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Function-First Ligandomics for Ocular Vascular Research and Drug Target Discovery

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Abstract

Human eyes may develop different vascular diseases with neovascularization and/or leakage, including wet age-related macular degeneration (AMD), diabetic macular edema (DME), proliferative diabetic retinopathy (PDR), retinopathy of prematurity, corneal neovascularization and intraocular tumors. A breakthrough in therapy is the advent and approval of vascular endothelial growth factor (VEGF) inhibitors. However, anti-VEGF drugs not only have limited efficacy to treat AMD, DME and PDR but also are not approved for other ocular indications. The key to addressing these unmet clinical needs is to develop novel therapies against VEGF-independent angiogenic factors or signaling pathways for alternative or combination therapy. We recently developed the first paradigm of ligandomics for global mapping of cell-wide ligands as well as disease-selective ligands. Therapies targeting disease-selective angiogenic or vascular leakage factors likely have high efficacy, minimal side effects, wide therapeutic windows and relatively low drug attrition rates. A critical challenge is how to distinguish between genuine drug targets and spurious hits identified by high-throughput ligandomics. Here we exploited the unique advantages of the eye and extracellular ligands by combining ligandomics with “function-first” and/or “therapy-first” analyses to efficiently characterize functional activity, disease selectivity, pathogenic role and therapeutic potential of identified ligands. The innovative function- or therapy-first ligandomics will systematically and reliably delineate disease-selective angiogenic or vascular leakage factors and markedly facilitate ocular vascular research and ligand-guided targeted anti-angiogenic therapy.

Graphical Abstract

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H.T. and W.L. are shareholders of Ligandomics, LLC and Everglades Biopharma, LLC. W.L. is inventor of issued and pending patents.

Keywords

Retina; vascular disease; ligandomics; comparative ligandomics; angiogenic factor; vascular leakage factor; drug target discovery; target validation

1. Introduction

Human eyes are nourished by multiple vasculatures, including the central retinal artery, posterior and anterior ciliary arteries, and choriocapillaries (Kiel, 2010). Under pathological conditions, human eyes may develop different vascular disorders, such as wet (neovascular, exudative) age-related macular degeneration (AMD) with choroidal neovascularization (CNV), diabetic macular edema (DME), and proliferative diabetic retinopathy (PDR) with retinal neovascularization (RNV) (Shao et al., 2016; Wong et al., 2016). Other ocular vascular diseases include retinopathy of prematurity (ROP) with RNV, polypoidal choroidal vasculopathy (PCV), retinal vein or artery occlusion, corneal neovascularization, neovascular glaucoma, and intraocular tumors (Abdelfattah et al., 2015; Amaro et al., 2017; Haymore and Mejico, 2009; Hellstrom et al., 2013; Ortiz and Dunkel, 2016). A breakthrough in the therapy of wet AMD and diabetic retinopathy (DR) is the approval of vascular endothelial growth factor (VEGF) inhibitors, such as ranibizumab and aflibercept (Diabetic Retinopathy Clinical Research Network et al., 2015; Group et al., 2011). Nonetheless, these drugs have a number of downsides, including limited efficacy, similar mechanisms of action, possible increase in the risk of geographic atrophy in wet AMD patients and inappropriateness for other ocular vascular diseases (see details in Section 6.1) (Dedania and Bakri, 2015; Grunwald et al., 2017). Novel therapies against other angiogenic factors are under intense investigation to address these limitations. However, the failure of platelet-derived growth factor (PDGF) and anti-angiopoietin-2 (Ang2) inhibitors in recent clinical trials highlighted daunting challenges to develop alternative anti-angiogenic therapies (2016; Dunn et al., 2017).

Endothelial ligands, including angiogenic factors, are traditionally identified on a case-by-case basis with technical challenges. It is even more formidable to delineate ligands with therapeutic potentials. Consequently, most known angiogenic factors regulate both diseased and healthy vasculatures (Gacche and Meshram, 2014). Therapies targeting these conventional ligands not only exert therapeutic benefit on diseased vessels, but also may trigger adverse side effects on normal vasculatures with narrow therapeutic windows and high drug attrition rates.

We recently developed the first paradigm of ligandomics as the only high-throughput technology to globally identify cell-wide endothelial ligands with simultaneous binding activity quantification (LeBlanc et al., 2017). Quantitative comparison of entire ligandomes

for diseased vs. healthy cells can systematically map disease-selective or restricted ligands, including angiogenic and vascular leakage factors. Therapies targeting disease-selective ligands have the advantages of high efficacy, minimal adverse side effects on normal cells or vessels, wide therapeutic windows and low drug attrition rates (Li et al., 2018). Owing to possible false positives identified by ligandomics, however, it is of importance to characterize the functional activity, disease selectivity, pathological roles and therapeutic potentials of identified ligands before committing to drug development.

Here we propose a new concept of “function-first” and “therapy-first” screenings to efficiently characterize identified targets by exploiting the uniqueness of the eye and extracellular ligands. We investigated the validity of this concept by not only performing new experiments but also recapitulating the process of drug target discovery by ligandomics in our recent study (LeBlanc et al., 2017). The combination of high-throughput comparative ligandomics with low-throughput function/therapy-first analyses will markedly improve our technical capability to efficiently and reliably identify disease-selective angiogenic and vascular leakage factors. The potential impact of function- or therapy-first comparative ligandomics on ocular vascular research and drug target discovery are discussed.

2. Materials and supplies

2.1. Animals

C57BL/6J mice (6 weeks old, male) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Male mice were chosen because they were more susceptible to developing streptozotocin (STZ)-induced hyperglycemia than female mice (Deeds et al., 2011). Mice were maintained and handled in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Miami.

2.2. Materials

Open reading frame phage display (OPD) cDNA libraries were generated from adult mouse eyes and mouse embryos at day 18 (E18) as previously described (Caberoy et al., 2009a; Caberoy et al., 2010). Clonal phage expressing human VEGF (hVEGF-Phage) or green fluorescent protein (GFP-Phage) was constructed as described (LeBlanc et al., 2017). Hepatoma-derived growth factor-related protein 3 (HRP-3) was expressed and purified as described (LeBlanc et al., 2015). Other materials and their respective suppliers are as follows: ketamine (Henry Schein Animal Health, Dublin, OH, USA); xylazine and Bacitracin zinc with polymixin B sulfate ophthalmic ointment (Akorn, Lake Forest, IL, USA); Alcaine® ophthalmic solution (0.5% proparacaine HCl; Alcon, Fort Worth, TX, USA); human secretogranin III (Scg3) (Sino Biological, Wayne, PA, USA); human VEGF165 (R&D Systems, Minneapolis, MN, USA); BLT5615 bacteria, isopropyl β -D-1-thiogalactopyranoside (IPTG), LB Broth, LB Agar, carbenicillin, CsCl, 1,1'-Diocetadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), Evans blue, STZ (MilliporeSigma, St. Louis, MO, USA); anti-Scg3 polyclonal antibody (pAb) (Cat. #10954-1-AP) and anti-Plekha1 control pAb (#10238-1-AP) (Proteintech, Rosemont, IL, USA);

Whatman filter paper (Grade 3) (GE Healthcare, Chicago, IL, USA); and aflibercept (Eylea) (NDC 61755-0005-02; Regeneron Pharmaceuticals, Tarrytown, NY, USA).

3. Detailed Methods

3.1. Animal model of diabetic retinopathy

Mouse model of DR was developed as previously described (Furman, 2015; LeBlanc et al., 2017). Briefly, mice were starved for 4 h and then injected intraperitoneally (i.p.) with STZ (40 mg/Kg body weight) or mock sodium citrate buffer (137 mM, pH 4.5) for five days. Mice were monitored for blood glucose biweekly with a glucometer by collecting a drop of blood from the tail vein and considered diabetic when blood glucose was ≥ 300 mg/dL. Hyperglycemic mice were aged for 4 months to develop DR.

3.2. *In vivo* ligandomics analysis

The procedures of *in vivo* ligandomics to identify retinal endothelial cells are depicted in Fig. 1 (LeBlanc et al., 2015; LeBlanc et al., 2017). Briefly, BLT5615 bacteria were grown in carbenicillin-LB Broth at 37°C to an OD₆₀₀ of 0.5, shaken for additional 30 min with 1 mM IPTG and stored at 4°C for up to 2 days (Caberoy et al., 2009b). The two OPD libraries were amplified in IPTG-induced BLT5615 bacteria until bacterial lysis and pooled together in equal titer to increase library representation (LeBlanc et al., 2015). hVEGF-Phage and GFP-Phage were amplified in IPTG-induced BLT5615 bacteria and diluted into the pooled libraries at 1:1,000 ratio. The mixed libraries were precipitated, purified by CsCl gradient centrifugation, and dialyzed against phosphate-buffered saline (PBS) (LeBlanc et al., 2015). Purified phage libraries were intravenously (i.v.) injected into diabetic or control mice (3 mice/group/round, 1×10^{12} pfu/mouse), which were anesthetized with ketamine/xylazine cocktail (100/10 mg/kg, intraperitoneally) (LeBlanc et al., 2017). After circulating for 20 min, unbound phages were removed by intracardial perfusion with PBS for 10 min. Retinas were isolated and homogenized in PBS containing 1% Triton X-100 to release endothelium-bound phages. Aliquots of retinal lysates were used to quantify phage titer by plaque assay (Caberoy et al., 2009b). Phages in remaining retinal lysates were amplified in IPTG-induced BLT5615, repurified and used as input for the next round of *in vivo* binding selection. Given that each round of selection can amplify enriched clones $>10,000$ -fold, three rounds of binding selection were necessary and sufficient to amplify all clones, including low-abundant clones in the libraries, for ensuring enough ligands for receptor binding. After 3 rounds of selection, cDNA inserts of enriched phages were amplified by PCR, purified from agarose gel (400 – 1,500 bp) and identified by next-generation DNA sequencing (NGS), as described (LeBlanc et al., 2015).

3.3. Corneal pocket angiogenesis assay

Corneal angiogenesis assay was carried out as described (Fig. 2) (LeBlanc et al., 2015; LeBlanc et al., 2017). Briefly, a drop of Alcaine® ophthalmic solution was applied to the eye of anesthetized diabetic or age-matched control mice for 5 min. A gentle cut was made in the middle of the cornea 1.2 – 1.4 mm from the corneal limbus with a von Graefe cataract knife without cutting through the cornea. A pocket was made under the epithelium layer of the cornea by horizontally inserting the knife into the middle of the cornea and extending the

knife toward the limbus carefully. Whatman filter paper (Grade 3) was autoclaved, cut into pieces (0.125 mm²/piece) and soaked in the solution of Scg3 (0.25 µg/µl), VEGF165 (0.1 µg/µl) or HRP-3 (1 µg/µl) for 2 h at 4°C. Soaked papers were implanted into corneal pockets of anesthetized mice (1 paper/pocket/cornea) with PBS-soaked paper for the fellow eye. Eyes were covered with a thin layer of Bacitracin after the surgery. After 6 days, corneal angiogenesis was analyzed using a slit-lamp microscope and photographed. The number of new sprouting vessels into the cornea, their branching points and semiquantitative score were quantified and normalized against PBS fellow eyes (LeBlanc et al., 2015). Mice were euthanized by CO₂ inhalation and immediately perfused intracardially with fluorescent DiI dye (Li et al., 2008). Isolated corneas were fixed, flat-mounted and analyzed by confocal microscopy to detect DiI-stained blood vessels.

3.4. Retinal vascular leakage assay

Blood-retinal barrier integrity was evaluated using the method of Evans blue extravasation in the retina, as described with minor modifications (Scheppke et al., 2008; Xu et al., 2001). Briefly, Evans blue dye was dissolved in saline at 20 mg/ml, sonicated and filtered through a 0.2-µm disc filter and stored at room temperature for up to 1 week. Reagents were intravitreally injected into one eye of anesthetized diabetic or age-matched control mice with PBS for the fellow eye (1 µl/eye). After 24 h, Evans blue dye was injected into retro-orbital venous sinus of anesthetized mice (120 mg/kg, half dose/sinus, 30 G needle) and allowed to circulate in the blood for 4.5 h. Mice were euthanized by CO₂ inhalation. After collection of an aliquot of the blood by cardiac puncture, mice were perfused intracardially for 30 min with sodium citrate solution (0.1 M, pH 3.5, pre-warmed to 37°C). Retinas were isolated, quickly rinsed in PBS, dried in a SpeedVac concentrator to remove excess liquid for 15 min at room temperature, and weighted. Dried retinas were incubated with 50 µl formamide at 70°C overnight, and centrifuged at 180,000 g for 1 h at 4°C. Evans blue in supernatants were quantified at 620 nm (signal) and 740 nm (background, subtracted). The collected blood was centrifuged at 3,550 g for 10 min, diluted in formamide and quantified at 620/740 nm. Evans blue in series dilution in formamide was used as a standard curve. The leaked dye was calculated as follow: [leaked EB concentration (mg/ml)/retinal weight (mg)]/[blood EB concentration (mg/ml) x circulation time (h)]. Data were normalized to PBS in fellow eyes.

3.5. Data analysis

For comparative ligandomics data analysis, cDNA sequences identified by NGS were aligned against NCBI CCDS database to identify enriched endothelial ligands. Copy numbers of cDNA inserts detected by NGS are the equivalent of the relative binding activities of the enriched ligands to retinal vessels and were quantitatively compared by Chi-square (χ^2) test to identify disease-related ligands. Briefly, all identified ligands and their copy numbers (i.e., counts) for diabetic and control retina were compiled into a single Excel spreadsheet. Each identified ligand had two copy numbers, one for diabetic retina (diabetic_count) and the other for control retina (control_count). Chi-square analysis to compare diabetic vs. healthy retina for individual ligands was calculated with the following formula:

$$\begin{aligned} \$chi &= (\$a * \$d - \$b * \$c) * (\$a * \$d - \$b * \$c) * (\$a + \$b + \$c + \$d) / ((\$a + \$b) * (\$b + \$d) * (\$a + \$c) * (\$d + \$c)) \\ \$a &= \$diabetic_count \\ \$b &= \$control_count \\ \$c &= \$total_diabetic_count - \$a \\ \$d &= \$total_control_count - \$b \end{aligned}$$

$\$total_diabetic_count$ and $\$total_control_count$ were total copy numbers of NGS reads matched for diabetic and control retina. In this study, $\$total_diabetic_count$ was 489,126, and $\$total_control_count$ was 473,965. Namely, $\$c = 489,126 - \a , and $\$d = 473,965 - \b . p value was calculated from Chi-square value ($\$Chi$) with the formula of $p = CHIDIST(\$Chi, 1)$.

Non-ligandomics data are expressed as mean $\pm$ SEM and compared by ANOVA with a Tukey post hoc test or Student's t-test.

4. Results

4.1. Global mapping of disease-selective endothelial ligands by comparative ligandomics

Detailed results of ligandomics analysis were recently reported (Fig. 1) (LeBlanc et al., 2017) and briefly described here. NGS analysis identified 489,126 and 473,965 valid sequence reads that aligned to 1,548 (diabetic) and 844 (healthy) ligands bound to retinal endothelium, respectively. Quantitative comparison of the entire ligandome data for diabetic vs. control retina revealed 353 “disease-high” ligands with increased binding to diabetic retinal vessels and 105 “disease-low” ligands with decreased binding (Fig. 1D). Among 1,772 all identified endothelial ligands, secretogranin III (Scg3) was uncovered as a novel diabetes-high ligand with the highest binding activity ratio to diabetic vs. healthy retinal vessels (1,731:0) and the lowest binding to control vessels (Fig. 1E). HRP-3, a previously discovered angiogenic factor (LeBlanc et al., 2015), was identified as a diabetes-low ligand (48:11,140), and VEGF was a “diabetes-unchanged” ligand (408:2,420) in 4-month-diabetic mouse retina. It is worth noting that these binding activity changes reflected the up- or down-regulation of their cognate receptors on diabetic retinal endothelium, not the expression level of ligands themselves.

4.2. Function-first validation

4.2.1. Disease-selective angiogenesis—We recapitulate our recent analysis of the angiogenic activity of Scg3 and its disease selectivity using corneal pocket assay in diabetic and control mice as previously described (Fig. 2 and 3) (LeBlanc et al., 2017). The results showed that diabetes-high Scg3 selectively induced corneal angiogenesis in diabetic but not healthy mice (Fig. 3). In contrast, diabetes-low HRP-3 promoted angiogenesis only in healthy but not diabetic corneas. As a control, VEGF induced corneal angiogenesis in both diabetic and control mice. These findings were supported not only by quantifying corneal vessel numbers, branching points and semiquantitative scores but also by staining corneal vessels with fluorescent DiI dye.

4.2.2. Disease-selective retinal vascular leakage—A major limitation of the above validation is the tissue inconsistency; namely, Scg3 was initially discovered as a diabetes-

high endothelial ligand in the retina by ligandomics, but independently verified as a diabetes-selective angiogenic factor in the cornea (LeBlanc et al., 2017). It is critical to validate Scg3 disease selectivity in diabetic vs. control retina, instead of the cornea. Intravitreal administration of angiogenic factors, such as VEGF, often induced retinal vascular leakage rather than retinal angiogenesis (Scheppke et al., 2008). As a result, we performed vascular leakage assay *in vivo* (Fig. 4A). Our results showed that intravitreal injection of Scg3 significantly induced retinal vascular leakage in diabetic but not healthy mice (Fig. 4B). As a control VEGF stimulated retinal vascular leakage in control mice. These findings suggest that indeed Scg3 is a highly diabetes-selective vascular leakage factor.

4.3. Therapy-first validation

We reported therapy-first analysis to validate Scg3 as a promising therapeutic target before developing a Scg3-neutralizing monoclonal antibody (mAb) for anti-angiogenic therapy in a recent study (LeBlanc et al., 2017). Briefly, we searched online and found that antigen-affinity purified pAbs against full-length human Scg3 were commercially available (see pAb selection criteria in Section 5). We confirmed the neutralizing activity of the pAbs to inhibit Scg3-induced endothelial proliferation assay *in vitro* (LeBlanc et al., 2017). We replaced the pAb buffer with PBS, concentrated and intravitreally injected the antibodies into 4-month-diabetic mice. Evans blue assay showed that Scg3-specific pAbs, but not mock pAb, significantly alleviated retinal vascular leakage in diabetic mice (Fig. 5). These findings led to the subsequent development of Scg3-neutralizing mAb (Fig. 5) (LeBlanc et al., 2017), which is currently under antibody humanization for clinical translation.

5. Potential pitfalls and troubleshooting

The proposed function- and therapy-first analyses are designed to be coupled to comparative ligandomics for efficient and reliable discovery of drug targets. Similar to functional proteomics (Meyer and Selbach, 2015), ligandomics may identify false positives, which will lead to negative results for target validation. Therefore, the key to successful validation of drug targets is to minimize spurious hits identified by comparative ligandomics.

Another potential pitfall is that not all identified endothelial ligands are angiogenic or vascular leakage factors. Thus, the proposed angiogenesis or vascular leakage assays are not applicable to all identified endothelial ligands. Different functional assays need to be developed for endothelial ligands that regulate other functions, such as adhesion, thrombosis, inflammation, secretion, vasoconstriction and vasodilation (Li et al., 2016; Rajendran et al., 2013; Sandoo et al., 2010; van Hinsbergh, 2012). In this regard, negative results by a specific function- or therapy-first assay do not disqualify identified proteins as endothelial ligands. An alternative solution is to perform cell-based ligand binding analyses, including *in vivo* binding assays (Cooper et al., 1999), before a specific function/therapy-first analysis.

Functional activity of disease-high ligands may depend on disease severity, as a small percentage of C57BL/6J mice treated with STZ may fail to develop diabetes. The severity of STZ-induced hyperglycemia also varied from mouse to mouse (Furman, 2015; Graham et

al., 2011). Whenever possible, fellow eyes treated with PBS should be used for data normalization to minimize the difference among individual diseased animals.

Another critical issue is how to choose commercial antibodies for therapy-first assay. Nowadays, antibodies against many proteins are commercially available, but not all of them have neutralizing activity. The following are the technical considerations to choose commercial antibodies: i) In the absence of neutralizing data, pAbs are more likely to have neutralizing activity than mAbs. ii) pAbs raised against full-length proteins are more likely to have neutralizing activity than those against only peptide fragments. iii) Ideally, pAbs should be antigen-affinity purified to minimize off-target effects. Minimally, pAbs should be purified using protein G or A columns to remove irrelevant serum proteins. iv) pAbs should be washed to remove preservatives in a filter spin unit with PBS before *in vitro* or *in vivo* therapy-first assays (LeBlanc et al., 2017).

Recombinant ligands or specific pAbs may not be always commercially available or could be technically difficult to be prepared. In these cases, function- or therapy-first analyses may not be both performed or even not at all. Nonetheless, with hundreds of disease-selective ligands identified, we estimated that more than two-thirds of identified ligands should have their recombinant proteins and/or pAbs commercially available for function- or therapy-first validation.

Comparative ligandomics is a high-throughput technology capable of identifying hundreds of disease-selective ligands. The key to drug target discovery is to develop efficient, reliable and cost-effective validation screening assays. The assays presented in this study required hyperglycemic mice to be aged for 4 months to develop diabetic vascular complications, including DR leakage. Thus, ligandomics and validation analyses presented in this study were truly “low-throughput” assays in terms of total time required. Perhaps animal models with other ocular vascular diseases, such as laser-induced CNV, could offer less time-consuming alternatives for high-throughput *in vivo* ligandomics profiling as well as subsequent efficient target validation.

6. Discussion

6.1. Unmet clinical needs for new anti-angiogenic therapies of ocular vascular diseases

Several VEGF inhibitors, including ranibizumab, aflibercept and pegaptanib, have been approved for the therapy of wet AMD and/or DR. Nonetheless, novel therapies against other angiogenic factors, such as PDGF, Ang2, integrin $\alpha_v\beta_3$, erythropoietin and endoglin, are under intense development (Cabral et al., 2017), because anti-VEGF drugs have the following limitations. First, VEGF inhibitors have limited efficacy to treat wet AMD and DR (Dedania and Bakri, 2015; Rosenfeld et al., 2006). Patients with poor response to one anti-VEGF drug are often switched to another VEGF inhibitor, in spite of their similar mechanisms of action (Mira et al., 2017; Moisseiev et al., 2015). Alternative or combination therapy against different angiogenic factors or signaling pathways may improve treatment efficacy. Second, systemic administration of VEGF inhibitors for cancer therapy may trigger severe adverse effects, including gastrointestinal perforation, delayed wound healing, fatal hemorrhagic or thromboembolic events, hypertension and proteinuria (Chen and Cleck,

2009; Falk et al., 2015). This is because VEGF regulates both diseased and healthy vasculatures. Although intravitreal administration of anti-VEGF drugs induces few short-term side effects, emerging clinical evidence suggests that these drugs may increase the long-term risk of geographic atrophy in wet AMD patients (Grunwald et al., 2017). Finally, a number of other ocular diseases, such as ROP, corneal neovascularization and intraocular tumors, still lack an FDA-approved anti-angiogenic therapy, probably due to side effects of VEGF inhibitors (Chen and Cleck, 2009; Kim et al., 2008; Lepore et al., 2014). Compared to adult retina, developing retina and vasculature in preterm ROP infants may be particularly susceptible to anti-VEGF therapy (Lepore et al., 2014).

6.2. Extracellular ligands as promising drug targets

Plasma membrane receptors are the most valuable drug targets, accounting for approximately 40% of 1,578 FDA-approved pharmacological agents (Santos et al., 2017). One of the possible reasons for this preference is that extracellular or cell surface targets are fully accessible by both small molecules and biologics, whereas intracellular targets can be accessed only by hydrophobic small molecules (Li et al., 2018). Additionally, biologics, such as antibodies, have higher target specificity, less side effects and lower drug attrition rates than small molecules (Li et al., 2018). Another possible reason is that, in contrast to intertwined intercellular signaling pathways (Logue and Morrison, 2012; Natarajan and Berk, 2006), ligand-receptor interactions often regulate well-defined cellular functions. Consequently, pharmaceutical agents targeting such extrinsic pathways likely modulate specific cellular responses with therapeutic benefits.

In this regard, we might assume that extracellular ligands as the counterparts of plasma membrane receptors would have similar high preference as therapeutic targets. Surprisingly, only a limited number of ligands have been successfully exploited as drug targets, including insulin and VEGF (Santos et al., 2017). This discrepancy is probably because receptors can be readily identified based on their transmembrane domains and cell surface expression. In contrast, it is technically difficult to identify unknown cellular ligands.

We recently developed ligandomics as the only technology to globally profile cell-wide ligands, and comparative ligandomics as the only approach to systematically map disease-selective targets, including angiogenic factors (LeBlanc et al., 2017; Li et al., 2018). Of all ligands categorized by comparative ligandomics in the binding activity plot (Fig. 1D), disease-high ligands are the most valuable drug targets. Drugs directed at disease-high ligands have the advantages of ligand-guided targeted therapies, including minimal side effects on normal cells, wide therapeutic windows and relatively low safety-related drug attrition rates (Li et al., 2018). Furthermore, drug doses could be increased within wide therapeutic windows to improve efficacy. Drugs targeting ligands that regulate both diseased and healthy vessels, such as VEGF, may trigger adverse side effects with narrow therapeutic windows, as highlighted by the side effects of bevacizumab for cancer therapy (Chen and Cleck, 2009; Falk et al., 2015). Disease-low ligands with reduced receptor expression have diminished therapeutic potential. If their receptor expression is completely silenced in diseased conditions, no agonists or antagonists can exert any therapeutic activity regardless of their potency. In this regard, the higher the binding activity ratio, the more promising the

ligand as a therapeutic target. Therefore, this study mainly focused on disease-high Scg3 with the highest binding activity ratio to diabetic vs. healthy vessels among thousands of identified endothelial ligands (LeBlanc et al., 2017). Appropriate cutoff threshold of binding activity ratio as criteria to choose disease-high ligands as therapeutic targets is yet to be determined based on drug development success rates.

6.3. Target validation for ligandomics vs. functional proteomics

Ligandomics shares a number of similarities with mass spectrometry-based functional proteomics, as elaborated in our recent review (Li et al., 2018). Briefly, both technologies are capable of identifying cellular binding proteins in large scales. Additionally, both high-throughput technologies cannot delineate the functional role of identified binding proteins and may discover a large number of false positives (Meyer and Selbach, 2015). Therefore, subsequent validation and functional characterization are critical to drug target discovery.

However, in contrast to ligandomics for globally mapping of cell-binding ligands, functional proteomics is mainly designed to efficiently identify intracellular protein-protein interaction complexes or interactomes, but not extracellular ligands. Consequently, there are major differences for target validation between these two technologies in terms of efficiency, convenience and *in vivo* applicability. First, it is much more efficient and convenient to verify the functional activity of extracellular ligands than intracellular proteins. Purified ligand proteins are often commercially available and can be directly added to cultured cells or administered into animals. Extracellular ligands, either exogenously delivered or endogenously expressed through gene transfer, can travel and regulate different cells. In contrast, intracellular proteins have to be overexpressed with technical difficulties to ensure gene transfer efficiency for all cells of interest, particularly in animals. Second, extracellular ligands can be conveniently blocked by neutralizing antibodies. By contrast, intracellular proteins can only be inhibited by either hydrophobic small molecules or gene silencing, because antibodies in general cannot efficiently penetrate through the plasma membrane. However, small molecule inhibitors may not be readily available for novel drug targets. Thus, gene silencing and deletion may become primary approaches for functional blockade to validate intracellular targets, but could be time-consuming, labor-intensive and technically challenging, particularly in animal models. Taken together, it is much more efficient and convenient to characterize functional activity, disease selectivity, pathogenic role and therapeutic potential for extracellular ligands than intracellular proteins, as demonstrated in this study. Consequently, technical difficulties for target validation in a large scale has significantly compromised the capacity of functional proteomics for efficient target discovery and drug development.

6.4. Eye as a special organ for target validation

This study exploited the unique advantages of the eye for *in vivo* target validation with the following considerations. First, the eye is a compartmentalized organ with the blood-retina barrier for optimally long pharmacokinetic retention of intravitreally injected ligands or neutralizing antibodies. This is supported by the therapeutic duration of intravitreally injected anti-VEGF drugs ranibizumab and bevacizumab for up to one month in patients (Diabetic Retinopathy Clinical Research Network et al., 2015). As a result, no repetitive

injections are necessary for angiogenesis and vascular leakage assays. However, this advantage may not be applicable to ligands with small molecular weight. In this case, purified ligands as fusion proteins with carriers could be used to increase retention time, unless fusion protein carriers may interfere with ligand function. Second, the eye is a small organ. A small amount of ligands or neutralizing antibodies purchased commercially should be sufficient for functional or therapeutic studies in more than half dozen mice for statistical analyses. Third, vitreous fluid serves as a medium for injected ligands or antibodies to diffuse and access to different cells. Penetration of full-length antibody bevacizumab (~149 kDa) or Fab fragment ranibizumab (~48.4 kDa) into the subretinal space is well supported by their therapeutic activity to treat wet AMD with CNV. Fourth, the eye is a conveniently accessible organ for precise intravitreal injection, even though appropriate training is required for the injection into the small mouse eye. Finally, the eye is a transparent organ, in which pathological neovascularization and vascular leakage can be monitored non-invasively in anesthetized animals by various imaging techniques, including funduscopy, fluorescein angiography, optical coherence tomography (OCT), OCT angiography (OCT-A) and two-photon microscopy (Egawa et al., 2013; Giannakaki-Zimmermann et al., 2016; Mugisho et al., 2018; Wang et al., 2017). Although these analyses were not performed in this study, they are particularly valuable to characterize the pathogenic role and therapeutic potential of angiogenic and vascular leakage factors.

The above described advantages of the eye as a special organ for target validation are applicable to various ocular vascular diseases, including neovascular AMD, DR, ROP, retinal vein and artery occlusion or ocular tumors. Furthermore, non-ocular tumors may be heterotopically transplanted into the subretinal space or anterior chamber to take the advantages of the eye with immune privilege for drug target discovery and validation (Taylor, 2016; Wenkel et al., 1999).

7. Concluding Remarks

To our knowledge, function- and therapy-first comparative ligandomics proposed and demonstrated in this study is the only technology to not only systematically map but also reliably validate disease-selective ligands (Fig. 6). Although this study focused on an angiogenic/vascular leakage factor, ligandomics is broadly applicable to different cells or diseases in *in vitro* and *in vivo* settings (Li et al., 2018). For example, ligandomics may systematically map neurotrophic factors to promote neuron survival, axon growth factors to stimulate growth cones and axon regeneration, niche factors to maintain the stemness of progenitor cells, and immunoregulatory ligands for immunotherapy. Similarly, function/therapy-first ligandomics is broadly applicable to different cells, diseases and organs beyond ocular or vascular research. Developing appropriate function- and/or therapy-first assays is the key to efficient and reliable discovery of therapeutic targets. Targeted therapies directed at highly disease-selective ligands confer the advantages of optimal efficacy, minimal adverse side effects, wide therapeutic windows, low drug attrition rates and reduced R&D costs.

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Highlights

- A new concept of function- and/or therapy-first comparative ligandomics
- Ligandomics is the only technology to globally map cell-wide ligands
- The only high-throughput approach to map disease-selective cellular ligands
- Efficient and reliable identification and validation of disease-selective ligands
- Disease-selective ligands are high-quality drug targets with minimal side effects

Fig. 1.

Comparative ligandomics to systematically map diabetes-selective endothelial ligands. **(A)** Multi-round *in vivo* binding selection by open reading frame phage display (OPD) to enrich retinal endothelial ligands in diabetic and healthy mice. **(B)** Global identification of all enriched ligands. After 3 rounds of selection, cDNA inserts of enriched ligands were amplified by PCR and identified by next generation sequencing (NGS) with simultaneous binding activity quantification for all identified ligands. **(C)** Quantitative comparison of entire ligandome profiles for diabetic vs. healthy retina to systematically identify diabetes-selective endothelial ligands. **(D)** Binding activity plot for diabetic vs. healthy retina. All binding ligands are categorized into diabetic retinopathy (DR)-high, DR-low, DR-unchanged ligands and background binding. Pearson correlation coefficient $r=0.489$. **(E)** Enrichment of DR-high Scg3, DR-low HRP-3 and DR-unchanged VEGF. GFP was minimally enriched. (Adapted and modified with permission from Ref #(LeBlanc et al., 2017)).

Fig. 2.

Corneal pocket angiogenesis assay. **(A)** Whatman filter paper was cut into small pieces (~0.125 mm²). **(B)** Filter papers were soaked in a drop of solution with different growth factors or PBS mock control on parafilm. **(C)** Making an incision on the cornea of anesthetized mice. **(D)** Inserting filter paper into corneal pocket. **(E)** Quantifying corneal angiogenesis 6 days post implantation.

Fig. 3.

In vivo function-first validation of Scg3 as a diabetes-high angiogenic factor. (A)

Representative photographic images of corneal pocket angiogenesis assay in diabetic and healthy mice. (B) DiI staining of corneal blood vessels. * in A,B indicates the position of corneal implant. Scale bar = 500 μ m in (A,B). (C-E) Quantification of corneal angiogenesis in a blinded manner. (C) Total number of corneal vessels. (D) Number of branching points. (E) Total angiogenesis score. Diabetes-high Scg3 selectively stimulated corneal angiogenesis in diabetic but not control mice. In contrast, diabetes-low HRP-3 induced angiogenesis in healthy but not diabetic mice. VEGF promoted angiogenesis in both healthy and diabetic mice. Sample sizes (# of cornea) are indicated at the bottom of the graphs. $\pm$ SEM, one-way ANOVA test. n/s for not significant (Reproduced with permission from Ref # (LeBlanc et al., 2017)).

Fig. 4.

In vivo function-first validation of Scg3 as a DR-high retinal vascular leakage factor. (A) Schematics of Evans blue assay. (B) Scg3 selectively induced retinal vascular leakage in diabetic but not control mice. In contrast, VEGF stimulated vascular leakage in control mice. These data suggest that Scg3, but not VEGF, is a DR-selective retinal vascular leakage factor. $\pm$ SEM, $n=7$ mice (VEGF), $n=5$ mice (diabetic or healthy Scg3); paired Student's *t*-test. n/s, not significant.

Fig. 5.

In vivo therapy-first approach to validate the therapeutic potential of Scg3 before committing to development of Scg3-neutralizing mAb. Antigen-affinity purified pAbs against full-length Scg3 were purchased commercially. pAbs were intravitreally injected into one eye of diabetic mice with PBS for the fellow eye. Data are normalized to PBS fellow eye. After therapy-first validation, Scg3-neutralizing ML49.3 mAb was developed and verified with the similar therapeutic activity. $n = 5$ mice (except $n = 3$ for mock pAb) (Reproduced with permission from Ref #(LeBlanc et al., 2017)).

Fig. 6. Function- and therapy-first comparative ligandomics for systematic and reliable mapping of disease-selective ligands as drug targets. **(A)** *In vivo* comparative ligandomics for high-throughput mapping of disease-selective or restricted endothelial ligands (LeBlanc et al., 2017). **(B)** Low-throughput function- and/or therapy-first screening to validate identified ligands.