2020a), the hepatoma cell line HepG2 (Olsson et al., 2007), and primary keratinocytes (Allhorn et al., 2003) have been shown to internalize A1M from the culture medium. Intracellularly, A1M was shown to be localized to the mitochondria, and specifically to Complex I of the respiratory chain (Olsson et al., 2013). Mitochondrial localization, and an uptake of exogenously added A1M to mitochondrial Complex I of skin keratinocytes, blood cells and liver was shown by immunofluorescence, fluorescence-activated cell-sorting, and electron microscopy. Furthermore, mitochondrial A1M could be purified and identified by mass spectrometry. The functional role of A1M in the mitochondria is not fully understood, but since the cellular uptake of the protein is strongly intensified during apoptosis, it may be involved in assisting the mitochondria to maintain its energy delivery during apoptosis and cell death. A1M may also, at the same time, counteract and eliminate the ROS generated by the mitochondrial respiration to prevent oxidative damage to surrounding healthy tissue. As a result, it was suggested that A1M has a role in maintaining mitochondrial redox homeostasis (Olsson et al., 2013).

MOLECULAR MECHANISMS

Comprehensive studies of the A1M protein over the last decades have revealed that A1M has a role as a physiological cell- and tissue protective antioxidant, and as described above three molecular mechanisms contribute to its function. These are reductase activity, radical scavenging and heme binding (Åkerström and Gram, 2014; Gunnarsson et al., 2017; Kristiansson et al., 2020c), and are summarized briefly below and schematically outlined in **Figure 4**.

Reductase Activity

Owing to the free cysteine residue in position 34, A1M has the capacity to reduce several biological substrates, including metHb, cytochrome c, oxidized collagen I, oxidized low density lipoprotein, free iron and the synthetic radical ABTS (**Figure 4**, reaction 1). In addition to the thiol group of cysteine 34, the reductase activity appears to be dependent on three lysyl residues in position 92, 118, and 130 (Allhorn et al., 2005). The reduction potential of A1M has been observed to be catalytic in the presence of strong electron-donators such as nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH) and ascorbate. However, since it has been shown that A1M can reduce physiological substrates both intra- and extracellular it is likely that there are additional physiologically electron-donating co-factors (Olsson et al., 2008, 2011; Rutardottir et al., 2013).

Radical Scavenging

As shown with the synthetic radical ABTS, A1M retain the capacity to react with and "trap" radicals in a reaction that involves the reducing potential of C34 (**Figure 4**, reaction 2) (Åkerström et al., 2007). It has been demonstrated that 8–9 ABTS radicals are consumed per A1M molecule, with reduction of 5–6 ABTS radicals and covalent trapping of 2–3 radicals. The trapped ABTS radicals are localized to at least two different tyrosine residues, Y22 and Y132, confirming that each A1M molecule can covalently trap several ABTS molecules. As described above, a number of modifications of A1M side chains have been found on A1M isolated from urine (Åkerström et al., 1995; Berggård et al., 1999a; Sala et al., 2004), suggesting that the radical-trapping mechanism of A1M also operates *in vivo*. In addition, a recent report identified plasma conjugates between A1M and infused cancer-targeting drug compounds, and the authors speculated that the free thiol of C34 on A1M is involved in adduct formation (Su et al., 2019). Based on the observation that both the radicals and the A1M protein are electroneutral after the above-described reactions and consequently do no longer constitute an oxidative threat to tissues, cells and proteins, etc., the term "radical sink" was proposed (Åkerström et al., 2007; Åkerström and Gram, 2014).

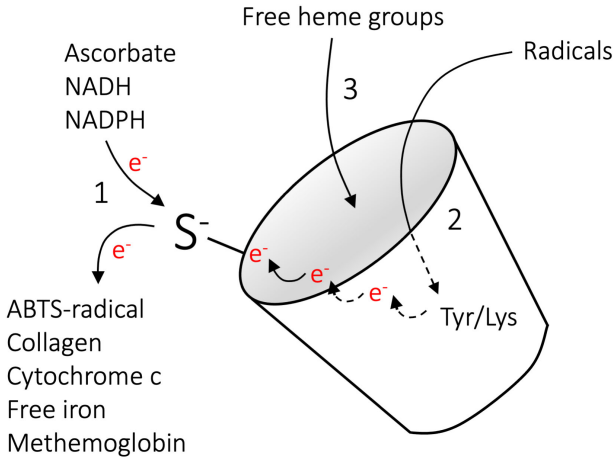

FIGURE 4 | Molecular mechanisms of A1M. The molecular mechanisms implicated in the actions of A1M include (1) reductase activity (2) radical scavenging and (3) heme binding. The reductase activity is driven by the free thiol group of C34, is catalytic in the presence of the electron-donating co-factors NADH, NADPH, or ascorbate, and several substrates have been identified (Allhorn et al., 2005). The radical-scavenging reaction is also driven by C34, which, in the absence of electron-donating co-factors, can oxidize Y32 and Y132 to tyrosyl-radicals via intramolecular electron-transfer. This is followed by reactions with external radicals, ultimately leading to covalent trapping of the radicals and modification of the side-groups (Åkerström et al., 2007). *In vivo*, covalent modifications have been shown on the side groups of K69, K92, K118, and K130, suggesting a similar radical trapping mechanism on lysine residues. Binding of heme-groups were shown on two separate binding sites (Allhorn et al., 2002; Siebel et al., 2012; Rutardottir et al., 2016). Reproduced from Gunnarsson et al. (2017).

Heme Binding

α_1-microglobulin binds heme with a $K_d = 10^{-6}$ M and at a molar ratio of 2:1, such that two heme groups are bound to each A1M molecule (**Figure 4**, reaction 3) (Allhorn et al., 2002; Larsson et al., 2004; Siebel et al., 2012). The heme-binding property of A1M is evolutionarily conserved, and has been demonstrated for human, mouse, rat, guinea pig, cow, chicken and plaice

TABLE 1 | Summary of animal studies performed to examine the therapeutic potential of A1M against oxidative stress related diseases.

Species	Disease	Pathology	Treatment/route of admin	Effect of treatment	References
Rat	AKI	Infusion of HbF Increased glomerular permeability	A1M *i.v.* infusion 22.4 μg/min	Restored glomerular permeability	Sverrisson et al., 2014
Mouse	AKI	PRRT-induced kidney damage, DNA damage, upregulation of apoptotic- and stress-related genes, proteinuria, kidney lesions and death	A1M *i.v.* injection 7 mg/kg body weight, single dose	Decreased DNA damage and upregulation of apoptotic- and stress-related genes Reduced proteinuria and kidney lesions Less animal deaths	Kristiansson et al., 2019
Mouse	AKI	Rhabdomyolysis-induced mild AKI Upregulation of stress genes HO-1 and Hsp70 in kidneys	A1M *i.v.* injection 7 mg/kg body weight, single dose	Reversed upregulation of stress genes	Åkerström et al., 2019
Rabbit	IVH	IVH induced by glycerol injection Structural tissue and mitochondrial damage Expression of pro-inflammatory genes	A1M intracerebroventricular injection 0.235 mg, single dose	Decreased structural tissue and mitochondrial damage Reduced expression of pro-inflammatory genes	Romantsik et al., 2019
Sheep	PE	PE-like symptoms after starvation Structural damage to placenta and kidneys Increased glomerular permeability	A1M *i.v.* injection 1.8 mg/kg, two doses	Decreased structural damage to placenta and kidneys Restored glomerular permeability	Wester-Rosenlöf et al., 2014
Rabbit	PE	PE-like symptoms after HbF infusion Proteinuria and increased glomerular filtration Structural damage to placenta and kidneys	A1M *i.v.* injection 6 mg/kg, five doses	Reversed proteinuria and structural changes to placenta and kidneys Restored glomerular permeability	Nääv et al., 2015
Mouse	PE	PE-like symptoms (STOX1 transgenic) Gestational hypertension, proteinuria and organ alterations	A1M *i.p.* injection 0.27 mg, six doses	Decreased gestational hypertension Lowered hypoxia and nitrative stress in placenta Reduced cellular damage to placenta and kidneys	Erlandsson et al., 2019

A1M (Larsson et al., 2004). In addition to binding heme, A1M has been described to react with lysed red blood cells, purified Hb or the heme-containing enzyme myeloperoxidase (MPO). In this process, a truncated form of A1M, denoted t-A1M, was described to be formed (Allhorn et al., 2002; Cederlund et al., 2015). Interestingly, this processed form of A1M, lacking the four most C-terminal amino acids leucine-isoleucine-proline-arginine, was shown to be capable of degrading the heme group into a heterogeneous chromophore associated with the protein. A1M has been shown to bind and degrade heme both in its free monomeric form and when complex bound to IgA (Allhorn et al., 2002; Larsson et al., 2004).

ASSOCIATION WITH DISEASES

Based on its antioxidant functions, A1M has been proposed as a therapeutic agent in diseases where free radicals and heme groups are involved in the pathology, such as preeclampsia (Gunnarsson et al., 2017) and hemolytic conditions (Kristiansson et al., 2020c). A modified recombinant version of A1M named ROSgard® is currently being evaluated in phase I clinical trials as a therapeutic agent against acute kidney injury (AKI) after open chest cardiac surgery. ROSgard is developed by the company Guard Therapeutics International. The use of A1M in therapy has been evaluated in a number of animal models and the studies are described below and summarized in **Table 1**.

Kidney Damage

Kidney damage can occur through a multitude of different external or internal processes. As a result of a relatively high level of A1M in the kidneys, the use of it as a kidney protector in various conditions and as a biomarker for tubular damage (Yu et al., 1983; Nordberg et al., 2007) has gained attention. In fact, in a number of different animal models of renal damage, A1M has been observed to counteract or significantly reduce the renal damage. This includes (i) a study in rats where infused fetal Hb (HbF) induced increased glomerular permeability, (ii) a study in rabbits where renal damage was induced by infusion of HbF, (iii) a mouse study infusing radiopeptides, and (iv) a mouse rhabdomyolysis study where glycerol-injections triggered mild renal damage. In these studies, A1M was observed to reduce the damage-induced renal upregulation of stress genes and restore the compromised renal function (Sverrisson et al., 2014; Nääv et al., 2015; Åkerström et al., 2019; Kristiansson et al., 2019). Taken together, these studies suggest a protective effect of the kidneys by A1M from a variety of insults inducing oxidative stress-related injuries.

Hemolysis

Hemolysis, i.e., the rupture of red blood cells, results in Hb- and heme-induced toxicity on cells and tissues and it is associated with a wide range of diseases. A1M has been shown to have erythroprotective effects (Kristiansson et al., 2020a), which could be a potential role *in vivo*, but may also offer a therapeutic opportunity in certain conditions [reviewed in Kristiansson et al. (2020c)]. Intraventricular hemorrhage (IVH) of prematurely born infants, is a severe hemolysis condition where fragile vessels in the brain rupture, leading to accumulation of toxic metabolites, such as free heme and Hb within the brain (Ley et al., 2016; Agyemang et al., 2017). In a preterm rabbit pup model of

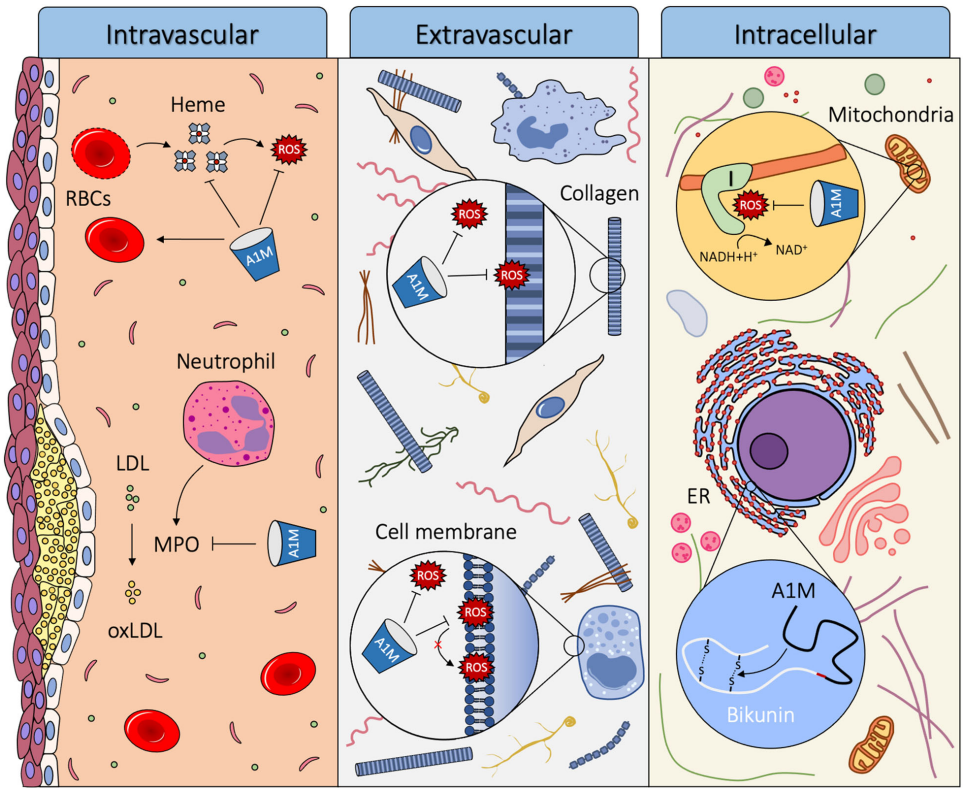

FIGURE 5 | Proposed biological roles of A1M. Using its heme binding, radical scavenging and reductase activity, A1M can act as a housekeeping protein in three different compartments of the body: intravascular, extravascular and intracellular. Intravascularly, A1M acts a stabilizer of red blood cells, it reduces damage from hemolytic events by binding heme and reducing Hb and it inhibits oxidation of LDL by MPO. Extravascularly, A1M protects tissues, cells and macromolecules from oxidative insults, including reducing oxidation products formed on ECM structures and lipid peroxidation of cell membranes. Intracellularly, A1M is bound to Complex I of the respiratory chain in the mitochondria where it may have a radical scavenging function and preserves ATP-production. A1M also acts as a chaperone during the folding of bikunin in the ER.

IVH, intracerebroventricular administration of A1M displayed a strong co-localization with Hb and resulted in a decreased structural damage and a normalized/reduced expression of pro-inflammatory genes (Romantsik et al., 2019).

Preeclampsia

Preeclampsia is a pregnancy related disease associated with high blood pressure and albuminuria as defining clinical features (Young et al., 2010). The disease has been described to be associated with elevated concentrations of HbF in the maternal blood plasma during the second and third trimester, and in the fetus and placenta at term, and it was suggested that oxidative stress induced by the HbF-iron is critically involved in development of PE (Centlow et al., 2008; Olsson et al., 2010a; Anderson et al., 2011). Since oxidative stress, Hb and free heme are targets of the protective activities of A1M, it was suggested that A1M may be used as a therapeutic agent in PE (Gunnarsson et al., 2017). Further support for this notion is that human A1M plasma levels were observed to be increased and the expression of the gene was upregulated in liver and placenta of PE patients, suggesting that the protein is involved in the natural protection against PE (Olsson et al., 2010a; Anderson et al., 2011, 2016). In order to study the therapeutic effect of A1M, three different animal models of PE have been evaluated. First, starvation of pregnant ewes resulted in development of PE-like structural damages to both placenta and kidneys (Wester-Rosenlöf et al., 2014). Second, infusion of HbF in pregnant rabbits resulted in PE-like symptoms, i.e., proteinuria and an increased glomerular sieving coefficient as well as intra- and extracellular tissue damage in the kidneys (Nääv et al., 2015). Third, overexpression of the transcription factor Storkhead box 1 (STOX1) led to PE symptoms in pregnant mice, including hypertension, proteinuria and structural impairments of kidneys and placenta (Erlandsson et al., 2019). In these three studies, administration of A1M reduced structural and functional injuries closely linked to the PE pathology.

PROPOSED BIOLOGICAL ROLES

α_1-microglobulin is primarily produced in the liver and secreted into the circulation. From the blood, it is rapidly transported across the capillary walls of all organs, and equilibrated between intra- and extravascular compartments, from where it also

can be internalized by cells. A1M has the ability to reduce oxidation products, scavenge radicals and bind free heme molecules, which indicate a role as a housekeeping protein that contributes to the antioxidation defense and detoxification and repair of oxidized macromolecules (Åkerström and Gram, 2014). A1M is thought to exert its function in the three different compartments described: intravascular, extravascular and intracellular. The proposed biological roles of A1M are summarized in **Figure 5**.

Intravascular

Although the half-life of A1M in the blood is relatively short, several observations indicate that key functions of A1M, including reduction, heme binding, heme degradation and protection of red blood cells against hemolysis, are important intravascularly. Heme groups are present in various parts of the body, but mostly abundant in the blood as the oxygen binding molecule in Hb. If hemolysis occurs, extracellular Hb can oxidize nearby molecules and release its heme molecules causing further oxidative damage and trigger inflammation. A1M can counteract this in three ways: firstly, it stabilizes the red blood cells and protects them against various forms of stress to reduce the hemolytic event (Kristiansson et al., 2020a). Secondly, it can reduce oxidized extracellular Hb (Allhorn et al., 2005) to decrease its toxicity. Thirdly, A1M can bind and degrade free heme groups (Allhorn et al., 2002).

Another reported physiological function of A1M is to prevent MPO from oxidizing low density lipoprotein (LDL). Oxidation of LDL plays a central role in the pathogenesis of atherosclerosis (Heinecke, 1997), and MPO is one of the key mediators involved in the oxidation of LDL. A1M inhibits MPO from oxidizing LDL, and when interacting with MPO A1M is proteolytically cleaved, forming t-A1M (Cederlund et al., 2015). This suggests a role of A1M as a protector against oxidation of LDL, which may result in a decreased formation of atherosclerotic plaques.

Extravascular

Extravascular A1M is often co-localized with structural components of the extracellular matrix (ECM), *c.f.* elastin and collagen (Bouic et al., 1985; Ødum and Nielsen, 1994; Olsson et al., 2011), and it has been shown to bind collagen *in vitro* (Santin and Cannas, 1999). A1M both inhibits the destruction of collagen fibrils exposed to Hb, heme or Fenton reaction-generated radicals, and repairs the collagen fibrils after the damage has already taken place (Olsson et al., 2011; Rutardottir et al., 2013). Additionally, perfusion of *ex vivo* placentas with A1M results in a large increase in the amount of collagen fibrils in the placenta (May et al., 2011). Moreover, evidence of ECM repair mechanisms has been found in studies on sheep, rabbits and mice with induced PE-like symptoms (Wester-Rosenlöf et al., 2014; Nääv et al., 2015; Erlandsson et al., 2019). Treatment with A1M resulted in reduced damage to structural components in the extravascular space of the kidney and placenta, including collagen fibrils. This suggests a biological role of A1M in protection and repair of the ECM and its structural components.

α_1-microglobulin can bind to the surface of several different cell types, such as keratinocytes (Olsson et al., 2011), erythrocytes (Kristiansson et al., 2020a), neutrophils (Méndez et al., 1986), peripheral lymphocytes (Fernandez-Luna et al., 1988; Babiker-Mohamed et al., 1990b; Wester et al., 1998, 2000) and blood cell lines (Olsson et al., 2007, 2008). The phospholipid bilayer of the cell membrane is susceptible to oxidation by free radicals causing lipid peroxidation. The lipid peroxidation results in structural derangement of the membranes which alters the membrane fluidity, but can be inhibited by antioxidants such as vitamin C and E (Tai et al., 2010). The presence of A1M at the cell surface may enable both protection through radical scavenging and reduction of oxidation products formed in, or in the proximity of the cell membrane. Indeed, A1M has been found to protect a variety of cell types against oxidative stress-induced cell death, including keratinocytes (Olsson et al., 2011), erythroid cells (Olsson et al., 2008), erythrocytes (Kristiansson et al., 2020a), kidney cells (Kristiansson et al., 2020b) and liver cells (Olsson et al., 2010b; Rutardottir et al., 2013), supporting the proposed biological role as a protector against free radicals in the plasma membrane of cells.

Intracellular

α_1-microglobulin has been shown to bind to a subunit of Complex I of the mitochondria, which is a significant source of intracellular free radical production. This co-localization of A1M to Complex I was shown to preserve mitochondrial ATP-production during oxidative stress conditions (Olsson et al., 2013). The functional role of A1M in the mitochondria is not fully understood, but it has been suggested that A1M has a role in maintaining mitochondrial redox homeostasis (Olsson et al., 2013).

The co-synthesis of A1M and bikunin has been a mystery since its discovery over 30 years ago (Kaumeyer et al., 1986). Development of an A1M knockout mouse ($A1M^{-/-}$) with a selective removal of the A1M exons and intact bikunin exons resulted in correct translation of signal peptide-containing bikunin but a defective synthesis of the high molecular weight forms of bikunin (Bergwik et al., 2020). The $A1M^{-/-}$ mice showed signs of hepatic ER-stress, most likely due to misfolding of bikunin. A novel intracellular function of A1M as a chaperone during bikunin synthesis was therefore proposed (Bergwik et al., 2020).

CONCLUSION

Approximately 45 years have passed since the discovery of A1M and since then new information about the protein has been published continuously. In this work, we have given an overview of the multifaceted functions of A1M and put them in a physiological relevant context. In brief, the lipocalin A1M employs three molecular mechanisms, chemical reductase activity, radical scavenging and heme binding to execute a physiological function as an antioxidant and tissue cleaning factor.

AUTHOR CONTRIBUTIONS

JB and BÅ: conceptualization and writing – finalization and submitting, and visualization. JB, AK, MA, MG, and BÅ: writing – original draft preparation and reviewing. AK, MG, and BÅ: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest: BÅ and MG are co-founders and BÅ, MA, and MG are share-holders of Guard Therapeutics International AB, which holds the patent rights for medical uses of A1M.

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Into the Labyrinth of the Lipocalin α1-Acid Glycoprotein

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α_1-acid glycoprotein (AGP), also known as Orosomucoid (ORM), belongs to the Lipocalin protein family and it is well-known for being a positive acute-phase protein. AGP is mostly found in plasma, with the liver as main contributor, but it is also expressed in other tissues such as the brain or the adipose tissue. Despite the vast literature on AGP, the physiological functions of the protein remain to be elucidated. A large number of activities mostly related to protection and immune system modulation have been described. Recently created AGP-knockout models have suggested novel physiological roles of AGP, including regulation of metabolism. AGP has an outstanding ability to efficiently bind endogenous and exogenous small molecules that together with the complex and variable glycosylation patterns, determine AGP functions. This review summarizes and discusses the recent findings on AGP structure (including glycans), ligand-binding ability, regulation, and physiological functions of AGP. Moreover, this review explores possible molecular and functional connections between AGP and other members of the Lipocalin protein family.

Keywords: α1-acid glycoprotein, orosomucoid, inflammation, metabolism, ligand-binding, Lipocalin

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INTRODUCTION

α_1-acid glycoprotein (AGP), also known as Orosomucoid (ORM), is member of the Lipocalin protein family and well-known for being a positive acute-phase protein. Approximately 70 years have passed since the discovery of AGP (Schmid, 1950; Weimer et al., 1950) and thousands of studies have been performed since then. In the big picture, AGP is commonly defined as a transport protein in plasma whose main function is to modulate the immune system, including cytokine secretion (Hochepied et al., 2003). Numerous *in vitro* and *in vivo* activities such as the inhibition of platelet aggregation (Costello et al., 1979), modulation of cell proliferation/differentiation (Chiu et al., 1977; Qin et al., 2017; Lee et al., 2021; Shi et al., 2021), and drug transport have been reported (Israili and Dayton, 2001). However, the exact molecular mechanism of AGP function remains to be elucidated. AGP is mostly found in plasma from hepatic origin, but other tissues/cells such as the adipose tissue, the nervous system, endothelial cells, and immune cells also express AGP, especially during inflammatory conditions (Dente et al., 1988; Sorensson et al., 1999; Hochepied et al., 2003). Indeed, a large number of pathological conditions (including many types of cancers, infection, obesity, and cardiovascular diseases) raise AGP levels in plasma (Israili and Dayton, 2001).

This work represents an overview of the multiple faces of AGP and focuses on its physiological roles. Furthermore, this review explores possible molecular and functional connections between AGP and other members of the Lipocalin protein family (summarized in **Table 1**).

TABLE 1 | Summary of the associations/common features of AGP and other members of the Lipocalin protein family discussed in this mini-review. Note many other examples might exit.

Other Lipocalins	Topic discussed	Section
Several	Immunocalin group	AGP, Definition, and Molecular Characteristics
ApoD and ApoM	Progesterone binding	Endogenous Ligands
ApoM	PAF, barrier permeability	Endogenous Ligands
Lipocalin 1 and 2	Siderophore binding	*In vivo* Approaches, Transgenesis, and Knockouts
ApoD	Leptin Receptor	*In vivo* Approaches, Transgenesis, and Knockouts
RBP4 and several others	Retinol Binding	*In vivo* Approaches, Transgenesis, and Knockouts
ApoD and Lcn2	Lipocalins and Astrocyte responses	*In vivo* Approaches, Transgenesis, and Knockouts

AGP, DEFINITION, AND MOLECULAR CHARACTERISTICS

Human AGP (hAGP) is actually not a single unique protein. Instead, two main forms of AGP coexists in humans. They are encoded by a cluster of genes: AGP1 is encoded by the *ORM1* gene and AGP2 by the *ORM2* gene. Both genes have identical structures with 5 introns (Sanchez et al., 2003), and AGP1/2 sequences only differ in 22 amino acids. Besides this complexity, *ORM1* gene has three common variants: F1, F2, and S (collectively referred as F*S). Equally, AGP*2* is sometimes referred as variant A. Interestingly, other mammals have a different number of *Orm* genes. For instance, AGP is coded by three *Orm* genes in mouse, whereas rats have a single gene. The different number of "AGP-genes" in laboratory animal models could be turned into a research advantage, though this has not been much exploited yet.

The crystals of unglycosylated AGP1/2 (produced in *E. coli*) revealed a typical Lipocalin fold comprising an eight-stranded β-barrel which is flanked by a C-terminal α-helix (**Figure 1A**; Schonfeld et al., 2008; Nishi et al., 2011). Four loops connect the β-sheets and a tryptophan is buried inside of the cavity. This is one of the few, but key, amino acid mostly conserved across the Lipocalin family (Flower et al., 2000; Ganfornina et al., 2000; Schiefner and Skerra, 2015). AGP2 ligand-binding region is narrower than that in AGP1, explaining the different compound-binding affinities reported for AGP1/2 (several examples can be found in Herve et al., 1993; Herve et al., 1996, 1998; Kuroda et al., 2003; Zsila et al., 2008; Nishi et al., 2011). Two disulfide bridges stabilize the structure of hAGPs (Schonfeld et al., 2008; Nishi et al., 2011). Interestingly, hAGP2 has an extra free Cys that could form a covalent binding with other proteins or participate in redox reactions, though this is merely a speculation.

α_1-acid glycoprotein is heavily glycosylated, five N-linked glycans are present in hAGPs. These glycans represent around 45% of the AGP molecular weight and contain a high proportion of sialic acid, giving AGP its characteristic acid isoelectric point (p*I* = 2.7–3.2). In contrast, the unglycosylated AGP p*I* was calculated to be 4.97. Glycosylation increases AGP solubility but, importantly, increases its molecular weight such that it escapes glomerular filtration in the kidneys. AGP glycosylation pattern is rather complex and heterogeneous [reviewed in detail by Fournier et al. (2000); and further studied by Fernandes et al., 2015]. Multiple glycan combinations have been detected in the plasma of healthy humans (Treuheit et al., 1992; Ongay and Neususs, 2010; Baerenfaenger and Meyer, 2018; Keser et al., 2021) and changes under pathological states have been reported (De Graaf et al., 1993; Liang et al., 2019; Keser et al., 2021). More specifically, branches with sialic acid are also fucosilated creating highly biologically active sialyl-Lewis X epitopes (sLe^x). It is not fully clear how glycosylation affects AGP binding toward endogenous ligands, but the fact that Asn-75 localizes near to the entrance of the binding pocket should be considered (Nishi et al., 2011). Additionally, there are documented examples where branching and fucosylation do limit drug-binding affinity (i.e., Wu et al., 2018).

Evolutionary, Lipocalins were classified in fourteen clades (I-XIV), where AGP clusters among the modern ones and included in clade XII (Gutierrez et al., 2000). AGP is only found in vertebrates and classified as an "outlier Lipocalin" because it contains only one of the three Lipocalin structurally conserved regions (SCRs) (Flower et al., 2000). Besides the traditional way of classifying Lipocalins, the term Immunocalins was proposed some time ago (Logdberg and Wester, 2000). Immunocalins would be a group of proteins sharing the Lipocalin fold and involved in immune system regulation. The founder group included: AGP, α_1-Microglobulin, Glycodelin, and Lipocalin 2 (Lcn2 and also known as Siderocalin, NGAL, or 24p3), Complement Factor 8, γ-subunit, Tear Lipocalin (also known as Lcn1 or Von Ebner's gland protein) and Lipocalin Prostaglandin D Synthase (L-PGDS). Even though the concept is still valid, we now know that many other Lipocalins modulate the immune response. Examples include Apolipoprotein D (ApoD) (Dassati et al., 2014) and Apolipoprotein M (ApoM) (Frej et al., 2017; Ruiz et al., 2017). Altogether then, there is a mounting evidence that the first ancestral Lipocalin may have had general defensive functions.

AGP LIGANDS AND RECEPTORS

A powerful approach to investigate a Lipocalin's physiological function(s) is often to identify its ligand(s). Such an approach would be suitable for some Lipocalins, such as RBP (Retinol Binding Protein 4), L-PGDS or ApoM. However, AGP is much more complex: it has a promiscuous ligand-binding behavior and is capable of binding hundreds of molecules from endogenous or exogenous origin. AGP has one primary high-affinity binding-site -"the classical Lipocalin binding-site"- but other sites with different capacities and lower affinity exists. Binding data for more than 300 drugs and endogenous substances were compiled several years ago and the list of compounds keeps growing

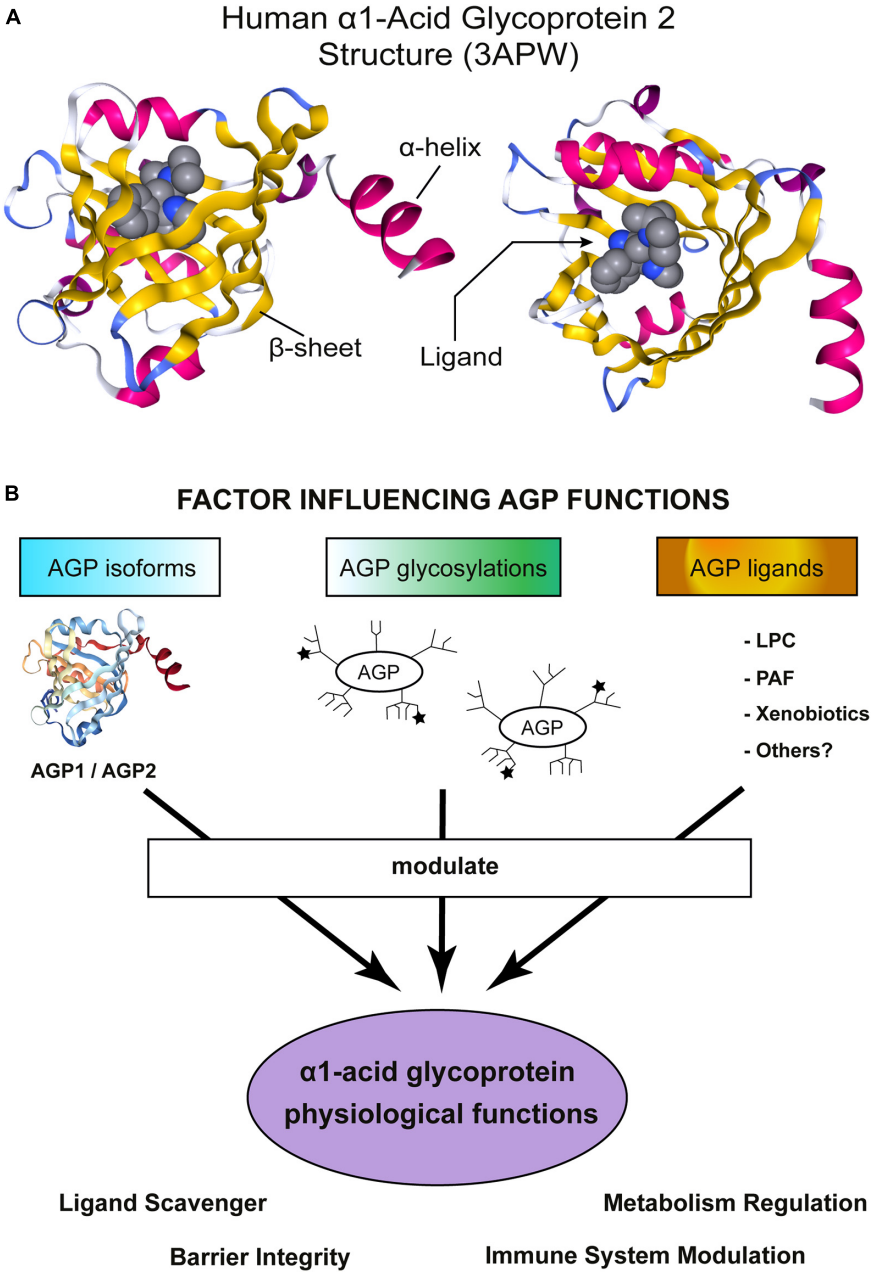

FIGURE 1 | α1-Acid Glycoprotein (AGP) structure and functions. **(A)** Schematic representation of hAGP2 structure in complex with a drug in the Lipocalin cavity. In this case the ligand is the antiarrhythmic medication disopyramide (3APW) [originaly published by Nishi et al. (2011)]. The left model is a lateral view of AGP2 and the right model show the view from the top, the open side of AGP β-barrel. The secondary structures are colored: β-sheets in yellow and α-helix in magenta. The ligand (disopyramide) is represented in a bulky format to highlight the cavity inside of AGP2. The figure was generating using NGL viewer (Rose et al., 2018). **(B)** Schematic representation showing important factors influencing AGP functions. AGP is highly heterogenic and numerous activities *in vitro* and *in vivo* have been reported. AGP is an acute-phase protein that modulates the immune system and, recently, AGP has also been shown to regulate the metabolism. AGP tissue/cell type of expression determines which isoform is expressed: AGP1 or AGP2. Then, each tissue counts with a specific set of enzymes to glycosylate proteins and, indeed, multiple glycosylation patterns have been detected in AGP. The ★ symbols represent the possible presence of sLe^x groups. Note that the schematic cartoon of the glycosylation does not represent the actual complex and heterogeneous AGP glycosylation pattern. More detailed information can be found in Baerenfaenger and Meyer (2018), Keser et al. (2021). Finally, the environment where AGP is secreted allows to bind/scavenger a different set of ligands, Indeed, an enormous number of compounds are efficiently bound by AGP. The sum of all the above-mentioned factors would contribute to AGP physiological functions.

(Israili and Dayton, 2001; Wishart et al., 2006; Zsila et al., 2006; di Masi et al., 2016; Smith and Waters, 2018). AGP binds mainly to basic molecules, given its highly acidic nature, but it is also able to bind neutral and acidic drugs (Taguchi et al., 2013).

Endogenous Ligands

Several endogenous molecules bind to AGP. Catecholamines are long-known but are low-affinity ligands of AGP (Sager et al., 1987). Similarly, the ability of AGP to bind progesterone with low affinity was documented earlier (Westphal et al., 1961; Ganguly et al., 1967), and further confirmed with modern methods (Albani, 1997; Ojala et al., 2006; Huang and Hudgens, 2013). It was suggested that progesterone sequestration by AGP would represent a buffer system (Westphal et al., 1961), but not much have been experimentally demonstrated. Curiously, plasma AGP is not the only Lipocalin able to bind progesterone. ApoD was isolated as a progesterone-binding protein from mammary cystic fluid (Pearlman et al., 1973; Rassart et al., 2000) and even the complex crystalized (Eichinger et al., 2007). Additionally, recombinant ApoM showed certain ability to interact with progesterone *in vitro* (Ahnstrom et al., 2007), but no evidences of progesterone being an endogenous ApoM ligand have been presented so far.

Another report strongly argued for biliverdin as the endogenous ligand of hAGP1. Even though convincing *in vitro* data supports the binding, no evidences of biliverdin as endogenous ligand were presented (Zsila and Mady, 2008). The authors speculated that inside of the β -barrel, biliverdin might be transiently protected from enzymatic oxidation, thereby preventing accumulation of toxic bilirubin (Zsila and Mady, 2008).

In the search of physiological AGP ligand(s), a big effort was implement in Finland a few years ago (Ojala et al., 2006). Large amounts of AGP were isolated from plasma, followed by lipid extraction and mass spectrometry analysis. Significant amounts of lysophospholipids, and more specifically lysophosphatidylcholines (LPC) with unsaturated acyl chains, were identified. Further *in vitro* ligand-binding assays confirmed the highest affinity for LPC20:4, LPC18:3, and LPC18:1. However, AGP was also able to efficiently bind free fatty acids and platelet activated factor [(PAF), 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine, (as also previously reported in McNamara et al., 1986)]. Finally, to highlight the biological relevance of AGP-LPC and AGP-PAF complexes, it was shown that AGP prevented LPC-induced priming and PAF-induced activation of human granulocytes (Ojala et al., 2006). Systemically, several studies have shown that AGP contributes to maintain cellular barriers in the kidneys, lungs, brain and vessels (i.e., Haraldsson et al., 1992; Johnsson and Haraldsson, 1993; Muchitsch et al., 1996, 1998, 2000; Sorensson et al., 1999) whereas LPC and PAF induce permeability (Huang et al., 2005; Hudry-Clergeon et al., 2005); suggesting that AGP could be a LPC/PAF scavenger. Interestingly, the expression of another Lipocalin, ApoM, is induced by PAF (Xu et al., 2002) and ApoM is fundamental to maintain barrier function (Christoffersen et al., 2011; Ruiz et al., 2017; Mathiesen Janiurek et al., 2019). In conclusion, the work by Ojala et al. (2006) could potentially explain the anti-inflammatory/protective effects of AGP and it probably has been the best attempt to explain the physiological relevance of AGP ligand(s).

Xenobiotics-Binding

Many compounds show potential therapeutic capacity when examined *in vitro* or in animal models. However, the impressive ability of AGP to bind drugs sometimes represents a limitation for their clinical use (as discussed on Smith and Waters, 2018; Bteich, 2019; Bteich et al., 2021). UCN-01 (7-hydroxystaurosporine) is an anti-cancer drug and its sequestration by AGP is a classic example of AGP affecting drug pharmacokinetics and pharmacodynamics. hAGP displays an unexpected high affinity for UCN-01 and hence increases drug plasma concentration, while blocking its distribution and elimination. In contrast, canine AGP has lower affinity for UCN-01 and therefore has little effect on the pharmacodynamics of UCN-01 and rat AGP exhibited only weak and nonspecific binding to UCN-01 (Fuse et al., 1998, 1999). Interestingly, encapsulation of UCN-01 in liposomes has been proposed to reduce the impact of AGP on UCN-01 pharmacodynamics (Yamauchi et al., 2005, 2008).

Examples of recent studies on drugs bound by AGP include: Warfarin (Hanada, 2017), Pinometostat (Smith et al., 2017), Aripiprazole (Nishi et al., 2019), Imatinib (Mic et al., 2020), Voriconazole (Yuan et al., 2020), ONO-2160 (Kono et al., 2019, 2021), SCO-272 (Ebihara et al., 2018, 2019), and Brigatinib (Wang et al., 2020).

AGP Receptors

The paragraphs above discussed the binding capabilities of AGP. But it is unclear if AGP is a passive scavenger protein or, alternatively, whether AGP delivers its cargos to particular receptors. Several membrane proteins have been reported to interact with AGP. For instance, AGP binds to the C-C chemokine receptor type 5 (CCR5) in the plasma membrane of macrophages and skeletal muscle cells (Atemezem et al., 2001; Lei et al., 2016). Both, AGP polypeptide and glycans are important for AGP-CCR5 interaction. Speculatively, the authors suggested that AGP-CCR5 association could block the infection of macrophages by the HIV-1 virus (Seddiki et al., 1997).

The presence of numerous sLe^x groups in AGP have led to depict AGP as an interacting partner of the endothelial adhesion molecules P-selectin and E-selectin. In this way, AGP binding would block the adhesion of circulating leukocytes to the endothelium upon inflammatory stimuli (Jorgensen et al., 1998; Hochepied et al., 2000). A structural model of AGP and P-selectin interaction have been calculated *in silico* (Fernandes et al., 2015). Furthermore, AGP sLe^x groups mediate the interaction of AGP with immunoglobulin-like lectins (Siglecs) (Gunnarsson et al., 2007) and modulate reactive oxygen species (ROS) generation in neutrophils (Gunnarsson et al., 2010).

Finally, the liver-expressed asialoglycoprotein receptor binds molecules of AGP in which the terminal sialic groups are missing and efficiently clears asialo-AGP from circulation (Kindberg et al., 1990; Matsumoto et al., 2010). It has been suggested that another, yet unknown, receptor would mediate AGP uptake (with sialic acid residues) (Matsumoto et al., 2010; Taguchi et al., 2013).

However, it is not known if AGP is simply targeted for degradation or has also intracellular functions.

Remarkably, AGP binds to membranes and undergoes a pH-induced conformational change (a unique transition from a β-sheet-rich structure to an α-helix-rich structure) which caused a decrease in AGP affinity for progesterone (Nishi et al., 2002, 2004, 2006). This has been interpreted as a mechanism to release molecules inside of the cell (illustrated in Taguchi et al., 2013). However, this has also been interpreted in the opposite way: as a mechanism to sequester LPC/PAF from the plasma membranes where they are generated (Ojala et al., 2006). Interestingly, this unique β-sheet to α-helix transition has also been reported for Tear Lipocalin (Gasymov et al., 1998). Follow-up investigations to address the relevance of AGP β-sheet to α-helix transition *in vivo* would be highly valuable.

IN VIVO APPROACHES, TRANSGENESIS, AND KNOCKOUTS

α_1-acid glycoprotein being an acute-phase protein, most of the early *in vivo* studies were related to inflammatory insults. For that, different transgenic mouse models were initially created to study AGP. Bacterial lipopolysaccharide (LPS) strongly induced expression and liver secretion of hAGP1 in mice carrying the whole hAGP gene cluster (*ORM1*, *ORM2*, and *ORM3* genes) or a fragment with only the *ORM1* gene (Dente et al., 1988). Later, another transgenic mouse in which the rat *Orm* gene was over-expressed was made. LPS, IL-1, IL-6, or glucocorticoids were used to trigger the inflammatory response and this boosted rated AGP expression several folds (Dewey et al., 1990). In general, AGP has shown to be protective *in vivo* against inflammatory insults (as summarized in Hochepied et al., 2003). One example is that the intraperitoneal injection of hAGP (but also rat and bovine) protected against lethal shock induced by TNFα (Libert et al., 1994). However, the overexpression of rat AGP led to a more aggressive development of acute colitis (Hochepied et al., 2002).

Another example of a protective effect is that pre-administration of exogenous bovine or hAGP or transgenic over-expression of rat AGP in mice, significantly increased survival against a lethal infection with the Gram-negative bacteria *Klebsiella pneumoniae* (Hochepied et al., 2000) or *Bacillus anthracis (*Shemyakin et al., 2005). However, the molecular mechanism involved is unknown. One explanation could be the reported capacity of AGP to form complexes with LPS (Moore et al., 1997). However, AGP-LPS complexes cannot explain the documented protection against the Gram-positive *Bacillus anthracis*. Alternatively, a recent paper proposed a direct action of AGP on bacterial growth. Siderophores are small molecules secreted by bacteria to secure their iron supply (a scarce and essential micronutrient) and growth. The authors reasoned that many bacteria secrete stealth siderophores that escape Lcn2 recognition (the archetype Siderocalin), and suggested that AGP may be a "Siderocalin" and hence able to bind siderophores (Samsonov et al., 2021). Interestingly, Tear Lipocalin, a highly abundant Lipocalin in secretions, interferes with microbial growth by scavenging of siderophores (Fluckinger et al., 2004), Tear Lipocalin is, however, not present in plasma. Given the similarities between Lcn2, Tear Lipocalin and AGP, one may speculate that AGP can also neutralize siderophores escaping Lcn2 entrapment and inhibit *K. pneumoniae* or *B. anthracis* growth. Unfortunately, AGP computational experiments were inconclusive for *K. pneumoniae* siderophores (Samsonov et al., 2021). However, the results were more positive about petrobactin (one of the siderophores secreted by *B. anthracis*) being a candidate ligand for AGP (Samsonov et al., 2021). In any case, the ability of AGP to bind siderophores and inhibit bacterial growth needs to be experimentally demonstrated.

AGP1 is highly abundant in plasma (∼0.075 g/dl; ∼15 μM) (Kremer et al., 1988; Gannon et al., 2019; McDonald et al., 2020), easy to purify and represents a relatively affordable commercial source of AGP protein for *in vitro* and *in vivo* experiments. However, there are some limitations that can complicate the interpretations of the results. First, different batches of protein come from different donors and likely have distinct glycosylation patterns. Importantly, mouse and human livers possess a different set of fucosyltransferases and hAGP produced in mice lacks sLe^x (Havenaar et al., 1998). sLe^x groups can be important to efficiently modulate hAGP function. Thus, hAGP might be not fully functional in murine experimental models. Additionally, isolated AGP will likely come with uncharacterized ligand(s) in its cavity and variations in the purification protocols may impact their presence and nature.

The absence of AGP-KO animal model was a strong limitation to understand AGP functions, until, for the first time, an Orm1-KO mouse was published in 2016 (Lei et al., 2016). The newly created mouse mutants were first used to demonstrate that AGP1 binds to CCR5 on skeletal muscle cells to increase muscle endurance (Lei et al., 2016). Later, the same group showed that AGP1-CCR5 increased the activity of glycogen synthase (the rate-limiting enzyme in the glycogen synthesis pathway) via AMPKα2 (Qin et al., 2016). Further, they identified estrogens (as a negative) and erythromycin (as a positive) regulators of the AGP1-CCR5 pathway (Sun et al., 2018; Wan et al., 2020).

Interestingly, *Orm1*-KO mice show altered metabolic parameters, such as increased levels of insulin and leptin together with impaired glucose tolerance (Sun et al., 2016) and AGP1 deficiency increases the expression of genes related to fibrosis in adipose tissue (Wang et al., 2021). Previously, and in agreement with the *Orm1*-KO mouse model, the continuous systemic infusion of hAGP1 improved glucose and insulin tolerance in obese/diabetic mice (Lee et al., 2010). Further explorations in the *Orm1*-KO mouse led to the discovery that AGP inhibits food intake. Mechanistically, AGP1 interacts with the leptin receptor (Lepr) in the hypothalamus and activates the JAK2-STAT3 pathway to inhibited food intake (Sun et al., 2016). However, it is not clear how AGP1, the main isoform in circulation, crosses the blood brain barrier to interact with the Lepr in the hypothalamus. Additionally, the main isoform in the brain is AGP2 and its levels do not change under metabolic stress (Sun et al., 2016). Even though, the nature of the AGP-Lepr interaction model is not completely understood, its existence is certainly an interesting observation. ApoD, the most ancestral Lipocalin in vertebrates,

has also been shown to interact with the Lepr (Liu et al., 2001). ApoD interaction is thought to take place with the cytosolic domain of the Lepr (Liu et al., 2001), whereas AGP interaction was modeled to occur via the leptin-binding domain of the Lepr (Sun et al., 2016).

The first complete AGP-KO (*Orm1*, *Orm2* and *Orm3*-KO) was finally published last year, 2020 (Watanabe et al., 2020). The AGP-KO mice did not show any obvious defects in appearance or growth. However, the AGP-KO animals had exacerbated fibrosis, inflammatory response and macrophage infiltration in a model of renal fibrosis (Watanabe et al., 2020, 2021). Accordingly, AGP administration reduced renal fibrosis and inflammation (Bi et al., 2018). Interestingly, all-trans retinoic acid treatment boosted AGP serum concentration in plasma and required AGP to protect against renal fibrosis. So, how do all-trans retinoic acid and AGP damper renal fibrosis and the immune response? It is noteworthy that all-trans retinoic acid is a classical Lipocalin ligand and binds to AGP with micromolar affinity (Breustedt et al., 2006; Ruiz et al., 2013). Therefore, all-trans retinoic acid might just induce AGP expression that then transports it to the damaged area? Interestingly, the major transporter of retinol in plasma, the Lipocalin RBP4, is a negative acute-phase protein (Rosales et al., 1996). Thus, AGP could take the place of RBP4 and transport retinols during inflammation.

Interestingly, AGP1-KO did not affect the infarct area in a model of ischemic stroke (even when the blood brain barrier was compromised). Instead, the expression of AGP2 was induced in the ischemic tissue (Wan et al., 2016, 2019). Unfortunately, an AGP2-KO model was not available at that time. Therefore, the availability of a full AGP-KO is now a great tool to explore anew the role of AGP in the central nervous system. Expression of AGP2 in the brain is induced upon systemic inflammation, astrocytes being the main source of AGP. Mechanistically, AGP2 inhibited CCL4-induced microglial activation by blocking the interaction of AGP with CCR5 and reduced microglia-mediated neurotoxicity (Jo et al., 2017). Noteworthy, other Lipocalins are also expressed in glial cells. For instance, astrocytes express ApoD upon stress conditions to promote neuronal survival (Bajo-Graneras et al., 2011; Pascua-Maestro et al., 2018). Oppositely to AGP and ApoD, Lcn2 is an autocrine mediator of astrocytosis and renders astrocytes more sensitive to cell-death signals (Lee et al., 2009). To add one extra level of complexity, only apo-Lcn2 (no-ligand bound) sensitized activated astrocytes to cell-death (Lee et al., 2009). Therefore, it would be relevant to investigate if any ligand mediates the protection by AGP in the brain upon inflammation.

CONCLUDING REMARKS

α_1-acid glycoprotein expression is strongly up-regulated during the acute-phase response probably as a counter-balance to damper an excessive inflammatory response. Thus, AGP is typically associated with protection. Interestingly, AGP investigations are not limited to inflammation and new studies reported an active role of AGP in metabolic regulation. One of the most interesting features of AGP is its heterogeneity, from the amino acid sequence to the glycosylation pattern (**Figure 1B**). Multiple AGP forms are possible which suggests the existence of fine-tuned mechanisms to regulate AGP functions and highlights AGP versatility to participate in multiple process. The best example of AGP versatility is its ability to bind hundreds of small molecules (**Figure 1B**). Despite thousands of publications about AGP, its molecular functions are not fully understood. Hopefully, the newly created AGP-KO mice will help to shed light on AGP physiological roles.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Tear Lipocalin and Lipocalin-Interacting Membrane Receptor

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Tear lipocalin is a primate protein that was recognized as a lipocalin from the homology of the primary sequence. The protein is most concentrated in tears and produced by lacrimal glands. Tear lipocalin is also produced in the tongue, pituitary, prostate, and the tracheobronchial tree. Tear lipocalin has been assigned a multitude of functions. The functions of tear lipocalin are inexorably linked to structural characteristics that are often shared by the lipocalin family. These characteristics result in the binding and or transport of a wide range of small hydrophobic molecules. The cavity of tear lipocalin is formed by eight strands (A–H) that are arranged in a β-barrel and are joined by loops between the β-strands. Recently, studies of the solution structure of tear lipocalin have unveiled new structural features such as cation-π interactions, which are extant throughout the lipocalin family. Lipocalin has many unique features that affect ligand specificity. These include a capacious and a flexible cavity with mobile and short overhanging loops. Specific features that confer promiscuity for ligand binding in tear lipocalin will be analyzed. The functions of tear lipocalin include the following: antimicrobial activities, scavenger of toxic and tear disruptive compounds, endonuclease activity, and inhibition of cysteine proteases. In addition, tear lipocalin binds and may modulate lipids in the tears. Such actions support roles as an acceptor for phospholipid transfer protein, heteropolymer formation to alter viscosity, and tear surface interactions. The promiscuous lipid-binding properties of tear lipocalin have created opportunities for its use as a drug carrier. Mutant analogs have been created to bind other molecules such as vascular endothelial growth factor for medicinal use. Tear lipocalin has been touted as a useful biomarker for several diseases including breast cancer, chronic obstructive pulmonary disease, diabetic retinopathy, and keratoconus. The functional possibilities of tear lipocalin dramatically expanded when a putative receptor, lipocalin-interacting membrane receptor was identified. However, opposing studies claim that lipocalin-interacting membrane receptor is not specific for lipocalin. A recent study even suggests a different function for the membrane protein. This controversy will be reviewed in light of gene expression data, which suggest that tear lipocalin has a different tissue distribution than the putative receptor. But the data show lipocalin-interacting membrane receptor is expressed on ocular surface epithelium and that a receptor function here would be rational.

Keywords: tear lipocalin, lipocalin interacting membrane receptor, lipocalin 1, von Ebner's gland protein, gene sharing, human tears, limb development membrane protein-1, phospholipid transfer protein

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INTRODUCTION

Tear lipocalin is a member of the calycin superfamily, which includes fatty acid binding proteins, avidins, and the lipocalin family. The lipocalins share a highly conserved lipocalin fold formed by eight antiparallel β strands that are continuously hydrogen-bonded and folded in the shape of a flattened pita bread (Flower, 1996). These strands in concert with conserved 3_{10} and α-helices form the internal ligand-binding site for small hydrophobic ligands. Lipocalins are small; the main isoform of tear lipocalin has a molecular mass 17,446 Da (Glasgow et al., 1998b). The published solution and crystal structures of tear lipocalin are shown in **Figure 1** (Gasymov et al., 2001; Breustedt et al., 2005). As with other lipocalins, tear lipocalin contains the highly conserved regions of the lipocalin family, including the 3_{10} helix (preceding the A strand), the FG loop, and the α-helix preceding the I strand. The structures show a capacious and flexible pita-shaped cavity. Hydrophobic residues line the internal cavity including a highly conserved tryptophan (**Figure 2**). Tear lipocalin is a multifaceted protein with a variety of functions. At least four prior reviews updated its functions. The most recent was 10 years ago (Redl, 2000; Glasgow et al., 2002c; Dartt, 2011; Glasgow and Gasymov, 2011). As noted in one of the reviews, the functions in tears need to be rectified with the potential receptor on the ocular surface (Dartt, 2011). Recent advances have been made in our understanding of the structure and functions of tear lipocalin as well as its putative receptor. This review will update the prior reviews with a focus on new information, including some unpublished data. The methods and discussion section will present some new data regarding the lipocalin-interacting membrane receptor.

Nomenclature

Tear lipocalin was originally discovered and named according to its electrophoresis profiles of tears. Initially referred to as "tear albumin" (Erickson et al., 1956), later the name was changed to "anodal tear protein" to reflect a migration pattern that was different from albumin. Immunoelectrophoresis indicated that the protein was present in tears but absent in blood and other body fluids (Bonavida et al., 1969). It was renamed "specific tear prealbumin." But tear lipocalin was unrelated to transthyretin and it migrated faster than albumin, and so it was rechristened "protein migrating faster than albumin" (Gachon et al., 1979). Later the primary sequence indicated the protein was a member of the lipocalin family. The sequence identity to family members was a maximum of 58% to von Ebner's gland protein of the rat, with only 27% identity to β lactoglobulin (Redl et al., 1992). For other human lipocalins the sequence identity is about 20–25% but specific residues such as tryptophan are highly conserved (**Figure 2**). The name was changed to "human tear lipocalin," but was also referred to as von Ebner's gland protein as the same protein is secreted from lingual glands around circumvallate papillae (Delaire et al., 1992; Redl et al., 1992; Bläker et al., 1993; Lassagne et al., 1993). The glands had been named after the Austrian histologist, Victor von Ebner. Later, as one of the first lipocalins to be cloned, the gene for tear lipocalin was designated as "lipocalin-1," while the name for the protein was retained as human tear lipocalin. This was a consensus decision made at Benzon Symposium #50 of the Lipocalin Protein Superfamily in 2003. The nomenclature is not strictly followed, so the protein is variously called tear lipocalin, lipocalin 1, and von Ebner's gland protein. Initial two-dimensional gel electrophoretic profiles revealed at least six published isoforms of tear lipocalin (Fullard and Kissner, 1991; Delaire et al., 1992; Glasgow, 1995). Later mass spectrometry demonstrated that these "isoforms" appear to be mainly truncated versions of the only protein predicted by a single known mRNA (Glasgow et al., 1998b). Native purified tear lipocalin contains all of these molecular species.

The nomenclature of the putative receptor for tear lipocalin is also relevant. Lipocalin-interacting membrane receptor was described as comprising of 647 amino acids, and the gene has 17 exons and at least five isoforms (Wojnar et al., 2001a). Several reports identified a nearly identical protein, limb development membrane protein-1, comprising of 649 amino acids. Most studies reported six sequential bases at the start of exon 3 that are missing in the original paper (Wojnar et al., 2001a). The isoform without the six bases is now considered as isoform 2, Q6UX01-2. The bases code for valine and aspartic acid in positions 53 and 54, respectively of the 649 amino acid receptor sequence. In almost all of the published variations of the sequence, the bases coding for valine and aspartic acid are present. Some other isoforms are reported as a result of theoretical and identified alternative splicing. The term limb development membrane protein-1 appears to be a misnomer. Initially, the genetic locus was linked to congenital limb malformations. Later it was shown that the malformation was due to the disruption of a long range sonic hedgehog enhancer located in an intron of the gene (Lettice et al., 2003). For simplicity, limb development membrane protein-1 will be considered synonymous with lipocalin-interacting membrane receptor. Here the term, lipocalin-interacting membrane receptor, will encompass the putative isoforms.

METHODS

Gene Expression of Tear Lipocalin and Lipocalin-Interacting Membrane Receptor

To compare the mRNA expression profiles for tear lipocalin and lipocalin-interacting membrane receptor, the gene expression omnibus (GEO) repository was searched for human tissue profiles that contained either a variety of normal tissues and/or ocular surface tissues (Barrett et al., 2013). Each profile can include data derived from various sources such as expressed sequence tags (ESTs), microarrays, high throughput sequencing, nanostring methods, or reverse transcriptase polymerase chain reactions. Calculations are based on the original submitter-supplied expression measurements presented as "values" in the sample record. The submitters to the GEO repository are required to normalize the values of expression of each gene to the total number of expressed transcripts, as measured by their method. There is great diversity in the data values and ranges provided by GEO submitters. Each set may have different types of tissues and vary in types as well as number of transcripts

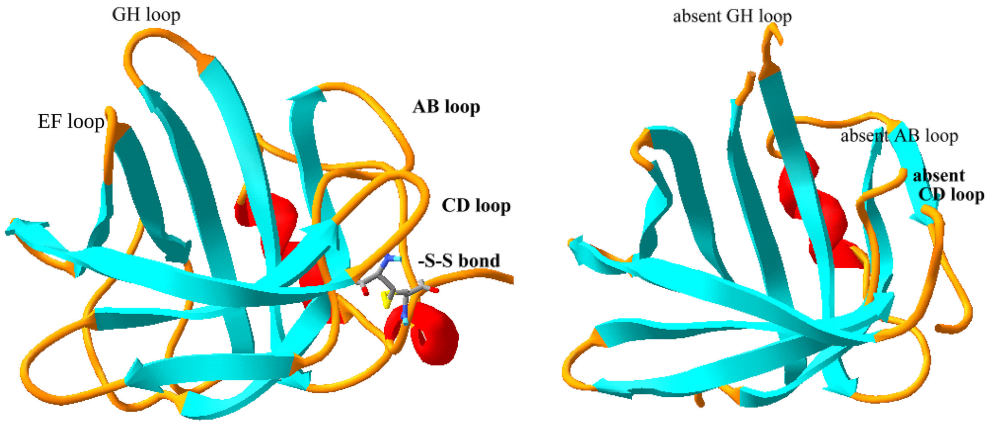

FIGURE 1 | Comparison of solution and crystal structure of tear lipocalin. The solution structure is shown on the left (Gasymov et al., 2001) and the crystal structure is shown on the right (Breustedt et al., 2005), PDB file, 1XKI. The loops (yellow) were evident from the solution structure, but were not resolved by the crystal structure. The strands or β sheets are shown in aqua and the α-helices are shown in red. The loops are named (black) by the adjoining strands for orientation.

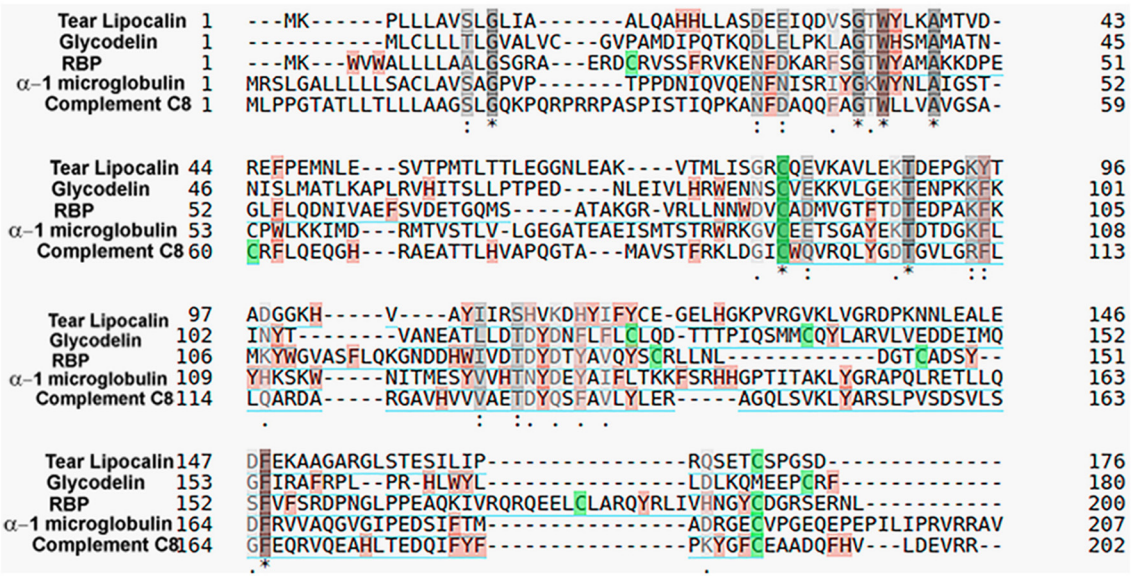

FIGURE 2 | Comparison of representative human lipocalins for amino acid sequence identity. Colors indicate disulfide bonds (green), aromatic residues (tan), and similar amino acid properties (gray). Asterisk (*) indicates a fully conserved sequence, colon (:) indicates groups of strongly similar properties and period (.) indicates groups of weakly similar properties. Sequence identity was determined by the Universal Protein Resource (https://www.uniprot.org/align/) with protein accession numbers: P31025-tear lipocalin, P09466-glycodelin, P02753-retinol-binding protein 4 (RBP), and P07360-complement component C8 gamma chain.

that are tagged or identified. The presentation of data in the GEO repository can be variably formatted as relative values on linear or log base 10 scales or as log10 ratios representing the abundance of expressed transcripts. In each data set, the values are dimensionless. Submission of data to the GEO does not require the assessment of background noise in the data sets to ensure that a given threshold value represents true expression. Therefore, the expression of various tissues can be compared within a data set but are more difficult to compare between data sets. Specific data sets, GDS (GEO data set) (423,3834, 3113, 1085), were queried for expression values of the gene for lipocalin-interacting membrane receptor (Shyamsundar et al., 2005; Yanai et al., 2005; Dezs et al., 2008; She et al., 2009). In addition, GDS 2682 provides a comparison of gene expression in the conjunctiva and the cornea (Turner et al., 2007). In order to obtain the relative amounts of transcript expressed by lipocalin-interacting membrane receptor and tear lipocalin, the normalized data from a single data set were analyzed. However, in order to compare multiple data sets for the transcript expression of lipocalin-interacting membrane receptor rank means were used. This basic method avoids statistical misassumptions such as the presence of normal distributions. The relative order (rank) of the

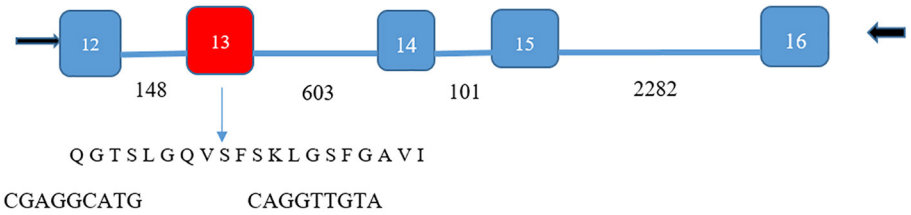

FIGURE 3 | Isoform identification of lipocalin-interacting membrane receptor in ocular surface epithelium. PCR products of 499 and 559 base pairs spanned five exons (numbers shown in white) for the lipocalin-interacting membrane protein. Number of bases in the introns are shown by black numbers. The 60-base pair deletion in exon 13 (red) was verified by the sequencing of the 499 base pair product (DNA sequence cga ggc atg –cag gtt gta bridging the missing sequence). The missing 60-base pair sequence corresponds to amino acids missing from isoform 3, Q6UX01-3, an alternative splice variant (https://www.uniprot.org/uniprot/Q6UX01). This translates to the transmembrane helical segment of the protein sequence shown. The 559-base pair product contained the missing sequence.

mean of the normalized values given for transcript expression of lipocalin-interacting membrane receptor was calculated for each type of tissue in a data set. A percentile rank was calculated for the expression of lipocalin-interacting membrane receptor for the various tissues of each data set. The percentile ranks of expression for each tissue type from the multiple data sets were then averaged to give a mean percentile rank to provide a broader view of the relative expression of lipocalin-interacting membrane receptor. Generally, a minimum of three samples were available for each tissue in any one data set. In one data set, the authors suggested a threshold value, below which it was considered noise (Yanai et al., 2005). Tissue types that had expression values below the threshold value of this data set were not considered for statistical analysis in other data sets without a threshold value. The analysis of such data is more useful if there is agreement across the data sets for a particular percentile rank of expression for a given tissue. The standard error of the mean of the mean percentile ranks is provided.

Since the lacrimal gland was not one of the tissues in the GEO data sets, data from the now discontinued Human UniGene (*http://www.ncbi.nlm.nih.gov/unigene)* libraries were included. The libraries were searched for tear lipocalin and lipocalin-interacting membrane receptor ESTs, and filtered with a minimum cut-off of 1000 transcripts. Expression levels were calculated by taking the sum of all ESTs in a given category divided by the sum of all transcripts.

Identification of Lipocalin-Interacting Membrane Receptor in Ocular Surface Epithelium

Although quite useful as a guide, the GEO repository data for cornea and conjunctiva were not provided with threshold levels. Therefore, direct confirmation of transcript expression for lipocalin-interacting membrane receptor was undertaken. Reverse transcription polymerase chain reactions (PCR) were performed on cDNA from a human cornea epithelial library (ScienCell, Carlsbad, CA) as well as from discarded surgical samples of the cornea and conjunctiva in accordance with the Declaration of Helsinki. The study was approved by the UCLA Institutional Review Board for Human Subjects. Samples had generally been fixed in acidified ethanol for about 48 h. Cornea and conjunctival epithelium were microdissected, rinsed in RNAase free water, lysed, and homogenized in sterile microfuge tubes. RNA was extracted according to the instructions of the manufacturer (RNeasy Fibrous Tissue Kit (Qiagen). First strand cDNA was synthesized from the purified RNA using the iScript™ kit with RNase H+ reverse transcriptase (BioRad). Both first strand synthesis and PCR were done using a GeneAmp PCR system 2400 (Perkin Elmer). The strategy was to use lipocalin-interacting membrane receptor specific primers (forward 5′-GTGCTTGCTGGTGCTGACGG-3′ and reverse 5′-TCACTGGTGCTGGGTCTTCCTAGATG-3′) from exons that would have intervening introns (**Figure 3**). This ensured that genomic DNA was not misidentified as cDNA. In addition, some isoforms could be identified. The PCR parameters included 35 cycles of denaturation at 95°C, annealing at a step gradient including 60°C, 51°C, and 42°C for 20 s each, followed by extension at 72°C, with 1 min at each step. Products were immediately ligated in PCR 2.1TOPO vectors for 5 min and transformed in chemically-competent cells (Invitrogen). Selection of colonies and plasmid purification were performed as previously described (Gasymov et al., 1997). Sequencing was performed on an Applied Biosystems® 3730 Capillary DNA analyzer (Life Technologies) using T7 and T3 promoter sas primers. Analysis of products from PCR and subcloned plasmid inserts was performed on 1.5–1.7% agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

Tissue Localization of Tear Lipocalin

Initially, tear lipocalin was proffered to be tear specific. Immunofluorescence studies demonstrated tear lipocalins in acinar cells of main and accessory lacrimal glands (Glasgow, 1995; Ubels et al., 2012), but not in the cornea, meibomian glands, or conjunctiva (Inada, 1984). Tear lipocalin was identified by the group of Redl in the Western blots of saliva, sweat, and nasal mucus (Holzfeind et al., 1996; Wojnar et al., 2001a), as well as by immunofluorescence/immunohistochemical studies of the tracheobronchial tree, prostate, and pituitary (Holzfeind et al., 1995, 1996; Redl et al., 1998; Wojnar et al., 2002). Tear lipocalin could not be confirmed in eccrine glands of skin (Glasgow, 1995). This finding appears consonant with the

TABLE 1 | Relative rank order of tissue expression of lipocalin-interacting membrane receptor from multiple GEO data sets.

Tissue	Mean of means of percentile rank ± standard error
Adrenal	6.4 ± 0.6
Testis	17.2 ± 10.4
Brain	21.2 ± 4.4
Thyroid	22.5 ± 10.1
Diaphragm	25.1 ± 2.6
Brain (fetal)	29.4 ± 1.9
Pituitary	30.0 ± 5.6
Lung	33.3 ± 10.0
Spinal cord	33.4 ± 11.6
Ovary	35.0 ± 8.5
Thymus (fetal)	37.5 ± 6.2
Breast	37.5 ± 12.5
Kidney	45.4 ± 6.2
Thymus	47.2 ± 20.3
Bladder	48.1 ± 13.1
Pancreas	50.0 ± 9.6
Uterus	51.9 ± 20.0
Blood (or lymphocytes)	53.1 ± 7.3
Prostate	55.0 ± 8.1
Pancreas	59.6 ± 9.6
Spleen	62.5 ± 19.7
Kidney (fetal)	62.8 ± 2.8
Noise threshold (Yanai et al., 2005)	64.7
Heart	
Retina	66.9
Bone marrow	67.7
Liver	67.7
Salivary gland	71.4
Small intestine	72.2
Skin	78.8
Trachea	79.4
Tonsil	82.6
Placenta	84.7
Colon	89.1
Liver (fetal)	93.8
Vagina	94.4
Stomach	99.0
Skeletal Muscle	100.0

The means of percentile ranks of normalized expression values for each tissue in every data set were averaged and ordered across the available data sets. Most abundant transcripts values have the lower ranks.

expression value below threshold levels for noise (**Table 1** and **Figure 4**). Immunoelectronmicroscopy studies of secretion of tear lipocalin in the lacrimal gland show that the protein appears to be packed in secretory granules often colocalizing with other proteins (Glasgow, 1995; Wojnar et al., 2002). The distribution was indicative of regulated secretion (Glasgow, 1995).

Tear Lipocalin Is a Multifunctional Protein

Gene-sharing is a common theme in evolution (Piatigorsky, 1998, 2007). Efficient conservation dictates that evolutionary selection pressure will favor proteins that are multifunctional. Tear lipocalin is paradigmatic for the multifunctional nature of proteins. The documented functions for this protein are shown in **Table 2** and range from lipid-binding and transport, enzymatic activity, enzymatic inhibitor, and polymer formation. Virtually all the functions of the of tear lipocalin stem from interaction with other molecules. In most cases these are small hydrophobic lipids, proteins, or substrates such as DNA. Tear lipocalin is a promiscuous protein with a broad array of native ligands and the potential to bind an enormous suite of molecules. **Table 3** shows many of the known ligands and interacting molecules of tear lipocalin. Direct comparison of the dissociation constants is often hindered by the methods used for calculation. Many have been calculated by displacement of a fluorescent ligand such as DAUDA, but some have been calculated directly. **Table 3** shows that binding affinities are usually in the micromolar range. Tear lipocalin has an internal hydrophobic binding site with capacity for a C18 alkyl chain, although other hydrophobic groups can bind with less affinity (Abduragimov et al., 2000). The binding strength correlates with the length of the chain (Glasgow et al., 1995). The stoichiometric parameter (n) is close to 1 for the ligands of tear lipocalin. Data variation can be attributed mainly to preoccupied binding sites (Glasgow and Abduraguimov, 2018).

The Functions of Native Tear Lipocalin in Tears

The functions of a protein can be defined by the tasks that the ligand or the protein performs in native environments. Therefore, discovery of the native ligands provided important clues to functions. Tear lipocalin is highly concentrated in tears, ∼60–100 μM, and is second only to lysozyme in concentration (Yeh et al., 2013). For tears the native ligands of tear lipocalin were extracted from the protein and include phospholipids, fatty acids, fatty alcohols, and cholesterol (Glasgow et al., 1995; Dean and Glasgow, 2012). Therefore, tear lipocalin obligatorily acts to solubilize, transport, and/or modulate these substances in tears. Most of the ligands have long alkyl chains and have negligible solubility in aqueous solutions. Transport of these ligands in tears to their targets allows the ligands to perform their functions. Conformational changes are triggered by local pH changes that promote ligand binding and release (Gasymov et al., 2007b). The following functions have been posited for tear lipocalin.

Antimicrobial Activity

Tear lipocalin facilitates antimicrobial activity by transport of antimicrobial lipids that would be otherwise be insoluble in tears. For example, lauric acid is a potent antimicrobial for *Propionibacterium acnes, Streptococcus group A*, *Nocardia* sp., *Micrococcus,* and *Candida* sp. (Kabara et al., 1972; Nakatsuji et al., 2009; Yang et al., 2009). Fatty alcohols have activity against herpes simplex virus (Sands et al., 1979). Tear lipocalin also has some bacteriostatic activity but it is not known if this is ligand-related or intrinsic to the protein (Selsted and

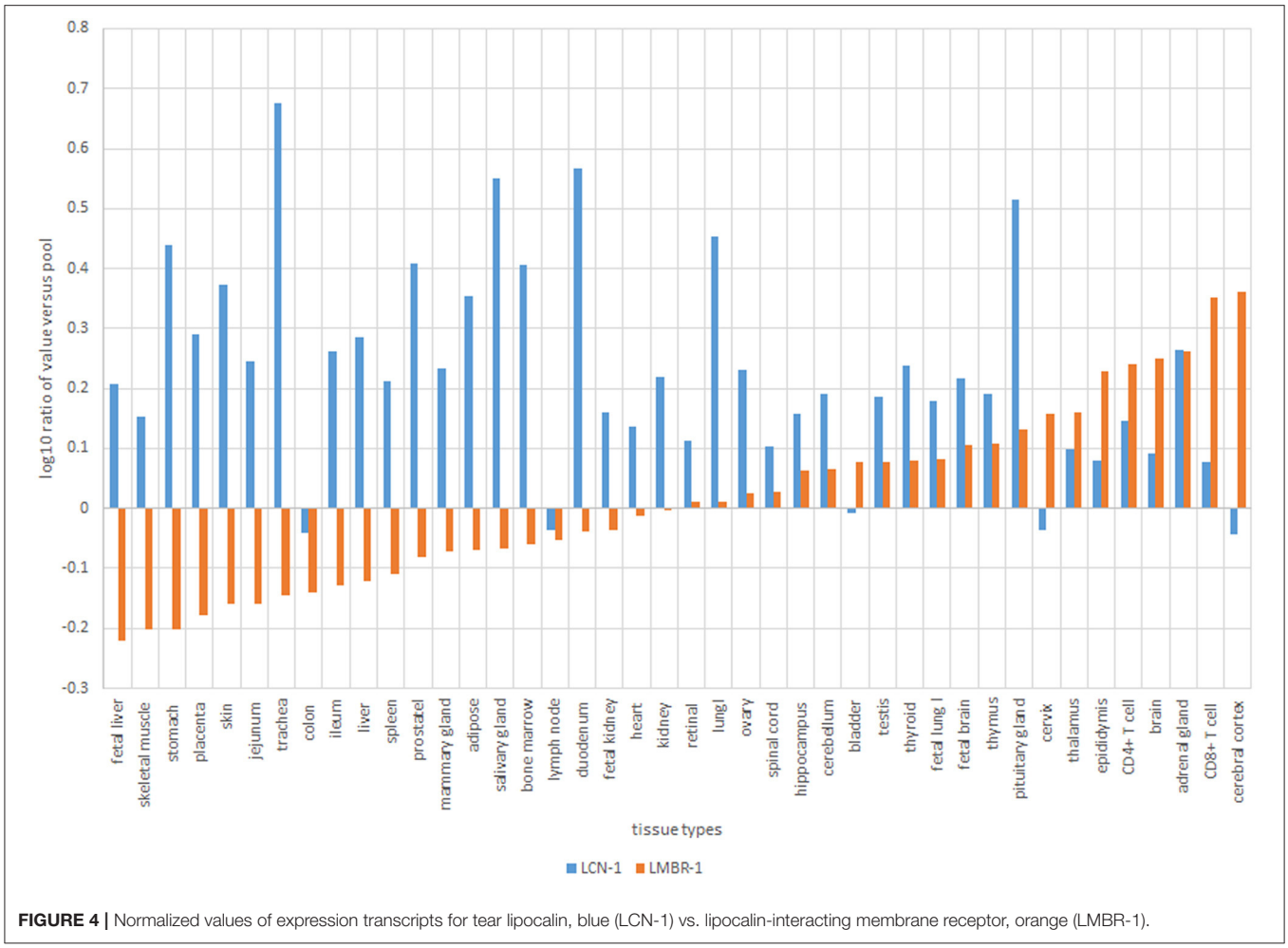

FIGURE 4 | Normalized values of expression transcripts for tear lipocalin, blue (LCN-1) vs. lipocalin-interacting membrane receptor, orange (LMBR-1).

Martinez, 1982). Tear lipocalin also interacts with lysozyme, which has a separate bacteriolytic function (Gasymov et al., 1999b). Interestingly, neither do the major lipids of the tear film, namely, wax and cholesterol esters, bind to lipocalin nor have they been shown to have significant antimicrobial activity (Glasgow and Abduragimov, 2018a). Tear lipocalin has antifungal activity. This action is related to binding fungal siderophores. The fungal siderophores compete with human lactoferrin for iron in tears. Iron is crucial for metabolic activities of microbes and host cells (Fluckinger et al., 2004). Antimicrobial activity might also be conferred by the ability of tear lipocalin to inhibit cysteine proteases, i.e., papain (Van't Hof et al., 1997; Wojnar et al., 2001b). Cysteine proteases cleave peptide bonds of proteins at a thiol group adjacent to a basic amino acid, commonly histidine. Microbial cysteine proteases cleave inactive precursors of microbial proteins to create active forms. Inhibition of cysteine protease prevents cleavage and may disable microbial functions. The prototypical cysteine protease inhibitor is cystatin, which shares some common sequences with tear lipocalin. The N terminus of tear lipocalin confers the inhibition of the protease activity, perhaps by blocking substrate access. At high concentrations of papain, the inhibition by tear lipocalin was vitiated due to cleavage of the N terminus of tear lipocalin. Some forms of cystatin have greater inhibitory activity than tear lipocalin (Wojnar et al., 2001b). Inhibition of cysteine proteases by tear lipocalin has not yet been related to the inhibition of any specific microbes. The relative importance of cysteine protease inhibition of tear lipocalin remains uncertain.

Tear lipocalin may have an antimicrobial role in the mouth. However, it has also been posited that tear lipocalin carries small molecules to receptors for taste, but experiments testing the binding of tastants have not been successful (Schmale et al., 1993).

Transport of Vitamins E and A

Tear lipocalin has been shown to bind retinol and vitamin E. Vitamin E has been extracted from purified fractions of tear lipocalin from tears. About half of the Vitamin E content is bound to protein and 86% of the protein bound fraction is complexed to tear lipocalin (Glasgow et al., 2002b). Vitamin E is a potent antioxidant and potentially useful to the exposed lipid layer of the tear film as well as to ocular surface epithelium.

TABLE 2 | Functions of tear lipocalin.

Function	Interacting partner	References
Scavenger of toxic compounds	Prostaglandins, ceramides, phospholipid, thioredoxin	Redl et al., 1999; Lechner et al., 2001; Wojnar et al., 2002; Gasymov et al., 2005; Glasgow and Abduragimov, 2018b
Transport of vitamins, nutrient	Vitamins A and E	Hong, 1986; Redl et al., 1992; Gasymov et al., 2002a; Glasgow et al., 2002a
Endonuclease activity	DNA (human/microbial)	Yusifov et al., 2000, 2008
Anti-microbial activity	Lipids, siderophores, lysozyme	Josephson and Wald, 1969; Kabara et al., 1972; Selsted and Martinez, 1982; Miller et al., 1988; Bibel et al., 1989; Fluckinger et al., 2004
Acceptor protein for phospholipid transport protein	Phospholipid transport protein, phospholipid	Glasgow and Abduragimov, 2021
Inhibitor of cysteine protease activity	Cysteine protease	Holzfeind et al., 1995; Van't Hof et al., 1997; Wojnar et al., 2001b
Viscosity (Confers non-newtonian sheer thinning behavior)	Polar lipids, lysozyme	Pandit et al., 1999; Tiffany and Nagyová, 2002; Gouveia and Tiffany, 2005
Drug delivery native tear lipocalin	Rifampin	Gasymov et al., 2004a; Staudinger et al., 2014
Drug delivery (Anti and duo-calins)	(e.g.,Vascular endothelial growth factor)	Eyer et al., 2012; Richter and Skerra, 2016

Retinol, retinal, and retinoic acid have been shown to bind lipocalin but have not been extracted successfully from purified lipocalin in tears (Redl et al., 1992; Gasymov et al., 2002a). Retinol binding protein has a higher affinity and specificity for retinol than lipocalin. It remains to be seen what the role for tear lipocalin is in the transport of retinol from the tear film to the ocular surface epithelium.

Scavenger for Lipid Peroxidation Products

Tear lipocalin was studied in a teratocarcinoma cell line and found to have increased expression when treated with ferrous sulfate or hydrogen peroxide (Lechner et al., 2001). In the same study, centrifugal concentration followed by anion exchange chromatography was used to enrich fractions of tear lipocalin from the cell culture. An enzyme immunoassay of chloroform extraction products of fractions with tear lipocalin was positive for F2-isoprostanes. *In vitro* DAUDA displacement assays confirmed that arachidonic acid and several lipid peroxidation products including 7β hydroxycholesterol, 8-isoprostane, and 13-hydroxy-9, 11-octadecadienoic acid were bound to tear lipocalin. The conclusion was that tear lipocalin has an essential function in scavenging harmful lipid peroxidation compounds. Further, this scavenger function was related to the interaction of tear lipocalin with thioredoxin. This interaction was discovered by the phage display of a cDNA library of prostate tissue. Thioredoxin appears to promote oxidation of the conserved disulfide bond in tear lipocalin. The proposed function is that the promotion of disulfide oxidation results in an increased affinity of tear lipocalin for a number of toxic ligands. This would have an impact on circumstances where disulfide reduction is favored, such as anaerobic conditions (Redl et al., 1999). However, the disulfide bond of tear lipocalin is normally intact in tears and proof of this mechanism has not been substantiated (Glasgow et al., 1998b).

Scavenger for Lipids From the Surface of the Cornea and in Tears

Tear lipocalin has been shown to remove native lipids from a variety of surfaces including the ocular surface (Glasgow et al., 1999; Gasymov et al., 2005; Yeh et al., 2013). Many meibomian lipids are hydrophobic due to long alkyl chains, and often these lipids are insoluble in aqueous solutions. Lipid contamination of the corneal surface, either because of loss of mucin or contamination of a mucinous surface, lowers the surface tension and renders the cornea unwettable (Sharma, 1993). This situation is possible whenever the tear film thins, such as in dry eye disease. The types of lipids shown to be removed from the ocular surface include fatty acids and phospholipids. Tear lipocalin also binds avidly to ceramides (Glasgow and Abduragimov, 2018b). Ceramides may destabilize the tear film. Ceramides have been shown to increase hysteresis in Langmuir trough experiments and can induce eventual collapse of the lipid film (Arciniega et al., 2013). Ceramides comprise about 7% of the total lipids in chalazia (Nicolaides et al., 1988). Elevation of ceramides has been noted in moderate dry eye disease (Lam et al., 2011). The evidence suggests that a principal function of tear lipocalin is to solubilize, sequester, and shuttle potentially destabilizing lipids from the ocular surface to the nasal lacrimal duct.

Acceptor for Phospholipid Transfer Protein and Modulator of Phospholipids

Recently, tear lipocalin has been shown to accept phospholipids from micelles in tears in concert with phospholipid transfer protein (Glasgow and Abduragimov, 2021). Since micelles have not been found in tears one cannot be sure whether micelles were present in the first place. However, the lipid composition in tears has been modeled to be conducive to forming both normal phase and inverse micelles (Wizert et al., 2014). The concentration of these lipids in tears exceeds the critical micellar concentration. Micelles scatter light. The intensity of the light scatter is a

TABLE 3 | Interacting molecules (ligands) native and non-native of tear lipocalin with binding constants.

Ligand	Kd (μM) and/or special conditions	References
Palmitic acid	8.3–13.5 tritiated palmitic acid apo/holo. 1.5–3.2 Ki	Glasgow et al., 1998a; Gasymov et al., 2011
Lauric acid spin label	2.4–8.3 apo/holo,	Gasymov et al., 1999a; Glasgow et al., 1999
Lauric acid	9.1 Ki apo	Gasymov et al., 1999a
Stearic acid	1.3 Ki apo	Gasymov et al., 1999a
Fatty alcohols C14-C18	NA	Glasgow et al., 1995, 1998b
16-(9-anthroyloxy)palmiticacid(16-AP)	0.8	Gasymov et al., 1999a
Cholesterol	15.9 Ki apo	Gasymov et al., 1999a
L-α-lysophosphatidylcholine	1.2–1.5 Ki and IC50	Gasymov et al., 1999a, 2005
2-(6-(7 nitrobenz-2-oxa,1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD C6-HPC)	0.15	Gasymov et al., 2005
Ceramide C6 NBD	0.08–0.32 (various methods)	Glasgow and Abduragimov, 2018b
Ceramide C12 NBD	0.1–1.23 (various methods	Glasgow and Abduragimov, 2018b
Retinol (Vitamin A)	0.13–0.19 apo pH7, pH3; 0.6 holo	Redl et al., 1992; Gasymov et al., 2002a; Breustedt et al., 2006
Retinoic acid	1.8	Breustedt et al., 2006
Retinal	0.39	Gasymov et al., 2002a
Vitamin E	>2.8 (displacement methods)	Glasgow et al., 2002b
Arachidonic acid	0.35 (IC50)	Lechner et al., 2001
7β-hydroxycholesterol	0.27 (IC50)	Lechner et al., 2001
8-isoprostane	0.94 (IC50)	Lechner et al., 2001
13-hydroxy-9,11-octadecadienoic acid	1.1 (IC 50	Lechner et al., 2001
4-Hydroxynonenal	16.8	Lechner et al., 2001
Lactoferrin and lysozyme	NA (shown by EPR	Gasymov et al., 1999b
Triacetylfusarinine C	0.5–1.5 (various methods)	Fluckinger et al., 2004
Rhodotorulic acid	∼0.13(IC 50)	Fluckinger et al., 2004
Rifampin	128 (circular dichroism)	Gasymov et al., 2004a; Staudinger et al., 2014
Rifalazil	2.3 (centrifugal precipitation)	Staudinger et al., 2014
Rifabutin	22.3* (gel filtration)	Gasymov et al., 2004a; Staudinger et al., 2014
Rifaximin	38.4*	Gasymov et al., 2004a; Staudinger et al., 2014
Rifamycin SV	63.8*	Gasymov et al., 2004a; Staudinger et al., 2014
Rifapentine	38.4*	Gasymov et al., 2004a; Staudinger et al., 2014
DAUDA(11-(((5-(dimeth-lyamino)−1-naphthalenyl)sulfonyl)amino)undecanoicacid)	1.0–2.8	Gasymov et al., 1999a; Lechner et al., 2001; Breustedt et al., 2006
ANS (8-anilino-1-naphthalenesulfonic acid)	0.5–10	Breustedt et al., 2006; Gasymov et al., 2008
1NPN,N-phenyl-1-naphthylamine	9.1	Gasymov et al., 2008
Fluorescein-labeled octadecyl ester	NA	Yeh et al., 2013
TNS, 6-(p-toluidino)-2-naphthalenesulfonic acid	7.6	Gasymov et al., 2008

NA not available.

**Calculated from gel filtration relative to rifampin.*

function of the square of the difference of the index of refraction of micelles from aqueous solution (Rayleigh, 1899). Elimination of micelles would reduce scattered light and maintain clarity of tears, a critical requirement for vision. Phospholipids have been shown to exist at the surface of the lipid layer of the tear film (Glasgow, 2020). Despite their surface activity, phospholipids are probably not present in enough concentrations to form a monolayer on tears (Glasgow, 2021). Evidence for the amount of phospholipid at the tear surface is based on the absorption from native phospholipid in stimulated tears compared with a known monolayer of phospholipid (Glasgow, 2020). But even small amounts of phospholipids will aid in the spreading of the main lipid components, such as cholesterol and wax esters, of the tear film (Rantamäki and Holopainen, 2017). A greater amount of phospholipid could theoretically displace gel-forming lipids from the tear film. Modulation of phospholipid concentrations by tear lipocalin could provide a more stable tear film.

Modulation of Viscosity in Tears Through Heteroprotein Polymer Formation

Tear lipocalin may have a role in modulating the viscosity of the tear film, although the data are confusing. The tear film behaves as a non-Newtonian fluid, meaning that the viscosity of tears is dependent on the sheer stress. Viscosity in Newtonian fluids, such as water, is independent of sheer stress. The source of sheer stress for tears is the blinking of the eyelids over the ocular surface. Tears are sheer thinning such that the viscosity decreases with increased blink speed (Gouveia and Tiffany, 2005). Sheer thinning with lower viscosity may vitiate damage to the ocular epithelium during blinking. The usual basis for sheer thinning is polymer separation often from the reduction in hydrogen bond interactions. One would posit that polymers exist in tears that are separated with blinking. The observation made by the group of Tiffany was that the removal of lipids from tears (most lipids are bound to tear lipocalin) results in Newtonian or sheer independent behavior, suggesting that the presence of lipids contributes to polymer formation. However, recombinant tear lipocalin in the work of Tiffany, presumably apo-tear lipocalin, showed non-Newtonian sheer thinning behavior. This seeming contradiction may be explained by the observation that recombinant tear lipocalin expressed in *E. Coli* has bound lipids (Gasymov et al., 2007c; Tsukamoto et al., 2009). But what is unexplained is that holo-tear lipocalin (with tear lipids added back to recombinant tear lipocalin) also showed Newtonian behavior (Gouveia and Tiffany, 2005). This appears contradictory to data that show recombinant and holo-tear lipocalin are monomeric, whereas aggregation occurs with delipidation (Gasymov et al., 2007c). The molecular basis of the polymer interaction in tears remains unclear. Perhaps, the relatively weak electrostatic interaction documented between tear lipocalin, lysozyme, and lactoferrin may have some influence (Gasymov et al., 1999b).

Surface Activity in Tears and Reduction of Evaporation

Tear lipocalin, like many proteins including some tear proteins, has been shown to unfold at an aqueous–air interface (Glasgow et al., 1999; Tragoulias et al., 2005; Mudgil and Millar, 2008). Further, tear lipocalin was shown to insert into meibomian film layer (Miano et al., 2005). The rate of penetration seems to differ between bovine and human meibomian layers and the reason for the difference is not entirely clear (Mudgil and Millar, 2008). Compared to other tear proteins, lipocalin was able to penetrate even at 30 mN/m of surface pressure, the highest surface pressure attained by whole tears. One possible mechanism that has been proposed for penetration of the surface by tear lipocalin is a proton conduction gradient, which forms at interfacial planes between lipids and aqueous (Prats et al., 1987). Phospholipids have been shown to produce a pH gradient when present at an aqueous interface. Phospholipids are present at the surface of tears (Gabriel et al., 1991; Glasgow, 2020). Acidic pH induces structural changes in proteins, and in this case it particularly facilitates loop motion of tear lipocalin. Changes in loop conformation result in low affinity for lipids, for example, some lipids may be offloaded at the surface (Gasymov et al., 1998, 2010a,b). Lipids, particularly long chain fatty alcohols, are known to reduce evaporation (La Mer and Healy, 1965; Saggaï and Bachi, 2018). The data for the effect of tear lipids and proteins on evaporation are somewhat disparate and controversial (Borchman et al., 2009; Herok et al., 2009). Tear lipocalin was not specifically tested in these studies. While proteins appear to contribute marginally to reducing evaporation in tears, surface active lipids should have a significant effect.

Endonuclease Activity

Tear lipocalin has been shown to be the major endonuclease in tears, accounting for about 75% of total endonuclease activity (Yusifov et al., 2000, 2008). Tear lipocalin acts as a Mg^{+2}-dependent nonspecific endonuclease. Activity has been related to a conserved protein sequence motif, LEDFXR, which is shared by *Serratia marcescens*. The LEDFXR motif is found in other lipocalins with similar activity (e.g., bovine β-lactoglobulin). Those lipocalins lacking the motif show no activity (e.g., retinol-binding protein). Glutamine at position 128 in tear lipocalin is critical for endonuclease activity. The functional relevance of endonuclease activity may be to degrade human DNA from exfoliated epithelial cells of the ocular surface. In addition, degradation of microbial DNA by tear lipocalin would serve as a useful function for destroying potentially infectious DNA of viruses. Such activity may work in concert with lysozyme in tears that binds viral DNA (Lin et al., 2008).

Drug Delivery

Because of promiscuity for ligands, tear lipocalin and mutant proteins derived from tear lipocalin have been used as drug binders and transporters. Tear lipocalin binds avidly to several members of the rifamycin family (**Table 3**). This raises the possible application of treatment for tuberculosis. Not only do these drugs bind lipocalin, but some rifamycins that are susceptible to oxidative degradation are protected when bound to tear lipocalin (Gasymov et al., 2004a; Staudinger et al., 2014).

Mutations of the loops at the open end of the cavity of lipocalins may result in specific changes in binding. Using combinatorial libraries the Skerra's group has made mutants of

TABLE 4 | Structure-function of critical motifs of tear lipocalin.

Structural Motif	Amino Acid, Strand	Function	References
Hydrophobic patches	• W17/F99 **A** & **G** internal • I98 **G** external	Stabilizes ligand binding through rigidity, conserved	Gasymov et al., 1998, 2002a,b
Disulfide bond	• C79 & C171 • **D** strand & C terminus	Induces protein rigidity, aromatic asymmetry; modulates conformational selection, conserved	Glasgow et al., 1998b
N terminus segment	L4-S7 & Q12-G16 **A**	Cysteine protease inhibition	Van't Hof et al., 1997; Wojnar et al., 2001b
Titratable trigger residues	R27 (**AB**)	Protonation triggers loop motion regulates pH dependent ligand binding	Breustedt et al., 2009; Gasymov et al., 2010b. PDB files: 1XKI, EYC
Calyx entry loops	**AB** and **GH**	Critical for conformational selection of ligands, pH modulated	Gasymov et al., 2009, 2010b
Calyx entry loop	**CD**	Conformational selection, constrained by disulfide	Gasymov et al., 2004b
Calyx entry loop	**EF** (hairpin)	Ligand specificity of cavity, short length add promiscuity; large range of motion	Gasymov et al., 2001, 2004b
Closed end loop	**FG**	Possible receptor recognition site, highly conserved	Gasymov et al., 2000, 2001, 2009
Cation-π	• K108-F28 **H** &**AB** • R118-W17 **H** &**A**	Stabilization of holo-conformation, conserved	Gasymov et al., 2012a
Trigonal cluster	K114 **H**, H84 **F**, E34 **AB**	External binding site for charged residues	Gasymov et al., 2007a, 2008
Mg^{+2} H_20 cluster	E128 **α**	Endonuclease activity, divalent cation dependent, conserved	Yusifov et al., 2000
α- helix strand	F130 **α**- V113&I115 **H**	Interaction modulates strand motion for long range residue interactions	Gasymov et al., 2013

*Amino acids are designated with single letter and numbered residue position. β strands, loops, and α-helices are designated in bold font with single letters, two letters or **α**, respectively.*

several lipocalins for specific drug-like action. For example, a mutant of tear lipocalin can act to bind vascular endothelial growth factor, and it is in the pipeline as a possible therapy (Hohlbaum and Skerra, 2007).

Biomarkers

Tear lipocalin has been touted as a biomarker for several diseases including dry eye disease (Zhou et al., 2009; Karnati et al., 2013; Yeh et al., 2013), breast cancer (Yang et al., 2019), chronic obstructive pulmonary disease (Jessie et al., 2010; Nicholas et al., 2010; Wang et al., 2014), diabetic retinopathy (Csosz et al., 2012; Guzman et al., 2020), glaucoma and pseudoexfoliation syndrome (Pieragostino et al., 2012, 2013), Alzheimer's disease (Kall et al., 2016), and keratoconus (Pannebaker et al., 2010; Acera et al., 2011). Most of these studies are based on proteomic correlations or expression profiles with elevated or depressed lipocalin levels. In the case of dry eye disease, one would expect that protein secretion of tear lipocalin as well as lysozyme and lactoferrin would be impacted together by the destruction of lacrimal gland acini. These proteins are packaged and secreted together in the lacrimal gland (Glasgow, 1995). Further testing of large population groups is warranted to validate the use of tear lipocalin as a biomarker in these settings.

STRUCTURE FUNCTION RELATIONSHIPS OF TEAR LIPOCALIN

The solution structure (by site directed tryptophan fluorescence) and later the crystal structure (by X-ray crystallography) of tear lipocalin have been reported (Gasymov et al., 2001; Breustedt et al., 2005). There is a remarkable concordance between the published structures (**Figure 1**). The solution structure was obtained by deducing the molecular environment of sequentially substituted tryptophans to yield an accurate map of secondary structural elements throughout the entire protein. The map was transposed to align secondary elements and the sequence compared with a close relative in the lipocalin family, whose structure was known. Computer-calculated energy minimization for favored conformations resulted in the most probable 3D structure. The solution structure is especially informative in defining loop regions that were not sufficiently resolved by crystallography (**Figure 1**). The functional features of various structural elements (**Table 4**) were defined by studies in solution, but residue interactions were confirmed by their proximity with crystallography. Unlike other lipocalins, tear lipocalin has the capacity to bind a broad array of ligands (**Table 3**). The cavity is 10 Å in diameter and 15 Å deep. Binding affinity increases for alkyl chains up to 18 carbons in length. The loops are critical to both the affinity and specificity of ligand binding. In some lipocalins, the length of the overhanging hairpin loop EF constrains the ability of the ligand for conformational selection to enter the cavity. Retinol binding protein is relatively specific for retinol due to the constraints of a long EF loop. The EF loop is short in tear lipocalin, allowing greater-sized ligands access to the cavity. The motion of the AB, GH, and CD loops, as detected using fluorescence quenching techniques, have been shown to be critical for pH-driven ligand binding (Gasymov et al., 2004b, 2010b). A protonated residue appears

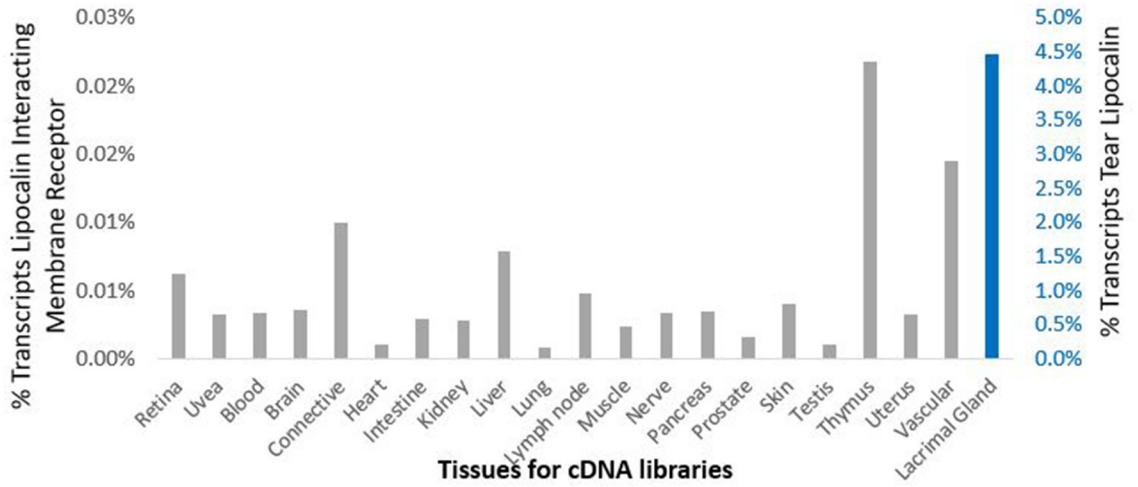

FIGURE 5 | Comparison of cDNA libraries from multiple human tissues for expression of lipocalin-interacting membrane receptor (gray) and tear lipocalin (red). The y-axis shows the percent of the total transcripts. The secondary *y*-axis, right is scaled differently for tear lipocalin because of the dramatic abundance of this transcript in the lacrimal gland library such that none of the other values appear in the bar graphs. The next most abundant value of percent total transcripts for tear lipocalin was in the testis 0.003%. Data was provided in the Unigene data base.

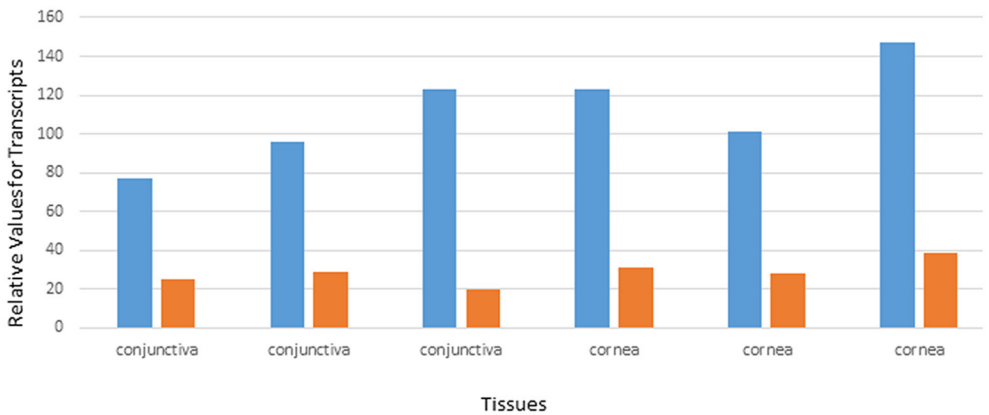

FIGURE 6 | Relative values of expression transcripts for tear lipocalin (orange) vs. lipocalin-interacting membrane receptor (blue) for cornea and conjunctiva samples. The data are adapted from GDS profile 2682 in the gene expression omnibus.

to be the trigger for loop motion at low pH. This leads to a low affinity conformation state and favors the release of the ligand.

Recently, cation-π interactions between positively charged residues and the negative charge cloud in the benzyl ring of aromatic amino acids were found (Gasymov et al., 2012a). Cation-π interactions are highly conserved among all lipocalins. The interactions stabilize the binding cavity, particularly in the holo state. Tear lipocalin contains motifs composed of one or more amino acids that are in proximity in the three-dimensional structure, but separated by long distances along the polypeptide chain of the protein. Substituting a residue to change either the charge, hydrophobicity, or size may greatly alter the binding affinity and function of tear lipocalin without significantly altering the secondary structure. For example, substituting alanine with tryptophan at residues 51, 66, and 86 in tear lipocalin reduced binding affinity for ligands by 3–4-fold (Gasymov et al., 2001). Substitution of E128 with tryptophan in tear lipocalin, reduced endonuclease (DNA nicking) activity by 80% (Yusifov et al., 2000).

Solution structure studies with ligands, both native and non-native, have been used to study quenching of single tryptophan mutants in tear lipocalin (Gasymov et al., 2009). Here the advantages of solution structure over crystal structure emerge. Dynamic loop structure is easily resolvable in the solution

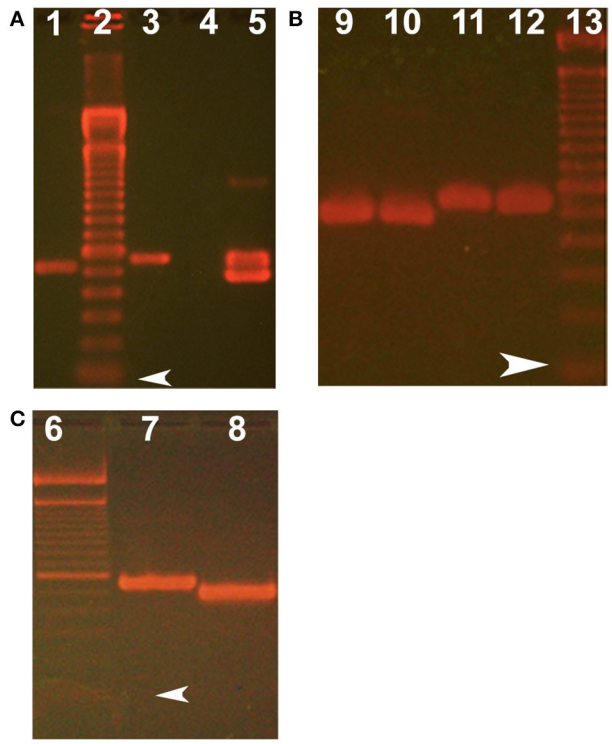

FIGURE 7 | Ethidium bromide stained agarose gels show size of products from PCR and subcloning lipocalin-interacting membrane receptor. **(A)** Shows RT-PCR products from corneal epithelium. **(B)** Shows plasmid inserts from single colonies from subcloning of RT- PCR of conjunctiva. **(C)** Shows plasmid inserts from single colonies from subcloning the PCR products of the corneal epithelial library. Lanes 2, 6, and 13 show 100 base pair molecular weight markers, arrowheads show the first marker at 100 base pairs. Target products lane 1 keratin 14 (control for cornea) using forward primer 5′-AGCCGCATTCTGAACGAGAT-3′ and reverse primer 5′-TCGTGCACATCCATGACCTT-3′, expected product size 529 bases; lanes 3, 5–10 show lipocalin-interacting membrane receptor using forward primer 5′-GTGCTTGCTGGTGCTGACGG-3′ and reverse primer 5′-TCACTGGTGCTGGGTCTTCCTAGATG-3′). Lane 3 uses plasmid with cloned lipocalin receptor as a positive control. Lane 4 is negative control (absence of template). The two sized products (e.g., Lane 5) were shown by sequencing to represent isoform, one which lacks a 60 base pair sequence corresponding to amino acids missing from isoform 3, Q6UX01-3, an alternative splice variant (as shown in **Figure 3**) (https://www.uniprot.org/uniprot/Q6UX01).

structure studies. One can probe the proximity to ligands to many amino acid residues of the protein in real time to provide a distribution of ligand binding sites. The distribution is reflective of multiple conformational states. Crystallography creates a model of a static structure. Only one conformation is usually sampled. Two crystal structures are available for tear lipocalin (Breustedt et al., 2005, 2009). The solution structure of tear lipocalin shows that residues on loops AB and GH as well as strands G and H are predominant interaction sites for both native and non-native ligands. Ligands move about the tear lipocalin cavity and loops; their positions are not static. Further, loops AB and GH at the open end of the cavity form the key portal for conformational selection by ligands in tear lipocalin (Gasymov et al., 2009). Based on ligand quenching of fluorescent residues, both static and dynamic, the major cavity-binding sites reside on the G and H strands in the cavity. The native ligand reports indirect quenching of mutant sites by amino acids in areas other than the binding site. For example, the FG loop moves to a more solvent-exposed conformation. An exposed conformation with ligand binding implicates the loop in potential receptor interactions (Gasymov et al., 2009). This is a plausible explanation for the highly conserved nature of this loop throughout the lipocalin family. Distance measurements between residues can be probed to the angstrom scale with variations of site-directed tryptophan fluorescence to detect subtle intramolecular changes such as rotamer conformations (Gasymov et al., 2012b, 2015).

The importance of portal loops was recognized by the group of Skerra (Schlehuber and Skerra, 2005; Gebauer and Skerra, 2012). Combinatorial methods facilitated variable mutations at positions of the loops in several lipocalin structures. The mutations can alter the conformations the lipocalins present to ligands. Anticalins, duocalins, etc. have been constructed for novel ligands with strong affinity. The strategy has worked well not only to create laboratory reagents for assays, but also for screening mutant proteins as potential treatments to target specific molecules involved in pathways in diseases. For example, tear lipocalin mutants with altered residues in the loops successfully target vascular endothelial growth factor, potentially useful in forms of macular degeneration and neoplasms. One caveat is that the loops of the lipocalin are exposed. The exposed nature of an altered sequence may pose a risk of increased antigenicity.

THE PUTATIVE RECEPTOR FOR TEAR LIPOCALIN, LIPOCALIN- INTERACTING MEMBRANE RECEPTOR

Receptors for the lipocalin family have been difficult to identify as is exemplified in the story of the receptor for retinol binding protein (Kawaguchi et al., 2007). A putative membrane receptor for tear lipocalin has important implications for potential functions. Lipocalin-interacting membrane receptor was discovered by biopanning a bacteriophage cDNA expression library from the human pituitary gland with purified tear lipocalin (Wojnar et al., 2001a). The genomic structure with 17 exons was also reported by the group of Redl (Wojnar et al., 2001a). The N terminus of the 487-amino acid protein interacted with purified tear lipocalin and was localized to the plasma membrane of NT2 cells derived from pluripotent human embryonal carcinoma (Wojnar et al., 2001a,b). The protein was modeled with nine transmembrane helical domains. The detailed structure is not known. Downregulation of lipocalin-interacting membrane receptor resulted in inhibition of cellular internalization (NT2 cells) of tear lipocalin (Wojnar et al., 2003). Therefore, lipocalin-interacting membrane receptor was considered an endocytic receptor. However, similar results

were obtained for internalization of bovine β lactoglobulin by lipocalin-interacting membrane receptor (Fluckinger et al., 2008). Curiously, forced expression of the lipocalin-interacting membrane receptor in COS-1 cells facilitated the binding of uteroglobin as well (Zhang et al., 2006). In contrast, surface plasmon resonance experiments with expressed lipocalin-interacting membrane receptor showed an interaction with tear lipocalin but not with β lactoglobulin or uteroglobin (Hesselink and Findlay, 2013). Recently, the function of lipocalin-interacting membrane receptor as an endocytic receptor for tear lipocalin has been challenged. An analogous protein to lipocalin-interacting membrane receptor in *Drosophila*, coined Lilipod, was shown to function in self-renewal of ovarian germ-line stem cells through enhanced signaling of bone morphogenetic protein (Dolezal et al., 2015). The functions described for these closely related proteins are not necessarily mutually exclusive. Comparison of various tissues for expression of both tear lipocalin and lipocalin-interacting membrane receptor may provide insight into their potential interactions.

Repository Data for Expression of Lipocalin-Interacting Membrane Receptor and Tear Lipocalin

Data from the GEO repository is useful to roughly assess the relative abundance of lipocalin-interacting membrane receptor transcripts in normal human tissues. An overall perspective from multiple data sets is provided in **Table 1**. The adrenal gland is at the top percentile ranking and the low standard error of the mean indicates that the expression of lipocalin-interacting membrane receptor was consistent across the data sets. The findings from brain tissue were similar to those of the adrenal gland. On the other hand, skeletal muscle and heart showed consistently lower percentile ranks of expression. In one study, a threshold below which values were considered noise and not associated with transcripts occurred for heart, liver, and skeletal muscle, as indicated in **Table 1** (Yanai et al., 2005). The adrenal gland and brain are not known either to express tear lipocalin or have access to these organs through the blood stream. The expression data question the notion whether lipocalin-interacting membrane receptor is a specific receptor for tear lipocalin. An attempt to specifically correlate the relative expression of lipocalin-interacting membrane receptor with tear lipocalin was possible in two large data sets. **Figure 4** shows the data for one such data set as log 10 values (She et al., 2009). Perhaps serendipitously, the log 10 format includes values from the heart at log values corresponding to the level of noise in **Table 1**. Adrenal gland and brain showed the highest expression of lipocalin-interacting membrane receptor. Furthermore the expression profiles of tear lipocalin (**Figure 4**) match the immunohistochemically identified expression of protein in tissues, such as trachea, prostate, and pituitary gland. However, for most tissues, the levels of expression were disparate for the two proteins. The lack of correlative expression was evident in another data set of 96 tissues (Dezs et al., 2008). Of course, expression profiles do not necessarily have to be similar to have a link in function (Yanai et al., 2006). Lacrimal gland tissue was not used in the studies retrieved from the GEO repository. However, the Unigene database included lacrimal gland tissue. The percentage of ESTs of tear lipocalin in the lacrimal gland library was three orders of magnitude more than the percentage of those for lipocalin-interacting membrane receptor in other tissue libraries (**Figure 5**). The percentages of tear lipocalin ESTs in libraries other than the lacrimal gland appear negligible. Cornea and conjunctiva tissues were not included in the aforementioned studies. However, these ocular surface tissues were present in one data set (**Figure 6**) in the GEO repository (Turner et al., 2007). The value of expression transcripts of lipocalin-interacting membrane receptor is greater than tear lipocalin in both conjunctiva and cornea. However, actual levels of expression relative to potential noise are not certain. The functional implications of the expression of lipocalin-interacting membrane receptor in ocular surface cells that are bathed in tears containing high concentrations of its putative ligand, tear lipocalin, calls for confirmation.

Ocular Surface Expression of Lipocalin-Interacting Membrane Receptor

The presence of lipocalin-interacting membrane receptor transcripts was queried in cultured human corneal epithelium cDNA (hCEPIC-ScienCell). The strategy was to amplify segments of the cDNA that spanned introns to exclude genomic contamination. The identification of a known alternative splicing isoform and the presence of large introns encompassed by the primer sets exclude the possibility of contamination by genomic DNA (**Figure 3**). The amplification strategy included two possible isoforms of lipocalin-interacting membrane receptor. The PCR products of individual colonies are shown in **Figure 7**. Two distinct sizes of 499 and 559 base pairs matched lipocalin-interacting membrane isoforms. and this was confirmed by sequence data (**Figure 3**). The agarose gel of PCR products from cDNA extracted from corneal epithelium is shown in **Figure 7**. Multiple products were similar in size to those seen from the corneal epithelial cell cDNA library as well as the conjunctiva. The expression of lipocalin-interacting membrane receptor at the ocular surface supports a possible receptor interaction with tear lipocalin. One possibility is that tear lipocalin functions to deliver vitamins or other molecules to the avascular central cornea epithelium through the receptor. Lacking here is immunohistochemical evidence of protein translation of lipocalin-interacting membrane receptor at the ocular surface. This was attempted but commercial antibodies as well as those obtained from Professor Redl did not show reactivity in tissues fixed in methanol, acidified ethanol, or formalin. Lipocalin-interacting membrane receptor, as well as tear lipocalin may be multifunctional proteins, which might explain some disparities. More investigation is needed to clarify the functions of this membrane protein, particularly in regard to its structure, distribution, and interactions with tear lipocalin.

DATA AVAILABILITY STATEMENT

The datasets for this study can be found in the Gene Expression Omnibus, https://www.ncbi.nlm.nih.gov/geo/, Protein Data Bank http://www.wwpdb.org/, The sequence data have been deposited in Gene Bank, accession numbers, MW841072 and MW841073.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UCLA Institution Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

The author confirms being the main contributor of this work and has approved it for publication.

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Lipocalin-2 in Diabetic Complications of the Nervous System: Physiology, Pathology, and Beyond

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Lipocalin-2 (LCN2) is a 25 kDa secreted protein that belongs to the family of lipocalins, a group of transporters of small hydrophobic molecules such as iron, fatty acids, steroids, and lipopolysaccharide in circulation. LCN2 was previously found to be involved in iron delivery, pointing toward a potential role for LCN2 in immunity. This idea was further validated when LCN2 was found to limit bacterial growth during infections in mice by sequestering iron-laden siderophores. Recently, LCN2 was also identified as a critical regulator of energy metabolism, glucose and lipid homeostasis, and insulin function. Furthermore, studies using *Lcn2* knockout mice suggest an important role for LCN2 in several biobehavioral responses, including cognition, emotion, anxiety, and feeding behavior. Owing to its expression and influence on multiple metabolic and neurological functions, there has emerged a great deal of interest in the study of relationships between LCN2 and neurometabolic complications. Thorough investigation has demonstrated that LCN2 is involved in several neurodegenerative diseases, while more recent studies have shown that LCN2 is also instrumental for the progression of diabetic complications like encephalopathy and peripheral neuropathy. Preliminary findings have shown that LCN2 is also a promising drug target and diagnostic marker for the treatment of neuropathic complications from diabetes. In particular, future translational research related to LCN2, such as the development of small-molecule inhibitors or neutralizing antibodies against LCN2, appears essential for exploring its potential as a therapeutic target.

Keywords: lipocalin-2, immunity, energy metabolism, diabetes, neuroinflammation, diabetic complications, nervous system

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INTRODUCTION

Diabetes is a syndrome defined by the presence of abnormally high blood glucose levels or hyperglycemia (Guthrie and Guthrie, 2004; Skyler et al., 2017). Hyperglycemia is known to cause a wide variety of metabolic disturbances and affect both the peripheral nervous system (PNS) and central nervous system (CNS), either directly or indirectly, which can lead to

several complications collectively referred to as diabetic neuropathy over a long period of time (Sima et al., 2004; Malone, 2016). Diabetes mainly affects nerves in hands, feet, legs, and arms and is long considered a disease of the PNS. However, there is now increasing evidence of diabetic effects on the CNS as part of a condition called diabetic encephalopathy (Selvarajah et al., 2011). The pathogenesis of diabetic encephalopathy has not been fully defined, yet appears to show similarities to the progression of diabetic peripheral neuropathy (DPN) (Manschot et al., 2008).

Recently, there has been wide agreement that excessive glial activation is a key mechanism in both CNS- and PNS-related complications of diabetes involving the release of proinflammatory cytokines. Glial cells play an essential role in maintaining the normal function of nervous tissues. In the PNS, neurons are intimately associated with numerous glial cells; the neuronal soma is enclosed by satellite glial cells (SGCs) and axons are covered along their length by Schwann cells (Goncalves et al., 2018). Similarly, neurons are in close contact with astrocytes, microglia, oligodendrocytes, and Müller glial cells in the CNS. These glial cells are responsible for the uptake and metabolism of glucose in the nervous system. Fluctuations in glucose levels activate them, causing activation of MAPK/PI3K/Akt/NF-κB signaling and release of proinflammatory factors (Quincozes-Santos et al., 2017; Hsieh et al., 2019). This inflammatory activation of glial cells triggers further metabolic deterioration and affects both small and large nerve fibers, resulting in nerve dysfunction that is characteristic of diabetic neuropathy (Goldberg, 2009; Pop-Busui et al., 2016).

Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL), siderocalin, and 24p3, is a member of the lipocalin superfamily and is a pleiotropic mediator of various physiological and pathological processes (Xiao et al., 2017; Bhusal et al., 2019b). LCN2 so far is known to act through two major membrane-bound receptors: megalin, also known as low-density lipoprotein-related protein 2 (LRP2), and 24p3R, also referred to as solute carrier SLC22A17 or brain-type organic cation transporter (BOCT). LCN2 functions through these receptors in an iron-dependent manner, where apo-LCN2 chelates iron inside the cell, releases to the extracellular medium, reduces intracellular iron concentration, and finally causes cellular apoptosis. On the other hand, holo-LCN2 increases intracellular iron concentration and prevents cellular apoptosis by decreasing the expression of the proapoptotic protein Bcl-2-like protein 11 (Bim) (Devireddy et al., 2005). However, this hypothesis has lately been challenged, as HeLa cells expressing BOCT receptors did not exhibit cellular iron efflux following LCN2 treatment. Moreover, LCN2, even at higher doses, did not induce apoptosis in hematopoietic cell line (Correnti et al., 2012). Studies also failed to show the interaction between LCN2 and BOCT (Bennett et al., 2011; Correnti et al., 2012). Similarly, conflicting views have also been reported regarding the role of LCN2 in several metabolic conditions, as reviewed earlier by our group (Bhusal et al., 2019b). In light of these findings, LCN2 has recently been proposed to play an important role in the development of diabetic complications. Here, we review the physiological as well as pathological role of LCN2 in the nervous system, and discuss the latest reports as to how it could be used as a target for the treatment of diabetic neurological complications. In this review, the term "diabetic neuropathy" is used throughout the manuscript in reference to a disease of the peripheral and central nervous system, unless otherwise specified.

THE ROLE OF LCN2 IN NERVOUS SYSTEM PHYSIOLOGY AND PATHOLOGY

Lipocalin-2 is produced by mammalian hosts to bind bacterial siderophores and sequester free iron as part of innate immune defenses against bacterial infection; however, thus far, its role in the nervous system is less well understood. The upregulation of LCN2 in the brain was first observed in response to peripheral turpentine-induced inflammation (Liu and Nilsen-Hamilton, 1995). In addition, the study of LCN2 function in various neuroinflammatory conditions largely began with the observation of LCN2 expression in microglia (Lee et al., 2007) and astrocytes (Lee et al., 2009) in the CNS. However, the physiological role of LCN2 has not been studied due to its low or undetectable expression in healthy adult brains (Ip et al., 2011; Chakraborty et al., 2012; Kim et al., 2017; Kang et al., 2018) or peripheral nerves (Jeon et al., 2013; Bhusal et al., 2020), although some studies have demonstrated LCN2 protein expression in the hippocampus (Mucha et al., 2011; Chia et al., 2015; Furukawa et al., 2017), cortex, and amygdala (Furukawa et al., 2017) of normal rodents. Furthermore, strong constitutive expression of the LCN2 receptor 24p3R in the brain of normal mice has been reported (Ip et al., 2011; Chia et al., 2015). It is, therefore, possible that basal expression of LCN2 in different regions of the brain may help in the defense of the CNS against pathogens. Beyond resisting infections, high levels of LCN2 in other regions of the brain may contribute to iron transport in these regions under normal conditions.

LCN2 in Stress, Anxiety, Depression, and Cognitive Function

Recent studies using *Lcn2* knockout (KO) animals have improved understanding of the role of LCN2 in the regulation of physiological conditions like stress, emotion, and memory. In one such study, *Lcn2* KO mice displayed increased anxiety and depressive-like behaviors and mild spatial reference memory impairments (Ferreira et al., 2013). These altered phenotypes were associated with hyperactivation of the hypothalamic-pituitary-adrenal axis, reflected in the increased levels of corticosteroids at both the morning and night periods in the *Lcn2*-deficient mice. Furthermore, the hippocampal neuronal morphology of *Lcn2* KO mice displayed hypertrophy of granular and pyramidal neurons at the ventral hippocampus, a region implicated in emotional behavior, as well as neuronal atrophy at the dorsal hippocampus, a region implicated in memory and cognition (Ferreira et al., 2013). Another study by the

same group found that *Lcn2* deficiencies lead to higher proportion of progenitor cells in hippocampus exiting the cell cycle and progressing toward apoptotic cell death (Ferreira et al., 2018). Furthermore, deletion of *Lcn2* in neural stem cells induced endogenous oxidative stress, cell cycle arrest, and cell death in an iron-mediated manner (Ferreira et al., 2018). In a later extension of their study, the impaired hippocampal neurogenesis observed in *Lcn2* KO mice was relieved by voluntary running, which counteracted oxidative stress and promoted cell cycling of neural stem cells, resulting in the partial reduction of anxiety and improved contextual behavior (Ferreira et al., 2019). In line with this, the ablation of *Lcn2* gene proved deleterious and promoted a stress-induced increase in spine density, which correlated with higher excitability of CA1 neurons and stress-induced anxiety (Mucha et al., 2011).

LCN2 in Food Intake Regulation

Mosialou et al. (2017) recently revealed an unexpected role of LCN2 regarding feeding behavior of mice. In that study, LCN2 secreted from bone crosses the blood-brain barrier, binds to melanocortin 4 receptors (MC4R) in neurons of the hypothalamus, and activates an MC4R-dependent anorexigenic pathway (Mosialou et al., 2017). This finding was extended to baboon, macaque, and human, where LCN2 acted as a satiety factor, and failure to stimulate postprandial LCN2 in individuals with obesity contributed to metabolic dysregulation (Petropoulou et al., 2020).

However, these observations should be carefully considered. In the study by Guo et al. (2010), indirect calorimetry measurements revealed no difference in food intake behavior between wild-type and *Lcn2* KO mice. In another study, *Lcn2*-deficient mice displayed no alteration in food intake upon Celastrol treatment, which is known to increase LCN2 levels in hypothalamus (Feng et al., 2019). Recently, LCN2-overexpressing transgenic mice showed an increased food intake (Principi et al., 2019). Similarly, food intake was increased when mice were injected with LCN2 protein (Paton et al., 2013). These controversial findings warrant further investigation into the mechanisms how LCN2 regulates food intake behavior.

LCN2 in Inflammatory and Other Neurological Disorders

Beyond the physiological roles of LCN2, recent studies have shown an increase in expression of and an important role for LCN2 in various pathological states. LCN2 has been found to regulate diverse cellular processes and phenotypes in the nervous system, including cell death and survival (Lee et al., 2007; Naude et al., 2012; Bi et al., 2013), cell migration and morphology (Lee et al., 2011, 2012; Rathore et al., 2011), and the functional polarization of microglia (Jang et al., 2013b; McPherson et al., 2014) and astrocytes (Jang et al., 2013a). These functional characteristics of LCN2 have been exploited by many researchers to study the role of LCN2 in different neurological disorders, including neuroinflammation (Lee et al., 2011; Jin et al., 2014a, 2018; Mondal et al., 2020), Alzheimer's disease (Naude et al., 2012; Dekens et al., 2018; Eruysal et al., 2019), ischemic stroke (Jin et al., 2014b; Xing et al., 2014; Hochmeister et al., 2016; Ranjbar Taklimie et al., 2019; Zhao et al., 2019), experimental autoimmune encephalomyelitis (Berard et al., 2012; Marques et al., 2012; Nam et al., 2014), brain and spinal cord injuries (Chia et al., 2011; Rathore et al., 2011; Dong et al., 2013), malignant gliomas (Suk, 2012), and pain hypersensitivity (Poh et al., 2012; Jeon et al., 2013; Jha et al., 2013, 2014). As a result of its active participation in the pathogenesis of various neurological diseases, LCN2 can be considered a promising therapeutic target for both prognostic and diagnostic purposes. Recently, the number of studies describing a role for LCN2 in metabolic homeostasis (Yan et al., 2007; Jun et al., 2011; Guo et al., 2012, 2016; Mosialou et al., 2020) and the pathogenesis of diabetes-related complications is on the rise, indicating similar importance of LCN2 in diabetes-related neurological disorders.

LCN2 IN NEUROLOGICAL COMPLICATIONS OF DIABETES

Recently, several clinical studies have demonstrated a close relationship between LCN2 expression and the risk of impaired glucose metabolism (Wang et al., 2007; Yang et al., 2009; Huang et al., 2012). Furthermore, LCN2 levels have been linked to diabetic complications like retinopathy (Chung et al., 2016; Wang et al., 2020) and nephropathy (Wu et al., 2014; Papadopoulou-Marketou et al., 2017; Sisman et al., 2020). However, the precise mechanisms underlying the role of LCN2 in diabetic complications remain unclear and several plausible explanations have been suggested. LCN2 has been reported to deliver iron to cells, causing intracellular iron overload and results in oxidative stress, cellular degeneration, and increased levels of advanced glycation end-product (AGE) receptors for AGE binding (Ciudin et al., 2010). LCN2 has been shown to activate metalloproteinase-9 (MMP-9) by forming a stable complex with MMP-9 (Yan et al., 2001). MMP-9 activation may then facilitate an increase in vascular permeability through the proteolytic degradation of tight junction proteins (Opdenakker and Abu El-Asrar, 2019). LCN2 may also be linked to the production of AGE (Chung et al., 2013; Petrica et al., 2015), which interacts with plasma membrane-localized receptors for AGEs (RAGE) to alter intracellular signaling, gene expression, and the release of proinflammatory molecules and free radicals (Singh et al., 2014). In addition, LCN2 is involved in immune reactions and inflammatory processes (Lee et al., 2011; Jin et al., 2014a, 2018; Mondal et al., 2020). These mechanisms involving LCN2 are all relevant in the nervous system, as LCN2 is significantly expressed in both CNS and PNS following the onset of diabetes, making LCN2 a potential focus in the study of diabetic neuropathy.

LCN2 in Diabetic Encephalopathy

Diabetic encephalopathy is a chronic complication of diabetes mellitus characterized by oxidative stress, impaired

microvascular permeability, neurogenesis, cognitive functions, and electrophysiological, neurochemical, and structural abnormalities (Cai et al., 2011; Ho et al., 2013; Chen et al., 2018). People with diabetes mellitus have increased levels of proinflammatory cytokines such as C-reactive protein, TNF-α, and IL-6 (Esposito et al., 2002; Tangvarasittichai et al., 2016). Recently, a study reported the upregulation and pathological role of LCN2 in the hippocampus of an insulin-deficient diabetes model created by streptozotocin injection (Bhusal et al., 2019a). In this study, deletion of the *Lcn2* gene ameliorated diabetes-induced reactive gliosis and expression of proinflammatory cytokines in the hippocampus of diabetic mice. Moreover, *Lcn2* KO diabetic mice showed decreased neuronal loss in the hippocampus compared to wild-type diabetic animals, an effect correlated with improved cognitive behavior (Bhusal et al., 2019a) (**Figure 1**). Previously, the same group reported increased plasma levels in patients with mild cognitive impairments (Choi et al., 2011), supporting the correlation between LCN2 and cognitive deficits. Another study conducted using four independent cohorts with a large number of samples concluded that cerebrospinal fluid LCN2 is a promising biochemical marker for the differential diagnosis of neurodegenerative dementias (Llorens et al., 2020). These findings indicate that LCN2 has significant translational potential for several brain-related complications of diabetes.

Recently, a study reported increased serum and hippocampal LCN2 levels in ob/ob mice. In this study, ob/ob mice showed impaired spatial learning behavior, which was improved following a calorie-restricted diet that correlated with decreased expression of LCN2 in the hippocampus (Park et al., 2019). Another study suggested that increasing LCN2-mediated iron uptake may be required for Toll-like receptor 4/endosome-related WD repeat and FYVE domain-containing 1 (Wdfy1)-signaling during hippocampal neuroinflammation in ob/ob mice (Jin et al., 2020). Furthermore, a study using a combination of high-fat diet and fructose showed that elevated hippocampal and peripheral LCN2 levels mediate the impact of chronic inflammation on the CNS, which is associated with behavioral dysfunction (de Sousa Rodrigues et al., 2017). Additionally, recent studies attempted to evaluate the role of a chronic high-fat diet in neuroinflammation with respect to myeloid sirtuin1 (SIRT1) and LCN2 function. It was found that SIRT1 promotes hippocampal inflammation in association with LCN2 levels (Kim et al., 2018; Park et al., 2019).

Obesity and high-fat diet are associated with an increased risk of developing insulin resistance and type 2 diabetes. Considering evidence that circulating LCN2 levels are associated with hyperglycemia, insulin resistance, and metabolic syndrome based on *in vitro* (Yan et al., 2007; Chan et al., 2016), *in vivo* (Wang et al., 2007; Guo et al., 2010), and clinical studies (Cakal et al., 2011), it is speculated that LCN2 may be involved in the regulation of insulin sensitivity in the brain, although the relationship between LCN2 expression and insulin resistance in the brain has not been investigated. Taken together, specific mechanisms

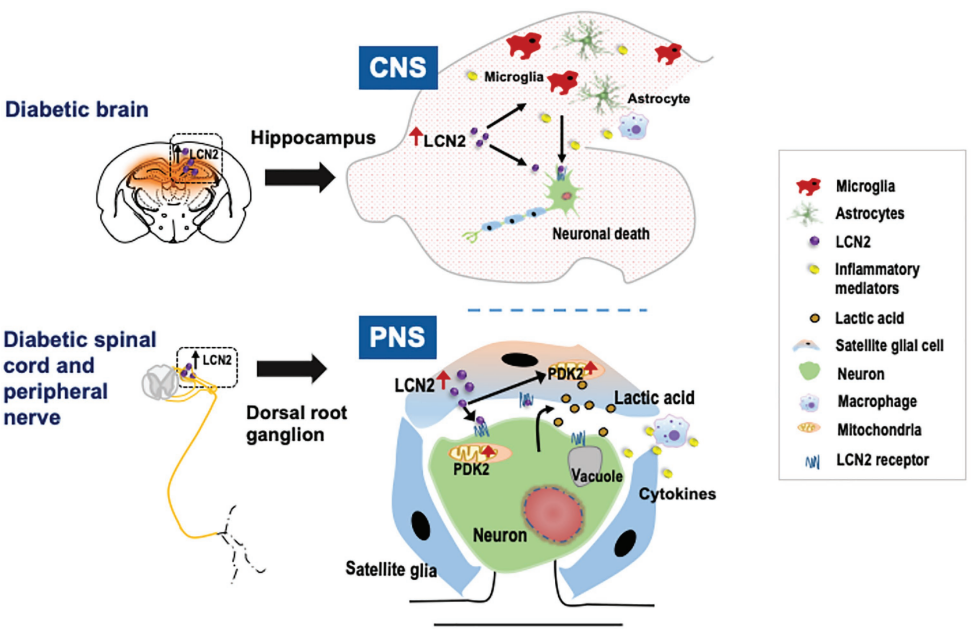

FIGURE 1 | Schematic representation of the role of lipocalin-2 (LCN2) in diabetic neuropathy. Diabetes leads to the upregulation of LCN2 in the hippocampus of the brain and in satellite glial cells (SGCs) of the dorsal root ganglion (DRG). In the central nervous system (CNS), induction of LCN2 causes activation of glial cells, macrophage infiltration, and upregulation of inflammatory cytokines leading to diabetic encephalopathy. In the peripheral nervous system (PNS), it has been suggested that LCN2 released from the SGCs in the DRG acts on SGCs as well as nearby neurons to induce pyruvate dehydrogenase kinase-2 (PDK2) expression. The LCN2-PDK2 axis-mediated glycolytic metabolic shift in the DRG results in the production of lactic acid, which causes an acidic microenvironment that eventually causes neuronal damage, leading to diabetic peripheral neuropathy.

relating to the association between LCN2 and IR in the brain can be additional factors aggravating diabetic encephalopathy.

LCN2 in Diabetic Peripheral Neuropathy

Diabetic peripheral neuropathy is the most common microvascular complication of diabetes mellitus characterized by inflammation, oxidative stress, and mitochondrial dysfunction (Roman-Pintos et al., 2016). Based on the previous observations of the induction of LCN2 expression and its role in neuroinflammation in the brains of mice with diabetes or various demyelinating diseases (Nam et al., 2014; Chun et al., 2015; Al Nimer et al., 2016), Bhusal et al. (2020) have recently provided evidence for a potentially novel role of LCN2 in the progression of DPN. They found that LCN2 is expressed by SGCs in the dorsal root ganglion (DRG) and by Schwann cells in the sciatic nerves of diabetic mice. This LCN2 expression was dependent on high glucose levels, as evidenced by the decrease in LCN2 levels in the DRG and improvement in nerve conduction velocity in diabetic mice following insulin treatment. Furthermore, their study using *Lcn2* KO mice showed a decrease in inflammation in the DRG and sciatic nerve and reduction in the consequent DPN phenotype; however, the mechanisms through which *Lcn2* deficiency attenuates DPN remain unclear.

Pyruvate dehydrogenase kinase-2 (PDK2), a mitochondrial enzyme, is known to drive a metabolic shift in dorsal root ganglia, which induces neuroinflammation, lactic acid build-up and ultimately produces painful DPN (Rahman et al., 2016). Similarly, multiple studies have reported that LCN2 increases mitochondrial reactive oxygen species, alters mitochondrial oxidative phosphorylation, and impairs overall mitochondrial activity (Yang et al., 2012; Song et al., 2018; Chella Krishnan et al., 2019). Given the proven role of LCN2 and PDK2 in mitochondrial activity, Bhusal et al. (2020) investigated whether there is any functional relationship between LCN2 and PDK2 in terms of neuroinflammation and lactic acid production in DPN. In their study, PDK2 overexpression using adenoviruses in the DRG in *Lcn2* KO mice potentiated inflammation and DPN, which was not observed when LCN2 was overexpressed in *Pdk2* KO mice. Furthermore, LCN2 increased expression of PDK2 in SGC culture, which was shown using pharmacological blockade to be dependent on peroxisome proliferator-activated receptors. From these findings, the authors concluded that LCN2 acts as an upstream regulator of PDK2 in the SGCs of DRG, which potentiates neuroinflammation, lactate surge, and consequent DPN (Bhusal et al., 2020) (**Figure 1**). These findings ultimately put forward a novel mechanism for an LCN2-PDK2-lactic acid axis in diabetes-induced neuroinflammation and consequent diabetic complications like neuropathy.

CONCLUDING REMARKS

During the past few decades, there has been an increased understanding of the relationships among metabolic syndrome, adipokines, and inflammatory diseases. In this regard, LCN2 can be considered one of the mediators responsible for inflammation in complications associated with diabetes. Recent studies have provided essential evidence of LCN2 as a major player linked to metabolism, inflammation, and neuropathy. LCN2 expression may be a useful early diagnostic biomarker for diabetic neuropathy; however, further validation using human samples derived from larger multi-institutional cohorts is needed. Further research into the function of LCN2 will guide our understanding of its potential use as a diagnostic and therapeutic agent and will create new opportunities for improving the care of patients with diabetic neuropathy.

AUTHOR CONTRIBUTIONS

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REVIEW
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Biological Roles of Lipocalins in Chemical Communication, Reproduction, and Regulation of Microbiota

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Major evolutionary transitions were always accompanied by genetic remodelling of phenotypic traits. For example, the vertebrate transition from water to land was accompanied by rapid evolution of olfactory receptors and by the expansion of genes encoding lipocalins, which – due to their transporting functions – represent an important interface between the external and internal organic world of an individual and also within an individual. Similarly, some lipocalin genes were lost along other genes when this transition went in the opposite direction leading, for example, to cetaceans. In terrestrial vertebrates, lipocalins are involved in the transport of lipophilic substances, chemical signalling, odour reception, antimicrobial defence and background odour clearance during ventilation. Many ancestral lipocalins have clear physiological functions across the vertebrate taxa while many other have – due to pleiotropic effects of their genes – multiple or complementary functions within the body homeostasis and development. The aim of this review is to deconstruct the physiological functions of lipocalins in light of current OMICs techniques. We concentrated on major findings in the house mouse in comparison to other model taxa (e.g., voles, humans, and birds) in which all or most coding genes within their genomes were repeatedly sequenced and their annotations are sufficiently informative.

Keywords: lipocalins, odorant, mouse, major urinary protein, odorant-binding protein, retinol-binding protein, LCN, microbiota

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INTRODUCTION

Initial mouse genome sequencing (Abril et al., 2002), re-sequencing of wild derived mice and other laboratory strains (Chang et al., 2017; Lilue et al., 2018) and now widely used OMICs techniques helped to link genotypes and phenotypes and provided new pieces of evidence that lipocalins are essential for life for their capacity to bind and transport biologically active as well as the toxic organic compounds in many fitness-related contexts. The lipocalins are small soluble proteins (typically 20 kDa) with an eight-stranded antiparallel β-barrel often with two α-helices (N-terminal, C-terminal) on both ends of the protein, reviewed in (Stopková et al., 2009, 2014; Phelan et al., 2014b). The structure of the lipocalin

β-barrel is open at one end allowing for the binding of various hydrophobic substances. There is yet another group of highly similar proteins that have β-barrels, though 10-stranded, and they include cellular retinol-binding proteins (CRBPs, depicted in **Figure 1A** as RBP1,2,5,7) and fatty acid binding proteins. The exception is extracellular RBP4 which, similarly as other lipocalins, have eight-stranded β-barrel. All together these proteins and lipocalins form the calycin superfamily (Sanchez et al., 2003; Álvarez et al., 2005). The lipocalin family is diverse accounting for at least 55 genes in the house mouse (Stopková et al., 2014) with little DNA sequence homology but with conserved tertiary structure thus forming β-barrel in all the mouse lipocalins but also in all metazoans over the evolutionary history (Ganfornina et al., 2000, 2006; Sanchez et al., 2003, 2006). Another interesting feature of lipocalins is their ability to bind and transport a wide spectrum of compounds including potentially toxic metabolic end-products such as ROS (reactive oxygen species) and also xenobiotics in many bacterial, plant, insect, avian, and mammalian species (Charron and Sarhan, 2006; Kwak et al., 2011, 2016; Bhatia et al., 2012).

From the evolutionary point of view, ancestral lipocalins can be found in many animal taxa including bacteria. The closest homologues of bacterial lipocalins are lazarillo in insects

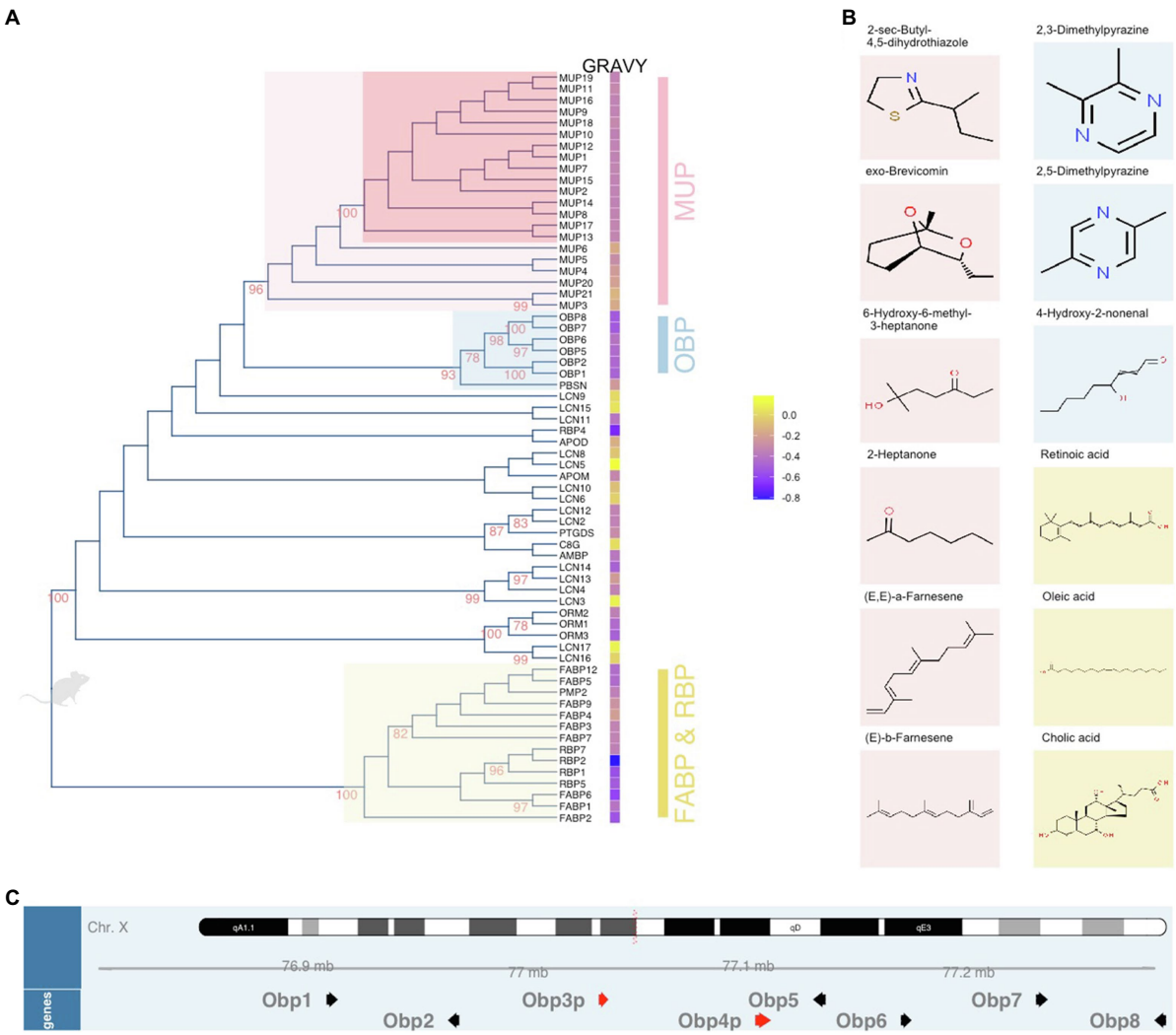

FIGURE 1 | Unrooted dendrogram of mouse lipocalins within the Calycin superfamily. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree **(A)** with the highest log likelihood (−17,639.2427) is shown. The percentage of trees in which the associated proteins clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 66 amino acid sequences. There were a total of 406 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 while the final figure was plotted with *ggtree*. Next to the tree, Gravy index is scaled from blue (hydrophilic) to yellow (hydrophobic). The colour code of highlighted subtrees is also used **(B)** to highlight the most common ligands with high affinities to particular protein groups. The position of Obp genes (black arrows) and pseudogenes (red arrows) on the X chromosome is visualized in **(C)** using Gviz Bioconductor package.

or vertebrate apolipoproteinD – APOD (Ganfornina et al., 1995, 2008; Bishop et al., 2006). Concerning the vertebrate calycins (incl. lipocalins), some members were found through all the taxa (fish, amphibians, birds, and mammals), for example, APOD, FABPs, C8G, AMBP, LCNs (Bilkova et al., 2018), while others are highly specific for mammalian species – major urinary protein (MUP), odorant-binding protein (OBP). Although many OBPs were detected in insect species, where they are involved in chemical communication, being highly abundant in antennae and having specific affinities to insect pheromones, there is no true homology between the vertebrate and insect OBPs (Pelosi et al., 2006; Vieira and Rozas, 2011). This supports the view that the radiation of OBPs happened independently during the evolutionary history.

The aim of this review is to further explore what features make the lipocalins essential for life from the mouse perspective but also from other vertebrates which makes the view on lipocalin functions more robust. Thus, in this review, we aim to extract biologically relevant features typical for different lipocalin proteins in the mouse based on amino-acid sequence similarities using a dendrogram (**Figure 1A**) that was generated from public data[1] and using other characteristics and expression sites of particular lipocalins. In addition, we also re-opened and explored proteomic datasets from our previous experiments that are also publicly available to discuss them in light of other studies published to date. We discuss the importance of lipocalins in three major biological areas in which lipocalins play crucial roles, namely in chemical communication, reproduction and development, and in the regulation of pathogens and natural microbiota.

CHEMICAL COMMUNICATION

Chemical communication is facilitated by behaviour-guiding olfactory signals, which in mice are abundant in body secretions such as tears, saliva, vaginal secretions, and urine (Thoß et al., 2015, 2016; Stopka et al., 2016; Cerna et al., 2017; Stopkova et al., 2017). These signals are often complex and provide information about multiple states of an individual including (sub-)species, kin, sex, health, and food sources (Hurst et al., 1997, 1998; Zala et al., 2004, 2015; Cheetham et al., 2007; Stopková et al., 2007; Bímová et al., 2009). Different types of molecules manifest the complexity of such signals which are detected *via* chemosensory G-protein coupled receptors of the main olfactory epithelia (MOE) and of the vomeronasal organ (VNO; Moss et al., 1997; Leinders-Zufall et al., 2000; Spehr et al., 2006; Wynn et al., 2012; Ibarra-Soria et al., 2014a; Nagel et al., 2018; Santoro and Jakob, 2018; Van Der Linden et al., 2018; Miller et al., 2020; Bansal et al., 2021). These receptors are tuned to particular signalling molecules including the volatile organic compounds – VOCs (Berghard and Buck, 1996; Malnic et al., 1999; Kwak et al., 2012, 2013), short peptides (Leinders-Zufall et al., 2004; Sturm et al., 2013) and non-volatile lipocalins, in mice, dominated by the male-biased MUPs (Chamero et al., 2007; Roberts et al., 2012). MUP like signals even have interspecific effects *via* the parallel mechanisms for kairomone detection. For example, lipocalins released from predators (cats, rats) are detected by mice and this induces aversive responses (Papes et al., 2010; Carvalho et al., 2015; Pérez-Gómez et al., 2015). Together, these secretory signals form signature mixtures, which generate stable representations in the accessory olfactory bulb (AOB) and, in other parts of the brain, these representations are linked to relevant behaviours (Bansal et al., 2021).

Major urinary proteins are essential for proper delivery of various volatile signals out of the body and for protecting and slowly releasing them from the urine marks (Hurst et al., 1998; Drickamer, 2001; Sharrow et al., 2002, 2003). Many of the ligands released from MUPs or just from the drying urine are directly detected by VNO neurons (Leinders-Zufall et al., 2000). MUPs were also demonstrated as signals themselves that are detected by basal, V2R-expressing sensory neurons in the VNO (Chamero et al., 2007) which due to small but detectable differences between different Mup genes and MUP protein structures (Phelan et al., 2014a,b) provide the basis for combinatorial coding of MUP signalling (Chamero et al., 2007; Kaur et al., 2014) which are then responsible for the display of specific mouse behaviours (Roberts et al., 2010, 2012; Dey et al., 2015; Demir et al., 2020). Importantly, these neurons are different from those that detect small hydrophobic compounds, which are apical V1R-expressing VNO neurons (Leinders-Zufall et al., 2000). In **Figure 1A**, MUPs are depicted in dendrogram as monophyletic group in which the associated proteins highly cluster together (bootstrap, BT = 96). This group can be further separated onto sub-recently duplicated and highly similar "central" MUPs (BT = 100) and the ancestral "outlier" MUPs 3, 4, 5, 6, 20, and 21 (Logan et al., 2008; Stopková et al., 2009, 2014; Phelan et al., 2014b; Sheehan et al., 2019). In **Figure 1A**, the Gravy index[2] is provided in heatmap next to the protein names indicating that both groups of MUPs have different numbers of hydrophobic residua and thus the affinity to bind different VOCs; this difference is significant while their isoelectric points are similarly acidic pI = ~4.9 (Stopková et al., 2016). This evolutionary differentiation of mouse MUPs has widened the spectrum of hydrophobic compounds that they protect, transport and release. This step was presumably crucial for the evolution of the house mouse as it enabled to "utilize" a wider and more informative odour space in individual recognition. Examples of typical MUP ligands (VOCs) previously studied in mice (Cavaggioni et al., 1987; Novotny et al., 1999; Zidek et al., 1999; Sharrow et al., 2003; Bingham et al., 2004) are presented in **Figure 1B**. For their complexity (volatiles, non-volatile proteins, and peptides), body secretions are now seen as signature mixtures rather than pheromones (Brennan and Kendrick, 2006; Nagel et al., 2018; Roberts et al., 2018; Bansal et al., 2021).

The system of signal detection has also diversified within the vertebrates thus evolving clear morphological structures (VNO, MOE) in tetrapods (except birds) while genetic components (V1R, V2R vomeronasal receptors) have already

[1]https://www.ensembl.org/

[2]http://www.gravy-calculator.de/

been found in teleost fish and sharks (Grus and Zhang, 2008). The transition from water to land was accompanied by a change in the ratio of V1R and V2R receptors such that the terrestrial vertebrates have more V1R receptors detecting smaller molecules including volatiles, whilst aquatic vertebrates have more V2R receptors for soluble and larger molecules including peptides and proteins. For example, amphibians (xenopus) have 21 V1Rs and 330 V2Rs, reptiles (python) have 4 V1Rs and 216 V2Rs, while the mouse system of olfaction is highly specialized for terrestrial life with 239 V1Rs (Tirindelli, 2021) with distinct evolutionary trajectories across mouse species (Miller et al., 2020), and 122 V2Rs (Tirindelli, 2021). Humans have lost functional VNO and the genes with remaining five intact V1Rs (Shi and Zhang, 2007). Profound differentiation of VNO and MOE in mice resulted in a highly specialized system being able to detect a wide spectrum of compounds including soluble (V2R) and volatile (V1R) compounds with VNO and general odorants with MOE. The signals detected by VNO are then processed in the AOB which maintain stereotypic sensory representations for broad types of stimuli, providing a substrate for relevant behaviours (Bansal et al., 2021). The responses of VNO and MOE are fast because odours are transported in turbulent plumes from the sites of origin and for example mice are able to detect these dynamically changing odours with a frequency of up to 40 Hz (Ackels et al., 2021). The main message from this study is that volatiles, released from MUPs in the urine marks, must directly interact with chemosensory receptors and not *via* nasal volatile-binding proteins that would slow down this interaction. This is also suggested in a study on elephants, who produce the OBPs along the whole trunks making it unlikely to fast transport the volatile signals to the vicinity of chemosensory receptors from distant trunk parts (Lazar et al., 2002).

The most interesting aspect of olfactory diversification is a co-evolutionary process during which the receptor diversification was accompanied by the duplication and neo-functionalization of lipocalins. Particularly, there is a group of highly similar lipocalins (LCN3, LCN4, LCN13, and LCN14) which cluster together (BT = 99, **Figure 1A**) and which are predominantly expressed in the mouse VNO thus forming of up to 36% of all transcripts in males and almost 29% in females with *Lcn14* being the most abundant transcript in VNO (Kuntova et al., 2018). Transcriptomic analysis of MOE provided similar results as what we have detected with proteomics of the nostrils with prevailing OBPs (*Obp2*, *1*, *5*, *8*, and *Mup4* transcripts). Interestingly, a total of 17 lipocalins were detected on the proteomic level in the mouse saliva including LCNs that were detected mainly in VNO (Stopka et al., 2016). Many of these proteins are presumably transported to the oral cavity by a system of tiny tunnels called naso-palatinal ducts which are required for proper pheromone signalling and functioning of VNO which is why they are concentrated near the VNO opening (Levy et al., 2020). It is likely that after the signals are detected in VNO, they are chelated by the VNO-specific LCNs with larger barrels and consequently pumped out and "released" to the oral cavity where they have been detected (Stopka et al., 2016) and where the digestion begins.

In mice, the neo-functionalization of lipocalins can be demonstrated by specific proteomic signatures, which in our case are presented as particular differences in the relative contribution to total protein content across different secretions. In **Figures 2A–C**,[3] we present the data from our previous experiments where we generated the whole proteomes from lavages of eyes (Stopkova et al., 2017), nostrils (Kuntova et al., 2018), and oral cavity (Stopka et al., 2016) using label-free proteomics. We used just a few proxies to see relevant signatures, namely lipocalins, proteins involved in immunity and antimicrobial defence, secretoglobins [SCGB or ABP – androgen-binding proteins, formerly suggested as putative semiochemicals (Bimova et al., 2011)], exocrine-gland secreted peptides (ESP, putative pheromones; Haga et al., 2010; Abe and Touhara, 2014), and many other proteins with structural, homeostatic or cellular functions depicted as 'other'. The most interesting result of this simple comparison is that the nostrils contain excessive amounts of lipocalins. In comparison with the urine which contains as much as 85% of lipocalins, mainly MUPs (Enk et al., 2016), the nostrils contain mainly the odorant binding proteins OBP8, OBP1, OBP5, OBP2 and a lipocalin named LCN11, **Figure 2B**.

The family of OBPs is coded by genes on the mouse chromosome X. To extend the knowledge on wild-living mice, we have sequenced all *Obp* transcripts and provided unique *Obp* sequences for feral *M. m. domesticus* (*Obp1* – KJ605385, *Obp2* – KJ605386, *Obp5* – KJ605387, *Obp6* – KJ605388, and *Obp7* – KJ605389), and *Mus musculus musculus* (*Obp1* – KJ605390, *Obp2* – KJ605391, *Obp5* – KJ605392, *Obp6* – KJ605393, and *Obp7* – KJ605394) now available in GenBank (Stopková et al., 2016). All the novel OBPs have a feature typical for the entire *Obp* cluster – a specific disulfide bond (Cys38–Cys42), which represents a strong OBP-diagnostic motif CXXXC (Cys-Xaa-Xaa-Xaa-Cys). OBPs are phylogenetically close to MUPs, **Figure 1A**. However, their biochemical properties are different from those of acidic MUPs in that their isoelectric points are closer to neutral and their hydropathy (Gravy) index is lower (Stopková et al., 2016). Thus, their affinity for less hydrophobic compounds, their prevalence in the proteomic profiles of eyes and nostrils and their close-to-neutral pI suggests that these proteins are important in removing the background organic molecules including odorants that are different from volatiles presented by MUPs in the urine. From the point of view of the "odour space" a fraction of odours and other compounds is being instantly removed from the nostrils and this presumably facilitates that the receptors preferentially bind relevant signals.

There is yet another perspective in the understanding of potential roles of OBPs in the mouse nostrils. They are sexually dimorphic similarly as the semiochemical responsive olfactory neurons (Vihani et al., 2020). This means that for some reason, being most likely a sharper detection of the ligands produced by males, females produce more OBPs (almost 50% of all soluble proteins) in the nostrils, which is likely to remove those VOCs that do not have the chemical properties of MUP

[3]http://string-db.org

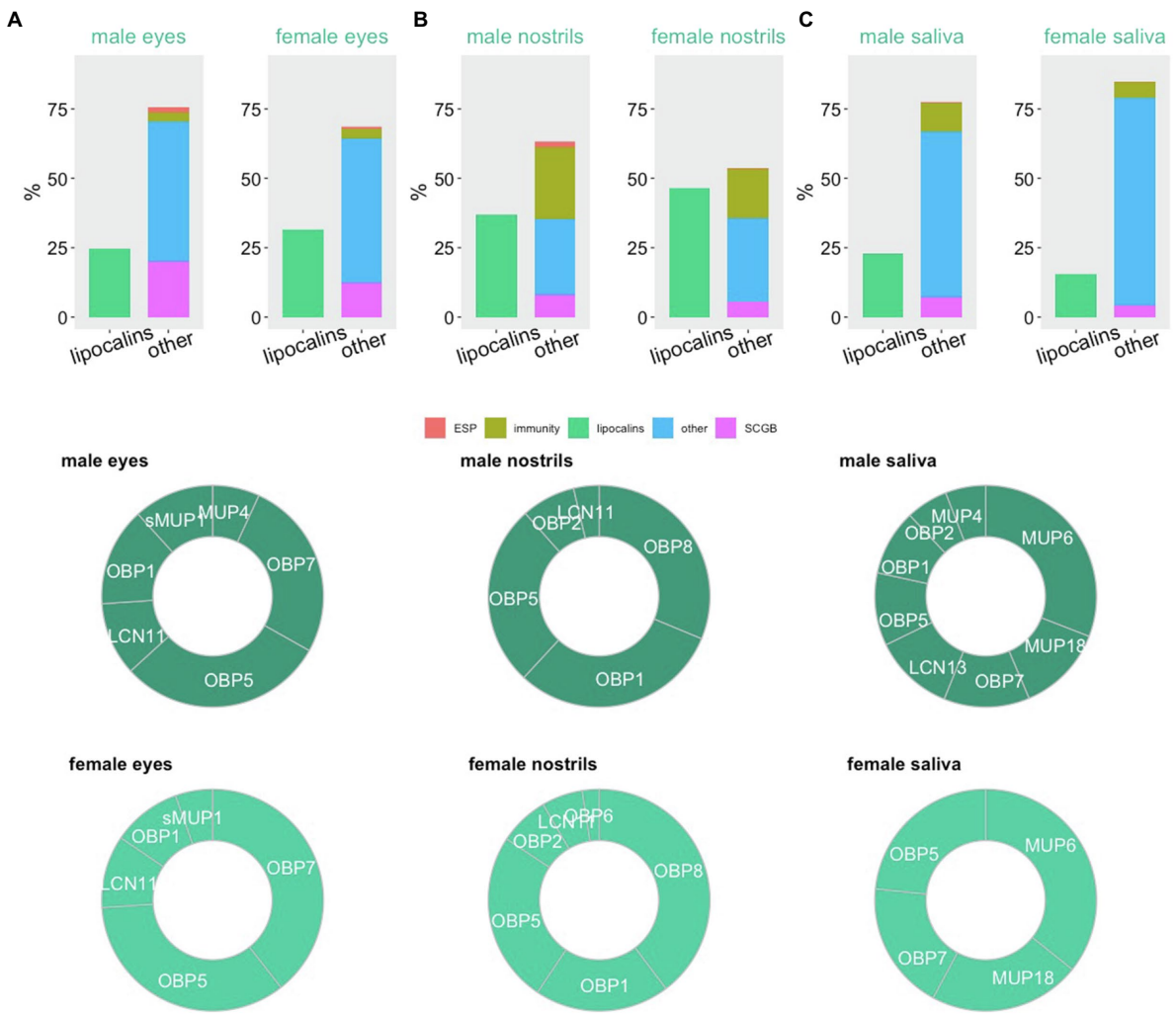

FIGURE 2 | Proteomic signatures in major orofacial secretions of the house mouse. Estimation of protein abundances is represented as a fraction (%) of all proteins in male and female eyes **(A)**, nostrils **(B)**, and oral cavity **(C)**. We have extracted this data from our previously published studies with publically available data. Proxy for functions (exocrine-gland secreted peptides – ESP, immunity, lipocalins, secretoglobins – SCGB) is based on public gene ontology tool.

ligands that males use for signalling. Similar pattern was detected in the eyes with similar levels of sexual dimorphism, **Figure 2A**. Because, eyes, nostrils and oral cavity are the inter-connected structures, with eyes being connected with nostrils *via* naso-lacrimal ducts, nostrils (VNO) with the oral cavity by the naso-palatinal ducts (Levy et al., 2020) and through naso-pharyngeal upper airways, OBPs may help removing the given spectrum of selected compounds from the majority of oro-facial mucosa. The selective forces that shaped the evolution of OBPs probably included the ability to remove organic toxins from nasal epithelia and upper airways. This has been shown in porcine OBPs, which bind with high affinity HNE (4-hydroxy-2-nonenal) depicted in **Figure 1B**, a toxic compound derived from lipid peroxidation. OBPs therefore protect living cells from damage caused by oxidative stress (Grolli et al., 2006). In non-rodent taxa, such as Artiodactyla (Ruminantia), the olfactory secretome becomes more complex during the reproductive period. For example in goats and sheeps, more OBP variants are expressed and the ligand reception by OBPs is often facilitated by protein modifications including O-GlcNAcylation (Cann et al., 2019), which was previously described also in pigs (Nagnan-Le Meillour et al., 2014).

In many mammals, Obp genes were selected for diverse functions across various taxa. Arvicolids (Cricetidae) are a parallel lineage to Murid rodents (Muridae) within the superfamily Muroidea and they seem to lack the genes for MUPs. However, they produce excessive amounts of OBPs in their urine and several studies provided evidence that they are produced by the liver. For example, the bank voles (*Clethionomys glareolus*) produce at least three OBPs coded by Obp1, Obp2, and Obp3 genes encoding the predominant lipocalins present in their urine and saliva (Stopkova et al., 2010), see also (Loxley et al., 2017). Similarly, the European water voles (*Arvicola terrestris*) produce one sexually dimorphic

OBP named arvicolin with transcripts being highly abundant in the liver and proteins present in the urine. The reception of pheromones by this particular OBP may be facilitated *via* protein modifications (e.g., phosphorylation and O-GlcNAcylation; Nagnan-Le Meillour et al., 2019) while the hamster OBP (aphrodisin) is *N*-glycosilated (Singer et al., 1986). All these arvicolid and also mouse OBPs are highly similar to hamster aphrodisin, which is vaginal OBP that was named after its effect upon male-mounting behavior *via* its natural OBP ligands (Singer et al., 1986; Briand et al., 2004) and is also expressed by other hamster species (Turton et al., 2010). When looking further from the muroid rodents (Myomorpha), OBPs have also been detected in the urine of highly social African mole rats (Hystricomorpha; Hagemeyer et al., 2011) thus providing evidence that the involvement of OBPs in chemical signaling was likely the ancestral mode of chemical communication in rodents. Taken together, the selective pressures that drove the evolution of chemical communication in rodents acted upon the function of a range of lipocalins and not on particular lipocalin gene. This is evidenced by the fact that those species that lack the genes for MUPs evolved other lipocalins for this function so the function of MUPs as carriers of chemical signals was supplanted by OBPs in many rodent taxa.

An interesting aspect of the systems of signalling and detection is that they are socially (environmentally) modulated. To our knowledge, we have been the first laboratory that provided evidence that the abundance of MUPs in the urine of female mice correlates with the estrous cycle, reaching the highest levels od MUP abundance in the urine (Stopka et al., 2007) and vaginal secretions (Cerna et al., 2017) near estrus. There is also a strong influence of social environment, whereby males of wild *M. m. musculus* increase their production of MUPs in the urine when presented with a female behind metal grid (Janotova and Stopka, 2011). Socially induced MUP variation has also been demonstrated in seminatural enclosures, where males doubled the excretion of MUPs after acquiring a territory and became socially dominant (Thoss et al., 2019). Higher concentrations of MUPs may then yield various behavioural responses in the receiver due to MUP detection by VNO neurons *via* progesterone signalling (Dey et al., 2015). Recent development of transcriptomic techniques helped to reveal that the fitness-related social dynamics of protein expression was also demonstrated in olfactory tissues. The expression of many receptor genes was altered when presented with different stimuli in several strains of the laboratory mice (Ibarra-Soria et al., 2017) and similarly when mice are separated, the isolation induces sex-specific differences in the olfactory sensory receptor repertoires (Santoro and Jakob, 2018; Van Der Linden et al., 2018). These social and reproductive effects are relevant examples of the expression dynamics that is regulated by benefits and costs of lipocalin and receptor production driven by social stimuli and the potential to mate. Until this point, we concentrated on lipocalins that function as an interface between external and internal worlds of an individual. However, many lipocalins have important functions within an individual homeostasis, reproduction and development.

ROLES OF LIPOCALINS IN REPRODUCTION AND DEVELOPMENT

The production of fully mature and motile sperm cells is essential for successful reproduction. The first key event happens in the seminiferous tubules where, through a complex process of spermatogenesis, germ stem cells differentiate into highly polarized spermatozoa. Along testosterone, vitamin A in the form of retinoic acid (RA) is an important signalling molecule that initiates spermatogonial differentiation and meiotic entry (Zhou et al., 2008). After the conversion from retinol, which is synthetized in Sertoli cells, RA specifically binds to intracellular heterodimeric receptors RARs and RXRs and regulates gene expression, e.g., of Stra8 gene inducing the synthesis of downstream markers of meiosis (Livera et al., 2002; Zhou et al., 2008). Signalization mediated by RA and its receptors was shown to be important also for the normal function of steroidogenic Leydig cells producing testosterone (Jauregui et al., 2018). However, as retinoids are not soluble in an aqueous environment, they require both extracellular and intracellular binding proteins. Interestingly, lipocalin-type prostaglandin D_2 synthase (L-PGDS or PTGDS) is expressed in many tissues, also including the mouse testis (Gerena et al., 2000). Since PTGDS has been reported to have a high affinity to RA and retinaldehyde, it was proposed that it might serve as a retinoid carrier supplying developing germ cells (Urade and Hayaishi, 2000; Rossitto et al., 2015). Moreover, Kido et al. (2005) showed the specific expression in transgenic mouse testis of fatty acid binding protein 9 (FABP9). Later study specified the FABP9 localization to advanced stages of spermiogenesis – from elongating spermatids on (Selvaraj et al., 2010). FABP9 shows high homology (58%) to FABP4, which binds long-chain fatty acids like stearate, palmitate, and oleate, but also RA, though with lower affinity (Oko and Morales, 1994; Richieri et al., 2000). Because spermatogenesis is a process demanding high levels of fatty acids (e.g., for membrane biogenesis), FABP9 likely contributes to providing them to the germ cells.

Fully developed testicular spermatozoa further undergo several maturation steps during their transit through epididymis, which is divided into three segments, each with unique luminal environment determined by proteins secreted from various epithelial cell types (Turner et al., 2003). The modification of sperm protein profile in region-specific manner is one of the proposed features of epididymal maturation. Support for this was provided by proteomic analysis of *M. musculus* whole sperm isolated from the caput, corpus and cauda regions (Skerget et al., 2015). The study showed that, among other proteins, lipocalins LCN2, LCN6, and LCN8 were downregulated in the caput sperm, whereas LCN12 was abundant in the corpus. On the contrary, LCN5 and FABP9 were common in sperm from all the segments. Moreover, Guyonnet et al. (2012) specified in CD 1 mice the FABP9, LCN5 and LCN12 localization to the matrix of sperm acrosome, while LCN2 and LCN6 signals were observed using fluorescent techniques to be associated with the mouse and human spermatozoa isolated from cauda epididymis (Chu et al., 2000; Hamil et al., 2003).

Despite the pieces of evidence, biological functions of many lipocalins in reproduction still remain unclear. LCN8 is hypothesized to bind and transfer retinoids (RA) similarly as LCN5 and thus might drive the development and maintenance of epididymal epithelium (Rankin et al., 1992; Costa et al., 1997; Lareyre et al., 2001). Based on an increased uptake of ferric ion by sperm mediated by LCN2, lipocalins are also suggested, by yet unknown mechanism, to undergo internalization into the sperm cell (Elangovan et al., 2004). In order to elucidate the role of lipocalins and cytosolic calycins in male reproduction, several knockout studies have been performed. Deletions of either of Lcn6, Lcn8, Lcn9, or Fabp9 did not cause abnormalities in the morphology of testis or epididymis nor in sperm production and fertility. However, the sperm from Lcn8$^{-/-}$ and Fabp9$^{-/-}$ double-knockout male mice showed abnormal head and tail morphology. An enhanced frequency of spontaneous acrosome reaction occurred in Lcn6 and Lcn8 deficient sperm (Selvaraj et al., 2010; Yin et al., 2018; Wen et al., 2021). All this evidence indicates that particular lipocalins are not essential for sperm development and its fertilizing ability because they can, to some extent, supplant each other. Very likely, it might be due to functional compensatory mechanism originating from their high structural and hence also binding homology. Therefore, double or triple knockout studies should be performed to undoubtedly elucidate this mechanism. The major significance of lipocalins in male reproduction thus remains in epididymal sperm maturation and events linked to capacitation and acrosome reaction occurring in the female reproductive tract.

The estrous cycle along with the oogenesis, which is linked to folliculogenesis are the most important fitness-related processes in female reproduction. In mice and other rodents, the cycle is generally divided into the four phases known as proestrus, estrus, metestrus, and diestrus. Interestingly, in some rodents such as the wood mice (*Apodemus sylvaticus*) these phases dynamically change as a reaction to the presence or absence of male mating partners (Stopka and Macdonald, 1998). Particular phases are characterized by the differential gene expression and by varying cellular types of the utero-vaginal epithelia (**Figure 3D**) reflecting the maturation of ovarian follicles (Byers et al., 2012; Yip et al., 2013; Cora et al., 2015). In detail (see **Figure 3D**), the proestrus phase is typical with predominant nucleated epithelial cells, whereas estrus phase with keratinized epithelial cells on which bacteria feed. The metestrus is overpopulated with neutrophils. Hormonal imbalance or pathogen

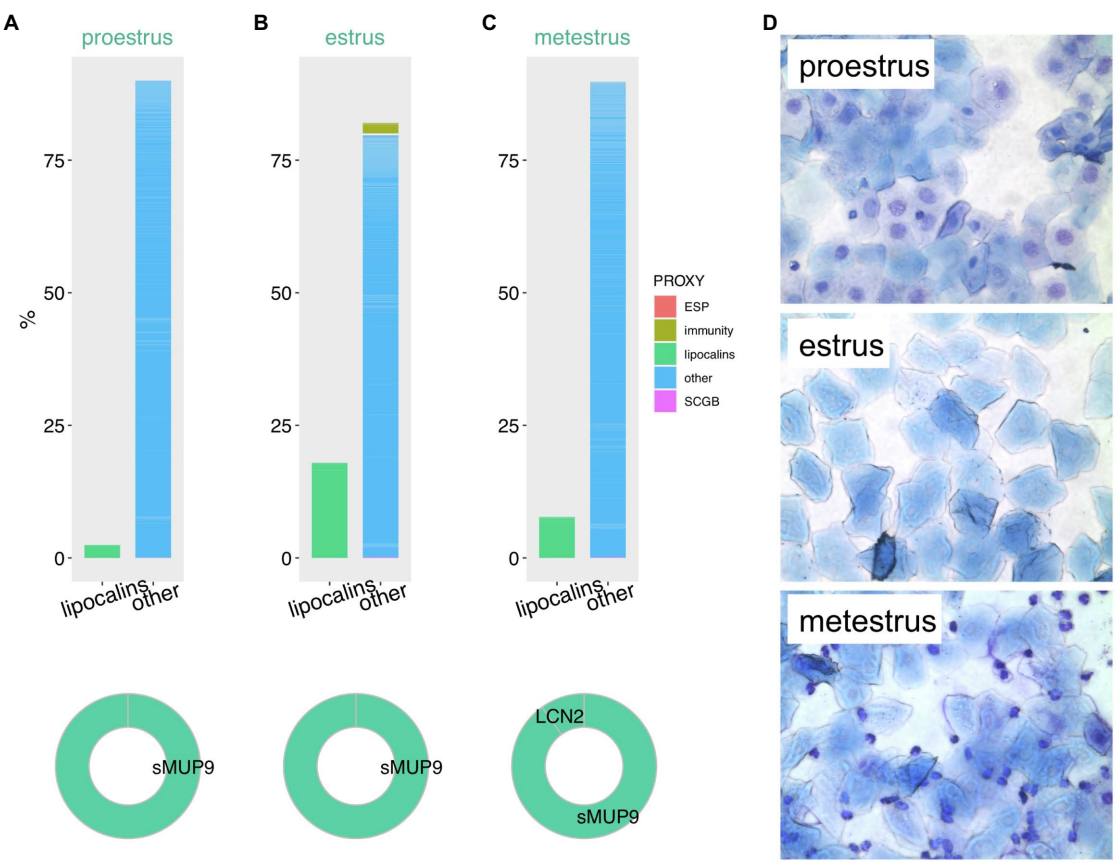

FIGURE 3 | Proteomic variation in vaginal secretions throughout the mouse estrous cycle. Estimation of protein abundances is represented as a fraction (%) of all proteins in vaginal lavages during proestrus **(A)**, estrus **(B)**, and metestrus **(C)**. We have extracted this data from our previously published study with publically available data. Proxy for functions (ESP, immunity, lipocalins, secretoglobins – SCGB) is based on public gene ontology tool. Representative microphotographs of the vaginal cytology were taken at magnification 100× after May-Gruendewald and Giemsa staining **(D)**.

exposure may lead to disruption of cycling. As shown in **Figures 3A–C** using the vaginal fluid proteomic data from *M. m. musculus*, lipocalins are significantly up-regulated in estrus and metestrus compared to proestrus (Cerna et al., 2017). This stage-specific elevation was proportionally most obvious in sMUP9 (i.e., group of highly similar MUPs: MUP6, MUP9, MUP16, and MUP19) while LCN2 only mildly varied, **Figure 3B**, and was upregulated only in metestrus (**Figure 3C**). Except these, MUP20, sMUP17, LCN11, and OBP5 were significantly up-regulated in estrus but their proportion to total protein content was lower. On the contrary, retinol-binding protein 1 (RBP1) had the lowest abundances during estrus. Given their proposed ability to internalize and transport various ligands, highly abundant lipocalins in these stages possibly detoxify the mouse vaginal environment while some of these ligands might have become the signals by which males recognize female receptivity (Stopková et al., 2009).

High abundances of LCN2 limits the bacterial growth during estrus by iron-chelating bacterial siderophores (Flo et al., 2004). Similarly to male reproduction, retinoids are essential also for the female genital system. Vitamin A sufficiency is important for the differentiation of meiotic germ cells, embryonic implantation and normal embryonic development. The significance of the uterine expression of retinoid binding proteins and their transcripts, e.g., for RBP1, RBP4, CRABP2 thus supports this view (Clagett-Dame and Knutson, 2011; Yip et al., 2013). Additionally to the LCN2 protective roles for female genital-tract epithelia, this lipocalin also functions as a sperm capacitation-promoting factor *in vitro* (Watanabe et al., 2014). Isolated sperm from the oviduct of $Lcn2^{-/-}$ knockout females showed a significant decrease in membrane lipid raft movement, which was retrieved after LCN2 addition. The association of LCN2 with sperm surface also enhances the progressive motility (Lee et al., 2003). The interaction between mammalian spermatozoa with seminal fluid, and female genital tract is known to be reciprocal. For instance, spermadhesins from ejaculate, apart from other functions, modulate the uterine immune responses. As lipocalins are contained in male genital fluid, they very likely co-participate on this direct interaction (Rodriguez-Martinez et al., 2010). To add, lipocalins are essential for various stages of reproduction both in males and females and similar lipocalins were detected also in avian ova (Bilkova et al., 2018), thus suggesting that the roles of lipocalins are conserved across distant vertebrate taxa.

REGULATION OF PATHOGENS AND NATURAL MICROBIOTA

Lipocalins are expressed in all body parts with the complex microbial communities (Seo et al., 2006; Stopka et al., 2016; Cerna et al., 2017; Xiao et al., 2017) and their typical β-barrel structure with a central cavity for binding of various hydrophobic molecules (Flower, 1996) provides several strategies for constant sensing and controlling both pathogens and commensal bacteria. There is growing evidence that each microbiome-populated body site hosts unique bacterial ecosystems (Lee and Mazmanian, 2010; Matějková et al., 2020; Moudra et al., 2021) and that those tissues permanently face the challenge of discriminating between commensal and pathogenic bacteria. A rapid destruction of potential pathogens is especially important in the sites where pathogen burden could have detrimental effects upon the host. Such places include the oral cavity representing the main route to the digestive tract, urogenital openings, and eyes and nostrils as a gate to the brain *via* the optic and olfactory nerves (Stopková et al., 2014).

Communication between the host and its microbiome is facilitated by molecules secreted by bacteria. Microorganisms (and especially bacteria) belonging to the same species are able to synchronize their activities *via* so-called quorum sensing, **Figure 4**.[4] Each quorum sensing is characterized by a signalling compound, usually a small molecule (QSM – quorum sensing molecule) which is released into the environment and recognized by other co-specific microorganisms (Rutherford and Bassler, 2012). When the concentration of QSM reaches a certain threshold, the whole population of microorganisms synchronizes its biosynthetic activity. QSMs can be inactivated by quorum quenching mechanism (QQ), which is usually mediated by specific enzymes (Uroz et al., 2009). However, the alternative strategy for QQ is the scavenging and removal of QSMs provided by the proteins with a QSM binding capacity. Ligands associated with bacterial infections and those from defeated bacteria during regulation of microbiota are also detected with MOE and VNO *via* the microorganism-associated molecular patterns (MAMPs), and they are sensed in many places in the body including specific chemosensory neurons in the mammalian nose (Bufe and Zufall, 2016). They also include the formyl peptide receptor-like proteins in VNO, which provide sensitivity to disease/inflammation-related ligands (Riviere et al., 2009) and presumably they are responsible for the activation of bactericidal proteins. Bactericidal proteins (i.e., such as BPI proteins) were previously detected in the olfactory transcriptomes of the mouse (Ibarra-Soria et al., 2014b) in tears (Stopkova et al., 2017) and saliva (Stopka et al., 2016).

Lipocalins have the ability to bind a broad spectrum of ligands, therefore it is likely that they are involved in the regulation of microbiota and of the function of olfactory neurons (Bryche et al., 2021). For example, a recent study provided the evidence that bovine (bOBP) and porcine (pOBP) odorant binding proteins can effectively bind farnesol, which is a terpenoid produced by *Candida albicans* as QSM and which is able to affect transformation from the mycelial to the yeast state (Bianchi et al., 2019). Moreover, both OBPs have the capability to almost completely remove bacterial toxin pyocyanin produced by *Pseudomonas aeruginosa*. To some extent, both OBPs have also the affinity to various types of *N*-acyl-homoserine lactones produced as QSMs by *P. aeruginosa* and some other microbial organisms. Despite the lower affinity of OBPs to *Pseudomonas* signalling molecules, OBPs might scavenge QSMs when they reach potentially dangerous concentrations that activate quorum sensing. Interestingly, assays testing the direct

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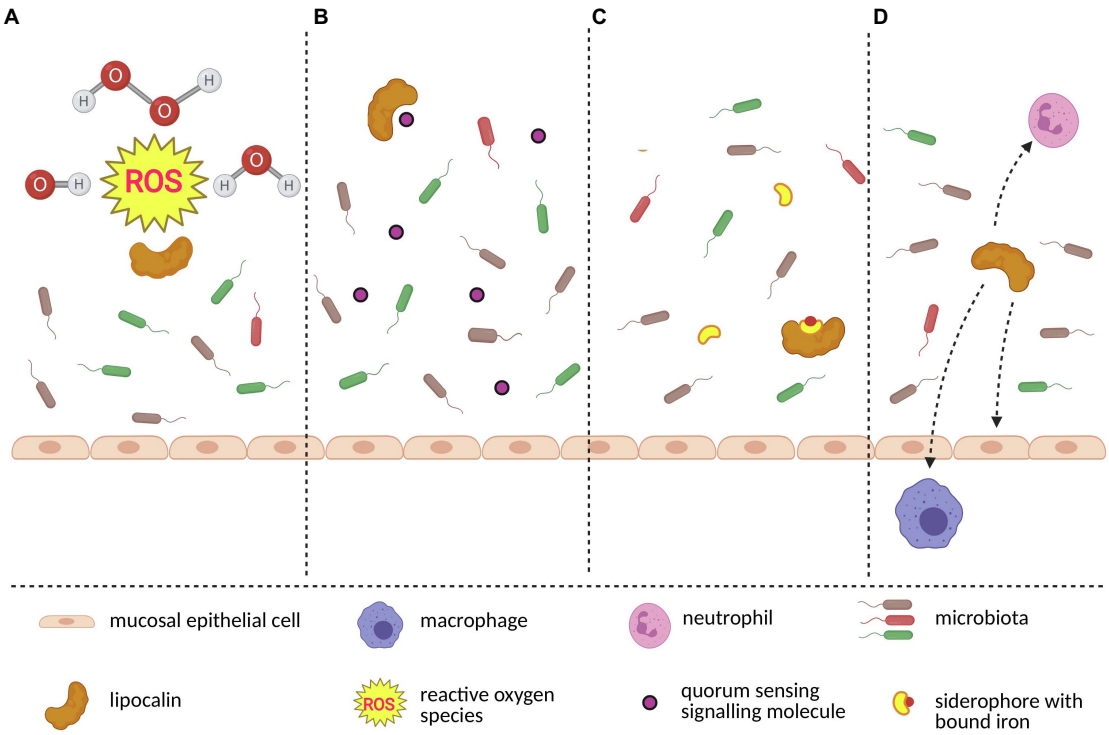

FIGURE 4 | A model of interactions between lipocalins and microbiota. Lipocalins protect host's epithelial cells and also commensal microbiota by scavenging toxic molecules such as reactive oxygen species which originate during oxidative stress **(A)**. Keeping microbiome homeostasis requires blocking the origin of bacterial monoculture. Lipocalins contribute to diversified microbiome by scavenging bacteria-produced quorum sensing signalling molecules **(B)**. Similarly, certain bacterial species use siderophores to gain iron and subseuently outcompete siderophore-lacking bacteria. By binding to siderophore-iron complex, lipocalins further contribute to stable microbiome **(C)**. Finally, lipocalins closely interact with other members of the immune system network (i.e., neutrophils, macrophages and epithelial cells) and together create a complex microbiota-surveillace system **(D)**. The figure was created in Biorender.

antimicrobial or antifungal activity revealed different efficiencies of the two OBPs. While pOBP has higher inhibitory activity on fungi, bOBP can better reduce the growth of various bacterial species (Bianchi et al., 2019). It is the first evidence that vertebrate odorant binding proteins exert antimicrobial activity through their scavenging capacity. Furthermore, OBPs also shape the microbiomes by scavenging toxic molecules (i.e., aldehyde 4-hydroxy-2-nonenal) that are produced during oxidative stress (Lacazette et al., 2000; Grolli et al., 2006) and whose higher concentrations cause damage not only to epithelial cells but also to commensal microbiota (Macedo-Marquez et al., 2014) which play a crucial role in keeping epithelial homeostasis (Koskinen et al., 2018).

Although the members of microbial communities are often described as commensals, the relationship between the host and a particular bacterial species can simply change to other forms of relationship as mutualistic or parasitic depending on the host's genetic background, nutritional status, or co-infection of the host (Belkaid and Harrison, 2017). One of the main aspects influencing the bacterial switch from commensal to pathogen is the availability of iron, which is strongly regulated by lipocalins (Lechner et al., 2001; Goetz et al., 2002; Fluckinger et al., 2004; Julien et al., 2019). Iron is one of the essential nutrients for almost all aerobic organisms. In mammals, the majority of iron is bound either in hemoglobin or in ferritin and transferrin. Therefore, the concentration of extracellular free iron is maintained below 10^{-24} M (Correnti and Strong, 2012). This low concentration of available iron serves as a protection of the host against the reactivity of free iron and also against the potentially pathogenic bacteria that would thrive in an iron-rich environment. To "steal" iron from host proteins (i.e., ferritin, transferrin, and lactoferrin), bacteria synthesize and secrete low-molecular-weight (<1.0 kDa) iron chelators called siderophores (Nairz et al., 2010). One of the most studied siderophores is enterochelin (Ent) whose affinity for iron allows it to effectively outcompete the majority of the host's iron-binding proteins (Fischbach et al., 2006). Typical lipocalin involved in iron homeostasis is LCN2 (alias 24p3, SIP24, siderocalin, NGAL), which is constantly present in low amounts in mucosa where it chelates Ent in order to inhibit overgrowing of Ent-producing bacteria. To prevent Ent from delivering iron back to the potential pathogens (e.g., to *Escherichia coli*, one of the major pathogens of gut-origin sepsis), LCN2 chelates the Ent-iron complex and forms a structure which is not capable of transferring iron back to bacteria (Goetz et al., 2002; Johnson et al., 2010; Wu et al., 2010; Mori et al., 2016). While LCN2-siderophore binding capacity is limited towards one group of siderophores – catecholate-type (Xiao et al., 2017),

human tear lipocalin (LCN1) is capable of binding a broader array of siderophores, including the bacterial catecholate and hydroxamate-type, and all major classes of fungal siderophores (Fluckinger et al., 2004). This physiological function of LCN2 and human LCN1 is widespread in vertebrates and for example, in birds, four proteins highly homologous to LCN2 (CALβ, CALγ, Ggal-C8GC and Ex-FABP) exerted almost identical roles in the protection against *E. coli* (Garénaux et al., 2013).

Protective function of LCN2 is documented in experiments with Lcn2-deficient mice, which are highly prone to bacterial infection and sepsis. In detail, the lack of LCN2 leads to significant changes in gut microbiota composition followed by microbiota dysbiosis with a disproportionate growth of gram-negative bacteria (Moschen et al., 2016; Singh et al., 2016, 2020). Reducing effects on bacteria were observed in a study focused on *in vitro* growth of *Aeromonas hydrophila* and *E. coli* in the presence of fish LCN2 (ortholog – 3nLcn2; Zhou et al., 2020). Similarly to *in vitro* experiments, there were lower bacterial loads in tissues of 3nLcn2-administered fish infected by *A. hydrophila* (Zhou et al., 2020). Homeostatic relationships with microbiota can be kept only when the contact between bacteria and the host's epithelial cells surface is minimized. This segregation of microbiomes from the host's cells is accomplished by combined action of epithelial cells, mucus and the involvement of both innate and adaptive immune systems (Shi et al., 2017). The observation that Lcn2-deficient animals are also susceptible to the siderophore-independent pathogens suggests that LCN2 protects the microbiome-host barrier not only as a siderophore scavenger but also plays an important role in a broader immune system network.

CONCLUSION

From the evolutionary point of view, lipocalins represent a fascinating protein family because, in vertebrates, many of their members underwent rapid evolution and neo-functionalization during or after the transition from water to land and this is well documented in rodents. At the same time, there is a group of lipocalins and other calycins that seem to be essential for life, because they are stereotypically expressed in almost all vertebrates and play important roles in spermatogenesis and embryonic development (FABP, RBP), regulation of oxidative stress (AMBP), and antimicrobial defence (e.g., LCN2). The transition from water to land was the major driving force that has driven the reorganization of sensory systems of detection (nose, eyes) of fitness-related cues such as food, predators and other individuals of the same species and particular sex. Mice are primarily dependent on olfactory cues. They use MUPs to protect and transport volatile pheromones that trigger many physiological, behavioural and reproductive responses *via* the vomeronasal and major olfactory systems. Various tissues in the oro-facial region, including lymphoid tissues, epithelia and lacrimal glands produce excessive amounts of OBPs which have different binding properties than MUPs (e.g., lower hydrophobicity) and thus they represent the major system of chelators of signals that are different from those presented by MUPs. In VNO these chelators include specific lipocalins LCN3, LCN4, LCN13, and LCN14, while MOE is overpopulated by OBPs. Interestingly, many lipocalins have similar barrels across the vertebrate taxa and thus they may have overlapping roles in tissue detoxification and in the regulation of bacterial growth. To conclude, for their capacity to bind various lipophilic and other organic molecules, lipocalins are essential for life, and this is documented by parallel evolution of their functions across animal taxa that inhabited the terrestrial ecosystems.

AUTHOR CONTRIBUTIONS

RS and PS wrote the first draft of the manuscript. TO prepared the involvement of lipocalins in reproduction while TM worked on the microbiota section. BK helped with discussions and analysis of literature searches. All authors contributed to the article and approved the submitted version.

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β-Lactoglobulin and Glycodelin: Two Sides of the Same Coin?

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The two lipocalins, β-lactoglobulin (βLg) and glycodelin (Gd), are possibly the most closely related members of the large and widely distributed lipocalin family, yet their functions appear to be substantially different. Indeed, the function of β-lactoglobulin, a major component of ruminant milk, is still unclear although neonatal nutrition is clearly important. On the other hand, glycodelin has several specific functions in reproduction conferred through distinct, tissue specific glycosylation of the polypeptide backbone. It is also associated with some cancer outcomes. The glycodelin gene, *PAEP,* reflecting one of its names, progestagen-associated endometrial protein, is expressed in many though not all primates, but the name has now also been adopted for the β-lactoglobulin gene (HGNC, www.genenames.org). After a general overview of the two proteins in the context of the lipocalin family, this review considers the properties of each in the light of their physiological functional significance, supplementing earlier reviews to include studies from the past decade. While the biological function of glycodelin is reasonably well defined, that of β-lactoglobulin remains elusive.

Keywords: β-lactoglobulin, glycodelin, *PAEP*, biological function, lipocalin

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INTRODUCTION

When β-lactoglobulin (βLg) was first isolated by Palmer, 1934 there can be little doubt that nobody realized that the protein would remain something of a puzzle 85 years later. βLg, a significant component of cow's milk, is a member of the ancient and widespread protein family that came to be named the lipocalins (Pervaiz and Brew, 1985). The protein is abundant and easily prepared so that it has served as a convenient test-bed for essentially every molecular technique from absorption spectroscopy (Townend et al., 1960) to X-ray crystallography (Crowfoot and Riley, 1938) and zeta-potential measurement (Chen and Dickinson, 1995) and pretty much everything else in between. Its ready availability has also led to redox (Conway et al., 2013; Corrochano et al., 2018), enzymic (Li et al., 1990; Gowda et al., 2017, 2018) and co-factor (Pérez et al., 1992) properties being ascribed though that is hardly surprising. What is not always clear, is whether any of these observations have any direct relevance to the physiological function.

The report by Futterman and Heller (1972) that βLg bound retinol was almost certainly unexpected as the focus of the paper was retinol binding to retinol-binding protein (RBP) and a convenient "blank" was required. The correct sequence of βLg was published the same year (Braunitzer et al., 1972), and the sequence similarity between βLg and α2u-microglobulin was noted by Unterman et al., 1981. However, it was not until the publication of the sequence (Rask et al., 1979) and structure (Newcomer et al., 1984) of retinol-binding protein that it became clear there was a close structural relationship (Godovac-Zimmermann et al., 1985; Sawyer et al., 1985). Since then all biological kingdoms have been found to contain family members, the lipocalins

(Pervaiz and Brew, 1985), the functions of the majority being associated with communication in its broadest sense: transport (serum retinol binding protein), camouflage (insecticyanin and crustacyanin), stress response (apolipoprotein D and α_1-acid glycoprotein), marking (mouse urinary protein and darcin), and some have enzymic activity (prostaglandin-D synthase, reductase, and plant epoxidase) (Flower, 1996; Åkerström et al., 2006). To date there are some 1,500 entries under "lipocalin" in the Protein Data Bank (Berman et al., 2003) and more than 46,000 in the UniProtKB database (The UniProt Consortium, 2019). However, this review will concentrate on a tiny subset of the family, βLg and glycodelin (Gd, PAEP) described by Gutierrez et al. (2000) and Sanchez et al. (2006) as Clade IV of a general lipocalin classification, paying particular attention to their physiological function (Pérez and Calvo, 1995).

Throughout the 1980s as comparison techniques became more robust, 3-dimensional molecular structures began to be used to infer homology as a complement to sequence-based techniques (**Figure 1**). This was particularly important when the pairwise identity of sequences dropped below 25–30% (Rost, 1999). βLg/RBP and βLg/α2u-microglobulin sequence comparisons (pairwise sequence identity of approximately 25%) highlighted their potential evolutionary relationships (Pervaiz and Brew, 1985). Other proteins, however, show even lower pairwise sequence identities and yet their membership of the same homology family became apparent when the folds revealed by the tertiary structure were found to be similar. The lipocalin fold is an 8-stranded up-down β-barrel open at one end forming what has come to be known as the calyx, with a 3-turn α-helix packing on the outer surface and usually a ninth β-strand located under the helix also on the barrel surface. As more members of the family emerged, three structurally conserved regions (SCR, **Figure 1**) were identified that served as signatures (formally, synapomorphies) and which are present in the vast majority of lipocalins: almost all possess SCR1 and SCR3, and many also contain SCR2. Intriguingly, all three of the SCRs are found on the solvent side of the foot of the calyx, implying some similarity of function (North, 1989). In addition, in many lipocalins including, Gd and βLg, there are conserved, intramolecular disulphide bridges. Now that DNA sequencing of whole genomes can be readily achieved, it is found that within the lipocalin family the intron/exon boundaries are also well conserved despite poor sequence identity (Salier, 2000; Sanchez et al., 2006).

Because members of the lipocalin family are found in all kingdoms, the ancestral lipocalin must have appeared long before the amniotes emerged around 250 million years ago (Mya) and it appears likely that there was already a form of animal skin secretion which was to develop into what is now referred to as lactation (Oftedal, 2002, 2013; Newman et al., 2018; Sharp et al., 2020). These skin secretions could provide sustenance and protection from infection for offspring and this process is still found in the egg-laying monotremes, animals whose young are produced in an immature form and require both feeding and protection. As the offspring matures, the composition of the secretion changes (Tyndale-Biscoe and Janssens, 1988; Lefèvre et al., 2010; Kuruppath et al., 2012; Sharp et al., 2020). The development of secretory cells associated with a specific organ, the teat, appears to have occurred around 165 Mya subsequently followed by the development of the true placenta and the emergence around 148 Mya of the eutheria or placentalia (Oftedal, 2002, 2013; Lefèvre et al., 2010). Many of the proteins that are present in the milk of today's placental species were present 160 Mya (Oftedal, 2013; Vilotte et al., 2013). The origin of βLg therefore must have been at least 160 Mya based on the secretions provided by the ancestral monotremes for their offspring (Oftedal, 2000; Joss et al., 2009; Lemay et al., 2009; Skibiel et al., 2013; Sharp et al., 2020). Gd, the other protein in Clade IV (Salier, 2000; Sanchez et al., 2006) is more recent as has been pointed out by Oftedal (2013). **Figure 2** shows a simple representation of the closer relationship of the lactoglobulins and the glycodelins than of the rest of the lipocalins, represented by RBP. It is the lineage leading to the placentalia upon which this review focuses.

Speculation that Gd might be the precursor of βLg (Kontopidis et al., 2004; Cavaggioni et al., 2006; Sawyer, 2013) is wrong, a mumpsimus, for several reasons. Lactation preceded placentation and, as βLg is found in monotremes as well as marsupials and eutheria, it was well-established before the emergence, no longer than 60 Mya, of the endometrial protein Gd (Oftedal, 2002; Lefèvre et al., 2010; Schiefner et al., 2015). Gd is glycosylated (Julkunen et al., 1988; Dell et al., 1995) and most, if not all, of its distinct functions depend upon this glycosylation (Halttunen et al., 2000; Seppälä et al., 2007; Lee et al., 2016) so that it is improbable that a post-translationally modified form could have arisen before the polypeptide itself! Further, Gd has no (Koistinen et al., 1999) or at least a significantly lower (Breustedt et al., 2006; Schiefner et al., 2015) affinity for hydrophobic ligands. Mutations Asp28Asn and Glu65Ser in βLg which form the glycosylation sites in Gd could have occurred earlier but there is little evidence, although glycosylation of βLg has been reported. There appears to have been a rare genetic mutation discovered only in the individual analysis of a large number of Droughtmaster animals (Bell et al., 1970, 1981) or possibly more commonly, in the milk of the domestic pig, *Sus scrofa* (Hall, 2010). However, in this latter case the glycosylation is O-linked through Thr4, unlike the N-linking in Gd (Dell et al., 1995), and in βLg-Dr (Bell et al., 1981). Another vertebrate lipocalin, lipocalin-2 (LCN2) or neutrophil gelatinase-associated lipocalin is N-glycosylated but at a site on the C strand, distinct from those in Gd (Holmes et al., 2005; Bandaranayake et al., 2011).

Lactation, a characteristic of mammals, produces a fluid rich in protein, fat and sugars, the exact proportions of which vary considerably across species and through lactation (see for example **Table 1** and Jenness and Sloan, 1970; Martin et al., 2013; Powers and Shulkin, 2016; Goulding et al., 2020). βLg is widely but not universally distributed – it is absent from the milk of rodents and lagomorphs (glires), camels, and humans although a pseudogene, *PAEPP1* (Hunt et al., 2018), is present but not expressed, close to *PAEP* on human chromosome 9. Some species, for example horses and cats, express paralogs and cows and goats have a pseudogene, related to one of these paralogs (Passey and Mackinlay, 1995; Folch et al., 1996). Most work on Gd has been on primates, especially humans and Schiefner et al. (2015) report its presence only in the old and new world

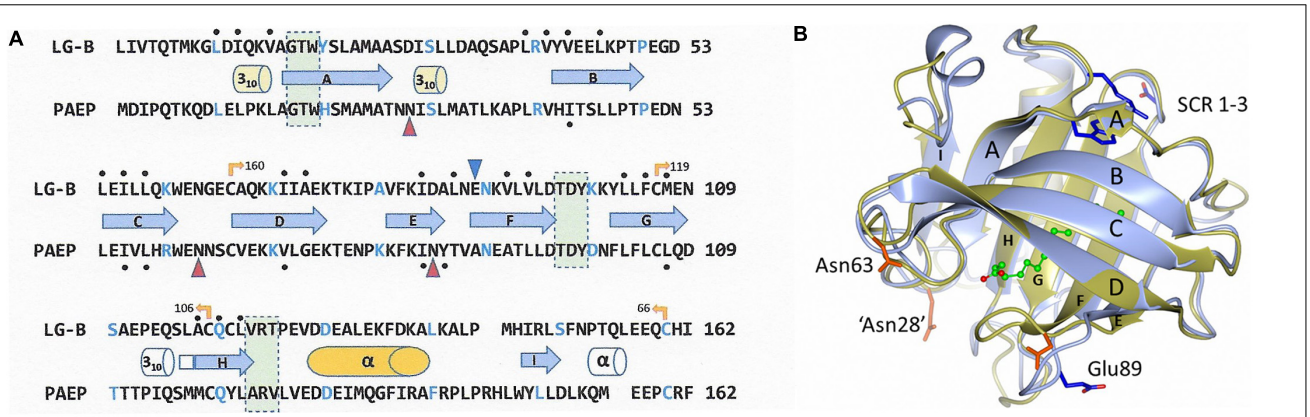

FIGURE 1 | (A) Alignment of the protein sequence of cow β-lactoglobulin B variant (LG-B) with that of human glycodelin (PAEP, Gd) showing the structurally conserved regions (SCR) in the lipocalin family boxed in pale green. Every 10th amino acid is colored blue and the secondary structural elements shown as cylinders for helices and arrows for β-strands, labeled as they are in the crystal structure. Uncolored structural elements appear in only one structure both structures and disulphide bridges are shown as yellow arrows with the partner residue number. Glycosylation sites in Gd are marked with red triangles, that at 85 being unused, while the ligand-gating Glu89 in βLg is shown as a blue triangle. The residues lining the central calyx are marked above/below with black dots, from which it will be seen that the cavity in Gd is very much smaller than that in βLg (Schiefner et al., 2015). **(B)** A cartoon showing the remarkable similarity of monomers of βLg (PDB: 1gxa, blue-gray) and Gd (PDB: 4r0b, olive). The βLg structure has a molecule of palmitate bound within the central calyx (carbon atoms in green, oxygen in red). The β-strands are labeled as in **(A)**. The helix is at the rear and the structurally conserved regions (SCR1-3) are indicated by residues Trp19, Asp98 and Arg124 in blue, all on the outer surface at the foot of the calyx. Glu89 in βLg which is on the EF-loop in the open position is close to the unglycosylated Asn85 in Gd shown in pink. The other residues in pink, Asn63 and Glu28, show the positions of the glycosylation present in native Gd. The mutation Asn28Glu was necessary to improve the solubility of the cloned Gd used in the X-ray analysis. Dimerization involves the I-strand in both proteins but there is significantly greater interaction in Gd (1170 Å^2 buried surface area) compared to βLg (530 Å^2) despite the remarkable overall similarity (the rmsd of the 160 Cα atoms is about 0.65Å). The carbon chain of palmitate is green. Figure drawn by CCP4mg (McNicholas et al., 2011).

monkeys and the hominids. However, there are isolated reports of its occurrence elsewhere. For example, in a proteomic study of dairy herd fertility (Koh et al., 2018), the plasma exosomes of heifers of low fertility contain the sequence of Uniprot G5E5H7 reported to be that of the gene *PAEP*, Gd. It is in fact the sequence of βLg-B. *In situ* hybridization on rat genital tract and PCR followed by sequencing has identified 100 bases of mRNA sharing "100% homology" with human glycodelin (Keil et al., 1999) and polyclonal antibodies to human Gd cross-react with rat reproductive and lung tissues (Kunert-Keil et al., 2005, 2009; Erdil et al., 2020). While false positive results arising in antibody cross reactivity experiments are not uncommon, the apparent presence of Gd in the rat by mRNA hybridization (Keil et al., 1999) remains something of a mystery. Putative *PAEP* pseudogenes have been identified in the genomes of tarsier, rat, rabbit and dolphin (Moros-Nicolás et al., 2018). Rodents and lagomorphs do not express βLg, though dolphin (Pervaiz and Brew, 1986) and tarsier (Schiefner et al., 2015) do. Lemay et al. (2009) report that there has been a loss of a section of DNA coding for amongst others, βLg, in glires since they were unable to find it and an evolutionary break point exists in the same region between the rodent and human genomes (Murphy et al., 2005). This reasoning would explain the absence of the milk protein in glires.

Since *PAEP* is now also used to describe the βLg gene, rather than *LGB* or *BLG* (e.g., Elsik et al., 2016; Hunt et al., 2018; HGNC, 2020), it has become difficult to be certain as to which protein is present without protein analysis. Schiefner et al. (2015) use the presence of the glycosylation sites at 28 and 63 to distinguish Gd from βLg which is a convenient method though it does not necessarily confirm expression. The reports by Azuma and Yamauchi (1991) and Kunz and Lönnerdal (1994) of a βLg-like protein with an Mr > 20,000 from the milk of the Rhesus monkey, *Macaca mulatta*, predate the comparative proteomic analysis of Beck et al. (2015) which describes this protein as Gd. However, the short N-terminal sequence (Azuma and Yamauchi, 1991) matches that of βLg in Schiefner et al. (2015), described in the UniProt database as, *inter alia*, 'Lipocln_cytosolic_FA-bd_dom domain-containing protein' and the two functional glycosylation sites are absent.

In summary, the ancestral βLg appeared sufficiently long ago (>250 Mya) for its presence to be detectable now in almost all mammals. Its loss from the glires (rodents and lagomorphs) occurred when they and primates diverged about 80 Mya and a similar event may explain its absence in the Camelidae which diverged from the other artiodactyls (even-toed, hoofed mammals) about 40 Mya (Price et al., 2005; Wu et al., 2014). Although there appear to be exceptions as noted above, Gd appears to be restricted to primates which started diverging 60–70 Mya (Dawkins, 2004; Schiefner et al., 2015).

Having now put the occurrence of βLg and Gd in context, it is the purpose of this article to review their properties relevant to their physiological functions.

β-LACTOGLOBULIN

β-lactoglobulin is a major component of bovine whey with properties that affect processing in the food industry. The

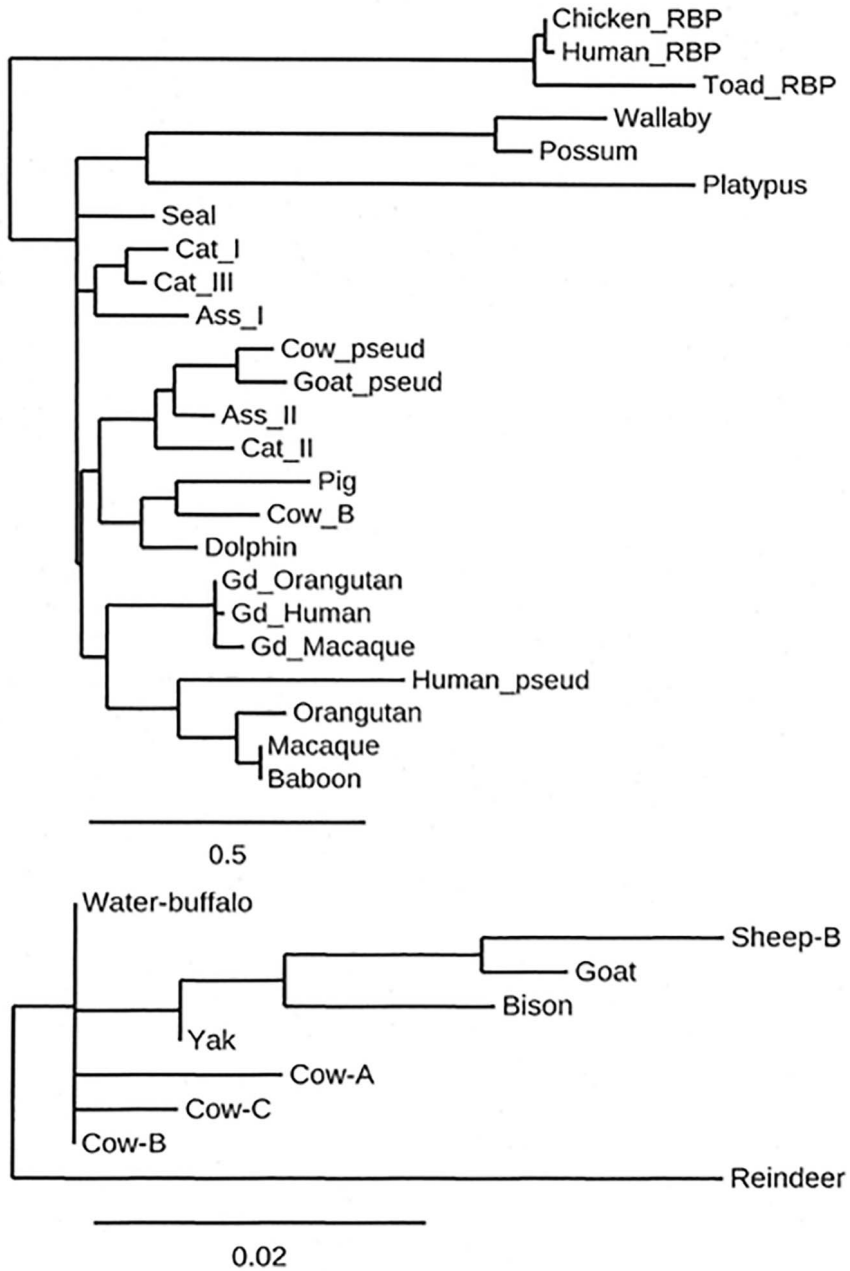

FIGURE 2 | A cladogram showing the relative amino acid sequence relationship of a selection of β-lactoglobulin (βLg), glycodelin (Gd) and retinol-binding protein (RBP) sequences, RBP being included as an outgroup to the βLgs and Gds. The inset shows the relationship between domesticated ruminant β-lactoglobulins. Note the difference in the branch-length scales. The amino acid sequences were aligned by CLUSTAL-Ω (Sievers et al., 2011) using its default parameters, and the cladogram was produced by the program PHYLOGENY.fr (Dereeper et al., 2008). The sequences used were UniProtKB (The UniProt Consortium, 2019) unless otherwise mentioned. *Lactoglobulins*: Platypus (F6SX48), Wallaby (Q29614), Possum (Q29146), Orangutan (NCBI: XP_002820419), Macaque (NCBI: XP_005580520), Baboon (O77511), Pig (P04119), Cow B variant (P02754), Beluga (NCBI: XP_022433973.1), Dolphin (A0A6J3RLE3), Cat I (P33687), Donkey I (P13613), Donkey II (P19647), Cat II (P21664), Cat_III (P33688), Fur Seal (A0A3Q7NUB2-1), Reindeer (Q00P86), Yak (L8J1Z0), Bison (NCBI: XP_010855058), Sheep B (P02757), Water buffalo (P02755), and Goat (P02756). *Glycodelins*: Gd_Human (P09466), Gd_Orangutan (NCBI: XP_00377753), Gd_Macaque (Q5BM07), *Pseudogenes*: Cow βLg pseudogene (Genbank: Z36937), Goat βLg pseudogene (Genbank: Z47079), Human βLg pseudogene (Ensembl: hg38_dna.[2298:6285].sp.tr), *Retinol Binding Proteins*: Toad RBP (P06172), Human RBP (P02753), and Chicken RBP (P41263).

extensive literature describing its behavior under these non-physiological conditions can be accessed through Boland and Singh (2020) and references therein. There have also been thorough reviews of βLg over the years, beginning with Tilley (1960), covering the properties and structure of βLg, sometimes alone but also as part of wider reviews on milk proteins, all of

TABLE 1 | Milk composition for a range of animals[1].

Source		Fat %[2]	Protein %[2]	Whey %[2]	βLg[2] mg/ml	PAEP[3]	References
Bos taurus	Cow	3.7	3.6	0.6	3.0	?	
Capra hircus	Goat	4.5	2.9	0.4	1.4		
Ovis aries	Sheep	7.4	5.5	0.9	2.8		
Cervus elaphus L.	Red deer	19.7	10.6		2.9		
Sus scrofa domesticus	Pig	6.8	4.8	2.0	0.6[2]		
Canis familiaris	Dog	12.9	7.9	2.1	10.1		
Felis catus	Cat	4.8	7.0	3.3			
Equus caballus	Horse	1.9	2.5	1.3	2.6		Wodas et al. (2020)
Tursiops truncatus	Dolphin	33.0	6.8	2.9	16.2		
Callorhinus ursinus	Fur seal	53.2	9.6	4.9	25[4]		Ashworth et al. (1966); Sharp et al. (2020)
Macropus rufus	Red kangaroo	3.4	4.6	2.3			
Macropus eugenii	Tammar wallaby		3.6	2.6	6.7[5]		Green and Renfree (1982), Lefèvre et al. (2007)
Didelphis marsupialis	Opossum	7.0	4.8	2.0			
Tachyglossus aculeatus	Echidna	9.6	12.5	5.2	15[6]		
Papio cynocephalus	Yellow baboon	5.0	1.6	0.5		Yes	Buss (1978)
Macaca mulatta	Rhesus monkey	4.0	1.6	0.5	2.8	Yes	Kunz and Lönnerdal (1994)
Homo sapiens	Human	3.8	1.0	0.6	∼0[7]	Yes	
Mus musculus	Mouse	13.1	9.0	2.0	0.0		
Rattus norvegicus	Rat	10.3	8.4	2.0	0.0	?	
Oryctolagus cuniculus	Rabbit	12.9	12.3	3.7	0.0		Maertens et al. (2006)

[1] *These data are mostly taken from Jenness and Sloan (1970) in which protein, in most cases, is listed as casein and whey protein. Here "protein" is given as the sum of the two. Figures are indicative as amounts vary within species and throughout lactation. βLg values are mostly from Sawyer (2003) and references therein.*
[2] *Protein is expressed as a percentage with no distinction made between g/100 g or g/100 mL, the specific gravity of milk being close to 1 g/mL.*
[3] *PAEP refers to whether the presence of glycodelin has been verified. A question mark means that it has been reported. A blank means there is no information.*
[4] *The βLg concentration is calculated based upon the percentage of nitrogen, relative to the total nitrogen in Ashworth et al. (1966).*
[5] *Estimated from the total protein and the relative abundance of expressed sequence tags.*
[6] *Estimated from the relative abundance of the expressed sequence tags and the crude skim milk protein concentration.*
[7] *An unexpressed coding sequence for human βLg has been identified (PAEPP1) but reports of its presence in milk are based upon immunological cross reactivity and it is known that lactoferrin can cross-react and ingested cow's milk peptides have been found in human milk.*

which rather mirror the state of protein chemistry at the time. Sawyer (2003, 2013) and Edwards and Jameson (2020) are three of the more recent.

Early work on the nature of βLg showed that it contained a good distribution of essential, or indispensable, amino acids (see for example, Forsum and Hambraeus, 1974; Smithers, 2008) in consequence of which it has a clear nutritional role. This is hardly surprising as colostrum and milk are the sole food source in the first few days for the newborn (Levieux and Ollier, 1999; Levieux et al., 2002; Hambræus and Lönnerdal, 2003) and it seems reasonable to assume that the composition is optimized for each species (e.g., Beck et al., 2015). This nutritional role includes its being a ready source of bioactive peptides (Pihlanto-Leppala, 2000; Korhonen and Pihlanto, 2006; Hernández-Ledesma et al., 2008; Nielsen et al., 2017) that appear to be important in neonate development (Park and Nam, 2015; Dave et al., 2016). However, most of the recent nutritional studies discuss milk proteins in the context of human nutrition (e.g., Sánchez and Vázquez, 2017) and studies on milk derived bioactive peptides with reference to human well-being (e.g., Marcone et al., 2017). Bioactive peptides with antibacterial (Pellegrini et al., 2001; Pellegrini, 2003; Chaneton et al., 2011; Sedaghati et al., 2015), opioid (Chiba and Yoshikawa, 1986; Pihlanto-Leppala, 2000; Teschemacher, 2003) and antihypertensive (Dave et al., 2016) activities may well have such effects in the neonate animal for which they have evolved but do not appear to have been reported specifically, despite the growing interest in their use in animal nutrition (Hou et al., 2017). Similarly, while studies of satiety are of considerable interest in maintaining well-being (Kondrashina et al., 2020), studying such a topic in neonate animals is less straightforward although there are studies on peptide production from whey and βLg in pigs (Barbé et al., 2014), rats (Hernández-Ledesma et al., 2007) and, of course, humans (Boutrou et al., 2015).

On the above theme, are there correlations between βLg and production traits that are beneficial for the calf? As livestock are valuable, farmers presumably select for traits that enhance their profit (more milk; better meat; and healthier offspring). A healthy immune system and gut microbiome are obvious consequences of satisfactory colostrum and milk ingestion but so too are normal behavioral characteristics as has been examined in for example, cows (Krohn et al., 1999) or piglets (Prunier et al., 2020). The recent reports of the generation of animals in which the βLg gene has been switched off or knocked out (Lamas-Toranzo et al., 2017; Sun et al., 2018; Yuan et al., 2020), should provide clues to any possible function in the neonate other than nutrition.

Transfer of immunity from mother to offspring is species dependent (Larson, 1992; Langer, 2009; Pentsuk and van der Laan, 2009; Hurley and Theil, 2011; Butler et al., 2015). In some species, like human and rabbit, mothers pass significant amounts of immunoglobulin, mostly IgG, *in utero* before birth

and the colostral antibody is mostly IgA. It has been shown that vaccinating pregnant women against tetanus, influenza and whooping cough (Lindsey et al., 2013) is generally beneficial for the infant but such immunization may interfere with the infant's own immune response (Bergin et al., 2018; Orije et al., 2020). Other animals like horse, pig and cow transfer mostly IgG in the colostrum after birth and there is a final group that includes dog, cat and rodents that transfer immunoglobulins both *in utero* and via the colostrum (Larson, 1992). The neonate intestine in those species where transfer occurs after birth, is permeable to immunoglobulins for periods of a day to a few weeks (Staley and Bush, 1985; Sangild et al., 1999) to facilitate this uptake. βLg is resistant to low pH and to pepsin so that it is able to pass through the stomach more or less intact (Miranda and Pelissier, 1983; Guo et al., 1995; Almaas et al., 2006; Rahaman et al., 2017). In the intestinal tract, the pH rises and the protein becomes less stable and susceptible to enzymic hydrolysis (Guo et al., 1995). That βLg might in some way be related to antibody transfer was suggested by Jenness (1979) though without much conviction. There is little on the topic until Fleming et al. (2016) found a positive correlation between levels of IgG and βLg in herds of cows being classified as low, medium or high immune responders but more convincingly, Crowther et al. (2020) have shown that βLg associates fairly specifically with the immunoglobulin fraction of both cow and goat milk, their thesis being that such an association would protect the immunoglobulins during their passage through the stomach. However, this novel finding needs to be investigated further by identifying the exact nature of the interaction in ruminant and in other species. Such an interaction might be expected to be with the constant rather than the variable regions of the immunoglobulins. In this regard, the only structure of a βLg complex with the Fab fragment of a monoclonal IgE molecule raised against the milk protein is not relevant (Niemi et al., 2007).

Conversely, there is a large body of evidence that milk allergy, especially in infants, arises from the presence of βLg (Tsabouri et al., 2014; Linhart et al., 2019). Indeed, βLg is also known as Bos d5 allergen (Breiteneder and Chapman, 2014; Pomés et al., 2018), one of 12 cow allergens of which, Bos d2, is another lipocalin (Rouvinen et al., 1999). A recent report on βLg's ability to promote proliferation of mouse hybridoma cells thereby enhancing an immune response (Tai et al., 2016), has not been shown in bovine cells but might be indicative of such a function in the immature calf intestine. Repeating such a study in a bovine mammary epithelial cell line (e.g., Huynh et al., 1991; German and Barash, 2002; Janjanam et al., 2013) would support this suggestion but the production of animals whose milk is without βLg (Lamas-Toranzo et al., 2017; Sun et al., 2018; Yuan et al., 2020) might better reflect the basis of their immunological well-being. Studies on the various epitopes identified on bovine βLg involve the use of antibodies raised in other species and consequently do not necessarily identify sites that are important in neonatal physiology (Williams et al., 1998; Clement et al., 2002; Cong and Li, 2012).

Although Davis and Dubos (1947) noted that βLg bound about half as much oleic acid as serum albumin, it was Groves et al. (1951) who showed that 2 mol/mol of sodium dodecyl sulfate not only bound but had a stabilizing effect on thermal denaturation. Since then, a large number of ligands for βLg has been identified and that number is still increasing (Sawyer, 2003; Tromelin and Guichard, 2006; Cherrier et al., 2013; Le Maux et al., 2014; Loch et al., 2015, 2018). To date, the only definitive ligand binding site is within the central calyx despite there being several experimental studies indicating that alternative sites may exist (Frapin et al., 1993; Dufour et al., 1994; Lange et al., 1998; Narayan and Berliner, 1998; Lübke et al., 2002; Yang et al., 2008, 2009; Edwards and Jameson, 2020). The crystal structures of many have been described (Qin et al., 1998; Wu et al., 1999; Kontopidis et al., 2002, 2004; Yang et al., 2008; Loch et al., 2012, 2013a,b, 2014; Rovoli et al., 2018) and some important NMR work has added to the description of the ligand binding site (Collini et al., 2003; Ragona et al., 2003, 2006; Konuma et al., 2007), in particular its pH dependence (Ragona et al., 2003). What is clear, however, is that the majority of molecules that bind are hydrophobic, or at least have significant hydrophobic moieties (Sawyer, 2013). This together with the similarity to other lipocalin transporters, most notably retinol-binding protein, has led to the speculation that βLg's function is as a transporter (e.g., Sawyer, 2013; Edwards and Jameson, 2020). Further weight is given to this idea by the identification of specific βLg uptake in part of the intestine of the neonate calf (Papiz et al., 1986), a process lost in more mature intestine. There is evidence, however, that not every species has a βLg that can bind a ligand (Pérez et al., 1993). What might be the natural ligand? Fatty acids seem unlikely as they are more efficiently carried in fat globules. Vitamins A and D have been shown to bind and the amounts required are more in keeping with the 125 μM βLg present in cow's milk but here too, hydrophobic vitamins are more likely found in the fat phase. Analysis of the ligands bound to βLg in milk showed only fatty acids (Pérez et al., 1989).

If transport is a function, then delivery implies some form of release mechanism as with RBP, or a receptor. Retinol is delivered by RBP/transthyretin to a surface receptor which internalizes the ligand only (Kawaguchi et al., 2007; Redondo et al., 2008). Papiz et al. (1986) reported the presence of specific βLg receptors in the neonate calf intestine, prompting speculation of the possible specific uptake of sparingly soluble ligands while Said et al. (1989) reported the βLg-enhanced uptake of retinol by suckling rats. Alternatively, the carrier plus cargo may be endocytosed. Reports of possible βLg receptors in rabbit ileum cells (Marcon-Genty et al., 1989), bovine germ cells (Mansouri et al., 1997), a CaCo-2 cell monolayer (Puyol et al., 1995) and a mouse hybridoma cell line (Palupi et al., 2000) have been followed by a description of the specific cellular uptake of βLg by the lipocalin-interacting membrane receptor (LIMR, Fluckinger et al., 2008). Although the LIMR used was human, there is a bovine receptor whose sequence is 59% identical (NCBI Reference Sequence: NP_001069254.2; Zimin et al., 2009). A more recent study of the human receptor, however, finds LIMR to be specific for human lipocalin-1 and nothing else (Hesselink and Findlay, 2013). There are receptors in the bovine intestine for various bioactive peptides generated by hydrolysis, but a specific receptor for βLg does not appear to have been reported since Papiz et al. (1986).

The pH dependent behavior of βLg was noted as early as by Pedersen, 1936 but it was the work of Tanford and

coworkers which identified an anomalous carboxylate, known now to be Glu89, that was revealed by a conformational change at about pH 7, the "Tanford transition" (Tanford et al., 1959; Tanford and Taggart, 1961). Crystallographic (Qin et al., 1998; Vijayalakshmi et al., 2008; Labra-Núñez et al., 2021) and NMR (Uhrinova et al., 2000; Sakurai and Goto, 2006; Sakurai et al., 2009) structural work identified the conformational change as being the EF loop moving away from the entrance to the calyx thereby facilitating ligand binding (**Figure 1B**; Ragona et al., 2003; Konuma et al., 2007). The cow protein now has complete 3-dimensional structural data from pH 2 to 8 (Khan et al., 2018; Yeates and McPherson, 2019). Interestingly, the transition is shifted to significantly higher pH in porcine βLg (Ugolini et al., 2001) while the EF loop is also mobile in the protein structures available for sheep (Kontopidis et al., 2014; Loch et al., 2014), goat (Crowther et al., 2014; Loch et al., 2015), reindeer (Oksanen et al., 2006) and pig (Hoedemaeker et al., 2002). As Glu 89 is very well conserved among the βLg homologues, it may be that there is functional significance in this observed gating (Qin et al., 1998; Ragona et al., 2003; Konuma et al., 2007; Loch et al., 2019), mimicked by simulation (Bello and García-Hernández, 2014; Bello, 2020; Fenner et al., 2020; Labra-Núñez et al., 2021), once again being consistent with a transport function (e.g., Sawyer, 2013; Edwards and Jameson, 2020).

The ruminant βLgs are dimers at around neutral pH but become monomeric at low pH (Timasheff and Townend, 1961; Zimmerman et al., 1970; Joss and Ralston, 1996; Mercadante et al., 2012; Khan et al., 2018). The dimer interface involves the antiparallel arrangement of β-strand I as well as other interactions and crystal structures reported over a wide range of pH show that the interface is flexible (Vijayalakshmi et al., 2008; Crowther et al., 2016). Porcine βLg on the other hand is dimeric at low pH and monomeric around neutrality (Ugolini et al., 2001) with a completely different, domain-swapping dimerization (Hoedemaeker et al., 2002). The final species for which there is some structural information is equine βLg which is monomeric over a wide pH range (Kobayashi et al., 2000). A chimeric version, Gyuba βLg, with cow core and equine loops dimerises like the ruminant proteins (Ohtomo et al., 2011). When the horse I strand and AB loop were replaced by the cow amino acids, no dimer formed (Kobayashi et al., 2002). While the calyx opening is away from the dimer interface, structural and modeling studies of the ligand binding behavior show some dependency upon the quaternary structure (Bello et al., 2011, 2012; Domínguez-Ramírez et al., 2013; Gutiérrez-Magdaleno et al., 2013; Labra-Núñez et al., 2021). However, it is not clear whether the quaternary structure is important for any functional property of βLg, as it is for Gd.

Finally, is there evidence of the involvement of βLg in the mammary gland before or during lactation? Reinhardt and Lippolis (2006) showed that βLg was not present in the milk-fat globule membrane (MFGM) while Bianchi et al. (2009) showed its presence in milk-fat globules. A subsequent study of the MFGM proteins in engineered and cloned animals found no greater changes in expression levels of βLg between the engineered animals expressing human proteins than between the cloned control and normally bred animals (Sui et al., 2014). These studies, however, have little bearing on whether βLg is providing any specific function in the mammary gland. Both Ca^{2+} and Zn^{2+} bind to βLg and both ions are important mediators of metabolic function. Farrell and Thompson (1990) suggested such a role for calcium ions but the idea does not appear to have been revisited. The dissociation constant for Ca^{2+} is around 5 mM (Jeyarajah and Allen, 1994) which is tenfold higher than the concentration of βLg in milk. However, that for Zn^{2+} is about 5 μM (Tang and Skibsted, 2016) which makes an intracellular association with βLg possible. It is not clear that this is physiologically important either in mammary metabolism or as a means of ensuring the neonate has sufficient zinc (McCormick et al., 2014). Removal of βLg by genetic manipulation in cattle does not appear to cause any functional problem although there is a compensating increase in the amount of casein and α-lactalbumin (Jabed et al., 2012; Wei et al., 2018). However, in a similar study with goat, removal of βLg also led to a lowering of amounts of casein and lactalbumin (Zhou et al., 2017). Thus, while it is probably too soon to rule out any functional involvement of βLg in the mother, that cannot be said of its close relative, Gd.

GLYCODELIN

The first reports of Gd appeared in the 1970s although, since the name tended to reflect the tissue from which the isolation had been prepared, the protein was referred to variously as progesterone-dependent or progesterone-associated endometrial (glyco)protein (PEP or PAEP, Joshi et al., 1980a,b), placental protein 14 (PP14, Bohn et al., 1982), placental α_2-globulin (Petrunin et al., 1976), pregnancy-associated α_2-globulin (α_2-PEG, Bell et al., 1985a,b), chorionic or placental-specific α_2-microglobulin (Petrunin et al., 1978; Tatarinov et al., 1980), or $\alpha_{2-uterine}$ protein (Sutcliffe et al., 1980). Bell and Bohn (1986) presented a discussion of this variability in nomenclature but the name glycodelin was not coined until Dell et al. (1995) to reflect the importance of glycosylation in the activity of the protein and to avoid using names apparently restricting its expression to specific tissues, and that is the name by which it will be referred to here. *PAEP* is used to refer to the gene (Kämäräinen et al., 1991; Van Cong et al., 1991; Uchida et al., 2013). However, as already noted *PAEP* is now also used to describe the βLg gene, rather than *LGB* or *BLG* (e.g., Elsik et al., 2016; Hunt et al., 2018; HGNC, 2020).

Glycodelin is implicated in the immunosuppression, angiogenesis and apoptosis activities associated with the first trimester of human pregnancy (Lee et al., 2016) as well as the fertilization and implantation processes (Seppälä et al., 2005, 2007; Lee et al., 2009). Its synthesis is therefore tightly controlled by progesterone, and possibly other factors like human chorionic gonadotrophin (hCG), relaxin and histone acetylation (Seppälä et al., 2009; Uchida et al., 2013). There are four distinct characterized isoforms of Gd all based upon the same polypeptide chain but differing in their glycosylation: Gd-A is found in amniotic fluid, in the secretory and decidualized endometrium (Seppälä et al., 2002; Koistinen et al., 2003)

and in the serum of pregnant women (Bersinger et al., 2009), Gd-C is associated with the cumulus matrix (Chiu et al., 2007a), Gd-F occurs in follicular fluid and oviduct and Gd-S is expressed in seminal vesicles and found at high levels in seminal plasma (Yeung et al., 2006; Chiu et al., 2007b; Uchida et al., 2013). The differences in activity are dictated by the different oligosaccharides attached to Asn28, located in a loop at the end of β-strand A, and Asn63, located in the loop joining β-strands C and D (Schiefner et al., 2015). There are glycosylation differences not only between the tissues in which the Gd is found but also in the same tissue from different individuals (Koistinen et al., 1996, 2003). **Figure 3** shows both the structure of the protein dimer with modeled sugars, and the distinct glycosylation patterns of Gd-A and Gd-S reflecting a distinction between female and male post-translational processing (Dell et al., 1995; Morris et al., 1996; Lapid and Sharon, 2006; Clark and Schust, 2013). Although there is a putative glycosylation site at Asn85, this is not modified, possibly because it is situated near the C-terminal end of β-strand E rather than in a loop at the end of the strand (Aubert et al., 1981; Moremen et al., 2012). Some of the immunomodulatory activity of Gd, however, appears to be associated with the protein moiety (Jayachandran et al., 2004; Ponnalagu and Karande, 2013; Dixit and Karande, 2020; Hansen et al., 2020) and it has been shown that Gd-A has lectin-like behavior in its interaction with T-cells (SundarRaj et al., 2009). However, much Gd binding involves its glycosylation (e.g., Lee et al., 2019; Dixit and Karande, 2020; Vijayan et al., 2020). Two of the Asn residues in Gd, those at 28 and 85, are Asp in βLg while it is the Ser65Glu change that disrupts the third N-linked glycosylation site (e.g., Sawyer, 2013). When these positions in βLg are engineered to those of Gd and the protein expressed in *Pichia pastoris*, glycosylation is observed but only at positions 28 and 63, that at 85 remaining unmodified as in Gd (Kalidas et al., 2001). It is not perhaps surprising to find that there is significant overlap in the epitope sequences in Gd and βLg as these tend to be on exposed sections of the polypeptide: angiogenic activity in Gd between 68 and 83 which includes the loop between strands D and E, and immunosuppressive activity between 57 and 65 at the other end of strand D (Ponnalagu and Karande, 2013). In βLg, these same regions have been identified as epitopes for human IgE and IgG (Cong and Li, 2012) which may indicate possible interaction sites in the calf. Results from Tai et al. (2016) show cellular proliferation via IgM but no epitope is identified, though lysine modification abolishes the effect.

The development of the human placenta in early pregnancy depends, *inter alia*, upon Gd-A, secreted by the endometrium in response to progesterone, and perhaps also hCG and relaxin, and which interacts with various cell types, especially the trophoblast and immune cells, modifying their behavior to allow implantation and ensure maternal tolerance of the growing embryo (Seppälä et al., 2002, 2007; Lee et al., 2011, 2016). It has also been found (Uchida et al., 2005, 2013) that Gd expression is regulated by histone acetylation/deacetylation such that overall control of expression appears to be both hormonal and epigenetic. Gd-A can also bind to the sperm surface through fucosyltransferase (Chiu et al., 2007b) inhibiting the sperm's ability to penetrate the zona pellucida (Oehninger et al., 1995). Low levels of Gd-A are observed in both uterine flushings and the serum, during the fertilization window (e.g., Bersinger et al., 2009; Seppälä et al., 2009). Levels rise after the hormone burst at ovulation such that successful implantation only occurs in the presence of Gd-A (Kao et al., 2002), borne out by women with repeated implantation failure having low serum and endometrial levels (Bastu et al., 2015). The changes to the decidual leukocyte populations caused by Gd-A are also important for successful pregnancy (Erlebacher, 2013). T-helper type 1 cells are depleted along with B-lymphocytes while T-helper type 2 cells increase (Saito et al., 2010). Natural killer cells account for the majority of decidual leucocytes by the end of the first trimester but have a low cytotoxicity compared to those in blood (Erlebacher, 2013). During the preimplantation stage, monocytes migrate to the endometrium where they differentiate into decidual macrophages whence the interaction with Gd-A appears to maintain the survival of the fetus and placenta (Stout and Suttles, 2004; Lee et al., 2016; Vijayan et al., 2020). A decrease in the levels of Gd-A leads to an increase in interferon-γ causing a variety of problems from pre-eclampsia to fetal loss (Lee et al., 2016).

Gd-F is the isoform secreted into the follicular fluid, after synthesis in the granulosa cells (Tse et al., 2002; Chiu et al., 2003, 2007b). Through a high affinity site and a low affinity one (which also binds Gd-A), it binds to spermatozoa inhibiting both their interaction with the zona pellucida and the progesterone-induced acrosome reaction (Chiu et al., 2003; Yeung et al., 2009).

The oocyte-cumulus complex is released from the ovulatory follicle and transported, along with follicular fluid, through the oviduct after ovulation. Spermatozoa must pass through the follicular fluid and then negotiate the expanded cumulus complex, a sticky mass of cumulus cells and hyaluronic acid surrounding the zona pellucida and called the cumulus-oophorus, before they can bind to the zona pellucida and initiate fertilization. Gd-A and Gd-F both of which can bind to spermatozoa and prevent binding to the zona pellucida as noted, are present in the oviduct. However, it is a third form, Gd-C, generated in the cumulus cells by modification of the glycosylation of Gd-A and Gd-F that effectively removes their inhibition, allowing penetration and subsequent fertilization (Chiu et al., 2007a; Lee et al., 2009).

Gd-S is the male form of Gd found in large quantities in seminal plasma. It binds to spermatozoa inhibiting the loss of cholesterol which in turn would lead to premature capacitation before entry into the uterine cavity. Once in the uterine cavity, Gd-S is released, there is an efflux of cholesterol and capacitation occurs. Gd-F binds to prevent the acrosome reaction, the necessary prelude to zona penetration. Gd-S does not inhibit interaction between zona pellucida and spermatozoa and is not therefore contraceptive (Koistinen et al., 1996; Morris et al., 1996). There are two binding sites for Gd-S on the human spermatozoon which are distinct from those of the other isoforms (Chiu et al., 2005; Yeung et al., 2006). As noted above, the glycosylation is also distinct from the female forms of Gd in that it has no sialyl glycans but rather is fucose-rich (Morris et al., 1996).

The isoforms of Gd are therefore intimately associated with the human/primate reproductive process but in non-primate reproduction which must have arisen before the emergence of

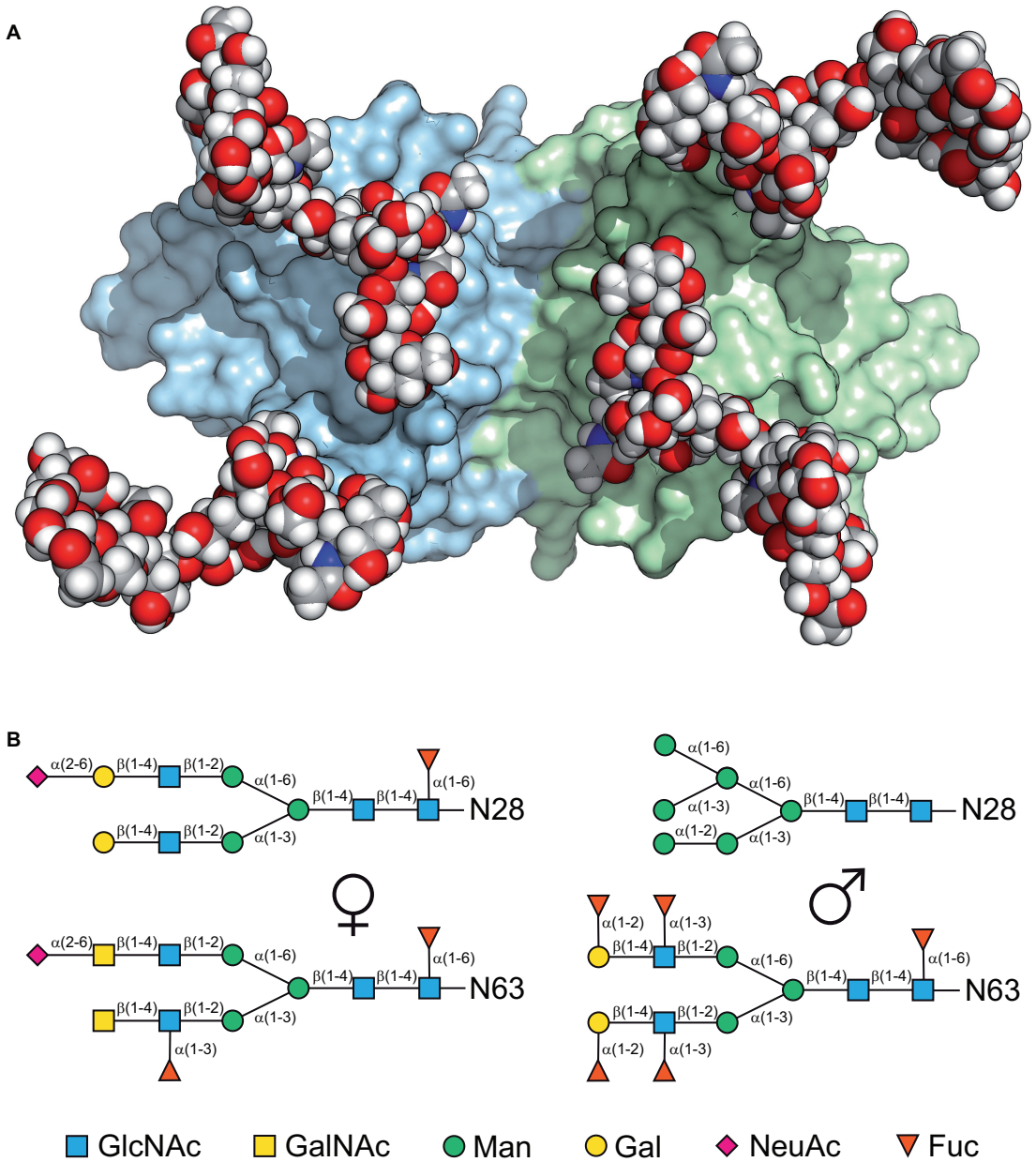

FIGURE 3 | (A) A model of the glycosylated physiological dimer of Gd-A viewed down its two-fold axis, showing the two subunits as surface representations in pale green and blue with the branched sugar moieties on Asn28 and Asn63 shown as space-filling with the conventional elemental coloring. **(B)** Schematic representation of the typical major glycan structures found in Gd-A, a female amniotic fluid form on the left with that of Gd-S, the male seminal fluid form on the right (Dell et al., 1995; Morris et al., 1996). **(A,B)** Reproduced from Schiefner et al. (2015) with permission.

Gd (Puga Molina et al., 2018) there exist lipocalin alternatives, though not necessarily carrying out similar functions. For example, progesterone-dependent uterine lipocalins are found in the endometria of pig (RBP, Stallings-Mann et al., 1993; SAL1, Spinelli et al., 2002; Seo et al., 2011), mare (p19 or uterocalin, Crossett et al., 1996, 1998; Suire et al., 2001) and cow (RBP, Mullen et al., 2011) though none is closely related to either Gd or βLg. However, both p19 and SAL1 are glycosylated. In all of these animals, and ungulates in general, there is a significant delay before implantation during which the uterine secretions supply a wide range of molecules for nutrition, protection and development (Artus et al., 2020). RBP has also been identified in cat conceptus (Thatcher et al., 1991) and dog endometrium (Buhi et al., 1995). Finally, mouse oviduct and endometrium secrete lipocalin-2 which leads to sperm capacitation (Watanabe et al., 2014). Would it be more than coincidence that another lipocalin, βLg, with binding properties similar to these uterine ones is involved at a subsequent developmental stage?

Glycodelin is also implicated in other biological processes (Seppälä et al., 2007, 2009) most notably cancer (Richter et al., 2007; Kölbl et al., 2014; Cui et al., 2017) where its presence can indicate a better outcome in some ovarian and breast cancers (Mandelin et al., 2003; Koistinen et al., 2009; Hautala et al., 2011), supported by functional studies (e.g., Kämäräinen et al., 1997; Hautala et al., 2008). However, in other cancers, high Gd expression indicates a poorer prognosis, also supported by functional studies (e.g., non-small cell lung cancer, Schneider et al., 2015, 2018; malignant pleural mesothelioma, Schneider et al., 2016; melanoma, Ren et al., 2010). It has also been shown that the glycosylation pattern of Gd expressed in cancer cells is not the same as that in normal tissue expression (Hautala et al., 2020) though whether this has any functional relevance is unknown at present. The method used involved both antibodies and specific lectins, and may be of much wider application (Hautala et al., 2020). As the presence of Gd in tissues can be monitored by antibodies, both mono- and polyclonal and by mRNA there is scope for apparently conflicting observations. For example, it was found that the presence of Gd was indicative of a better prognosis in endometrial cancer whereas the presence of the immunosuppressive Gd-A indicated a poorer overall survival rate (Lenhard et al., 2013). In ovarian cancer too, the presence of Gd-A has a positive correlation with other markers that indicate a poorer outcome (Scholz et al., 2012; Ditsch et al., 2020). The presence of Gd can be detected by immunohistological staining of tissue biopsies and also in serum samples but is just one of many proposed neoplastic markers, the clinical significance of which, if any, as a cancer marker remains to be established.

The other clinical area in which Gd has been implicated arises from the fact that Gd levels are very low during the ovulatory phase of the human menstrual cycle, only to rise in tandem with progesterone levels. This has possible implications for contraception (Yeung et al., 2006) though it is not yet clear whether deliberate attempts to control Gd levels might provide an alternative approach to those methods currently in use. Studies to date have monitored Gd, Gd-A and progesterone levels throughout the cycle in the presence and absence of progestogen contraceptive treatment to identify the mechanism of action (e.g., Durand et al., 2010). Much of the research has been focused on the effects of the so-called "morning-after pill" emergency contraception (Durand et al., 2001, Durand et al., 2005, 2010; Vargas et al., 2012; Mozzanega et al., 2014) and opinion is split as to the mechanism of action of either of the two common drugs, levonorgestrel (LNG) or ulipristal acetate (UPA). Taken before ovulation, LNG raises the Gd concentration in serum and in uterine fluid before ovulation possibly inhibiting fertilization (Durand et al., 2010). Taken at or after ovulation, its effectiveness may involve sperm motility, capacitation or interaction with the zona pellucida though Gd-A alone was unable to mimic the effects (Chirinos et al., 2017). UPA on the other hand, has a decreasing effect as ovulation approaches but, since it blocks progesterone receptors, prevents implantation (Mozzanega et al., 2014). Mozzanega and Nardelli (2019) consider the mechanisms of LNG and UPA from the viewpoint of informed consent since there is significant ethical concern if the action is directed toward the conceptus (Kahlenborn et al., 2015; Peck et al., 2016).

CONCLUSION

Glycodelin and β-lactoglobulin are two lipocalins with closely related sequences and 3-dimensional structures arising from homologous genes and both are involved in the reproductive process. The proposed (patho)physiological functions of Gd are several and well defined, at least in human cell models, and appear to be specifically dependent upon post-translational, N-linked glycosylation. βLg on the other hand appears much more widely distributed and is clearly important in the nutrition and health of the offspring, providing as it does, not only a balanced supply of amino acids, but also a series of peptides that have anti-oxidant and anti-bacterial properties which must help establish a good gut microbiome in the offspring. Several other activities have been associated with βLg, transport and, recently, immunoglobulin stabilization being perhaps the most likely. Now that animals can be produced which lack the protein, it should be possible to assess the nature of any problems that are manifest by its lack. However, today, the coin is still spinning!

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Regulation of Sexually Dimorphic Expression of Major Urinary Proteins

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Male house mice excrete large amounts of protein in their urinary scent marks, mainly composed of Major Urinary Proteins (MUPs), and these lipocalins function as pheromones and pheromone carriers. Here, we review studies on sexually dimorphic MUP expression in house mice, including the proximate mechanisms controlling MUP gene expression and their adaptive functions. Males excrete 2 to 8 times more urinary protein than females, though there is enormous variation in gene expression across loci in both sexes. MUP expression is dynamically regulated depending upon a variety of factors. Males regulate MUP expression according to social status, whereas females do not, and males regulate expression depending upon health and condition. Male-biased MUP expression is regulated by pituitary secretion of growth hormone (GH), which binds receptors in the liver, activating the JAK2-STAT5 signaling pathway, chromatin accessibility, and MUP gene transcription. Pulsatile male GH secretion is feminized by several factors, including caloric restriction, microbiota depletion, and aging, which helps explain condition-dependent MUP expression. If MUP production has sex-specific fitness optima, then this should generate sexual antagonism over allelic expression (intra-locus sexual conflict) selectively favoring sexually dimorphic expression. MUPs influence the sexual attractiveness of male urinary odor and increased urinary protein excretion is correlated with the reproductive success of males but not females. This finding could explain the selective maintenance of sexually dimorphic MUP expression. Producing MUPs entails energetic costs, but increased excretion may reduce the net energetic costs and predation risks from male scent marking as well as prolong the release of chemical signals. MUPs may also provide physiological benefits, including regulating metabolic rate and toxin removal, which may have sex-specific effects on survival. A phylogenetic analysis on the origins of male-biased MUP gene expression in *Mus musculus* suggests that this sexual dimorphism evolved by increasing male MUP expression rather than reducing female expression.

Keywords: major urinary proteins, MUP, house mice, *Mus musculus*, pheromones, sexual selection, differential sex gene expression, sexual dimorphism

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INTRODUCTION

> "Sexual dimorphism is common throughout the animal kingdom. However, a molecular understanding of how sex-specific traits develop and evolve has been elusive." Williams and Carroll, 2009, p. 797.

Sexually dimorphic traits are common and expected to evolve when they confer sex-specific effects on survival or reproductive success (Darwinian fitness). Such sex-specific fitness optima are expected to generate intra-locus sexual conflict, a form of sexual antagonism over allelic expression ("conflict over shared genes"; Pennell and Morrow, 2013). Because males and females share most of their genomes, such intra-locus sexual conflict can only be resolved by the evolution of sex-limited or sex-specific gene expression, that is, the repression or gain in gene expression in one sex. Until sexual conflict is completely resolved, sexual dimorphic traits will remain suboptimal for either sex. Investigating hypotheses about sexual dimorphisms at both proximate and evolutionary levels of analysis is challenging. Although the genes and physiological mechanisms controlling the expression of sexual dimorphic traits have been determined in a few model organisms, their adaptive functions and evolutionary origins are still unknown. And although the adaptive functions and evolutionary origins of sexually dimorphic traits have been studied in many non-model species, the molecular mechanisms controlling their expression are rarely known. The simplest route to addressing this challenge is to determine the adaptive functions and evolutionary origins of sexually dimorphic traits in model organisms—and their wild counterparts—rather than trying to identify the genes and proximate mechanisms controlling sexually dimorphic traits in non-model organisms (Badyaev, 2002; Williams and Carroll, 2009).

Here, we provide an integrative review of studies on sexually dimorphic expression of Major Urinary Proteins (MUPs) in house mice (*Mus musculus*; **Figure 1**). MUP genes are mainly expressed in the liver, they are the most highly expressed genes in the liver, and from the serum, MUPs are excreted in urine. MUP expression is a sexually dimorphic trait, and male mice excrete 2–8 times more protein in their urine than females. The molecular mechanisms controlling this sexual dimorphism are complex and provide a fascinating example of how the brain uses endocrine signals secreted by the pituitary gland to control the expression of genes in the liver and other target organs (Holloway et al., 2008). MUPs are also expressed in several secretory tissues, however, aside from lachrymal glands and nasal secretions, their expression is not sexually dimorphic (Shaw et al., 1983) and their functions are still unclear (Stopková et al., 2021). In contrast, the chemical signaling functions of urinary MUPs have been studied for many years. In males, MUPs bind and transport volatile pheromones, and they stabilize their evaporation from urinary scent marks (Robertson et al., 1993; Hurst et al., 1998; Cavaggioni et al., 2006, 2008). Through this time-release mechanism, MUPs are expected to prolong the influence of volatile male pheromones on conspecifics. Some MUPs also act as pheromones themselves, activating sensory neurons in the vomeronasal organ (VNO) and eliciting aggressive behavior from males (Mucignat-Caretta et al., 2004; Chamero et al., 2007, 2011; Kaur et al., 2014) and maternal aggression (Martín-Sánchez et al., 2015). For example, MUP20 ("darcin") increases female attraction to male versus female urinary scent (Roberts et al., 2010, 2012). Females are attracted to male urine spiked with MUPs during estrus when MUP-detecting sensory neurons are expressed (Dey et al., 2015). MUP20 in male urine also influences female behavior by inducing spatial learning (Roberts et al., 2010, 2012) and stimulating neural growth in their brain (Hoffman et al., 2015; Demir et al., 2020). Male scent marks and their male chemical components thus provide an interesting example of a sexually dimorphic extended phenotype and a male chemical signal that influences the physiology, brain, and behavior of females.

In addition to reviewing sexually dimorphic MUP expression in house mice, we also examine questions regarding (1) the proximate mechanisms that control hepatic MUP gene expression (physiology), (2) the development of these mechanisms (ontogeny), (3) their selective maintenance (adaptive functions), and (4) evolutionary origins (phylogeny; "Tinbergen's Four Questions"). Addressing these questions requires considering processes that operate over vastly different time scales and levels of biological organization (molecules cells, individuals, populations, and species). Before examining sex differences in MUP expression, we first provide more background on house mice and their MUPs.

BACKGROUND: ON MICE AND THEIR MUPS

Mus musculus Versus *Mus laboratorius*

Studies on MUPs and other chemical signals have mainly been conducted with domesticated, laboratory mouse strains, but these results do not always generalize to wild mice (*nota bene:* "wild type" laboratory mice, outbred strains, and wild-derived strains are not wild mice). Laboratory mice evolved under artificial selection in captivity, they are highly inbred, and they carry a variety of deleterious genes that cause neural, visual, auditory, and epithelial defects (Wahlsten, 1982; Sibilia and Wagner, 1995; Chang et al., 2013). Their behavior, sensory systems, physiology, immune system, and many other traits dramatically differ from their wild counterparts (Smith et al., 1994; Abolins et al., 2017). Laboratory mice are genomic mixtures of three *M. musculus* subspecies, derived mainly from *M. musculus domesticus*, though there is still some debate over their relative contributions. For these reasons, some propose that laboratory mice should be classified as a different species (e.g., *M. laboratorius* or *M. gemisch*; Didion and de Villena, 2013). Regardless, making conclusions about the MUPs or other traits of wild *Mus musculus*, their proximate mechanisms, adaptive functions, and evolutionary origins, require studies on *M. musculus*, and preferably in natural or seminatural conditions.

Wild male house mice are highly territorial and dominant males mark their territories with urinary scent marks. Males produce more scent marks than females and dominant males mark more than subordinates (Desjardins et al., 1973).

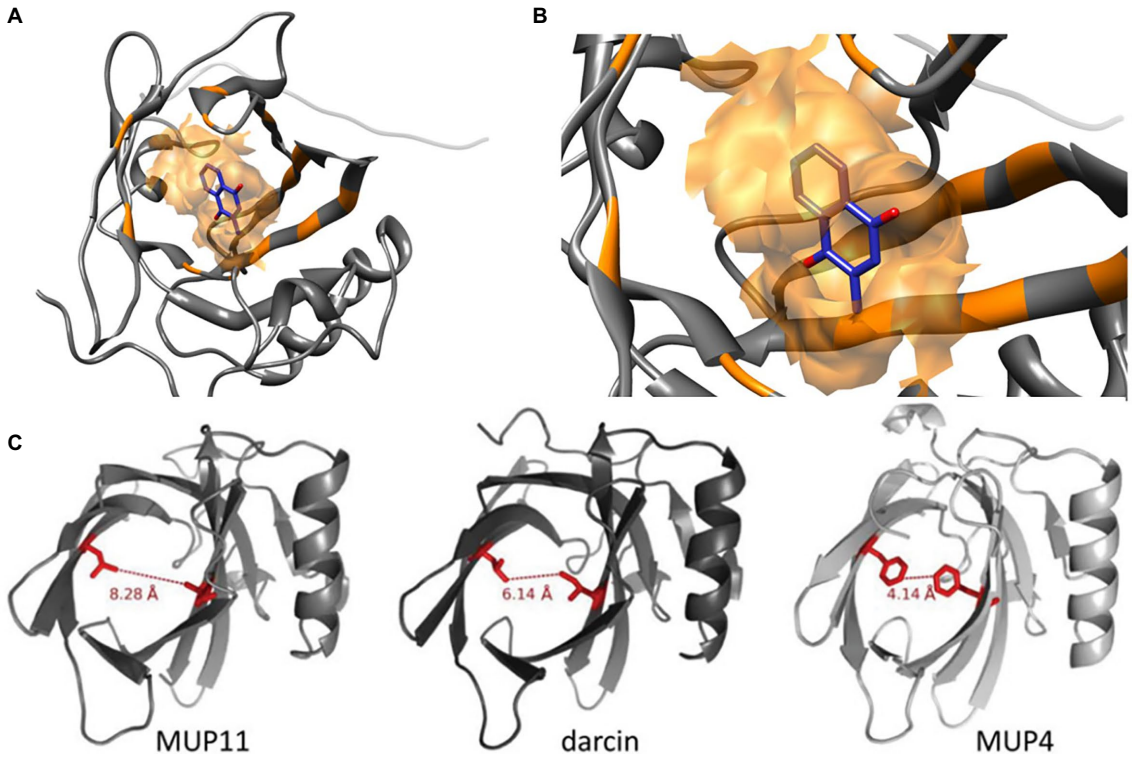

FIGURE 1 | Molecular Structures of MUP Proteins and Pheromone Ligands. MUPs are low molecular weight (18–40 kDa) proteins (162 amino acids) in the structural family of lipocalins, which have a barrel-shaped tertiary structure used for binding and transporting small molecules (Diez-Hermano et al., 2021). **(A)** The models shown here illustrate how each MUP contains a cavity that binds small hydrophobic ligands **(B)**, for example, menadione from Ricatti et al. (2019). These small molecules include male pheromones, such as 2-s-butyl-4,5-dihydrothiazole, 3,4-dehydro-exobrevicomin, and 6-hydroxy-6-methyl-3-heptanone (Böcskei et al., 1992; Žídek et al., 1999; Timm et al., 2001; Mucignat-Caretta and Caretta, 2014). **(C)** The binding cavities and interatomic distances between two similar residues are shown in red for a central MUP proteoform (MUP11), and the peripheral proteoforms of darcin and MUP4. MUP20 shows binding specificity for the pheromone 2-s-butyl-4,5-dihydrothiazole (SBT). Figure used with permission from Phelan et al. (2014).

Males increase scent marking in response to encountering females or female scent (Zala et al., 2004; Lehmann et al., 2013), and scent marking enhances male reproductive success when females can choose their mates (Thonhauser et al., 2013). The scent of male urine is attractive to females and exposure to male urine influences female behavior and physiology by accelerating puberty, synchronizing estrus, and inducing vaginal opening (Beynon and Hurst, 2003; Stopka et al., 2007, 2012; Stopková et al., 2009, 2014; Jouhanneau and Keller, 2013; Mucignat-Caretta and Caretta, 2014; Wyatt, 2014). The effects of male urinary odor on females are influenced by male MUPs or their volatile ligands (Jemioło et al., 1985; Harvey et al., 1989; Novotny et al., 1990; Jemioło et al., 1991). Thus, scent marks and sexual pheromones are secondary sexual traits, analogous to the colorful and conspicuous displays of peacocks (Penn and Potts, 1998; Zala et al., 2004; Thonhauser et al., 2013).

MUP Genes

House mice have *circa* 21 functional MUP genes and *ca*. 30 non-coding pseudogenes closely linked in a large cluster (Logan et al., 2008; Mudge et al., 2008; Charkoftaki et al., 2019; note that *Mup* is italicized whenever referring to a specific genetic locus or transcript, for example, *Mup1*; **Figure 2**). MUP genes are found in most placental mammals, though most species have only a single gene. Humans have one MUP gene, but it is dysfunctional and we are the only placental mammal lacking any active MUPs. MUPs likely evolved from another group of lipocalins, called odorant-binding proteins (OBPs; Charkoftaki et al., 2019; but see Igarashi et al., 1992). MUP genes are highly homologous and targeted methods, such as qPCR, do not necessarily amplify only one specific MUP locus (Holloway et al., 2006; Thoß et al., 2016). Only one study to our knowledge has measured genetic variation of MUPs within populations of wild house mice, and contrary to what is often suggested, MUPs have unusually *low* rather than high levels of individual variation (Thoß et al., 2016). MUPs show differences in expression across loci (Shi et al., 1989), but, contrary to what is often assumed, they do not show constitutive gene expression; as we show below, transcription is dynamically regulated and different MUPs are regulated in a different manner (Connerney et al., 2017).

Some MUPs are expressed in saliva, tears, vaginal and other glandular secretions (Shaw et al., 1983; Shahan et al., 1987; Stopka et al., 2016; Černá et al., 2017; Stopková et al., 2017, 2021), as mentioned above. For example, *Mup4* is expressed

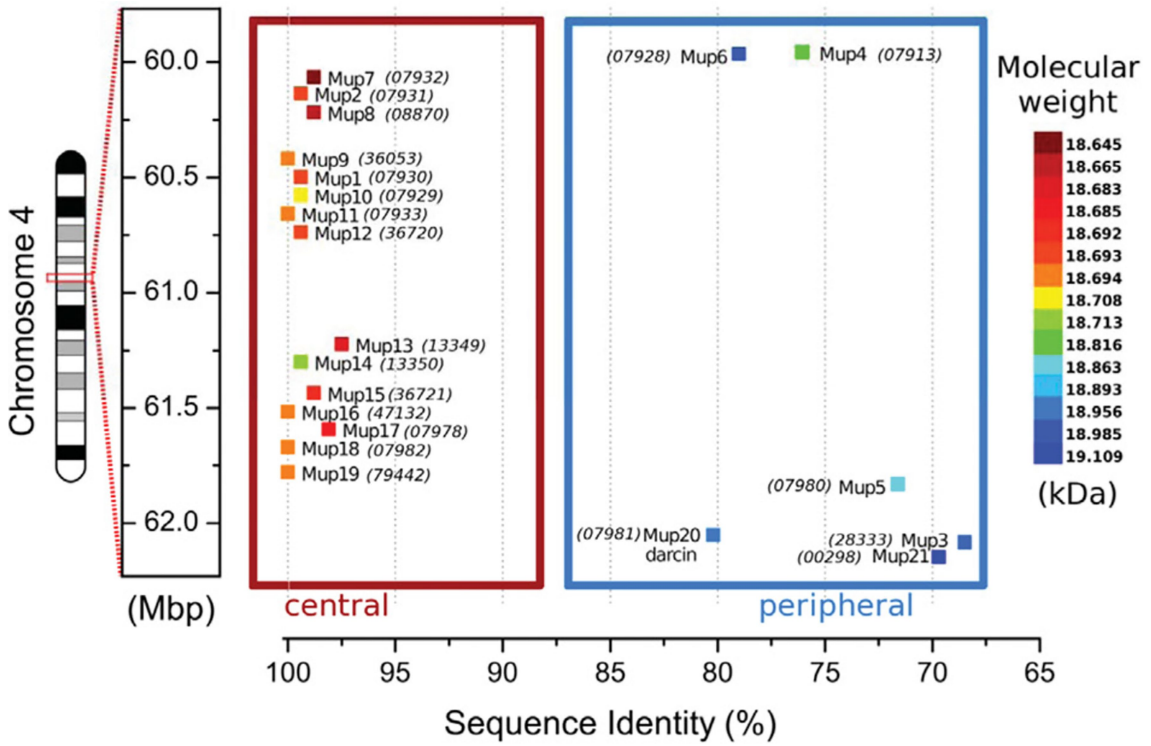

FIGURE 2 | MUP Gene Cluster of House Mice. MUP genes, indicated by colored squares, are closely linked in a large (2 Mb) region on chromosome 4 that contains up to 21 tandemly coding MUP genes and *ca*. 30 non-coding pseudogenes (Logan et al., 2008; Mudge et al., 2008; Phelan et al., 2014). MUP loci have been classified depending on their location inside of this cluster: the six Class A or "peripheral" MUPs (*Mup3, 4, 5, 6, 20,* and *21*, in the blue box) share *ca*. 88% similarity ("<82% mature protein sequence identity"), and these MUPs are likely more ancestral. The 15 Class B or "central MUPs" (in the red box) are nearly identical and show >97% mature protein sequence similarity and some differ by a single amino acid substitution. These highly homologous MUPs are likely recent duplications (Logan et al., 2008). Class A and B MUPs do not appear to differ in their gene expression levels in the liver (see **Figure 4** below). The coloration of the MUP gene corresponds to the molecular weight of the mature protein upon translation. The sequence identity percentage is relative to the most common mature amino acid sequence that is shared by genes *Mup9*, *11*, *16*, *18,* and *19*. Numbers in parentheses correspond to the MGI protein identification number. All numbers are prefixed with "OTTMUSP000000." Figure used with permission from Phelan et al. (2014).

in glands near the nasal cavity and in the nasal mucosa and the vomeronasal organ, where its proteins are suspected to transport ligands to olfactory receptors (Miyawaki et al., 1994; Cavaggioni and Tirindelli, 1999; Utsumi et al., 1999; Sharrow et al., 2002; Stopková et al., 2016). Thus, the original label "Major Urinary Proteins" turned out to be misleading because MUP proteins are not expressed only urine, which is also why the term "MUP protein" is used and not as redundant as it might seem.

MUP Proteins

MUPs are mainly synthesized in the liver (Finlayson et al., 1965), and they are among the most highly expressed genes in the liver: *ca*. 5% of the total hepatic mRNA in adult male mice consists of MUPs (Knopf et al., 1983). MUP are then released into the serum, filtered by the kidney, and excreted in the urine (Flower, 1996; Åkerstrom et al., 2000; Flower et al., 2000). It has long been known that male mice excrete high levels of protein in their urine (Parfentjev, 1932; Parfentjev and Perlzweig, 1933), and their urinary proteins are mainly composed of MUPs (Finlayson et al., 1963). It is often stated that around 95– 99% of urinary protein consist of MUPs (Humphries et al., 1999; Hurst and Beynon, 2004), but these may be overestimates. MUP-derived peptides accounted for 85% of the total urinary protein (from high-resolution mass spectrophotometry) of on wild-derived *M. musculus musculus* (Enk et al., 2016). Contrary to what was long assumed, gel-based methods do not separate different MUP proteoforms (Thoß et al., 2016), and quantifying the abundance of different proteoforms remains a challenge for proteomic methods (Enk et al., 2016).

MUPs undergo post-translational modifications in which a carbohydrate is attached (glycosylation), and there must be extensive modifications for these 162 amino acid proteins to expand to a mature protein 40 kDa in size. MUP15 has been shown to be glycosylated (Clark et al., 1985) and the resulting glycoprotein has a higher mass and exhibits a highly heterogeneous glycosylation pattern (Mechref et al., 2000). The relative ratio of protein masses predicted from mRNA generally matches the observed ratios of masses in protein data, suggesting that post-transcriptional modifications do not influence estimates of variation (Sheehan et al., 2016). Yet, MUP3 (referred to as "B6 gene18" Mudge et al., 2008), which is also glycosylated, does not show up on

standard analyses of urine protein content using mass spectrometry due to the change in its mass, even though it is detectable using other methods. Gel electrophoresis shows MUP expression in the urine of B6 males but not females and that lack of transcription analyses have probably misinterpreted expression patterns in wild populations (Sheehan et al., 2019). The effects of glycosylation on the functions of MUPs and their expression in different tissues are not understood and deserve more attention.

The cavity of each MUP20 protein has 14 amino acids associated with ligand binding, and a single amino acid substitution can alter ligand binding affinity and specificity (Ricatti et al., 2019). Yet, very few amino acid substitutions are found in the interior hydrophobic binding cavity of MUPs, as most occur on the protein surface (Darwish et al., 2001; Beynon et al., 2002; Sheehan et al., 2019). Surface substitutions do not likely influence ligand binding affinity, though they might alter the shape of the binding cavity (Darwish et al., 2001; Beynon et al., 2002), and variation in surface-exposed residues might influence detection by V2R receptors in the vomeronasal organ (Chamero et al., 2007, 2011; Phelan et al., 2014; Sheehan et al., 2019). Amino acid variations may also affect the stability of different MUP proteoforms, for example, MUP20 has been found to be more stable at higher concentrations of a denaturing agent (urea) compared to a central proteoform (MUP11; Phelan et al., 2014).

Levels of urinary protein output show differences between wild house mice versus laboratory mice, and between wild mice kept in standard cages versus seminatural conditions (Enk et al., 2016; Thoß et al., 2019; Luzynski et al., 2021). These results indicate that it is crucial to study MUP gene and protein expression in wild mice and preferably living in natural or naturalistic social conditions to understand their functions, as we show next in more detail.

Sexually Dimorphic MUP Expression

It has long been known that male laboratory mice excrete more protein in their urine (Wicks, 1941) and synthesize more MUP mRNA (Sampsell and Held, 1985) than females. MUP urinary excretion begins at puberty (Wicks, 1941; Thoß et al., 2015), and numerous studies have documented male-biased MUP expression in mice, though these estimates vary considerably. In laboratory mice, males express between 2 to 8 times more urinary protein (Stopka et al., 2007; Mudge et al., 2008; Cheetham et al., 2009; Novikov et al., 2009), and 5- to 10-fold more MUP mRNA in the liver than females (Szoka and Paigen, 1978; Hastie et al., 1979; Derman, 1981). The amount of protein excreted and the degree of sexual dimorphism varies among laboratory strains (Cheetham et al., 2009; **Figure 3A**). One strain, BALB/cJ, has unusually low levels of urinary protein excretion due to a mutation in a regulatory gene (Jiang et al., 2017; see more on gene regulation below).

Wild-derived house mice also show male-biased urinary protein excretion when housed in standard cages and in seminatural conditions (**Figure 3B**; Stopková et al., 2007; Thoß et al., 2019; Luzynski et al., 2021). Wild-derived *M. musculus musculus* show a 4:1 male-biased urinary protein excretion in the laboratory (Luzynski et al., 2021), which is equivalent to the grand mean sex bias found in laboratory strains, despite that wild-derived males excrete nearly three times more protein. *M. musculus musculus* males produce more urinary protein than *M. musculus domesticus* (Stopková et al., 2007; Hurst et al., 2017; see more below), though both European subspecies show a 3.5 to 4 fold male bias in urinary protein excretion in the laboratory. Thus, both sexes show higher mean protein output in seminatural social contexts and male-biased excretion is somewhat less pronounced than in laboratory conditions (3:1 versus 4:1 respectively; Thoß et al., 2019; Luzynski et al., 2021).

Some studies suggest that certain MUPs, such as *Mup7*, *11*, *20*, and *21*, show particularly high levels of expression in males and little if any in females (Norstedt and Palmiter, 1984; Hurst et al., 2017). However, because targeted methods, such as qPCR, do not necessarily discriminate different MUPs due to their high homology, it is usually unclear which MUP or

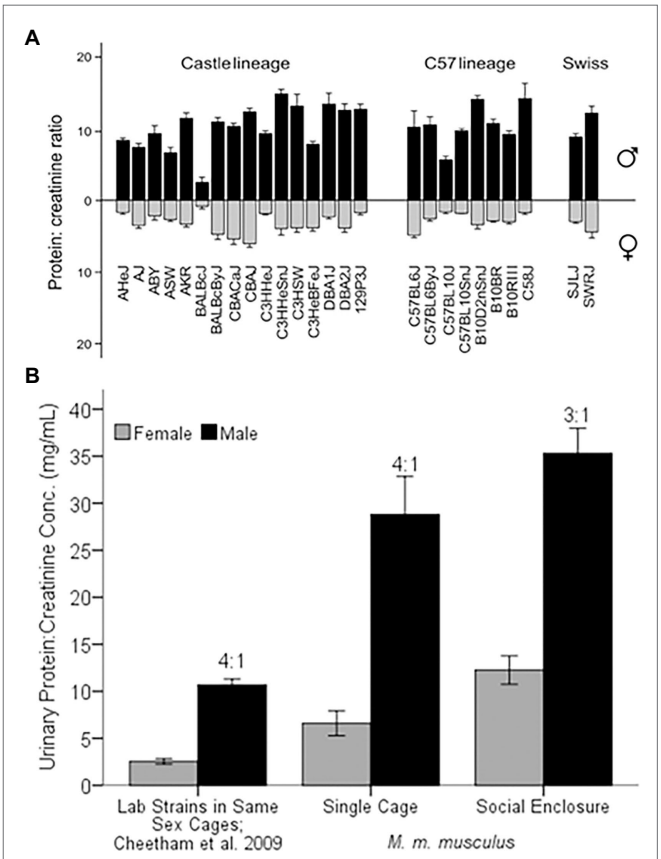

FIGURE 3 | Sex Differences in Urinary Protein Excretion. **(A)** Comparison of 26 strains of laboratory mice (protein:creatinine concentration in mg/ml) for males and females, represented as black and gray bars, respectively . Figure used with permission from Cheetham et al. (2009). **(B)** MUP excretion of laboratory versus wild-derived house mice. Laboratory strains were housed in same-sex cages of 5 individuals of the same strain (Cheetham et al., 2009). Wild-derived *M. musculus musculus* were either singly housed (Single Cage) or living in seminatural conditions (Social Enclosure) at 1:1 sex ratio (modified from Luzynski et al., 2021). The ratio above the black bars is the male:female ratio of protein excretion. Error bars indicate ±1 SEM.

MUPs are measured (e.g., see Holloway et al., 2006). Only one study to our knowledge has used RNA sequencing (RNA-seq), a more precise method for comparing MUPs (see Supplementary Table S2 in Connerney et al., 2017), and we plotted these results (**Figure 4**). Most MUP transcripts (17/19) showed significant male-biased expression (26-fold sex difference on average); however, there was enormous variation in the degree of sex-biased expression across loci (from 0- to 150-fold by our estimate). The most sexually dimorphic MUPs were *Mup7, 20, 11, 15*; only *Mup2* and *5* showed no significant sex differences. Males showed much variation in absolute expression levels across loci, as some MUPs had very high (*Mup7, 20, 17, 9, 3, 10*), whereas others had low expression (*Mup6, 15, 2, 5, 13*), comparable to females. Females had low expression for most MUPs, but also showed variation across loci and some (*Mup17, 9, 3, 10*) had higher levels than most MUPs in males. Only *Mup7* and *20* showed both large sex differences and high levels of male expression. We noticed that the same MUPs had either low (*Mup6, 15, 2, 5, 13*) or high expression (*Mup17, 9, 3* and *10*) in both sexes, and we found a correlation between male and female expression across loci ($r = 0.87$; $p = 2.6 \times 10^{-6}$; df = 18; not shown).

The results of this study show that most MUPs have sexually dimorphic expression, but that there are large differences in expression across MUP loci in both sexes and especially in males. The expression of different MUPs is correlated between the sexes, suggesting similarities as well as differences in their regulatory mechanisms. Since there is so much variation in expression across MUP loci, results from studies measuring the expression of a specific MUP are not likely to generalize to other loci and therefore should not be extrapolated (especially if the targeted method is specific). This RNA-seq study was conducted on one strain of domesticated mice in the laboratory, and therefore, more such studies are needed on wild mice living in more natural social contexts. The *main results* from this study reinforce the importance of this caveat: the expression of MUPs also showed dramatic changes following endocrine manipulation (Supplementary Table S2 in Connerney et al., 2017;

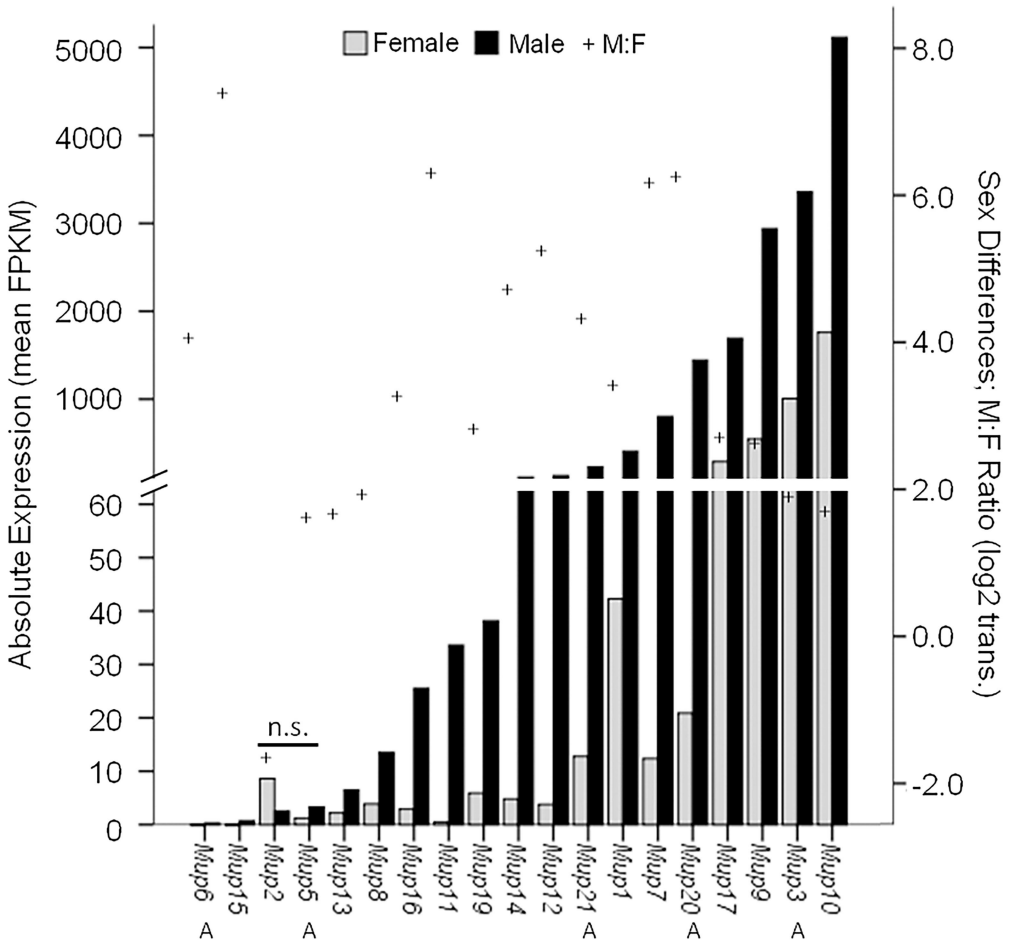

FIGURE 4 | Sex Differential MUP Gene Expression. MUP expression of male (black bars; $n = 18$) and female (gray bars; $n = 5$–9) was measured in normalized FPKM units (fragments per kb transcript per million mapped fragments) and MUP transcripts are shown in order of absolute expression levels of males; Swiss CD-1 mice, ICR strain. Our estimate of sex differential gene expression [log2(Male:Female); +] and Class A (peripheral) MUPs (A) are also indicated; others are Class B or central MUPs. Data from Connerney et al. (2017, Supplementary Table S2).

see more below). Before addressing hormonal mechanisms that control MUP gene regulation, including sex differences, we examine how MUP expression is dynamically regulated by several factors that can magnify or abolish sex differences.

FACTORS THAT INFLUENCE THE REGULATION OF MUP EXPRESSION

The amount and types of MUPs that mice excrete are regulated depending upon a variety of factors, including age, social interactions, social status, and health, and males and females show similarities and differences in how they regulate MUP expression (**Table 1**). For example, males upregulate MUP expression during puberty and after acquiring dominant social status, whereas only females regulate expression depending upon estrous cycle. It is premature to make general conclusions about sex differences in MUP regulation, however, because few studies have simultaneously compared males and females, and there are fewer studies on females than males. For example, male MUP expression is downregulated due to fasting (dietary restriction), infection, immune activation, microbiota depletion, and old age, and thus MUP output is a condition-dependent trait. Poor health or condition feminizes male MUP expression and can abolish sex differences. For most of these factors, however, it is still unclear whether females show similar condition dependence. Some factors, such as social status, infection, and immune activation, have been shown to result in perceptible changes in odor as well as MUP expression, though others have not yet been tested. The ecological relevence these findings are still unclear, as most studies come from the laboratory, and as mentioned above, mice alter how they regulate MUP expression in natural social contexts, as we examine next in more detail.

Social Status

After releasing wild-derived house mice kept in the laboratory conditions, males significantly increase their urinary protein excretion once they acquire a territory and become socially dominant in seminatural conditions (Thoß et al., 2019; Luzynski et al., 2021; **Figure 5**). Subordinate males, which do not acquire a territory, do not show a change in urinary protein excretion over time or compared to controls kept in the laboratory during the same time (nor do they differentially downregulate specific MUP proteoforms; Thoß et al., 2019). Dominant males also excrete higher levels of several MUPs, including MUP2, 5, 17, and 20, in their urine compared to subordinates

TABLE 1 | Factors affecting MUP expression.

Factor	Male regulation	Female regulation
Sexual maturity	↑ after puberty (Payne et al., 2001; Thoß et al., 2015)	Consistent expression from ages 20 to 100 days (Payne et al., 2001)
Housing (standard laboratory cages vs. social conditions)	↑ in seminatural conditions versus standard cages (Nelson et al., 2015; Enk et al., 2016; Lee et al., 2017; Thoß et al., 2019; Luzynski et al., 2021)	↑ in seminatural conditions versus standard cages (Stockley et al., 2013; Thoß et al., 2019; Luzynski et al., 2021)
	Solitary > group housed (6 per cage) at age 60 days (Mucignat-Caretta et al., 2014)	
Social status	↑ in territorial dominants but not subordinates (Nelson et al., 2015; Lee et al., 2017; Thoß et al., 2019; Luzynski et al., 2021)	No change (Thoß et al., 2019; Luzynski et al., 2021)
Intermittent or indirect agonistic and other social interactions	↑ in winners ("social dominants") in dyadic interactions (Guo et al., 2015; Lee et al., 2017)	↑ with intersexual indirect dyadic interactions (Stopka et al., 2007; Janotová and Stopka, 2011)
	↑ with territory defense (Garratt et al., 2012)	↑ with territory defense (Garratt, et al., 2011b)
	↑ with intersexual indirect contact and ↓ with intrasexual indirect contact (Janotová and Stopka, 2011)	↑ with aggressive behaviors (Stockley et al., 2013)
Estrous stage	N/A	↑ with estrus onset (Stopka et al., 2007)
Dietary restriction	↓ with dietary restriction (Hui et al., 2009; Giller et al., 2013; Mitchell et al., 2015)	↓ *Mup1* transcription with dietary restriction (Van Schothorst et al., 2006)
Cold stress	↑ with housing at 4°C ambient temperature (Liu et al., 2019)	No reports
Health (infection and immune activation)	↓ with infection and immune activation (Glibetic and Baumann, 1986; Isseroff et al., 1986; Gervois et al., 2004; Litvinova et al., 2005; Manivannan et al., 2010; Lopes and König, 2016; Deslyper et al., 2019; Ware et al., 2019; Oldstone et al., 2021; but see Lanuza et al., 2014)	↓ with infection (Isseroff et al., 1986; Deslyper et al., 2019)
	↓ transcription with immune activation (Glibetic and Baumann, 1986; Gervois et al., 2004; Lopes and König, 2016)	
Microbiota depletion	↓ transcription in germ-free mice (Weger et al., 2019)	↓ transcription in germ-free mice (Weger et al., 2019)
Toxin exposure	↑ with iron overloaded diet (Petrak et al., 2007)	No reports
Aging	↓ in old, senesced males (c. 26 mo) vs. middle-aged males (c.14 mo; (Garratt, et al., 2011a))	No reports

MUP gene expression in the liver and protein in the urine is regulated depending upon several factors, which can magnify or abolish sexual dimorphisms. Note that results based on targeted methods for measuring expression do not necessarily generalize to all loci.

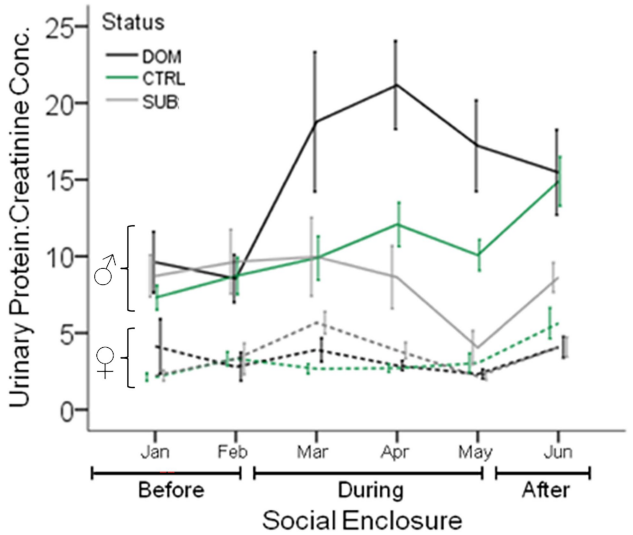

FIGURE 5 | Urinary Protein Excretion in Seminatural Contexts. The urinary protein excretion of wild-derived male and female house mice over time, before, during, and after their release into seminatural conditions. Lines show the mean protein excretion of males (solid lines) and females (dashed lines) caged singly in the laboratory (Before), and also while they were living in large enclosures for 12wk (During), and for 4 weeks after capture and being returned to single-caged housing (After). Dominant (DOM) versus subordinate (SUB) status is indicated by black and gray lines, respectively. Control mice (CTRL) are indicated by the green line. Error bars display ±1 SEM. Data from Thoß et al. (2019).

(Thoß et al., 2019). Interestingly, males upregulate the expression of some MUPs but downregulate others after being released into naturalistic social conditions (Enk et al., 2016). Protein excretion in the laboratory does not predict male social status in seminatural enclosures, and the differences in MUP excretion between dominant versus subordinate males in the enclosures are diminished after returning males to their cages. In contrast, females do not adjust their protein excretion depending upon their social status. Sociality has been observed to correlate with increased protein excretion in females (Garratt et al., 2011b; Stockley et al., 2013), and yet wild mice show lower sexual dimorphism in social contexts compared to the same mice in the laboratory (singly housed in cages; **Figure 3B**; Thoß et al., 2019; Luzynski et al., 2021). These findings indicate that, in addition to urinary protein excretion being male-biased, there are also sex differences in how mice regulate MUP excretion according to social status. MUPs are not only sexually dimorphic; in more natural conditions they also show a male dimorphism, like the secondary sexual traits of some other species (Számadó and Penn, 2018).

Agonistic Interactions

Studies of *M. musculus* and *M. laboratorius* indicate that MUP excretion depends on male aggression and competitive ability (Guo et al., 2015; Nelson et al., 2015; Lee et al., 2017; Thoß et al., 2019; Luzynski et al., 2021). Males that experienced repeated social defeat do not downregulate MUP excretion, but remained consistent with pre-interaction levels; any increase was to a lesser degree compared to dominant individuals (Nelson et al., 2015; Lee et al., 2017; Thoß et al., 2019). Two studies found that MUP excretion predicted subsequent aggression (Janotová and Stopka, 2011) or territorial dominance (Nelson et al., 2015), whereas other studies did not observe this pattern (Guo et al., 2015; Lee et al., 2017; Thoß et al., 2019; Luzynski et al., 2021). Male competitive ability is associated with the regulation of specific MUP isoforms: winners and socially dominant males upregulated MUP20 (Guo et al., 2015; Nelson et al., 2015; Lee et al., 2017; Thoß et al., 2019), as well as MUP2, MUP5, and MUP17 (Thoß et al., 2019). A downregulation of hepatic *Mup20* gene expression and MUP20 in urine has been observed in subordinate C57BL/6 males (Guo et al., 2015). Social hierarchies form within a few days (Guo et al., 2015; Lee et al., 2017) and are relatively stable in seminatural conditions (Thoß et al., 2019; Luzynski et al., 2021). However, MUP excretion changes in the laboratory depending upon social conditions or density, as evidenced by a decrease in MUP excretion by males upon moving from paired-housing to group-housing (Lee et al., 2017), and upon removal from social conditions to single-housing (Thoß et al., 2019). These studies are consistent with the hypothesis that MUP output is regulated depending upon social status. These findings suggest that the volatile ligands transported by urinary MUPs are important for signaling social status and that the persistence of male pheromones in scent marks should be prolonged by the high protein excretion of dominant males.

Thus, MUP excretion is more complex and dynamic in social contexts than in the laboratory, which raises caveats for studies conducted in the laboratory and also for surveys of wild house mice. After being trapped and housed in cages, dominant males reduced hepatic MUP20 protein expression by day 14 and urinary protein excretion by day 28 (Garratt et al., 2011b; Luzynski et al., 2021). Variation in the length of time that mice are kept in captivity can therefore affect MUP expression, and especially when animals are caged for more than 28 days (Thoß et al., 2019). To our knowledge, no studies on wild-caught mice have controlled for male social status or the amount of time in captivity. In the next section, we examine the complex mechanisms controlling the regulation of MUP expression in house mice.

MOLECULAR MECHANISMS CONTROLLING ADULT MUP GENE TRANSCRIPTION

The proximate mechanisms controlling sexually dimorphic MUP excretion are being worked out in remarkable detail and they are surprisingly complex, even in laboratory mice. These mechanisms are intensively studied because MUPs are used as a model protein for investigating sex differences in hepatic gene expression in physiology and biomedical sciences. These studies show how the pituitary gland regulates MUP gene

expression by releasing endocrine signals that trigger a complex cascade of biochemical changes in the liver.

Endocrine Mechanisms

Studies on the MUPs of laboratory rats (originally labeled *α2$_u$-globulins*) showed that sex differences in MUP expression are controlled by growth hormone (GH) and thyroxine [T_4; see references cited in (Knopf et al., 1983)]. Subsequent studies on laboratory mice showed that hepatic MUP synthesis is similarly under multihormonal control, involving testosterone (T), GH, and T_4 (Knopf et al., 1983). Surgical removal of the pituitary gland (hypophysectomy) makes it possible to examine the effects of pituitary hormones. Hypophysectomized female mice and GH-deficient *little* mice and have reduced MUP RNA expression in the liver, and administering either GH or T_4 increased MUP production in these mice. T had relatively little effect on the MUP RNA levels in hypophysectomized females, although it increased MUP production in normal females. GH and T_4 had the most pronounced effects on liver MUP RNA of hypophysectomized mice when administered together (even above normal physiological ranges). T, T_4, and GH appear to differentially regulate the expression of distinct MUPs (Knopf et al., 1983; Kuhn et al., 1984). A study using RNA-seq, mentioned above, found that hypophysectomy reduced gene expression of MUPs in both sexes (6-fold drop on average), and found variation in regulation across MUP transcripts (Connerney et al., 2017).

Many studies have shown that it is not the amount of circulating GH, but rather *the pattern of its pituitary release* that explains baseline sex differences in MUP expression. Here, we focus on GH- and T-mediated MUP expression, the best understood mechanisms, though additional regulatory mechanisms have been found that deserve more attention. For example, prolactin (also secreted by the pituitary gland) triggers milk production upon pregnancy, inhibits the expression of male-predominantly expressed hepatic genes, including *Mup1*, and upregulates mRNA expression of female-predominant genes (Sato et al., 2017). This finding could explain the increased MUP output of females in seminatural conditions (Stockley et al., 2013; Thoß et al., 2019; Luzynski et al., 2021). Prolactin did not reduce the levels of serum *levels* of GH and T in males (Sato et al., 2017), but its effects were not necessarily independent of hormones, contrary to what was suggested, because changes in the pulsatile secretion of these hormones were not investigated.

Growth Hormone

The effects of GH on growth and metabolism are well known, though interestingly, it is the *pulsatile* GH secretion in the pituitary that is necessary for normal postnatal growth, and especially accelerated growth during the peri-pubertal period. This peptide hormone has many pleiotropic effects as it affects reproduction, as well as growth, even though it is not usually considered to be a sex hormone. GH plays a key role in regulating MUP gene transcription and sex differences in the expression of MUPs and many other genes in the liver.

Studies on rats and mice have shown that the pattern of pituitary GH secretion is the key regulator of sex differences in the expression of MUPs and other genes in the liver (Mode et al., 1982; Norstedt and Palmiter, 1984; McIntosh and Bishop, 1989; Waxman and O'Connor, 2006; Zhang et al., 2012). Males have a highly pulsatile release of GH, whereas GH secretion in females is nearly continuous (Tannenbaum and Martin, 1976; MacLeod et al., 1991; Painson et al., 1992). In male mice, GH ultradian rhythms (rhythms that occur within a 24 h interval) exhibit regular periodicity with peak secretion periods occurring soon after the start of the light phase (2.5 h after lights on; Steyn et al., 2011). Males release *ca.* 5 secretory GH bursts per hour, and these multicomponent peaks last *ca.* 2 h and have an amplitude of *ca.* 200 ng/ml. The liver is the most sensitive target tissue for GH, and pulsatile release of GH release generates a male pattern in MUP gene expression and hundreds of other genes in the liver, which control metabolism of steroids, lipids, and toxins (Mode et al., 1982; Norstedt and Palmiter, 1984; Macleod and Shapiro, 1989; MacLeod et al., 1991; du Sert et al., 2020). Studies in rats found that it is the long inter-pulse interval with low plasma GH levels, rather than changes in pulse amplitude, duration, or frequency, that generates male versus female hepatic gene expression profiles (Pampori et al., 1991; Le Tissier et al., 2018). Pulsatile GH secretion is difficult to study in mice, though an alternative method has been developed for mice, which confirmed that plasma GH concentration patterns in mice are similar to other mammals (Xu et al., 2011). Additionally, experiments using continuous GH (cGH) infusion, which generate a female-like GH pattern, also suppress MUP output of male mice, and conversely intermittent GH administration results in male levels of MUP output in females (Gustafsson et al., 1983; Norstedt and Palmiter, 1984; Al-Shawi et al., 1992; Johnson et al., 1995; Metcalf et al., 2000). Infusing males with continuous GH repressed 86% of male-biased genes and induced 68% of female-biased genes within 4 days of infusion (Lau-Corona et al., 2017). This method of manipulating GH secretion has helped to unravel the molecular mechanisms through which GH regulates MUP gene transcription.

GH Regulation of MUP Gene Transcription

GH pituitary secretion controls sex differences in hepatic MUP gene expression through the JAK2-STAT5 signaling pathway in target cells (**Figure 6**; Holloway et al., 2008). This signaling pathway is an example of signal transduction, that is, the conversion of one type of signal to another type. It begins with GH binding to GH receptors (GHR) on target cells in the liver, which activates a key transcription factor, STAT5 (signal transducer and activator of transcription), which then enters the nucleus and initiates MUP gene transcription. Activation of STAT5 triggers a biochemical cascade of reactions (signaling cascade), so that the effects of a few GH molecules can be amplified through positive feedback to induce transcription of large numbers of MUPs (and other sex-biased genes), and the effects of GH can be dampened through negative feedback loops.

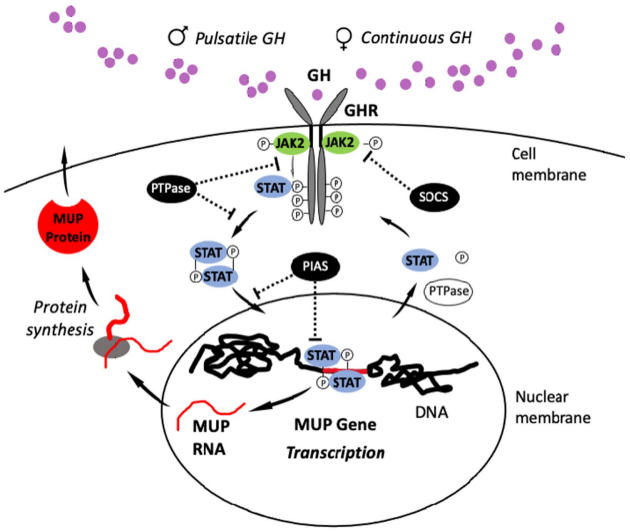

FIGURE 6 | JAK2-STAT5 Signaling Pathway. The pulsatile pattern of GH release in male mice activates the JAK2-STAT5 signaling pathway (or "signaling cascade"): Circulating GH binds to transmembrane GH receptors (GHR), which are members of the cytokine receptor family and widely expressed on the surface of target cells in the liver. GH binding induces a conformational change in GHRs that activate Janus kinase 2 (JAK2), and then this enzyme phosphorylates the cytoplasmic domain of the GHR, generating docking sites for the transcription factor STAT5b. After STAT5b binds to these sites, it undergoes JAK2-catalyzed tyrosine phosphorylation and dissociates from the GHR to form homodimers (e.g., STAT5-STAT5) or heterodimers with other STAT proteins. STAT5 dimers enter the nucleus and bind response elements in gene regulatory regions, initiating transcription (Udy et al., 1997; Teglund et al., 1998). After transcription, STAT5b is deactivated by phosphotyrosine phosphatase (PTPase). STAT5b can then enter into another cycle of JAK2-catalyzed phosphorylation, and it can undergo multiple rounds of this cycle in response to a single male GH pulse (Gebert et al., 1999). In response to female-like patterns of GH release, STAT5b cycles are terminated more rapidly through STAT5b inhibitors, including PTPase, PIAS (protein inhibitors of activated STATs), and SOCS (suppressor of cytokine signaling)/CIS. MUP gene expression is repressed in females by CUX2, a small interfering RNA (siRNA; Conforto et al., 2012). Figure adapted from Waxman and O'Connor (2006).

GH pulse rate regulates the expression of MUP genes *via* the JAK2-STAT5 pathway, and approximately 1,000 other genes in the liver (Udy et al., 1997; Teglund et al., 1998). The deletion of one or both Stat5a and Stat5b genes dramatically reduces male MUP gene expression levels (Udy et al., 1997). The deletion of Stat5a has no effect on female MUP levels, whereas deleting Stat5b reduces MUP gene expression, and the deletion of both Stat5a and Stat5b abolishes MUP protein synthesis (Teglund et al., 1998). STAT5-knockout (KO) mice have reduced hepatic MUP expression, especially on males (Clodfelter et al., 2006; Holloway et al., 2006). STAT5b appears to inhibit expression MUP expression in females (Waxman and O'Connor, 2006). Continuous GH infusion (cGH) overrides the normal pulsatile GH pattern of males and abolishes male-specific, pulsatile pattern of hepatic STAT5 activity (Zhang et al., 2012). cGH downregulates MUPs and other male-biased genes, whereas it upregulates female-biased genes in the liver (Holloway et al., 2006). There is much variation in how expression is regulated across MUP loci in response to changes in GH pulses, as mentioned above, and GH pulse rates regulate the expression of some MUPs (*Mup1, 2, 6,* and *8*) more rapidly than others (Waxman and O'Connor, 2006; Connerney et al., 2017).

There are other transcription factors that regulate MUP gene expression, such as the regulatory protein zinc fingers and homeoboxes 2 (Zhx2). Protein excretion varies among strains of laboratory mice, and BALB/cJ mice have the lowest protein output (**Figure 3**). Their low protein excretion is due to a mutation caused by the insertion of an endogenous retroviral element into the Zhx2 promotor on chromosome 15 (Jiang et al., 2017). Zhx2 suppresses expression of other genes in the liver, whereas it promotes the expression of a number of MUP genes by binding and activating promotors (Jiang et al., 2017). Zhx2 is necessary for the high levels of hepatic MUP expression of males, and several MUP genes (*Mup20, Mup3,* and class B *Mup7, Mup10,* and *Mup19*) show differential responsiveness to Zhx2. It is not known whether Zhx2 influences normal physiological variations of MUP output between or within the sexes. These findings raise the question: how do the transcription factors, STAT5 and Zhx2, initiate transcription in the nucleus?

GH Regulates Chromatin Accessibility

Pulsatile GH release controls transcriptional regulation of MUPs and other male-biased genes by dynamically regulating chromatin accessibility, histone modification, and binding of transcription factors (Connerney et al., 2017; Lau-Corona et al., 2017). Chromatin in genomic regions that are transcriptionally active loses its condensed structure and DNA is exposed. These open sites are sensitive to cleavage by DNase I and DNase I hypersensitive sites (DHSs), which are used as markers for active regulatory regions. DHSs contain key regulatory elements, including enhancers, promoters, insulators, and silencers, and they are often flanked by histone modifications. Experimental cGH closes many male-biased DHSs in the liver of male mice and opens female-biased DHSs (Ling et al., 2010). Mapping DHSs has revealed that sex differences in chromatin accessibility are associated with sex differences in gene expression (Ling et al., 2010; Sugathan and Waxman, 2013). Sex-biased STAT5 chromatin binding is enriched at sex-biased DHSs and positively correlated with sex-biased activating histone marks and negatively correlated with repressive marks (Zhang et al., 2012; Sugathan and Waxman, 2013). These studies show how the endogenous rhythms of male GH pulsatile release open and then close chromatin at regulatory sites of MUPs and other sexually dimorphic genes in association with temporal changes in transcriptional activation (Connerney et al., 2017). A cGH infusion study examined global gene expression in the liver over time to determine the transcriptional events that result in the feminization of MUPs and other male-biased genes (Melia and Waxman, 2019). As expected cGH infusion induced male-biased gene repression and female-biased gene derepression, and these changes occurred in distinct waves over time. These waves of transcription were initiated by a hierarchical

transcriptional network involving several sex-biased transcription factors. More recently, sex-dependent binding of STAT5 to chromatin has been shown to be closely linked to the sex-dependent demethylation of distal regulatory elements that map to genes that show sex-biased expression (Hao and Waxman, 2021).

Thus, GH-mediated regulation of MUP gene expression involves complex interactions between transcriptional networks, genomic regulatory elements, and epigenetics. Given its key role in controlling MUP expression, we next examine how GH secretion is regulated in adult mice.

Regulation of Pulsatile GH Secretion

GH pituitary release is regulated by two neuropeptide hormones, the stimulatory GH-releasing hormone (GHRH) produced by neurons in the hypothalamus, and the inhibitory somatostatin (SST) released by neurons in the pituitary (**Figure 7**). These two hormones influence pulsatile GH release by regulating each other's secretion. GHRH stimulates GH release in the pituitary, which activates inhibitory signals from short-loop feedback inhibition (SST). Additional evidence that the hypothalamus–pituitary axis (HP axis) controls MUP production in the liver comes from a study on SST knockout (*Smst*$^{-/-}$) mice: SST expression is greater in males than females, and *Smst*$^{-/-}$ male mice showed feminized hepatic MUP gene expression (which was not due to changes in T or T_4 levels; Low et al., 2001). It turns out that this textbook model of GH regulation through two hormones is more complex than previously assumed, as several mechanisms regulate the HP axis and subsequent MUP excretion.

For example, ultradian GH secretion is paced by the circadian clock regulators (*Cryptochromes*, *Cry1* and *Cry2*), and double mutant male mice (*Cry1*$^{-/-}$ *Cry2*$^{-/-}$) lack a functional circadian clock and show female-like growth rates and body mass (Bur et al., 2009). Double mutants also have dramatically decreased *Mup1* gene expression and urinary MUP protein compared to controls (Bur et al., 2009). Sex differences in MUP gene expression were found to decline with aging (2-year-old C57BL/6 mice) due to altered GH profiles, which can be reversed by reinstating GH pulses in mutant *Cry*$^{-/-}$ male mice. Deleting the circadian clock gene, *Bmal1,* disrupted the GH axis and reduced MUP expression (Schoeller et al., 2021), and *Mup2* expression is regulated by the circadian clock and glucocorticoids (Cho et al., 2011).

GH pulse rate is modulated by an array of neurotransmitters from the brain, including serotonin, acetylcholine, GABA, opioids (endorphins and enkephalins), and dopamine (Noaín et al., 2013; Ramirez et al., 2015; Brie et al., 2019), and also by peripheral hormones (long-loop feedback; Steyn et al., 2016; Le Tissier et al., 2018). For example, insulin-like growth factor 1 (IGF-1) provides negative feedback of GH release in the pituitary. GH stimulates IGF-1 synthesis in the liver (*via* the JAK2/STAT5 pathway), which negatively regulates GHRH and GH release (long-loop feedback inhibition). Knockout mice with liver-specific deletion of IGF-I (LI-IGF-I$^{-/-}$) have low circulating IGF-1, which increases GH levels. Male LI-IGF-I$^{-/-}$ mice reduced urinary MUP output compared to controls, whereas the MUP output of female LI-IGF-I$^{-/-}$ mice was unaffected (Wallenius et al., 2001). Thus, disrupting the expression of IGF-I results in increasing GH levels (which is why it is used as a biomarker for pathological GH deficiency), and feminizing male MUP expression. However, experimental cGH administration does not significantly alter liver IGF-1 expression, unlike elevated GH from pathology (Lau-Corona et al., 2017). We are not aware of any evidence that physiological IGF-1 levels influence variation in MUP output, however. Some cytokines bind to GHRs in the liver, activate the JAK–STAT pathway, and then induce suppressor of cytokine signaling (SOCS) and CIS proteins that generate negative feedback and inhibit the signaling pathways that initiate their production (Matsumoto et al., 1999; Ram and Waxman, 1999; Krebs and Hilton, 2001). Deleting SOCS-2 genes (socs2$^{-/-}$) disregulates GH signaling and reduces MUP levels in the urine (Metcalf et al., 2000). Other peripheral hormones that regulate GH secretion include insulin, leptin, ghrelin, nesfatins, and klotho (Devesa, 2021), though none have been shown to influence MUP production to our knowledge.

Factors Affecting GH Pulsatile Secretion

Several studies have investigated various factors that influence GH signaling and sex differences in pituitary hormone release (**Figure 7**). The first measurements of GH pulsatile patterns in mice (Steyn et al., 2011) were obtained in a study of male (C57BL/6) mice housed at *ca.* 20°C and placed on an *ad libitum* diet. This study showed that one overnight fast resulted in a striking decrease in pulsatile GH secretion (reduced mass of GH secreted per burst, pulsatile and total GH secretion rate, and increased irregularity of GH pulses), whereas mean GH levels did not show a significant difference between fasted treatments versus controls. Subsequent studies have shown that fasting, obesity, microbiome depletion, and aging can feminize male GH secretion, but the underlying regulatory mechanisms are complex and still unclear (Huang et al., 2019). Some of these studies have also analyzed MUP expression. For example, an RNA-seq study found that germ-free mice have reduced levels of MUP genes and proteins in urine compared to controls (Weger et al., 2019). It was concluded sexually dimorphic MUP expression in the liver requires microbiota, which is likely due to their effects on sexual development and GH release.

Changes in male GH pulsatile release might also explain how acquiring dominant social status triggers increased urinary protein excretion in males but not females (Thoß et al., 2019; Luzynski et al., 2021). To our knowledge, no studies have investigated whether changes in social status or other behaviors influence GH pulsatile release, though GH *levels* have been found to influence behavior. A study on the male offspring of wild-caught mice examined changes in behavior following daily GH administration (Matte, 1981). Increased GH triggered isolation-induced aggression by reducing latency to fight and extending fighting duration. Another study compared the aggressive behavior of GH-sufficient males (heterozygous for the GHRH-KO allele) to homozygous knock-outs, when the mice were challenged with another male (Sagazio et al., 2011). The mice were divided in three groups: untreated controls, recombinant GH administration,

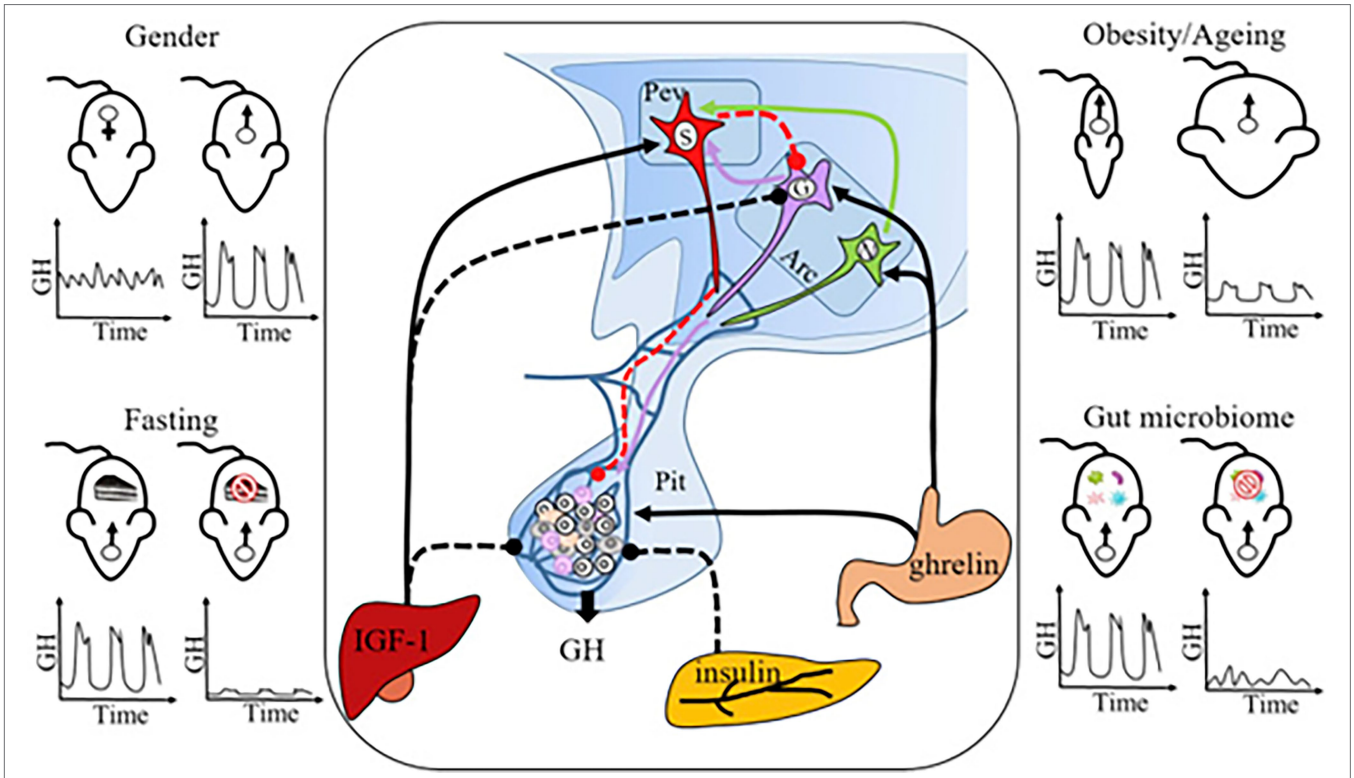

FIGURE 7 | Neuro-Endocrine Mechanisms Regulating Sexually Dimorphic GH Secretion. Pulsatile GH secretion is directly regulated by stimulatory GH-releasing hormone (GHRH), expressed by neurons in the arcuate nucleus (Arc), and inhibitory somatostatin, expressed by neurons in the periventricular nucleus (Pev) of the hypothalamus. GH release is also regulated through feedbacks from peripheral factors, including IGF-1 (inhibitory), insulin (inhibitory), and ghrelin (stimulatory). Sexually dimorphic GH release is the normal physiological pattern (**top left panel**), but some challenges, including fasting (**bottom left panel**), obesity and aging (**top right panel**), and gut microbiome depletion (**bottom right panel**), feminize GH secretion. GH, growth hormone; Pev, periventricular nucleus; Arc, arcuate nucleus; Pit, pituitary gland; S, SRIF (somatotropin release inhibiting factor) neuron; G, GHRH neuron; N, NPY (neuropeptide Y) neuron; and IGF-1 = insulin-like growth factor-1. Solid lines: stimulatory effect; dashed lines: inhibitory effect. Figure used with permission from Huang et al. (2019).

or sham controls (with vehicle, Veh). The study found that the homozygous KO mice showed significantly reduced aggression compared to heterozygous males. GH (but not Veh) administration restored aggressive behavior of KO mice, despite not restoring serum IGF-I. There was no difference in serum T levels among these groups at any time. This study showed that GH-deficient males are less aggressive, that GH replacement normalizes aggressive behavior, and that these behavioral changes are not related to an increase in serum T. Thus, GH level can influence aggressive behavior of males, and studies are now needed to determine whether social status (winning fights or increased scent-marking) affect MUP production by influencing GH pulsatile release.

Although the mechanisms that regulate MUP expression in adult mice are becoming clear, it is still unclear how these mechanisms develop in early life.

ONTOGENY: ORGANIZATIONAL EFFECTS FROM GONADAL STEROIDS

Sexual dimorphic MUP expression was originally thought to be controlled solely by testosterone (T). Urinary MUP excretion begins at puberty (4–7 weeks of age), which is when serum T concentration begin to rise in males, and therefore, early studies on MUPs focused on measuring T. Castrated male mice were shown to excrete less urinary protein than intact males, and T-treated females excreted more protein compared to controls (Thung, 1956, 1962). Administrating T to females increased the amount of urinary protein they excreted and altered the electrophoretic pattern of these proteins (incidentally, the term "Major Urinary Protein" was first coined in these studies on mouse urine; Finlayson et al., 1963). T also influences the amount of MUP mRNA in the liver of castrated males and females (Osawa and Tomino, 1977; Szoka and Paigen, 1978; Hastie et al., 1979; Clissold et al., 1984). Implanting adult females with T increased hepatic MUP gene expression to levels similar to males, though the expression of some MUPs appeared to be more dependent upon T than others (Szoka and Paigen, 1978; Clissold et al., 1984), and it induced the excretion of MUP mRNAs with a male pattern (Knopf et al., 1983). T influences the expression of MUPs in the lacrimal gland, as well as in the liver (the only two tissues found to have male-biased expression in the study), but not in other tissues (Shaw et al., 1983). Another study found that serum T concentration was positively correlated with

urinary protein excretion in male (CD-1) laboratory mice (Mucignat-Caretta et al., 2014), and studies are needed to confirm this result in naturalistic social conditions. It is unclear how T influences MUP production, though one possible mechanism is by programming the development of pathways in the hypothalamus and pituitary gland that control the release of growth hormone (GH).

Studies conducted on laboratory rats indicate that neonatal sex steroid hormones have organizational effects on the development of neural pathways and the GHRH and SST hormones in the hypothalamus that control ultradian GH secretion (Toews et al., 2021). Gonadal steroids continue to influence sex differences in GH secretory profiles during adulthood (Painson et al., 1992, 2000), which could explain the effects of T on MUPs (see above). The few studies on mice found similarities to rats. For example, administering T influenced hepatic MUP gene expression in adult mice by modulating the distribution of receptors of GHRH neurons (Bouyer et al., 2008). T also influenced the development of networks of GH cells in the pituitary that are dynamically regulated in adulthood (Sanchez-Cardenas et al., 2010). Administering T to neonatal females had organizational effect on the hypothalamus (GHRH) and IGF-1, as expected, however, exposure to T did not increase female hepatic MUP expression (Knopf et al., 1983; Ramirez et al., 2010). Thus, gonadal steroids may program and maintain sex-dimorphic patterns of the GH axis in mice, as with rats, but studies are still needed to clarify how gonadal hormones influence MUP production in mice.

ADAPTIVE FUNCTIONS

The selective advantage of producing MUPs has long posed an interesting challenge to explain. The researchers who discovered MUPs were surprised to find so much protein in the urine of male mice, as protein in the urine is a pathological condition in humans (*uremia*). They were especially baffled to find that male mice synthesize tens of milligrams of protein per day in the liver, apparently only to excrete it. This seemed to be a "wasteful" and "irreversible loss" of protein. Subsequently, many studies have shown that MUPs provide a signaling mechanism for males to influence the brains and behavior of females. Sexual dimorphic traits are expected to evolve when traits have sex-specific fitness effects and generate intra-locus sexual conflict, but studies on how MUPs affect survival and reproductive success have only just begun.

Chemosensory Signaling Functions: Sexual Selection

Most research on MUPs has focused on their chemosensory functions (Hurst and Beynon, 2004; Stopka et al., 2012; Mucignat-Caretta and Caretta, 2014). These studies have shown that MUPs influence male chemical signals and function as both pheromones and carriers of volatile pheromones. For example, MUP20, which is mainly expressed by males, attracts females and several volatile male pheromones are MUP ligands (**Figure 1**). In contrast, we are not aware of any studies that have shown that MUPs or MUP ligands influence female odor or its attractiveness to males. Studies on MUP-mediated chemical signaling, however, have often assumed that MUP expression is fixed, and ignored evidence that expression is phenotypically plastic (**Table 1**). There are also sex differences in the olfactory detection of MUPs, since MUP-detecting sensory neurons are selectively expressed in females during estrus (Dey et al., 2015). Different MUPs may have different roles on chemical communication, and the same MUP may have different signaling effects depending upon the sex of the sender and receiver. It is premature to make conclusions about sex differences in MUP-mediated chemical communication, however, because most studies have focused on males and comparable studies on females are lacking.

Male-biased MUP output is expected to be maintained by sexual selection on males for several reasons. First, the high levels of MUP output by males appear to mediate male–male competition for territories and access to females (intrasexual selection). Male house mice are more territorial and scent mark more than females, and male MUPs mediate aggressive interactions (Hurst and Beynon, 2004; Kaur et al., 2014). In seminatural populations, dominant territorial males have higher urinary protein output and MUP20 excretion than subordinates (**Figure 5**; Thoß et al., 2019; Luzynski et al., 2021). Some studies suggest that MUP output determines male social status, whereas others found that males upregulate urinary protein excretion after acquiring a territory (Thoß et al., 2019; Luzynski et al., 2021). Either way, elevated MUP output is expected to facilitate a male's ability to defend a territory, which is a major determinant of male mating and reproductive success (Meagher et al., 2000; Luzynski et al., 2021). Females increase urinary protein excretion during aggressive female–female interactions (Garratt et al., 2011b; Stockley et al., 2013); but unlike male mice, dominant females in seminatural populations do not excrete higher levels of urinary protein compared to subordinates (**Figure 5**; Thoß et al., 2019; Luzynski et al., 2021).

Second, MUP output may also enhance male mating success through female mate choice (also called "intersexual selection," which is an unfortunate term since the sexes are not competing). MUP20 in male urine influences female attractiveness to male odor, increases sex discrimination of scent marks, it induces spatial learning (Roberts et al., 2010, 2012), and after detection, it stimulates neural growth in the brain (Hoffman et al., 2015; Demir et al., 2020). Females are attracted to male urine spiked with MUPs when females are in estrus (Dey et al., 2015). Increased overall urinary protein concentration of males housed in laboratory conditions does not influence the attractiveness of females to male scent (Roberts et al., 2010), nor does it explain female preferences for the scent of dominant over subordinate males living in seminatural conditions (Thoß et al., 2019). Nevertheless, in natural conditions, high MUP output is expected to prolong the release of volatile male pheromones, increase the attraction of females to a male's territory, and induce an acceleration of female puberty and estrous cycling (Mucignat-Caretta et al., 1995; Marchlewska-Koj et al., 2000; Morè, 2006; Flanagan et al., 2011).

These two types of sexual selection are not mutually exclusive, as female sexual preferences are influenced by the outcome of male–male competition: estrous females are more attracted to the urinary scent of dominant, territorial males, which excrete higher levels of urinary protein and MUP20 than subordinate males (Thoß et al., 2019). Thus, studies on chemical communication predict that MUPs mediate sexual selection in male mice and that MUPs are expected to enhance male mating and reproductive success.

In contrast, there is no evidence that MUP expression levels influence female odor, the outcome of female–female competition or their sexual attractiveness to males. Female urinary protein excretion increases just before and during estrus (Stopka et al., 2007), however, it is not known whether estrus-dependent MUP regulation influences female odor.

Olfactory experiments on the attraction of females to male odor provide insights into the adaptive functions of MUP production, but they are insufficient to test hypotheses about sexual selection. Only one study to our knowledge has examined the effect of MUPs on reproductive success. This study found that urinary protein of wild-derived male mice (*M. musculus musculus*) is correlated with the reproductive success of males but not females (**Figure 8**; Luzynski et al., 2021) and that urinary protein excretion was the strongest correlate of male reproductive success. These findings support the hypothesis that male-biased MUP production is maintained by sexual selection in males. Studies are needed to determine whether these results are due to direct male–male competition, female mate choice, or both. Testing the mate choice hypothesis requires controlling for the effects of male–male competition (Thonhauser et al., 2013). Interactions between these two types of selection, however, make it difficult to examine their independent effects.

Studies are needed to explain why MUPs influence male but not female reproductive success, and why males regulate MUP output according to their social status, health, and condition (**Table 1**). To explain why males produce honest signals of their quality, it is often been suggested that MUPs function as a "handicap signal" (Malone et al., 2001; Stockley et al., 2013; Nelson et al., 2015). Zahavi's Handicap Principle proposes that costly signals provide honest indicators of quality, not despite their costs, but because they are costly to produce. This hypothesis can be rejected for many reasons, however (Penn and Számadó, 2020). For example, it is illogical and it assumes that animal signals, unlike other traits, evolve under a non-Darwinian process of "signal selection" that favors waste rather than efficiency. The widespread acceptance of the Handicap Principle was due to Grafen's (Grafen, 1990) "strategic choice" signaling model being misinterpreted as validating this idea, despite the fact that signals in this model are neither wasteful nor costly; on the contrary, they are efficient investments. It predicts that MUP output can provide honest signals of male social status, health, and other aspects of quality, if high-quality males have lower survival costs (or greater potential reproductive benefits) for producing MUPs than low-quality males. It is not known, however, whether male mice incur such differential fitness costs (or benefits) for MUP production.

It is often assumed that MUP production increases the absolute energetic costs of scent-marking, but the costs and benefits are likely to depend on a male quality or condition. Male MUP expression is condition-dependent (**Table 1**), suggesting that males in poor condition are less able to afford the energetic and other costs of producing MUPs. MUP production may *reduce* the net energetic costs of scent-marking of dominant males by reducing the effort, time as well as fitness costs (from predation and aggressive interactions) necessary to replenish territorial scent marks. However, almost nothing is known about how MUP production influences survival.

Viability Effects: Metabolic and Other Physiological Functions

Only one study to our knowledge has investigated whether MUP production affects survival: MUP knockout mice (KO's lacking MUP genes by deleting the entire 2.2 Mbp MUP gene cluster using CRISPR) were healthy for 2 years and did not show altered body mass or detrimental health effects compared to controls (Yang et al., 2016). This study was conducted in the laboratory, and therefore, studies are still needed to evaluate the fitness (longevity and reproductive success) of MUP-KO mice of both sexes living in more natural ecological conditions and exposed to physiological and other challenges.

Such experiments are crucial because MUPs provide physiological functions, including regulating metabolism (Petrak et al., 2007; Hui et al., 2009; Zhou et al., 2009) and eliminating harmful metabolic waste and toxic xenobiotics (Kwak et al., 2011, 2016; Stopka et al., 2016; Stopková et al., 2017; but see Nault et al., 2017). These findings are consistent with results from studies on the mechanisms that regulate MUP gene expression. GH controls the expression of many other genes in the liver that influence lipid and glucose metabolism and metabolism of xenobiotics. Their common regulatory pathways suggest that MUPs share common functions ("guilt by association"). Moreover, JAK2 and STAT5 deficiency results in hepatic lipid accumulation, and thus the regulatory proteins that control MUP expression may be crucial for survival in the wild. These findings can potentially help to explain the function of MUPs in female house mice, and why caged female mice elevate their MUP excretion after they are released from cages into naturalistic conditions (Thoß et al., 2019). And, if these physiological functions are more important for males than females, or more important for dominant than subordinate males, then this would help to explain differences in MUP gene expression between the sexes and among males. These results might also elucidate the original (ancestral) function of MUPs in rodents (see below), and help to explain the expansion of MUP loci in certain species (Charkoftaki et al., 2019). For example, multiple MUP loci might have enabled mice and rats, which are kleptoparasites (rather than commensals), to better cope with toxins when foraging on human refuse. No studies to our knowledge, however, have tested whether any of the proposed physiological functions of MUPs differ between the sexes, or whether they affect survival.

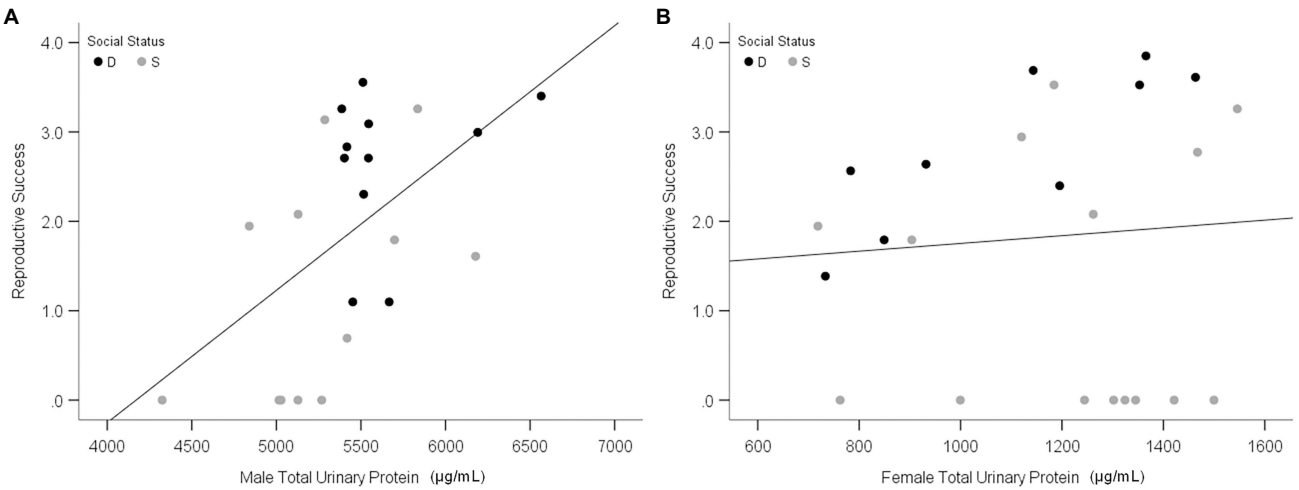

FIGURE 8 | Relationship between urinary protein output and reproduction success. Scatterplots show the total urinary protein output of males **(A)** and females **(B)** in relation to individual reproductive success in the large, seminatural enclosures. Black and gray data points indicate dominant (D) and subordinate (S) social status, respectively. Reproductive success was calculated as the Ln (number of offspring per mouse + 1). Adapted from Luzynski et al. (2021).

EVOLUTIONARY ORIGINS AND POTENTIAL EFFECTS ON DIVERGENCE AND SPECIATION

Determining the origins of sexually dimorphic MUP expression requires comparing MUP expression in both sexes among rodent species. The phylogeny MUP genes have been described (Stopka et al., 2012); however, few studies have compared urinary protein output (Nazarova et al., 2018) or MUP gene expression between the sexes in different *Mus* species (Sheehan et al., 2019; Matthews et al., 2021). Several studies have compared the MUPs of two European *Mus* subspecies and examined the hypothesis that divergence of these genes among populations promotes speciation.

The first study to provide a statistical comparison of MUP expression between *Mus* subspecies was conducted on wild-caught house mice from several populations near the European hybrid zone (Stopková et al., 2007). Quantitative differences between subspecies were found in hepatic MUP mRNA expression and total urinary MUP concentration (**Figure 9**). Male *M. musculus musculus* expressed more MUP mRNA than females, and more than *M. musculus domesticus* mice of either sex, and total urinary MUP concentration showed a larger sex bias in *M. m. musculus* than *M. musculus domesticus*. No differences were detected between females of these two subspecies.

A recent comparative study found that the magnitude of sexual dimorphic gene expression varies among *Mus* species and subspecies (Sheehan et al., 2019). This study compared seven species of *Mus* and three *M. musculus* subspecies. *M. musculus* had the most pronounced sexually dimorphic MUP gene expression and protein output, as well as having more duplicated MUP loci, and *M. musculus musculus* showed the largest sexual dimorphism (**Figure 10**).

These results confirm that *M. m. musculus* show greater male-biased MUP expression than *M. m. domesticus* (Stopková et al., 2007; **Figure 9**) and other *Mus* species. They also indicate that sexually dimorphic MUP output of *M. musculus* is the derived rather than the ancestral trait, and that sexual dimorphism evolved by males increasing MUP expression rather than females reducing MUP expression. These findings raise questions about the underlying mechanisms explaining this pattern. For example, do *M. musculus musculus* males have higher rates of GH pulsatile secretion or sensitivity to GH pulsatile release than other species? They also raise questions about the ecological and social factors that correlate with species differences in MUP sexual dimorphisms. For example, are *Mus musculus* males more territorial or do they deposit more scent marks than males in other *Mus* species? To understand whether and how selection explains the evolution of sexually dimorphic MUP expression in some *Mus* species, studies are needed to experimentally test the effects of manipulating MUP expression of the fitness of both sexes in different species.

Assortative Mating and Reinforcement

Stopková et al. (2007) suggested that differences in male MUP expression between subspecies might control subspecies recognition and explain assortative mating preferences in female *M. musculus musculus* observed in mice near the hybrid zone (Smadja and Ganem, 2002). Once perceptible differences in sexual characters evolve in diverging populations, and hybrids have reduced fitness, selection is expected to favor the evolution of assortative mating and further divergence and speciation (reinforcement hypothesis). In other words, the evolutionary divergence in male MUP expression among *Mus* species might drive further divergence between subspecies of mice through assortative mating.

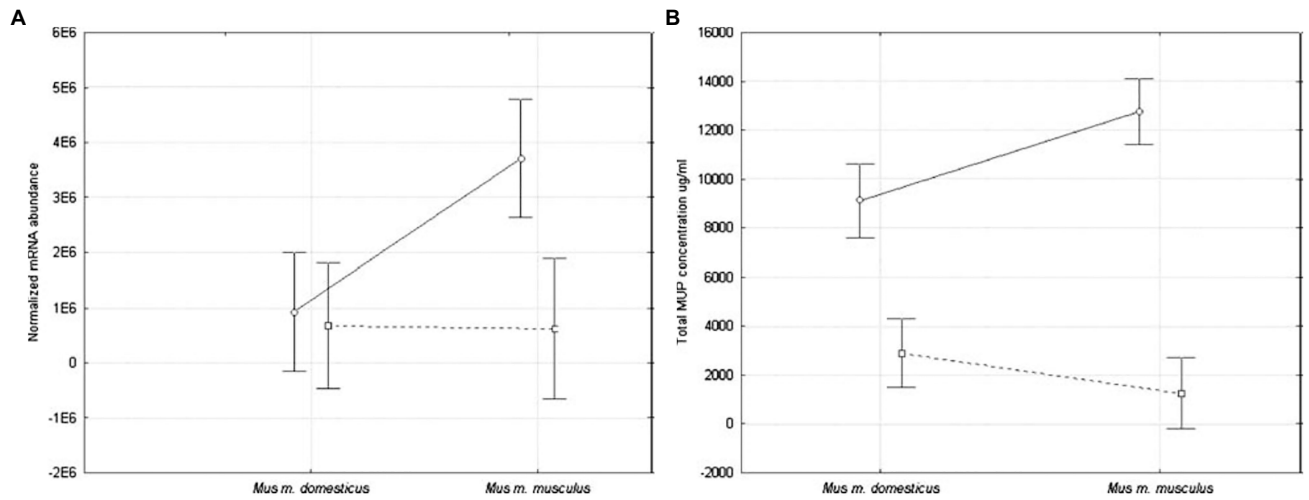

FIGURE 9 | MUP expression in two *Mus musculus* subspecies. MUP output was compared using **(A)** normalized hepatic MUP mRNA abundance, and **(B)** concentration of urinary MUP protein circles connected by solid lines show differences between males, and squares connected by dashed lines show differences between females of these subspecies (error bars show 95% confidence intervals). Figure used with permission from Stopková et al. (2007).

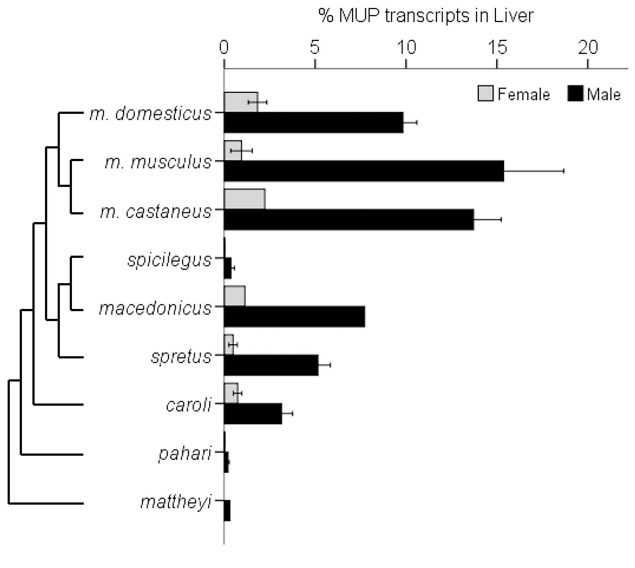

FIGURE 10 | Sexually Dimorphic MUP Gene Expression in *Mus* Species. The graph illustrates sexually dimorphic expression of MUP genes in relation to a *Mus* species from Steppan and Schenk (2017) and *M. musculus* subspecific phylogeny from Macholán et al. (2012). Sex differences in MUP expression among *Mus* species and subspecies [mean percentages of total MUP gene expression in liver tissue composed of *Mup3, Mup20*, and central MUPs; data from Sheehan et al. (2019)]. Error bars are ±1 SEM ($N = 1$ where error bars are absent). *Mup20* is strongly male-biased in *M. musculus*, but it is not found in most other species, and although *Mup3* was thought to be male-specific, it is expressed in female *M. m. domesticus* and some other species. Data from Sheehan et al., (2019).

Evidence for olfactory-mediated assortative mating preferences has been found in house mice trapped near the hybrid zone, and especially *M. musculus musculus* females (Smadja and Ganem, 2002). Yet, the mice were able to distinguish subspecies, regardless of whether the urine originated from mice in allopatric populations or the contact hybrid zone, suggesting that the divergence of the signal between subspecies originated in allopatry, contrary to the assumptions of the reinforcement hypothesis.

There have been several attempts to test the predictions of the MUP-mediated reinforcement hypothesis, including the following: (1) A genetic survey of wild house mice found considerable introgression between these two European subspecies for markers closely flanking the MUP cluster on both sides of a hybrid zone (Bímová et al., 2011), which contradicts assortative mating predictions. However, some alleles showed asymmetric introgression, as expected if only *M. musculus musculus* showed assortative preferences. (2) Another study scanned MUP and candidate olfactory (vomeronasal receptor or VR) genomic regions of mice from the hybrid zone and allopatric areas using microsatellite loci to detect recent selective sweeps (Smadja et al., 2015). Some MUP or VR loci displayed the expected reduction in variability in populations near the hybrid zone, but no strong conclusions could be made. (3) A detailed proteomic study compared the amount and types of urinary MUPs between these subspecies and their location near to the hybrid zones (Hurst et al., 2017). Urinary protein output was sexually dimorphic (overall males had *ca*. 3.5 times higher protein excretion than females), and *M. musculus musculus* had higher protein output than *M. musculus domesticus*; as previously shown (Stopková et al., 2007). Differences in male MUP excretion between subspecies were found in allopatric, but not contact zones, contrary to the reinforcement hypothesis. Subspecies divergence in the total urinary protein concentration in the contact zone was found in females, but not males due to the low concentration of protein in *M. musculus musculus* females. Several MUPs were expressed in one but not the other subspecies. The sample sizes were insufficient to make strong conclusions, however. (It was not possible to determine whether mass peaks that were

shared between subspecies represent identical MUP isoforms due to the technical difficulties in resolving such highly homologous isoforms, even with peptide mass fingerprinting). The authors concluded that the differences in the expression profiles of urinary MUPs might have the potential to convey information about subspecific identity, and MUPs showing differential expression in the contact zone were suggested to provide candidates for assortative mating preferences and reinforcement.

No studies to our knowledge have tested whether MUPs mediate assortative mating, but if so, this hypothesis contradicts the proposal that house mice show disassortative mating preferences for MUPs (Sherborne et al., 2007). Thus, it is still unclear whether MUPs play a role in driving evolutionary divergence and speciation in house mice.

DISCUSSION

Here we summarize the key findings from studies on sexual dimorphic MUP expression and highlight questions that need to be addressed in the future. Our review shows that MUP production is generally male-biased, that most MUP genes have sexually dimorphic expression, and that are large differences in gene expression among MUP loci in both sexes. Moreover, we show that MUP output is male-biased in seminatural social conditions, as well as in the laboratory, and the regulation of MUP expression is more complex and dynamic in natural social contexts than in the laboratory (Enk et al., 2016; Thoß et al., 2019; Luzynski et al., 2021). MUP expression is not constitutive or fixed, and instead, it is regulated depending on a variety of different factors, including social status, caloric intake, infection, immune activation, and senescence, and males and females can differ in how they regulate MUP expression (**Table 1**). For example, male mice upregulate urinary protein output in response to acquiring a territory and dominant social status, unlike females (Thoß et al., 2019; Luzynski et al., 2021), and females regulate MUP excretion depending on their estrous stage (Stopka et al., 2007), which might explain why wild female mice show increased urinary protein excretion in more natural social conditions. More studies are needed on female mice in general, which is why funding agencies are beginning to require researchers to include both sexes in their grant proposals (Klein et al., 2015). Most studies on MUP expression have focused on *Mup1* and *20*, and research is needed to investigate the regulation of other MUP loci, which is challenging because MUPs are so highly homologous (Enk et al., 2016; Thoß et al., 2016), and longitudinal analyses are needed to determine whether MUPs are up- or downregulated (Thoß et al., 2019; Luzynski et al., 2021).

We also examined studies on the proximate mechanisms and evolution of sexual dimorphisms in MUP expression. First, studies on the proximate mechanisms controlling gene expression in the liver have shown that male-biased MUP gene expression is due to sex differences in GH pulsatile secretion from the pituitary, which induce MUP gene expression through the JAK2/STAT5 signaling pathway (Udy et al., 1997; Teglund et al., 1998; Holloway et al., 2008). Studies are still needed to confirm that normal physiological variations in GH secretion explain male-biased MUP output under more natural conditions, to determine how social status, caloric restriction, aging, and other factors male MUP production, and to compare MUP regulation in both sexes (**Table 1**). Many MUPs are expressed at low levels or silenced in females, and CUX2, a female-specific transcription factor, suppresses MUPs other sexually dimorphic genes in the liver (Conforto et al., 2012). GH regulates the expression of hundreds of other hepatic genes, but it is unclear whether their co-expression is inextricably linked or functional. It is also unclear how variation in GHR expression affects MUP synthesis, or how GHR expression is regulated. MUP expression is regulated by several hormones, and different MUPs are regulated by different endocrine mechanisms, but it is unclear how or why.

Second, GH release is controlled by neurons and hormones in the hypothalamus and pituitary, which appear to be organized by gonadal steroids, though studies are needed to explain their development (ontogeny).

Third, many studies show that male MUPs function as pheromones and pheromone carriers, though only two studies have investigated their fitness consequences: (1) One study found that increased levels of urinary protein excretion of wild mice living in seminatural conditions correlated with the reproductive success of males but not females (Luzynski et al., 2021). Studies are needed to determine whether this result is due to direct male–male interactions, female choice, or both. For example, estrous females are attracted to the scent of dominant territorial males, which excrete higher levels of MUP20 and several other MUPs compared to subordinates (Thoß et al., 2019; Luzynski et al., 2021). Taken together, these findings suggest that the regulation of MUP expression influences male reproductive success, such as through effects on the sexual attractiveness of urinary scent marks (Roberts et al., 2010, 2012) or other effects on the physiology and behavior of females (Hoffman et al., 2015; Demir et al., 2020). It is unclear why mice regulate MUP production (**Table 1**), and why males honestly signal their social status, health, and condition to rivals and potential mates; however, MUPs are not a "handicap signal." It is often emphasized that MUPs are costly to produce, though MUPs may function to reduce the net costs, as well as to enhance the reproductive benefits of scent-marking by dominant males. (2) Another study examined whether MUP production influences survival, and though no effects were detected in MUP knockout mice of either sex, studies are needed to assess fitness effects in more natural conditions. MUPs provide physiological functions which may enhance survival, and perhaps more for males than females. If sexual dimorphisms are adaptive, then MUP expression should show differential fitness effects on the sexes, such that experimentally suppressing expression of males should impair their reproductive success, whereas elevating female MUP production to the levels of dominant males should reduce female survival or reproductive success. Such studies would help to test the hypothesis that sex differences in expression evolved due to intra-locus sexual conflict over allelic gene expression (Pennell and Morrow, 2013).

Fourth, studies have only just begun on the evolutionary origin of sexually dimorphic MUP expression in house mice. A recent comparative study of MUP gene expression in *Mus* species and *Mus musculus* subspecies (Sheehan et al., 2019). This analysis suggests that the ancestral state of MUP expression in *Mus* is sexually monomorphic, and that male-biased expression evolved by increasing male rather than reducing female MUP expression. However, the sample sizes of some species in this study were very small and housing conditions were not controlled. Studies are also needed to determine the underlying mechanisms controlling MUP gene expression and their functions in other *Mus* species. Comparative analyses are also needed to investigate whether sexual dimorphism co-evolved with the expansion of MUP loci in *Mus* and *Rattus*. MUPs could be used to test the hypothesis that sexual conflict favors the evolution of gene duplication (Cox and Calsbeek, 2009; Connallon and Clark, 2011; Gallach and Betrán, 2011). Other mammalian genera need to be investigated, as there are substantial differences in the sexual dimorphism in urinary protein excretion among different rodents, and house mice are not the most sexually dimorphic (Nazarova et al., 2018). It will be possible to begin reconstructing the evolutionary transitions that explain sexually dimorphic MUP expression once the genes that control MUP gene expression in *Mus musculus* and other *Mus* species are identified. It has been suggested that sex differences in MUP expression mediate assortative mating preferences, which subsequently drives further evolutionary divergence and speciation between house mice subspecies, but the evidence is mixed and direct tests are lacking.

AUTHOR CONTRIBUTIONS

DP wrote the first drafts, most text, and made most revisions. SZ and KL wrote the first draft of some sections and helped with the table, figures, and writing revisions and final editing. All authors contributed to the article and approved the submitted version.

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