Review Article

Lgr4 in Ocular Development and Glaucoma

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Received 25 March 2013; Accepted 15 May 2013

Academic Editor: Sahin Afsun

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The leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4, also called GPR48) plays a key role in multiple developmental processes, and mice lacking Lgr4 display anterior segment dysgenesis leading to early-onset glaucomatous retinal ganglion cell loss as well as defective eyelid formation. This paper will review Lgr4 signaling and its regulation of the Axenfeld-Rieger syndrome gene Pitx2, a crucial developmental transcription factor. In addition, Wnt signaling plays an important role in eye development, with Norrin functioning to activate the Wnt receptor Frizzled 4 required for proper retinal vascularization. Recent discoveries identifying Lgr4 as a receptor for Norrin highlight the potential for Lgr4 function in retinal vascularization. Finally, several unanswered questions impeding a full understanding of Lgr4 in glaucoma are considered as avenues for further research.

1. Introduction

Glaucoma is the second leading cause of blindness worldwide, but its etiology is complex and only partially understood. Frequently associated with elevated intraocular pressure (IOP) leading to a stereotypical pattern of retinal ganglion cell loss and cup excavation, glaucoma may result from closure of the iridocorneal angle, blockage of the trabecular meshwork responsible for aqueous humor outflow, pupil block of the space between the lens and iris necessary for humor circulation between the anterior and posterior chambers, and several other causes. Loss of the leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4, also called GPR48) plays a key role in multiple developmental processes, and mice lacking Lgr4 display anterior segment dysgenesis leading to early-onset glaucomatous damage in humans [1]. Recently published work identifies Lgr4 as a receptor for Norrin, a secreted protein with established roles in retinal neuron protection and retinal vascularization and therefore suggests an additional mechanism by which Lgr4 functions to prevent glaucoma [2]. This paper will provide an overview of Lgr4 signal transduction and its role in a wide variety of developmental processes, followed by a focus on recent developments in the role of Lgr4 in glaucoma.

2. Lgr4 Signaling

The leucine-rich repeat domain-containing G protein-coupled receptors (LGRs) feature a large N-terminal extracellular domain containing multiple leucine-rich repeats and are subdivided into three groups. One group consists of the three glycoprotein hormone receptors: luteinizing hormone receptor, follicle-stimulating hormone receptor, and thyroid stimulating hormone receptor, and thyroid stimulating hormone receptor. The second group contains LGR4–6, three receptors sharing high homology (~50% sequence identity) that were recently found to act as receptors for the Wnt-potentiating R-spondins, indicating that this group can signal through both G protein-coupled as well as Wnt-signaling pathways. The third group is comprised of the relaxin receptors LGR7 and 8.

Lgr4 has been shown to signal through classical Gαs-mediated signaling in multiple systems. In this pathway (Figure 1), ligand binding to Lgr4 leads to G protein activation through GTP binding. Coupled Gαs then dissociates
Figure 1: Signaling pathways downstream of Lgr4. Left: binding of unknown ligands (?) leads to $G_{\alpha}$ activation of adenylyl cyclase (AC) and resulting increase in intracellular cyclic AMP levels (cAMP). Elevated cAMP activates protein kinase A (PKA), which phosphorylates CREB inducing its nuclear translocation and regulation of CRE target genes such as Pitx2. Right: Norrin or R-spondin (Norrin/RSPO) binding to Lgr4 augments the response of Frizzled (Fzd) and LRP5/6 to Wnt binding, leading to activation of Disheveled (DSH). DSH blocks the GSK3/Axin2/APC-mediated degradation of $\beta$-catenin ($\beta$-cat), allowing $\beta$-catenin accumulation and nuclear translocation (dashed arrow) where $\beta$-cat binds TCF/LEF (TCF) family transcription factors to regulate Wnt target gene expression. Dikkopf (Dkk) is a competitive inhibitor of Wnt binding to Fzd.

from Lgr4 to activate adenylyl cyclase resulting in elevated levels of the second messenger cyclic AMP (cAMP); cAMP, in turn, activates protein kinase A (PKA), which phosphorylates the transcription factor Cre-binding protein, leading to elevated expression of target genes containing CRE binding motifs in their promoter. Known Lgr4 targets regulated through cAMP/PKA/CRE signaling include the mineralocorticoid receptor [3], estrogen receptor $\alpha$ in the male reproductive tract [4], ATF4 in bone development and definitive erythropoiesis [5, 6], and Pitx2 in eye development [1]. Significantly, the ligand(s) initiating cAMP/PKA signaling by Lgr4–6 remains unidentified. However, a constitutively active mutant, T755ILgr4, has been reported which results in elevated cellular cAMP levels and CREB activity [1, 7].

The Lgr4 family members Lgr5 and 6 have been implicated in stem cell maintenance in a variety of tissues. Lgr5 is a stem cell marker in the small intestine [8], stomach [9], liver [10], hair follicle [11], and, most recently, mammary gland [12, 13]. Lgr6 marks the most primitive kind of epidermal stem cell [14]. A single Lgr5 expressing colon cell was able to generate organoids that persist in culture and can engraft to form functional crypts in vivo [15]. Conditional loss of Lgr5 leads to depletion of stem cells in the mammary gland [12], implying that Lgr5 signaling has a functional role in stem cell self-renewal. The most likely mechanism for this role is by a second signal transduction pathway: Lgr mediation of Wnt signaling potentiation by R-spondin. R-spondin binding to LGR4–6 inhibits ZNRF3 and RNF43, negative regulators of Wnt signaling which promote degradation of the Wnt receptor Frz and the Wnt coreceptors LRP5/6 [16]. Thus, Lgr4 and its family members function to increase the membrane concentration of Wnt receptors in the presence of R-spondin, enhancing the signaling response to low levels of Wnt ligand. An alternative mechanism for R-spondin signaling has also been proposed, in which R-spondin-bound Lgrs bind directly to LRP6 to augment LRP6 phosphorylation in response to Wnt-Fzd binding [17]. Clathrin was also reported to be required for Lgr4 mediation of R-spondin in vitro [18]. Most recently, Lgr4 was shown to be a receptor for Norrin, another canonical Wnt signaling potentiator [2], providing another means by which Lgr4 modulates Wnt signaling.

3. Lgr4 in Development

Lgr4 mRNA expression in mice was first detected at E7 and in adult mice was the highest in liver, then kidney, with moderate expression in muscle, heart, and brain, and low levels in testes and lung [19]. Mazerbourg et al. (2004) [20]
first described the mouse expression pattern of Lgr4 protein, using both IHC staining of wild-type tissue as well as
transgenic mice expressing β-galactosidase from the Lgr4
promoter. They noted moderate Lgr4 expression in neonatal
kidney, adrenal, stomach, spine, ribs, brain, nasal cavity,
heart, and intestines, with lower levels in liver, lung, and
spleen. No Lgr4 was detected in skeletal muscle or pancreas.
Adults had a very similar pattern of Lgr4 expression, with
reduced heart Lgr4 and higher liver levels; also, no lung or
spleen expression was detected in adult Lgr4−/−[20]. Lgr4
mRNA expression in adult humans is the highest in the
pancreas, with moderate levels in liver, heart, and muscle, and
very low brain and kidney expression[19].

Two approaches have been used by different labs to
generate Lgr4−/− mice. In one approach, a gene trap cassette
was inserted in the first intron to generate a chimeric mRNA
containing the N-terminal Leucine-rich repeat of Lgr4 fused
to the CD4 transmembrane domain and the β-galactosidase
coding sequence[1, 20]. This approach showed that Lgr4 is an
essential gene for embryonic development; 60% of expected
Lgr4+/− pups died in utero, and the majority of remaining
Lgr4 null pups died perinatally in a C57Bl6/’ X Swiss Webster
background; modest (~10%) declines in expected numbers of
Lgr4−/− mice were also reported[20]. Lgr4−/− pups showed
embryonic growth retardation (14% decrease in Lgr4−/−
neonatal pup weight), with a significant decrease in liver and
kidney weight[20]. Background strain has a strong effect on
the embryonic and perinatal survival of Lgr4−/− mice, with
higher lethality in 129Ola x C57Bl6 mice[21], but 60% of
Lgr4 null mice on a CBA background survive[22]; most CD1
strain Lgr4−/− mice also survive to adulthood[23].
The alternative approach is to perform targeted knockout of
Lgr4 exon 18, containing the seven-transmembrane domain.
This approach revealed a similarly high level of embryonic
lethality, with embryonic kidney hypoplasia[21], later shown
to be accompanied by premature ureteric bud differentiation
[24].

A wide variety of developmental defects have been
reported in Lgr4−/− mice. In addition to the embryonic
lethality and decreased growth noted above and the eye
defects discussed below, midgestational erythropoiesis is
disrupted in Lgr4−/− embryos, with a 32% decrease in Ter119+
cells at E13.5 as compared to wild type[6]. Lgr4 loss delays
osteoblast differentiation, resulting in decreased embryonic
bone formation; bone formation kinetics and bone mineral
density were decreased through adulthood in Lgr4−/− mice
[5]. Lgr4−/− mice also lack a gall bladder and cystic duct,
although the common hepatic duct and intrahepatic bile duct
appeared unchanged[25]. Lgr4 regulates expression of the
mineralocorticoid receptor, implicating Lgr4 in electrolyte
homeostasis[3]. Both male[4, 22, 23, 26] and female[27]
reproductive tract formation is impaired in Lgr4−/−, leading
to infertility in homozygous null mice. Intestinal epithelial
cell proliferation was reduced in Lgr4−/− mice, with an 80%
decrease in crypt Paneth cells, suggesting that Lgr4 plays a
crucial role in intestinal stem cell maintenance[28]; Lgr4 also
appears to play a protective role against inflammatory bowel
disease[29].

Conditional knockout approaches have also been used to
avoid the embryonic lethality of Lgr4 loss and have revealed
additional developmental phenotypes. Mice with specific
ablation of Lgr4 in cells expressing keratin 5 showed focal
alopecia and fewer hair placodes, implicating Lgr4 action in
hair follicle development[30]. Loss of Lgr4 in keratin 5+ cells
also disrupted mammary gland branching morphogenesis
and delayed ductal elongation[31] and was proposed to
impair oviduct function in promoting embryo development
[27]. Therefore, Lgr4 is a key regulator of organ development
in a wide variety of extraocular systems.

3.1. Lgr4 in Eye Development. Lgr4 is normally expressed
in a finely tuned spatiotemporal pattern in the developing
eye. Using Lgr4-driven β-galactosidase expression to probe
for Lgr4 expression in Lgr4 heterozygotes, we found that at
E12.5, Lgr4 is primarily expressed in a layer of mesenchymal
cells between the surface ectoderm and the optic cup; limited
expression is also detectable in the lens and the outer layer of
the optic cup (Figure 2). By E16.5, high-level Lgr4
expression is seen in the tips of the optic cup and surrounding
mesenchyme, which later form the iris and ciliary body,
with lower levels in the cornea, retina, and lens. Neonatal
mice retain strong expression in the ciliary body, iris stroma,
and lens, and corneal epithelial cell layer, with lower expression
in keratoepithelial and endothelial cells. Adult Lgr4−/− mice
express Lgr4 in the lens epithelium, retinal ganglion and
inner nuclear layer, iris stroma, and outer ciliary body cell
layer[1].

Using the gene trap approach described above, we ge-
genreated Lgr4−/− mice which had multiple ocular defects. 25
out of 47 Lgr4−/− mice had microphthalmia, with a much
higher incidence in males (75%) compared to females (37%),
suggesting that Lgr4 interacts with a sex-linked factor in this
phenotype[1]. Partial corneal opacity, usually accompanied
by corneal neovascularization, was present in 18 of 47 mice.
A variety of keratopathies (corneal epithelial plug, corneal
inflammation, corneal cyst-like structures, and corneal vas-
cular pannus) were each found in 4 or more mice, with a
higher frequency in male mice[1]. Cataracts formed in 13 of
47 Lgr4−/− mice, which were correlated with higher levels of
insoluble aA-crystallin in the lenses of Lgr4−/− mice.

Furthermore, Lgr4 plays an essential role in eyelid develop-
ment. 85% of Lgr4−/− mice had a failure of eyelid fusion
in utero leading to eye open at birth, and adult Lgr4−/− mice
exhibited exposure keratitis[32]. In a separate paper using
an Lgr4 exon 18 deletion, Lgr4−/− mice had complete eye
open at birth penetration, but these mice did not survive to
adulthood[33]. Lgr4 is normally expressed in the eyelid tip
basal epithelium and mesenchyme at E14–E16. Lgr4−/− mouse
eyelids were morphologically similar to those of Lgr4+/− at
E12.5 but had decreased eyelid epithelial cell proliferation.
Eyelid extension towards the corneal center was noticeably
decreased by E15.5 in the absence of Lgr4[33], with fewer
Figure 2: Temporal and spatial expression of Lgr4 in mouse eyes. (a) Expression of Lgr4 at embryonic day E12.5 using β-galactosidase staining in Lgr4 heterozygous (+/−) and wild-type control (+/+ ) mice. (b) Expression of Lgr4 at postnatal day 0 (P0). Scale bar = 170 μm. (c) Temporal expression of Lgr4 during different stages of anterior segment development. At E12.5 days, Lgr4-expressing mesenchymal cells are located between the surface ectoderm and the lens and the inner layer cells of the optic cup. At E16.5–E18.5, Lgr4 expression is high at the tips of the optic cup and in the surrounding mesenchymal tissue. In newborn embryos, Lgr4 is highly expressed in the iris stroma, ciliary body, corneal epithelium, keratocytes, and endoepithelial cells. Wild-type (+/+) mice lack expression of β-galactosidase. Scale bars = 170 μm. (d). Expression of Lgr4 in adult mouse tissue. Staining was found in lens epithelial cells, retinal ganglion cells, inner nuclear layer, iris stroma, and the outer layer of the ciliary body. C: cornea; I: iris; R: retina; L: lens; CB: ciliary body; G: ganglion cells; IN: inner nuclear layer; ON: outer nuclear layer. Scale bars = 85 μm. Originally published PNAS 105(16):6081-6. Copyright 2008 National Academy of Sciences USA.

filopodia [32]. Cultured Lgr4−/− keratinocytes had decreased migration [33] and proliferation and decreased phospho-EGFR, suggesting that Lgr4 regulates EGFR activation [32]. Inhibition of EGFR reduced keratinocyte proliferation and migration to levels seen in Lgr4−/− cells, and inhibitory antibodies against HB-EGF also reduced wild-type keratinocyte proliferation to Lgr4−/− levels, suggesting that Lgr4 may activate EGFR signaling in keratinocytes through upregulation of
metalloproteases that generate HB-EGF; however, this model awaits confirmation in vivo [34].

Finally, anterior segment dysgenesis (ASD) was common in mice lacking Lgr4. Lgr4−/− mice displayed iris hypoplasia with decreased stroma and smooth muscle as early as postnatal day 4, diminished ciliary body size and folding, compressed trabecular meshworks with fewer and smaller beams, and a sharper iridocorneal angle that in some cases (13%) was completely closed. Lgr4−/− retinas had detectable loss of inner nuclear layer ganglion cells and disruption of the outer nuclear layer beginning at 6 months of age in 42% of mice examined (10 of 24), strongly implicating ASD resulting from Lgr4 loss in early-onset glaucoma [1]. Several lines of evidence implicate the Axenfeld-Rieger syndrome-related gene Pitx2 as a key mediator of Lgr4 in eye development. First, the ASD phenotype in Lgr4−/− mice closely matches that seen in Pitx2−/− mice, including iris hypoplasia and pericellular musculature defects. Second, Pitx2 was the only transcription factor out of a panel of fourteen key eye development genes to be downregulated in Lgr4−/− mice. Finally, Pitx2 was shown to be a direct downstream target of Lgr4 signaling through the cAMP/PKA/CREB pathway [1].

4. Pitx2 and Axenfeld-Rieger Syndrome

Axenfeld-Rieger syndrome (ARS) is a rare genetic disease generally with autosomal dominant inheritance characterized by ocular disorders (potentially including iris hypoplasia, corectopia, pseudopolycoria, posterior embryotoxon, and iris strands connecting to the trabecular meshwork or other angle structure anomalies) resulting in elevated intraocular pressure, sometimes accompanied by craniofacial abnormalities (telecanthus, hypertelorism) or dental defects (small or missing teeth). ARS patients have a high risk for glaucoma [35]. Cardiovascular abnormalities are also reported in ARS patients [36–39], as well as hearing loss in some cases [36, 40]. Mutations in either Pitx2 or FOXC1 have been estimated to account for 40% of ARS cases [35, 41–45]. Pitx2 is a paired-like homeodomain transcription factor. Mice heterozygous for Pitx2 display multiple anterior segment defects similar to ARS, including corneal endoderm and iris stroma agenesis, corneal mesothelial thickening, coloboma formation, and shortened ventral retina, and Pitx2−/− mice are embryonic lethal due to incomplete closure of the ventral body wall [46, 47]. Loss of extraocular musculature has also been reported in Pitx2 heterozygotes, with a more severe phenotype in Pitx2−/− mice [48]. Pitx2 is normally expressed in the neural crest during development beginning at E9.5, as well as in the developing eye mesoderm, hyaloid space, and eyelid mesenchyme [49, 50]. There is a strong dosage dependence of Pitx2 for proper development; transgenic mice overexpressing PITX2A in the corneal mesenchyme and iris show corneal hypertrophy, corneal opacification, iridocorneal attachments, and retinal degeneration [51]. Intriguingly, a downstream target of Pitx2 is the Wnt-signaling antagonist Dkk2, which plays a crucial role in regulating anterior segment morphogenesis [52].

5. Norrin and Wnt Signaling in Eye Development

Canonical Wnt/β-catenin signaling plays an essential role in development and in tissue homeostasis. Key components of the pathway are the Wnt proteins, a family of 19 secreted glycoproteins, the Frizzled (Fzd) receptors, which are seven membrane-spanning receptors for Wnt, and the Wnt coreceptors LR5 and 6. Wnt binding results in activation of the downstream transducer Dishevelled (Dsh), which phosphorylates glycogen synthase kinase 3 (GSK-3) resulting in inactivation of the Axin/APC complex that normally ubiquitylates β-catenin (Figure 1). As a consequence, β-catenin accumulates and translocates to the nucleus, where it associates with Tcf/Lef transcription factors to regulate gene transcription. Negative regulators of Wnt signaling include the Dikkopf (Dkk) and secreted Frizzled-related proteins (sFRP) which bind to Fzd or Wnt, respectively, to inhibit cascade activation. Loss of β-catenin in the pericellular ectoderm results in ectopic lenticoid body formation whereas presumptive lens ectoderm expression of dominant active β-catenin suppresses lens development [53], suggesting that low levels of β-catenin signaling are required for proper lens formation. Ectoderm-specific active β-catenin expression disrupts optic cup patterning resulting in a lack of cornea, conjunctiva, and eyelid development [54]. Dkk2−/− mice display a transformation of corneal epithelium into a stratified epithelium [55], iridocorneal adhesion, eye open at birth phenotype with hypomorphic eyelids and ectopic hair follicles in the presumptive conjunctiva, and ectopic corneal stroma vasculature [52]. Therefore, proper spatiotemporal control of Wnt/β-catenin pathway signaling intensity is essential for anterior segment development.

In addition to playing a crucial role in anterior segment development, Wnt signaling was reported to have a significant ongoing function in trabecular meshwork (TM) cell regulation. RNA differential display of cultured human TM cells identified elevated expression of the Wnt antagonist secreted Frizzled-related protein 1 (sFRP1) in cells from glaucomatous donors, and perfusion of sFRP1 into cultured human eyes resulted in decreased canonical Wnt signaling and reduced aqueous outflow. Intracocular injection of adenoviral vectors to express sFRP1 in BALB/c mice resulted in a twofold increase in intraocular pressure (IOP), an increase that was partially reversible through topical application of a GSK-3 inhibitor, suggesting that sFRP-1 increased IOP through inhibiting canonical Wnt/β-catenin signaling [56].

Intriguingly, Pitx2 had been reported to be a direct Wnt target gene in culture [57, 58], and Dkk2−/− mice have elevated Pitx2 expression in the corneal stroma, suggesting the existence of a negative feedback loop regulating Pitx2 expression during eye development [52]. We speculate that Lgr4 may play a crucial role in integrating these signaling circuits, as Lgr4 binding to R-spondin potentiates Wnt signaling, leading to Pitx2 expression, and activation of G protein signaling through a binding of an unknown ligand to Lgr4 results in Pitx2 activation and resulting Dkk2 expression, which then inhibits Wnt signaling even in the presence...
of both R-spondin and Wnt. Whether the failure of this signaling circuit is responsible for the ocular defects in Lgr4−/− mice remains to be determined.

5.1. Norrin in Retinal Vascularization and Function. Norrin has been shown to enhance Wnt signaling in vitro and to selectively bind to the Wnt receptor Fzd4 with high affinity to activate canonical Wnt/β-catenin signaling [59]. Mutations in the NDP gene encoding Norrin result in Norrie disease, an X-linked congenital syndrome characterized by retinal vascularization failure leading to blindness, often accompanied by microcephaly, deafness, hypogonadism, or mental retardation. Familial exudative vitreoretinopathy, a less severe disruption in peripheral retina vascularization, can also be caused by mutations in NDP, or alternatively by mutations in Fzd4 or LRPS. Loss of the mouse homologue, Ndp, causes defects in retinal vasculature which lead to blindness as well as cochlear vasculature, and results in female infertility due to defects in decidualization [59–63]. Curiously, a similar defect in retinal vascularization has been reported in mice lacking the Wnt receptor Fzd4 [59] or coreceptor Lrp5 [64]. Norrin has very recently been reported to be a ligand for Lgr4−6, suggesting that it plays a role in Wnt signal potentiation similar to that played by R-spondin family members [2]. Norrin is normally expressed by Müller glial cells of the mouse retina [65]; however, retinal vasculization defects in Ndp−/− mice are overcome by lens-specific expression of Norrin [66], implying a paracrine mode of action that does not require spatial concentration gradient formation. Systemic Norrin overexpression is embryonic lethal, marked by defective angiogenesis, but can be rescued on either an Fzd4−/− or an Lrp5−/− background [65]. Retinal pigment epithelial cell expression of Norrin ablated the extent of oxygen-induced retinopathy in young (P7–11) mice, implying a protective effect of Norrin on retinal vasculature [67]. Curiously, inhibition of Fzd4 signaling reduced the recovery from oxygen-induced retinopathy [68]. Angiopoietin-2, an angiogenic growth factor expressed in the retinal microvasculature endothelium, has been proposed to be a downstream mediator of Norrin’s role in angiogenesis, based on (1) a similar defect in retinal vascularization in Ang-2−/− mice, (2) evidence for Norrin regulation of Ang-2 expression in vivo, and (3) loss of Norrin-induced increased endothelial cell proliferation in the presence of Ang-2-blocking antibodies [67, 69, 70].

In addition to the retinal vascularization defects, Ndp−/− mice exhibit a loss of retinal ganglion cells (RGCs), with concomitant decrease in normal retinal function [60, 61, 66]. Lens-specific transgenic expression of Norrin increased RGC proliferation and resulted in thicker retinas in mice two days postnatal [66]. Norrin has also been reported to have a neuroprotective effect on retinal ganglion cells. Ocular injection of Norrin reduced the extent of RGC apoptosis and axon loss following NMDA injection [71], which is in agreement with a report demonstrating antiapoptotic effects of Norrin on a cultured RGC cell line [72]. The RGC protective effect of Norrin was partially mediated through induction of several growth factors (including fibroblast growth factor 2, ciliary neurotrophic growth factor, and brain-derived neurotrophic growth factor) by Müller cells, and partly through a direct effect of Norrin on RGCs [71]. Intriguingly, no activation of canonical Wnt/β-catenin signaling was detected in RGCs following Norrin treatment, suggesting that Norrin acts through a yet-undiscovered signal transduction pathway to prevent excitotoxicity in RGCs. Given the recent finding that Lgr4 is able to bind Norrin [2], this leads to the speculation that this neuroprotective effect of Norrin may be mediated by G protein-coupled signaling downstream of Lgr4.

6. Future Directions

In summary, there is strong evidence that Lgr4 is a key regulator of Pitx2 in anterior segment formation and that disruption of this signaling network can result in early-onset glaucoma in a mouse model. Furthermore, tantalizing new evidence suggests that Lgr4 functioning as a receptor for Norrin may be implicated in retinal vascularization and/or other functions of Norrin, such as retinal neuron protection from damage. However, a host of critical questions remain. First, what is the endogenous ligand activating Lgr4 signaling through the cAMP/PKA pathway resulting in regulation of Pitx2 and multiple other downstream targets? Identification of this unknown Lgr4 activator is essential for mapping the spatiotemporal profile of Lgr4 activity during development and may serve as a starting point for designing small-molecule agonists or inhibitors for potential therapeutic use. Second, which Norrin functions are mediated by Lgr4 or its close family members? Could Norrin be the unknown sex-linked factor responsible for the higher incidence of microphthalmia in male Lgr4−/− mice? Deciphering the relative importance of classical G protein-coupled versus Wnt/β-catenin signaling in Lgr4 function remains to be unraveled.

A third unresolved question concerns additional functions of Lgr4. An area of particular therapeutic interest is the use of stem cells for glaucoma treatment. Some evidence exists suggesting the existence of stem or progenitor cells in the region of Schwalbe’s line (called by several names including Schwalbe’s line cells, trabecular meshwork insert cells, and progenitors for endothelium and trabeculum, and functionally assayed using sphere formation in vitro; see [73] for an excellent review). Given the roles of Lgr5 and Lgr6 in stem cell self-renewal in a wide variety of tissues, together with the established function of R-spondin (and Norrin) to potentiate Wnt signaling through Lgr4−6, we speculate that Lgr4 or its family members may play a key role in maintenance of stem/progenitor cells in this region. Understanding such a role will be vital in progress towards using stem cells to regenerate a blocked trabecular meshwork as an approach towards relieving intraocular pressure.

References

[1] J. Weng, J. Luo, X. Cheng et al., “Deletion of G protein-coupled receptor 48 leads to ocular anterior segment dysgenesis (ASD)
through down-regulation of Pitx2,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 16, pp. 6081–6086, 2008.

[2] C. Deng, P. Reddy, Y. Cheng, C. W. Luo, C. L. Hsiao, and A. J. Hsueh, “Multi-functional norrin is a ligand for the LGR4 receptor,” *Journal of Cell Science*, 2013.

[3] J. Wang, X. Li, Y. Ke et al., “GPR48 increases mineralocorticoid receptor gene expression,” *Journal of the American Society of Nephrology*, vol. 23, no. 2, pp. 281–293, 2012.

[4] X. Y. Li, Y. Lu, H. Sun et al., “G protein-coupled receptor 48 upregulates estrogen receptor α expression via cAMP/PKA signaling in the male reproductive tract,” *Development*, vol. 137, no. 1, pp. 151–157, 2010.

[5] J. Luo, W. Zhou, X. Zhou et al., “Regulation of bone formation and remodeling by G-protein-coupled receptor 48,” *Development*, vol. 136, no. 16, pp. 2747–2756, 2009.

[6] H. Song, J. Luo, W. Luo et al., “Inactivation of G-protein-coupled receptor 48 (Gpr48/Lgr4) impairs definitive erythropoiesis at midgestation through down-regulation of the ATF4 signaling pathway,” *The Journal of Biological Chemistry*, vol. 283, no. 52, pp. 36687–36697, 2008.

[7] Y. Gao, K. Kitagawa, M. Shimada et al., “Generation of a constitutively active mutant of human GPR48/LGR4, a G-protein-coupled receptor,” *The Hokkaido Journal of Medical Science*, vol. 81, no. 2, pp. 101–109, 2006.

[8] N. Barker, J. H. van Es, J. Kuipers et al., “Identification of stem cells in small intestine and colon by marker gene Lgr5,” *Nature*, vol. 449, no. 7165, pp. 1003–1007, 2007.

[9] N. Barker, M. Huch, P. Kujala et al., “Lgr5<sup>tot</sup> stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro,” *Cell Stem Cell*, vol. 6, no. 1, pp. 25–36, 2010.

[10] M. Huch, C. Dorrell, S. F. Boj et al., “In vitro expansion of single Lgr5<sup>+</sup> liver stem cells induced by Wnt-driven regeneration,” *Nature*, vol. 494, pp. 247–250, 2013.

[11] V. Jaks, N. Barker, M. Kasper et al., “Lgr5 marks cycling, yet long-lived, hair follicle stem cells,” *Nature Genetics*, vol. 40, no. 11, pp. 1291–1299, 2008.

[12] V. Plaks, A. Brenot, D. A. Lawson et al., “Lgr5-expressing cells are sufficient and necessary for postnatal mammary gland organogenesis,” *Cell Reports*, vol. 3, no. 1, pp. 70–78, 2013.

[13] K. E. de Visser, M. Ciampricotti, E. M. Michalak et al., “Developmental stage-specific contribution of LGR5<sup>+</sup> cells to basal and luminal epithelial lineages in the postnatal mammary gland,” *The Journal of Pathology*, vol. 228, no. 3, pp. 300–309, 2012.

[14] H. J. Snippert, A. Haegerbath, M. Kasper et al., “Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin,” *Science*, vol. 327, no. 5971, pp. 1385–1389, 2010.

[15] S. Yui, T. Nakamura, T. Sato et al., “Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5<sup>+</sup> stem cell,” *Nature Medicine*, vol. 18, no. 4, pp. 618–623, 2012.

[16] X. Hao, Y. Xie, Y. Zhang et al., “ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner,” *Nature*, vol. 485, pp. 195–200, 2012.

[17] K. S. Carmon, X. Gong, Q. Lin, A. Thomas, and Q. Liu, “R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 28, pp. 11452–11457, 2011.

[18] A. Glinka, C. Dolde, N. Kirsch et al., “LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signalling,” *EMBO Reports*, vol. 12, no. 10, pp. 1055–1061, 2011.
[35] M. H. Strungaru, I. Dinu, and M. A. Walter, “Genotype-phenotype correlations in Axenfeld-Rieger malformation and glaucoma patients with FOXC1 and PITX2 mutations,” Investigative Ophthalmology & Visual Science, vol. 48, no. 1, pp. 228–237, 2007.

[36] E. T. Cunningham Jr., D. Elliott, N. R. Miller, I. H. Maumenee, and W. R. Green, “Familial Axenfeld–Rieger anomaly, atrial septal defect, and sensorineural hearing loss: a possible new genetic syndrome,” Archives of Ophthalmology, vol. 116, no. 1, pp. 78–82, 1998.

[37] J. Antevil, R. Umakanthan, M. Leacche et al., “Idiopathic mitral valve disease in a patient presenting with Axenfeld–Rieger syndrome,” The Journal of Heart Valve Disease, vol. 18, no. 3, pp. 349–351, 2009.

[38] N. A. Bekir and K. Gungör, “Atrial septal defect with interatrial septal aneurysm and Axenfeld–Rieger syndrome,” Acta Ophthalmologica Scandinavica, vol. 78, no. 1, pp. 101–103, 2000.

[39] J. C. Tsai and A. L. Grajewski, “Cardiac valvular disease and Axenfeld–Rieger syndrome,” American Journal of Ophthalmology, vol. 118, no. 2, pp. 255–256, 1994.

[40] S. Grosso, M. A. Farnetani, R. Berardi et al., “Familial Axenfeld–Rieger anomaly, cardiac malformations, and sensorineural hearing loss: a provisionally unique genetic syndrome?” American Journal of Medical Genetics, vol. 111, no. 2, pp. 182–186, 2002.

[41] E. V. Semina, R. Reiter, N. J. Leysens et al., “Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome,” Nature Genetics, vol. 14, no. 4, pp. 392–399, 1996.

[42] K. Kozlowski and M. A. Walter, “Variation in residual PITX2 activity underlies the phenotypic spectrum of anterior segment developmental disorders,” Human Molecular Genetics, vol. 9, no. 14, pp. 2131–2139, 2000.

[43] A. S. Borges, R. Susanna Jr., J. C. E. Carani et al., “Genetic analysis of PITX2 and FOXC1 in Rieger syndrome patients from Brazil,” Journal of Glaucoma, vol. 11, no. 1, pp. 51–56, 2002.

[44] N. Weisschuh, E. de Baere, B. Wissinger, and Z. Tümer, “Clinical utility gene card for: Axenfeld–Rieger syndrome,” European Journal of Human Genetics, vol. 19, no. 3, 2011.

[45] B. D’haene, E. Meire, I. Claerhout et al., “Expanding the spectrum of FOXC1 and PITX2 mutations and copy number changes in patients with anterior segment malformations,” Investigative Ophthalmology & Visual Science, vol. 52, no. 1, pp. 324–333, 2011.

[46] P. J. Gage, H. Suh, and S. A. Camper, “Dosage requirement of Pitx2 for development of multiple organs,” Development, vol. 126, no. 20, pp. 4643–4651, 1999.

[47] K. Kitamura, H. Miura, S. Miyagawa-Tomita et al., “Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism,” Development, vol. 126, no. 24, pp. 5749–5758, 1999.

[48] A. G. Diehl, S. Zareparsi, M. Qian, R. Khanna, R. Angeles, and P. J. Gage, “Extraocular muscle morphogenesis and gene expression are regulated by Pitx2 gene dose,” Investigative Ophthalmology & Visual Science, vol. 47, no. 5, pp. 1785–1793, 2006.

[49] A. L. Evans and P. J. Gage, “Expression of the homeobox gene Pitx2 in neural crest is required for optic stalk and ocular anterior segment development,” Human Molecular Genetics, vol. 14, no. 22, pp. 3347–3359, 2005.

[50] T. A. Hjalt, E. V. Semina, B. A. Amendt, and J. C. Murray, “The Pitx2 protein in mouse development,” Developmental Dynamics, vol. 218, no. 1, pp. 195–200, 2000.

[51] J. Holmberg, C. Y. Liu, and T. A. Hjalt, “PITX2 gain-of-function in Rieger syndrome eye model,” The American Journal of Pathology, vol. 165, no. 5, pp. 1633–1641, 2004.

[52] P. J. Gage, M. Qian, D. Wu, and K. I. Rosenberg, “The canonical Wnt signaling antagonist DKK2 is an essential effector of PITX2 function during normal eye development,” Developmental Biology, vol. 317, no. 1, pp. 310–324, 2008.

[53] A. N. Smith, L. D. Miller, N. Song, M. M. Taketo, and R. A. Lang, “The duality of β-catenin function: a requirement in lens morphogenesis and signaling suppression of lens fate in periorcular ectoderm,” Developmental Biology, vol. 285, no. 2, pp. 477–489, 2005.

[54] L. A. Miller, A. N. Smith, M. M. Taketo, and R. A. Lang, “Optic cup and facial patterning defects in ocular ectoderm β-catenin gain-of-function mice,” BMC Developmental Biology, vol. 6, article 14, 2006.

[55] M. Mukhopadhyay, M. Gorivodsky, S. Shromt et al., “Dkk2 plays an essential role in the corneal fate of the ocular surface epithelium,” Development, vol. 133, pp. 2149–2154, 2006.

[56] W. H. Wang, L. G. McNatt, I. H. Pang et al., “Increased expression of the WNT antagonist sFRP1 in glaucoma elevates intraocular pressure,” The Journal of Clinical Investigation, vol. 118, no. 3, pp. 1056–1064, 2008.

[57] P. Briata, C. Ilenge, G. Corte et al., “The Wnt/β-catenin→ Pitx2 pathway controls the turnover of Pitx2 and other unstable mRNAs,” Molecular Cell, vol. 12, no. 5, pp. 1201–1211, 2003.

[58] C. Kioussi, P. Briata, S. H. Baek et al., “Identification of a Wnt/Dvl/β-catenin→ Pitx2 pathway mediating cell-type-specific proliferation during development,” Cell, vol. 111, no. 5, pp. 673–685, 2002.

[59] Q. Xu, Y. Wang, A. Dabdoub et al., “Vascular development in the retina and inner ear: control by Norrin and Frizzled-4, a high-affinity ligand-receptor pair,” Cell, vol. 116, no. 6, pp. 883–895, 2004.

[60] K. Ruether, D. van de Pol, G. Jaisle, W. Berger, R. Tornow, and E. Zrenner, “Retinoschissielske alterations in the mouse eye caused by gene targeting of the Norrie disease gene,” Investigative Ophthalmology & Visual Science, vol. 38, no. 3, pp. 710–718, 1997.

[61] M. Richter, J. Gottanka, C. A. May, U. Welge-Lüßen, W. Berger, and E. Lütjen-Drecoll, “Retinal vasculature changes in Norrie disease mice,” Investigative Ophthalmology & Visual Science, vol. 39, no. 12, pp. 2450–2457, 1998.

[62] H. L. Rehm, D. S. Zhang, M. C. Brown et al., “Vascular defects and sensorineural deafness in a mouse model of norrie disease,” The Journal of Neuroscience, vol. 22, no. 11, pp. 4286–4292, 2002.

[63] U. F. O. Luhmann, D. Meunier, W. Shi et al., “Fetal loss in homozygous mutant Norrie disease mice: a new role of Norrin in reproduction,” Genesis, vol. 42, no. 4, pp. 253–262, 2005.

[64] J. Chen, A. Stahl, N. M. Krah et al., “Wnt signaling mediates pathological vascular growth in proliferative retinopathy,” Circulation, vol. 124, no. 17, pp. 1871–1881, 2011.

[65] X. Ye, Y. Wang, H. Cahill et al., “Norrin, frizzled-4, and Lrp5 signaling in endothelial cells controls a genetic program for retinal vascularization,” Cell, vol. 139, no. 2, pp. 285–298, 2009.

[66] A. Ohlmann, M. Scholz, A. Goldwich et al., “Ectopic Norrin induces growth of ocular capillaries and restores normal retinal angiogenesis in Norrie disease mutant mice,” The Journal of Neuroscience, vol. 25, no. 7, pp. 1701–1710, 2005.

[67] A. Ohlmann, R. Seitz, B. Brauner, D. Seitz, M. R. Bösl, and R. Tamm, “Norrin promotes vascular regrowth after oxygen-induced retinal vessel loss and suppresses retinopathy in mice,” The Journal of Neuroscience, vol. 30, no. 1, pp. 183–193, 2010.
[68] K. T. Paes, E. Wang, K. Henze et al., “Frizzled 4 is required for retinal angiogenesis and maintenance of the blood-retina barrier,” Investigative Ophthalmology & Visual Science, vol. 52, no. 9, pp. 6452–6461, 2011.

[69] N. W. Gale, G. Thurston, S. F. Hackett et al., “Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1,” Developmental Cell, vol. 3, no. 3, pp. 411–423, 2002.

[70] S. F. Hackett, S. Wiegand, G. Yancopoulos, and P. A. Campochiaro, “Angiopoietin-2 plays an important role in retinal angiogenesis,” Journal of Cellular Physiology, vol. 192, no. 2, pp. 182–187, 2002.

[71] R. Seitz, S. Hackl, T. Seibuchner, E. R. Tamm, and A. Ohlmann, “Norrin mediates neuroprotective effects on retinal ganglion cells via activation of the Wnt/β-catenin signaling pathway and the induction of neuroprotective growth factors in Müller cells,” The Journal of Neuroscience, vol. 30, no. 17, pp. 5998–6010, 2010.

[72] S. Lin, M. Cheng, W. Dailey, K. Drenser, and S. Chintala, “Norrin attenuates protease-mediated death of transformed retinal ganglion cells,” Molecular Vision, vol. 15, pp. 26–37, 2009.

[73] W. Y. Yu, C. Sheridan, I. Grierson et al., “Progenitors for the corneal endothelium and trabecular meshwork: a potential source for personalized stem cell therapy in corneal endothelial diseases and glaucoma,” Journal of Biomedicine and Biotechnology, vol. 2011, Article ID 412743, 13 pages, 2011.