Cellular and signaling dynamics in lacrimal gland development

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Contents

Layman's Summary

The lacrimal glands are the organs responsible for producing tears. Eye problems related to defective tears are very common, affecting about 1 in 11 people in the world. These eye problems are connected to dry eye disease and the lacrimal gland but have no cure besides artificial tears. For this reason, researchers study the lacrimal gland in humans and animals to create better treatments that do not require artificial tears. Lacrimal gland development describes how this organ forms from an embryo to an adult. Studying development can show us the lacrimal gland's biological history which is important for understanding the organ and creating better treatments.

In recent years, researchers have found new information that explains different parts of lacrimal gland development. One such example is recent progress about cell origins, also called cell lineages, which describes the history of how a cell became a certain cell type. Another example is cell signals, which describe the way cells communicate with each other and their surroundings. At the moment, there is no up-to-date overview that links this new information together during development. Because of that, this review describes how cell signals and cell types interact to form the lacrimal gland development.

During development, the lacrimal gland forms from cells in the embryo eye. These cells end up forming the lacrimal gland similar to how a plant bud becomes a tree with branches. The cells in the embryo divide and form a bud, made of many cells. This bud can extend by more cell division, ultimately forming a branch that consists of many buds. Branches of the lacrimal gland continue to grow after birth and until adulthood. Recent studies have shown some cell signals and cell types that are present in buds and branches.

Different cell signals work together as a complex web to form the lacrimal gland during development. For example, signal messengers of FGF proteins cause cell division in buds. On the other hand, signal messengers of the EGF protein cause cell differentiation into cell types found in adults. Although, only a few of these EGF protein messengers are known. Later in development, signal messengers of the BMP7 protein cause buds to become branches. However, BMP7 messengers do not use the same messengers as FGF proteins. This review links together the complex web of cell signals with each phase of lacrimal gland development.

This review also links the different cell types and origins to each phase of development. Cell types are quite diverse during development, but it remains unclear what defines each cell type and their origin. In conclusion, this review shows how the missing parts of lacrimal gland development relate to lacrimal gland biology and disease. This review innovates by showing how future research of cell signals, cell types, and cell origins is needed to better understand the lacrimal gland.

Abstract

The lacrimal gland (LG) is responsible for most tear production. Defective tear synthesis and secretion in the LG cause dry eye disease. This is a prevalent disease worldwide that causes eye irritation, and in severe cases, it can lead to eye damage. Understanding LG development is important for creating curative treatments for dry eye disease. Yet, cellular and signaling events during development remain partially uncharacterized. This review compiles an updated model of cell signaling, morphology, and cell lineage specification at each stage of LG development. These aspects of development are described together during presumptive bud formation, bud elongation, and branching. This review takes an innovative approach by further describing fetal and postnatal maturation in this model. Major signaling pathways in LG development are described and their recent characterization too. This review also highlights gaps in knowledge regarding cell heterogeneity, progenitor cells, and interspecies compatibility. In conclusion, this review describes cellular and signaling dynamics that coincide at each stage of LG development. Lastly, development and in vitro modeling are discussed to be of importance for guiding research to better understand LG physiology.

Introduction to lacrimal gland development: Shedding light on shedding tears

The lacrimal gland (LG) is the main exocrine gland that produces tears[1]. The LG is located above the eye[1]. The LG makes part of the ocular surface, which consists of multiple glands and epithelial tissues that maintain the apical surface of the eye[1]. The function of the LG is to secrete the tear film that bathes the ocular surface[1]. The tear film maintains ocular health and thus, good vision [1]. More specifically, the tear film lubricates and protects the ocular surface by neutralizing and washing away irritants [2]. The tear film also forms a refractive surface and supports immunity and metabolism of the ocular surface [2]. Three layers make up the tear film: the mucous, aqueous, and lipid layers [2]. These layers are produced by the meibomian gland, the LG, and the conjunctiva, respectively [2]. Each component of the tear film serves a different function: mucous supports immunity, water lubricates and refracts, and lipids prevent evaporation and uneven distribution[2].

Defective tear film secretion is caused by multiple widespread LG diseases[3]. For instance, dry eye disease occurs when the tear film provides inadequate eye lubrication due to wrong tear composition, scarcity, or autoimmunity [3]. Initially, dry eye disease causes itchiness and eye fatigue in patients and can lead to ocular surface scarring and impaired vision[3]. Moreover, dry eye disease affected about 1 in 11 people worldwide by 2021[4] and is currently uncurable since treatments are palliative, based on artificial lubrication. Therefore, understanding LG function and development at a cellular level is essential for designing curative treatments.

Secretory and supportive cell types participate in tear production in both human and mouse (Figure 1A)[3,5]. The LG epithelium is the largest cell population and is composed of three cell types: acinar, ductal, and myoepithelial cells [3]. These three cell types work together to synthesize and secrete the tear film. Acinar cells are polarized and form luminal cavities where they secrete water and tear-specific proteins[3,6]. Ductal cells form the secretory ducts that connect acinar lumens to the ocular surface, while also secreting water, electrolytes, and antimicrobial peptides [3,7]. Lastly, myoepithelial cells support acinar and ductal cell function and facilitate fluid secretion [3]. Other cell types regulate LG homeostasis, including nerve cells, fibroblasts, tissue progenitors, stromal cells, and immune cells [3]. LG physiology relies on cell lineage specification events that start during LG development [3]. Therefore, understanding LG development can guide future research on LG physiology.

Four prenatal stages and one postnatal stage best describe LG development in both human and mouse (Figure 1B) [5,8–10]. In mice, LG development of starts at embryonic day (E)12.5 and stretches until adulthood[9,10]. In comparison, human LG development occurs starts at day 41 of gestation and lasts longer than mice[5,8]. Chronologically, LG development is initiated with the presumptive glandular stage from E12.5 until E13.5 in mice[9]. During the presumptive stage, the epithelium at the temporal edge of the eye

thickens and forms a nodule surrounded by mesenchyme [9]. The presumptive stage is followed by the budding stage from E13.5 until E15.5 [9]. The budding stage is characterized by the formation of a singular round epithelial bud that extends into the mesenchyme [9]. Subsequently, the branching stage occurs from E15.5 until E19.5 [9]. Epithelial bud bifurcations form branched structures with lumens at the branching stage [9]. The exorbital and intraorbital lobes of the LG are formed at this time as well [9]. Finally, the fetal stage starts after branching and continues until birth, followed by the postnatal stage until adulthood[8,10]. The LG continues to grow in size during the fetal and postnatal stages accompanied by terminal cell differentiation and maturation until adulthood [8,10].

This review covers recent advances in cellular and signaling dynamics of LG development[6,11–15]. Furthermore, the link between current knowledge of signaling, morphology, and cell lineage specification is discussed. Overlaps are identified between missing knowledge of development and current research limitations. The updated model of LG development presented here aims to guide future research on LG physiology and disease.

The onset of lacrimal gland development

LG development starts after the first stages of eye development during embryogenesis. The eye invagination at E8.5 ends up forming the epithelial conjunctiva fornix (the deepest rim of invagination) above the murine eye at E11.5 [16]. Starting around E12.5, the presumptive LG bud forms from the epithelial fornix above the eye and marks the beginning of LG development [9]. Human and mouse LG development signaling and morphology are comparable during embryonic development and mostly uncharacterized after that[5,8,17]. Hereafter, we refer to murine development unless otherwise stated. The following sections review signaling pathways in each stage of LG development, from the presumptive stage to the fetal and postnatal stages.

Presumptive stage: Epithelium thickening and presumptive bud

The presumptive stage of LG development derives its name from the formation of the presumptive LG bud. An invagination of the ectoderm above the eye forms the epithelial fornix that later develops into the LG [16]. Starting at E12.5, the epithelial fornix at the temporal edge of the eye thickens and forms the presumptive bud by E13.5 (Figure 2A)[18]. This section will cover the main signaling pathways that drive presumptive bud formation.

Presumptive bud formation is driven by FGF10-FGFR2 signaling

Fibroblast growth factor (FGF) signaling is a known driver of presumptive bud formation during LG development [9,18–20]. *Fgf10* and, to a lesser extent, *Fgf7* are expressed in the mesenchyme adjacent to the presumptive bud epithelium [9]. *Fgf10*-null mice lack epithelial tissue by E18.5, while mesenchyme is intact [9]. Opposite to *Fgf10* loss, treatment with FGF10 and FGF7 beads induced presumptive bud formation in E13 LG explants [9]. FGF receptor 2 (FGFR2), a receptor of FGF10 and FGF7, is also

expressed in the epithelium of the presumptive bud, further confirming FGF signaling in LG development[9]. LGs explanted at E13.5 lack a presumptive bud after FGFR2 inhibition[9]. Therefore, FGF10-FGFR2 signaling is required for LG presumptive bud formation in mice.

Pax6 is a competence factor of FGF10 signaling

Independently of FGF signaling, *Pax6* is expressed in the eye at E12.5 in the epithelium[9]. *Pax6* is a transcription factor with DNA binding motifs dictating eye development in Drosophila, mouse, and human[21]. *Pax6* truncating mutation *Sey* allele inhibits presumptive bud formation by E13.5 without affecting *Fgf10* distribution. FGF10 induction forms *Pax6*+ presumptive buds in explanted LGs at E13[9]. Moreover, it is likely Pax6 permits FGF signaling to induce LG development from the epithelial fornix[9]. This indicates epithelial *Pax6* mediates LG development as a competence factor of FGF signaling.

Glycosaminoglycans limit in FGF10 diffusion

Extracellular matrix (ECM) glycosaminoglycans (GAGs) mediate FGF10 diffusion in the presumptive LG [19]. GAGs are polysaccharide compounds that participate in multiple signaling pathways and, in particular, GAG heparan sulfate binds to FGF10 to limit its diffusion [22]. As of E10.5, GAGs are expressed in the mesenchyme that would later surround the LG epithelium [19]. GAG synthesizing enzymes, like UDP-glucose dehydrogenase (*Ugdh*), are expressed in the LG mesenchyme during development too [19,23]. *Ugdh* loss eliminates the synthesis of GAGs in the LG mesenchyme from E10.5[19]. At E13.5, the *Ugdh* mutation causes normal *Fgf10* expression in the mesenchyme [19]. As for the LG epithelium at E13.5, the *Ugdh* mutation abolishes phosphorylation of downstream mediator extracellular signal-regulated kinase (ERK) and expression of downstream response genes *Erm* and *Dusp6. Ugdh* loss increases the diffusion of FGF10 in the mesenchyme in explanted LGs treated with FGF10 beads at E10.5[19]. The strong affinity between FGF10 and GAGs limits FGF10 dispersion and produces an FGF10 concentration gradient necessary for LG development.

Sox9 and *Sox10* signal downstream of FGF10

FGF10 downstream signaling involves *Sox9* and *Sox10* during the presumptive bud [18]. *Sox9*, a gene involved in embryonic development, was expressed as of E10.5 in the epithelium[18]. *Sox9* deletion stops presumptive bud formation by E13.5, while FGF10 expression and distribution in the LG mesenchyme are undisrupted [18]. *Sox10* is a downstream target of *Sox9* that localizes in the presumptive bud epithelium at E13.5 [18]. In the absence of *Sox9, Sox10* expression is lost at E13.5[18]. In contrast, *Sox10* loss mildly disrupts the formation of the presumptive bud at E13.5 without altering FGF10 expression or distribution [18]. The less severe phenotype after *Sox10* loss suggests additional downstream effectors of *Sox9* are also responsible for driving development. This indicates presumptive bud formation is influenced by *Sox9* and its downstream targets, such as *Sox10*.

miR-205 expression regulates AKT signaling

Lastly, microRNA miR-205 signaling is also identified in presumptive bud formation [20]. miR-205 is expressed in mammary gland stem cells and regulates neonatal skin cell proliferation [24,25]. miR-205 loss halts LG development mice [20]. *Fgfr2* and *Pax6* expression is unaffected after miR-205 loss in the epithelium at E12.5 [20]. This indicates miR-205 acts parallel or downstream to FGFR2 and *Pax6* signaling[20]. miR-205 loss also increases the expression of miR-205 target genes *Cadm1*, and *Inppl1* at E12.5 [20]. *Cadm1* and *Inppl1* are antagonists of AKT signaling [20]. More commonly, AKT signaling is involved in cell survival and growth but it is also a pathway downstream of FGF signaling that regulates epithelium differentiation[25–27]. miR-205 could cause epithelial metabolic switches during development since it induces a switch from amino acid to lipid metabolism in induced pluripotent stem cell (iPSC)-derived LG organoids[28]. Despite this, the interaction of epithelial miR-205 with other signaling pathways in LG development is unknown.

Cellular dynamics (e.g. proliferation, migration, differentiation) are shown in other stages of LG development but remain uncharacterized in the presumptive stage. Precursor cell heterogeneity is equally unclear since gene expression patterns in the presumptive stage are similar to later stages of development[9,18,19]. In comparison, these aspects of development and additional signaling pathways are studied during later of development but have yet to be validated in the presumptive stage.

Budding stage: Extension of the lacrimal gland bud

The LG budding stage encompasses the elongation of the presumptive bud epithelium into the surrounding mesenchyme in human and mouse[8,9]. The budding stage starts after the presumptive bud formation at E13.5 and ends with the bud elongation at E15.5 [9]. Much like during the presumptive bud formation, FGF10-FGFR2 signaling defines the morphological structure of the elongating bud (Figure 2B). This section discusses signaling pathways that drive the dynamic morphological features of LG bud extension.

FGF10 and ERK signaling define the bud and stalk morphology in LG budding

The mesenchyme surrounding the elongating LG bud expresses *Fgf10*, and to a less extent, *Fgf7* at E14.5, as identified by RNA in situ hybridization[9]. Loss of epithelial *Fgfr2* interrupts LG bud formation and lacks proliferation in the LG epithelium at E14.5 [29]. Epithelial loss of FGF signaling target *Sox9* at E14.5 decreases the expression of FGF downstream targets without affecting *Fgfr2* expression[18]. The expression of these FGF targets is not diminished after *Sox10* deletion [18]. These findings evidence that the FGF10-FGFR2-*Sox9* pathway also drives LG bud elongation between E13.5 and E15.5.

LGs explanted at E15.5 form a bud that elongates towards FGF10 beads but not other FGFs [30]. The LG bud has a defined distal bud region close to the FGF10 bead, and a proximal stalk region[30]. In the same LG explants, FGF3 causes an aberrant bud region with a normal stalk, and FGF7 induces an enlarged bud while lacking the stalk region[30]. Isolated stalk regions and whole LG explants cultured in FGF10 have high expression of LG differentiation markers associated with branching morphogenesis in comparison to the FGF7 and FGF10 buds[30]. Opposite to the differentiation markers, LG proliferation markers are downregulated in FGF10-treated stalk regions when compared to FGF7 and FGF10 buds [30]. This indicates isolated bud regions after FGF10 have a similar expression to the FGF7 buds [30]. Bud regions further express higher pERK in comparison to stalk regions, showing the presence of FGF10 and ERK activation gradients during LG budding at E15.5 [30]. Therefore, the FGF10 and ERK activation gradients drive proliferation in the distal bud and differentiation in the proximal stalk of elongating LG buds [30]. It is likely other FGF or FGFR proteins also have a function in budding.

Sulfation of heparan sulfate activates and guides FGF10-FGFR2-mediated bud elongation

The FGF10 gradient relies on ECM components, such as GAGs, to signal to the LG epithelium. The LG mesenchyme also expresses GAGs that limit FGF10 diffusion during LG bud elongation at E14.5 and E15.5, and among these GAGs is heparan sulfate [19,29]. The distal tip of the LG bud is particularly enriched with the N-sulfation 10E4 of heparan sulfate and heparan sulfate N-deacetylase/N-sulfotransferase 1 (*Ndst1*) gene at E14.5[29]. In comparison, the proximal bud region lacks enrichment of N-sulfation of heparan sulfate [29], indicating a role in bud-stalk morphology. *Ndst1* loss in the LG epithelium inhibits bud formation and lacks sulfation of heparan sulfate in the

LG epithelium but not mesenchyme at E14.5 [29]. The same *Ndst1* loss in the LG mesenchyme causes healthy bud elongation while having sulfation of heparan sulfate in the LG epithelium but not mesenchyme at E14.5. The LG epithelium requires *Ndst1*-mediated sulfation of heparan sulfate for driving bud elongation[29].

Heparan sulfate-mediated FGF10/FGFR2 and FGF7/FGFR2 complex localizes at the distal tip of the LG epithelial bud at E14.5 in wild-type LGs [29]. Staining of both of these heparan sulfate-mediate complexes is absent in *Ndst1* loss in the LG epithelium, while budding is disrupted, and *Fgf10* and *Fgfr2* expressions are unchanged [29]. In *Ndst1* loss in the LG epithelium, aberrant budding is nonetheless rescued after FGF10-bead treatment in LG explants. FGF10-beads treatment does not rescue budding in epithelial *Ndst1*/2 loss in LG explants at E14.5 [29]. These results evidence that sulfation of heparan sulfate via *Ndst* mediates the *Fgf10/Fgfr2* interaction that drives LG budding.

SHP2, Ras, and ERK mediate LG budding downstream of FGF10-FGFR2

Fgfr2 and *Ndst1* loss in the LG epithelium eliminates FGF signaling targets pERK, *Erm* expression, and phosphorylation of SHP2 (pSHP2) in the LG epithelium [29,31]. SHP2 is a protein tyrosine phosphatase that can be recruited by FGFR and positively regulates ERK activity [31]. *Shp2* loss in the LG epithelium blocks LG bud formation [31]. *Shp2* loss reduces sulfation of heparan sulfate, proliferation, pERK, and FGF target expression in the LG epithelium at E14.5 [31]. By E15.5, proliferation is absent in *Shp2* loss in the LG epithelium [31]. Ras signaling is among the possible downstream targets of *Shp2* [31]. A constitutively active *Kras* mutation in the *Shp2* loss partially rescues LG budding and pERK expression in the LG epithelium [31]. Thus, *Shp2* loss disrupts LG budding by inhibiting ERK signaling, but it is partially rescued by *Kras* activation.

Sprouty2 (*Spyr2*), is a dephosphorylation target of SHP2 and suppressor of receptor tyrosine kinase (RTK) signaling [31]. *Spyr2* expression and SPYR2 phosphorylation are lost in epithelial *Shp2* loss at E15.5[31]. The *Kras* activating mutation in *Shp2* loss, in contrast, hyperphosphorylates SPYR2 at E15.5. Aberrant budding and pERK expression are partly rescued by the *Kras* activating mutation fully rescue in the double *Shp2/Spyr2* loss[31]. This means SPYR2-Ras acts in a negative feedback loop on LG budding. Weakening the negative SPYR2-Ras feedback loop is necessary for LG budding. During budding, epithelial SHP2 phosphorylating Ras-ERK induces SPYR2 dephosphorylation.

FGF10-PI3K-mTOR-EGFR pathway crosstalk drives budding

The class IA phosphoinositide 3-kinase (PI3K) is also activated by FGF and insulin-like growth factor (IGF) signaling in LG budding[11]. IGF signaling via PI3K is a major metabolic signaling pathway in the developing LG[11]. PI3K is composed of a p85 regulatory subunit and a p110 catalytic subunit that can be recruited via Ras signaling[11]. p110 loss in the LG epithelium reduces downstream AKT phosphorylation (pAKT) and proliferation, abolishing bud formation at E14.5 without affecting *Sox9* expression[11]. Loss of both the Ras binding domain of p110 and p85 have similar effects, except p85

loss causes normal budding[11]. Constitutively activation of *Mek1*, downstream of *Ras*, leads to a partial rescue of budding and pERK expression in the absence of p110 at E14.5 [11]. In summary, FGF-Ras signaling recruits PI3K, which in turn activates Mek1-ERK and induces epithelial budding.

Additional Erk loss in the LG epithelium and p110 loss fail to develop an LG bud at E14.5 [11]. Erk loss and p110 loss mutants have downstream mTOR phosphorylation (pmTOR) despite the loss of phosphorylated mTOR targets p4EBP1 and pS6 at E14.5. Only when both PI3K and ERK are inhibited, pmTOR is completely absent in mouse embryonic fibroblast cultures at E13.5 [11]. Epithelial loss of the mTOR C1 subunit disrupts bud formation and decreases expression of *Sox10*, pERK, pmTOR, and mTOR downstream targets at E14.5 [11]. Similar to p110 loss, Constitutive activation of Mek1 partly rescues pERK expression and LG budding in the absence of mTORC1. mTOR activation, regulated by ERK and PI3K-Ras-Mek1, is necessary for LG budding and pERK and pAKT expression at E14.5.

Interestingly, the epidermal growth factor receptor (*Egfr*) is elevated after p110 loss at E14.5[11]. *Egfr* is expressed only in the proximal stalk region of the LG bud and not in the distal bud region of wild-type LGs [11]. Loss of either p110, *Fgfr2*, *Erk*, or mTORC1 causes uniform *Egfr* expression in the epithelium with disrupted budding at E14.5[11]. Moreover, EGF treatment disrupts LG budding in explanted E13.5 LGs. EGF likely induces proximal stalk cell cycle exit and differentiation, similar to other epidermal cell differentiation [32], although further confirmation is needed. This means inhibition of EGF signaling in the distal bud by the PI3K-Ras-ERK-AKT pathway allows bud proliferation and extension[11].

ALX4 initiates FGF signaling in the mesenchyme during LG development.

Upstream FGF10-FGFR2 signaling in LG development occurs via the mesenchyme. LG mesenchymal loss of *Frs2*, an adaptor protein that enables FGFR binding to SHP2, disrupts LG development at E14.5 [33]. Loss of *Fgfr1/2* or loss of *Frs2* binding sites in *Fgfr1* or *Fgfr2* in the LG mesenchyme also completely abolishes LG development by E14.5[33]. *Shp2* loss mutation in the LG mesenchyme deletes LG bud formation and *Shp2* expression at E14.5, further evidencing mesenchymal *Shp2* is necessary for LG development[33].

Shp2 loss in the LG mesenchyme downregulates FGF10 target pERK in the LG epithelium at E13.5 [33]. By E14.5, *Mek1/2* loss and *Erk1/2* loss in the LG mesenchyme abolish LG budding [33]. Earlier at E10.5, *Shp2* loss or *Mek1/2* loss in the neural crest mesenchyme, the later LG mesenchyme, downregulate FGF10 targets in the conjunctiva epithelium [33]. LG budding is rescued after mesenchymal *Shp2* loss by constitutive *Kras* activation in the absence of *Shp2* in the LG mesenchyme at E14.5 [33]. Loss of downstream *Etv1, Etv4,* or *Etv5* in the LG mesenchyme hinders LG budding but does not abolish it [33]. Together, these results show the SHP2-Ras-Mek-ERK pathway has additional downstream targets in the mesenchyme that induce LG budding.

Other downregulated genes in the mesenchymal *Shp2* loss include the homeodomain transcription factor *Alx4* at E13.5[33]. ALX4 expression is greatly reduced in the LG mesenchyme in *Shp2* loss, but it is rescued after constitutive *Kras* activation in mesenchymal *Shp2* loss[33]. In *Shp2* loss, mesenchymal expression of ALX4 is progressively lost from E12.5, and by E14.5 it is absent in the LG mesenchyme. *Alx4* null mutants have a drastic decrease in *Alx1, Fgf10, Etv4*, and *Etv5* expression by E14.5, and develop rudimentary LG buds by E16.5[33]. In addition, *Alx4* loss reduces proliferation and increases cell death within the LG epithelium[33]. Together, these results indicate FGF upstream signaling in LG budding is mediated by the FGFR1/2 and FRS that signal through SHP2 in the LG mesenchyme[33]. Ras-ERK signaling in the mesenchyme causes ALX4 to activate FGF10 expression and drive LG budding [33]. The FGF10 gradient induced by ALX4 also plays a role in the subsequent stages occurring after bud elongation.

Branching stage: Acquisition of glandular structures

Following the budding stage, the LG branching begins at E15.5 and ends at E19.5[9]. During the branching stage, the elongated bud forms an arborized structure with many acinar branches connected to a main duct (Figure 3A)[8–10]. Acinar structures arise from terminal end buds at the end of each branch[10]. Simultaneously, ducts arise from the formation of lumens in epithelial stalks[10]. From E16 onwards, branching establishes the proximal intraorbital and the distal exorbital lobes in the murine LG[8,9,34]. In human, the eyelid muscle expansion further separates the palpebral and orbital lobes, corresponding to intra- and exorbital lobes in mice[8]. Both in mouse and human, branching establishes the first acinar-ductal structural units of the LG. This section outlines the signaling pathways and cellular dynamics that define LG glandular structures.

Epithelial cell dynamics define glandular architecture

Beyond morphological features, the first acinar-ductal structural units are identified by their expression patterns of marker genes during branching. Indeed, acinar-like, ductal-like, and myoepithelial-like cells are identified by the expression of Keratin 14 (*Krt14*), Keratin 19 (*Krt19*), and alpha-smooth muscle actin (*αSMA*) at E16, respectively[10]. From E18, KRT14 expression is restricted to the basal and suprabasal epithelial cell layers, where acinar, basal ductal, and myoepithelial cells localize in adulthood[10]. On the other hand, KRT19 expression is restricted inside the proximal area of the bud stalk at E15[10]. This KRT19 expression pattern later re-arranges, marking luminal ductal cells by E18[10]. αSMA expression progressively increases in the LG epithelium from E15 to E18, overlapping with KRT14 expression[10]. At E18, αSMA is restricted to the suprabasal layer of terminal end buds, marking myoepithelial cells[10].

Cell proliferation of terminal end buds and duct cell apoptosis create LG branching[10]. The majority of the cells in basal and suprabasal layers of the terminal end buds are proliferative at E16. At E18, nearly all proliferation stops in the terminal end buds[10]. The lumen formation in LG ducts does not involve cell proliferation since ductal cells are, in their majority, in cell cycle arrest from E16 onwards[10]. Instead, LG ducts exhibit apoptosis as of E16, forming micro-lumens with apoptotic cells by E17, and having no apoptotic cells by E18 when the micro-lumens are fused[10]. In addition to cell proliferation, epithelial cell rearrangement via intercalation of different populations and probably mesenchymal-to-epithelial transition (MET) influence LG branch formation[10]. As for MET, mesenchymal cells expressing E-cadherin, a common MET marker, increase in numbers within the epithelial surface of terminal end buds from E16 to E18 [10]. Overall, cell lineage specification coincides with the formation of glandular structures in the branching stage. Branching is controlled by the interactions between multiple signaling pathways, including FGF signaling.

RTK signaling induces epithelium proliferation in terminal end buds during branching

FGF and additional RTK signaling pathways mediate cell proliferation during branching. The absence of FGF10-FGFR2 signaling components in the epithelium leads to incomplete budding and branching by E19.5 [9,18,20]. Other RTKs besides *Fgfr*, such as *Egfr* and hepatocyte growth factor (HGF) receptor (*Met*) are expressed in the LG mesenchyme and epithelium at E18[15]. Even though cell proliferation is drastically decreased during branching [10], inhibition of EGF and/or HGF further decreases proliferation in terminal end buds of explanted LGs at E16-E18[15]. EGF signaling also disrupts LG budding at E13.5, a process driven by cell proliferation [11]. Despite this evidence, in vivo studies are required to determine if EGF and HGF signaling mediates cell proliferation in vivo during LG branching.

BMP7 gradient drives branching through FOXC1

Like multiple organs, bone morphogenetic protein 7 (BMP7) signaling is required for LG branching. In the LG, BMP7 induces mesenchyme proliferation parallel to FGF10 signaling[34]. By E17, high levels of BMP7 are expressed in the mesenchyme surrounding terminal end buds and lacrimal ducts[34]. The rest of the mesenchyme and epithelium express low levels of BMP7 at this time too [34]. Embryos with genetic loss of BMP7 have decreased LG branching and mispositioned terminal end buds and lobes at E19.5, without affecting early budding at E13.5 [34]. This suggests that BMP7 is needed for late branching. Confirming this in LG explants at E15.5, BMP7 increases branching towards FGF10 beads while having similar bud elongation to the FGF10 beads control[34]. In fact, the branching effect of BMP7 is mediated by the mesenchyme[34]. BMP7 increases mesenchymal cell aggregation, proliferation, and their respective markers in a dose-dependent manner in LG explants at E15.5. The function of BMP7 was further confirmed by decreased mesenchymal cell aggregation and proliferation in BMP7 null mutants at birth[34]. Another effect of BMP7 is inducing mesenchymal cadherin expression, which is associated with MET in LG development[10,34]. It is likely that BMP7 also contributes to branching via MET, but further confirmation is required. These results show that BMP7 and FGF10 drive mesenchymal and epithelial cell proliferation and death during branching.

Forkhead transcription factor C1 (*Foxc1*) expression is required to induce BMP7 mesenchymal proliferation during LG branching[35]. *Foxc1* is expressed in the LG mesenchyme and epithelium by E14.5[35]. Genetic loss of *Foxc1* between E16.5 and E18.5 significantly reduces branching and bud elongation in the exorbital LG lobe and inhibits the formation of the intraorbital lobe[35]. Explanted LGs at E15.5 of *Foxc1* mutants still display epithelial proliferation and branching in response to FGF10 but lack mesenchymal aggregation and MET morphology in response to BMP7[35]. *Foxc1* expression is required for branching in response to BMP7, but not to FGF10.

WNT/β-catenin control FGF10- and BMP7-induced branching

WNT/β-catenin signaling controls LG branching by antagonizing BMP7 activity [36]. At E15.5-E16.5, the *Wnt2* ligand is expressed in the LG mesenchyme [36] WNT signaling activation via LiCl treatment in explanted LG at E15.5 decreases branching, inhibits epithelial and mesenchymal proliferation, and suppresses BMP7-induced proliferation [36]. In contrast, β-catenin loss increases branching in LG explants and *Fgf10* expression in the mesenchyme adjacent to the epithelium [36]. Wnt/β-catenin consequently control LG branching via the suppression of cell proliferation and regulation of *Fgf10* expression and BMP7 activity.

NOTCH1 regulates ductal-myoepithelial regions in branched structures

Similar to *Wnt*, *Notch* signaling, a common signaling pathway in development, also controls the tissue architecture of LG branching. By E16, NOTCH2 is expressed in the LG duct epithelium and the basal and suprabasal layers of terminal end buds [10], while NOTCH1 is highly expressed in the LG epithelium and mesenchyme [37]. NOTCH inhibition in E15.5 LG explants increases the number of terminal end buds in a dose-dependent manner and decreases their size without changing the overall LG size [10,37]. NOTCH inhibition also drastically decreases the expression of KRT19 in the LG epithelium and arrests the formation of ducts and lumens[10,37]. This indicates that NOTCH participates in the acquisition of ductal structures. Myoepithelial-like cells expressing αSMA in these explants also react to NOTCH inhibition by expanding further than the terminal end buds to the basal layer of almost all LG epithelium [10]. Changes in myoepithelial-like and ductal cell expression indicate NOTCH signaling is essential for epithelial cell lineage specification. Hinting at this, epithelial stalk differentiation and directional migration is also mediated by integrin β1 in LG explants at E15.5[30]. Epithelial differentiation likely occurs via the interaction of NOTCH and other signaling pathways, although this requires further investigation. In summary, NOTCH signaling draws the borders between LG ducts and terminal end bud regions during branching.

ECM remodeling is required for branching

Drastic structural and cellular changes during branching also depend on ECM remodeling. Among ECM remodeling aspects, ECM component fibronectin cleavage and matrix metalloproteinases (MMPs) are particularly required for branching[12,38]. A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS18) cleaves fibronectin during LG branching, and other organ branching [12]. At E16.5, *Adamts18* is expressed in the basal LG epithelium adjacent to the mesenchyme until its expression is lost by 14 days after birth. *Adamts18* loss decreases LG branching by E16.5, while fibronectin protein expression accumulates in the mesenchyme surrounding terminal end buds. These results show fibronectin cleavage via ADAMTS18 regulates LG branching[12].

A different network of MMPs, FGF10 and the transcription factor *Barx2* also regulates branching via ECM remodeling[38]. *Barx2* regulates MMP expression and epithelial migration in LG explants at E15.5 [38]. Similar to *Pax6* during early development, FGF10 particularly requires *Barx2* expression to induce branching [38]. Thus, FGF10 signaling interactions also control other processes of branching such as ECM remodeling.

First signs of cellular heterogeneity during embryonic development

While developmental signaling is relatively well understood, cellular heterogeneity in LG embryonic stages remains largely uncharacterized. For example, the proximal stalk region is specified by EGF signaling at E14.5, suggesting cell differentiation already occurs during budding[11]. Following E14.5, ductal cell commitment and myoepithelial-like population are also identified as early as E15 and E16, respectively[10,39]. However, the level of heterogeneity among LG cell types is uncharacterized before E16. In comparison, a dynamic cellular composition is present in different stages of salivary gland development, a digestive gland with a similar function and architecture to the LG[40]. Therefore, evidence in the LG and other organs suggests that the cellular heterogeneity and differentiation of the embryonic LG is more complex than our current understanding.

Interspecies differences in LG branching are another limitation to consider when studying the embryonic development. Mouse and human have vastly different branching architecture, even though the rest of LG morphology and expression are comparable[5,17]. Human LGs have a larger mesenchyme region, different acinar structures, and less branching in comparison to mouse[5]. Given these broad morphological differences, signaling pathways involved in human LG development could differ from the knowledge of murine development reviewed here. Alternatively, rabbit models are more representative of human LG tissue architecture than mouse[5]. Other models of development, such as iPSC-derived organoids, also recapitulate morphological aspects of human development in vitro but require further optimization[28,41]. Despite this, reviewing recent findings in postnatal murine models also benefits our understanding of human LG development.

Fetal and postnatal maturation in the lacrimal gland

The commonly established development timeline[8,9] omits fetal and postnatal development as defined stages of LG development. Since mouse gestation is only 20 days (E20), murine fetal development overlaps with the branching stage[42]. In comparison, human gestation is 40 weeks long. The end of the branching stage is after 16 weeks of gestation (W) in the human LG [8]. This is approximately comparable to mice E19.5 when lobes form in both species [8,9]. Human fetal LG development after W16 shows cell lineage commitment and an increase in LG size and branching until birth/postnatal day 0 (P0) [8,39]. This morphological difference makes it difficult to compare LG fetal development in human and mouse.

Further physiological maturation in fetal and postnatal human LGs might be indicative of similar events in mouse postnatal development. In human, infants born at W30-W27 secrete fewer tears than W38-W42 infants, and in both cases, tear secretion increases over time with weight after P0[43]. These events show functional maturation is linked to fetal and postnatal growth in human LGs. Similarly, the murine LG increases in size and exhibits cell maturation from P0 until the eyelids open at E13 and until adulthood at approximately P50[10]. Although further confirmation is required, the murine development until eyelid opening might recapitulate the human fetal development. This section shows the dynamic cellular and signaling changes that guide fetal and postnatal development in both murine and human LG (Figure 3B). In addition, their interactions with morphological changes during fetal and postnatal development are discussed.

Cell lineages arise during human fetal development

Terminal differentiation markers resembling the adult epithelium arise during human fetal development between W15 and W23 [39]. Ductal terminal differentiation genes *KRT19*, and ion transporter *NKCC1* are already expressed by W15 [39]. Fetal data shows duct cell differentiation earlier than fetal development agreeing with previous results[10]. Expression of secretory phenotype genes in acinar cells increases drastically between W17 and W23[39]. These include water channel *AQP5,* secretion regulator *MIST1,* and *SOX10* [39]. Acinar cell specification occurs between W17 and W23 [39]. At W23, *SOX10* and *MIST1* localize exclusively in acinar cells but are not expressed simultaneously in all cells, while *AQP5* marks both acini and small ducts[39]. Similar to acinar cells, expression of myoepithelial terminal differentiation genes *KRT5,* and *KRT14* increase drastically between W17 and W23[39]. At W23, *KRT14* colocalizes with *aSMA* in suprabasal cells [39] further suggesting myoepithelial differentiation between W17 and W23. Thus, the epithelium transitions from a largely undifferentiated cell population at W15 to a mixed population of acinar, myoepithelial, and ductal cells by W23. This transition evidences the dynamic nature of fetal LG development in human but remains unclear in mouse.

Fetal LG development in mice lacks a clear overlap with human expression changes between W15 and W23, and at P0. Signaling pathways involving FGF [18,19,29,33,38], EGF

[11,15], and BMP7 [34], among others, still participate in murine fetal development by P0 (Figure 3B). As for cell lineage commitment, mice LGs express ductal terminal differentiation markers already during the budding stage by E15 [10]. In human, it is unknown at what time point before the branching stage this happens[39]. Expression of terminal acinar and myoepithelial markers is only observed in postnatal development in mice, later than in human [39]. Similarly, murine acinar cells acquire protein synthesis and secretion genes between the branching stage and adulthood, as evidenced in a pseudo-time trajectory analysis [13]. These comparisons show cell lineages are defined at different time points in human and mouse development.

Major cellular heterogeneity emerges in postnatal development

Terminal cell differentiation and maturation occur during postnatal development in mice, coinciding with an increase in LG size. Postnatal development in mice starts at P0 when the epithelium slightly increases in size until P7 [10]. After P7, the epithelium expands rapidly until P13 when the murine eyelid opens [10]. The LG continues to moderately grow in size until adulthood at P50 [10]. From P0 to P50, changes in postnatal signaling pathways and cell composition lead to substantial cellular heterogeneity in the adult LG. In recent years, the diversity of the LG cell populations during mouse adulthood has been mapped using single-cell RNAseq[6,13].

Postnatal and adult LG cell populations are highly heterogeneous. The adult LG is composed of multiple different cell types including glandular epithelial cells, multiple fibroblasts subpopulations, vascular endothelial cells, mural endothelial cells, myeloid and lymphoid immune cells, pericytes, and Schwann cells (Figure 4A) [6,13,44]. The glandular epithelial cells consist of acinar cells, excretory ductal cells, basal ductal cells, luminal/intercalated ductal cells, and myoepithelial cells [6,13]. Additional subpopulations of acinar cells [6,13], epithelial progenitor cells [13], and injury-related muscle cells are also present in adult LG [45]. However, the progenitor cell population of the postnatal LG remains poorly understood.

Postnatal cell lineages and epithelial progenitors

The populations of epithelial progenitors in the postnatal LG are yet well identified (Figure 4B). Among these, only the luminal ductal cell progenitors are confirmed by cell lineage tracing [39]. The ductal cell lineage starts differentiating as early as E15 [10,39]. During postnatal development and adulthood, *Krt5*+ *Krt14*+ progenitor basal ductal cells divide asymmetrically to form *Krt5*+ luminal ductal cells [39]. In addition, fibroblast, immune and vascular cell populations already have a similar expression to the adult population at P4, suggesting their commitment [6]. As for the rest of the postnatal epithelium, acinar and myoepithelial cell differentiation also occurs during postnatal development [39].

Both acinar and myoepithelial markers are closely associated with each other at P4 in the undifferentiated epithelial population [39]. This indicates the possibility of a common cell progenitor. Within this population, myoepithelial-like cells are present from P3 in

acini structures. These cells further express maturation markers at P4 and assume their adult-like shape by P10[39]. Currently, myoepithelial cells are not exclusively associated with a progenitor cell population.

Acinar terminal differentiation markers are expressed in a subpopulation of the undifferentiated epithelium at P4[39]. Multiple progenitor populations in adults express acinar or epithelial markers, but progenitor differentiation is yet confirmed in vivo. Several of these progenitor populations express the RTK protein, *Kit* [6,13]. KIT+ cells are present in the epithelium by E16 [46] until adulthood[6,13]. For example, a *Kit*+ basal ductal progenitor population [6,13,47] can generate both ductal and acinar cells after transplantation in the LG [47]. Subpopulations like *Kit*+ cells are in a *Car6*+ population marking terminal acinar differentiation [6]. Moreover, a *Kit*+ population expresses *Ltf*, an acinar and ductal terminal differentiation marker [6]. Among the *Kit*+ *Ltf*+ cell population, a novel specific ion channel *Clic6*, marks ductal progenitors with a unique metabolic profile[6]. Another *Ltf*+ *Kit*- population in the LG also expresses pancreatic ductal progenitor markers[6]. In the branched salivary submandibular gland, *Sox10* expression can induce KIT- duct cells to become multi-potent KIT+ epithelial progenitors [46]. Despite that, these *Kit*+ and *Ltf*+ progenitor populations in the LG have yet to be confirmed to give rise to differentiated cells in vivo.

Postnatal LG expansion [10] coincides with the increase of differentiation markers expression of acinar cells, ductal cells, and progenitor subpopulations [6,39]. Although, associations between postnatal growth and cell differentiation require further proof. Like cellular dynamics, signaling pathways driving postnatal epithelium differentiation are poorly understood.

Eda signaling and extracellular matrix remodeling in postnatal development

Only a few pathways have been identified in postnatal development. Ectodysplasin-A (*Eda*) pathway and ECM remodeling via *Barx2* and fibronectin cleavage are implicated in postnatal developme [12,38,48] nt. *Barx2* decreases in expression after E19, but it still mediates ECM degradation by P7 [38]. Similarly, ADAMTS18 fibronectin cleaver is expressed only in acinar cells at P7, decreases significantly at P14, and is barely detectable in 10-month-old mice[12]. The timing of *Barx2* and ADAMTS18 expression [12,38] coincides with postnatal LG growth [10], indicating ECM cleavage might play a role. Also expressed in LG and mammary gland development, the transmembrane protein *Eda* affects the differentiation of acinar, ductal, and myoepithelial cells [48]. Loss of *Eda* disrupts postnatal LG development and leads to dry eye disease symptoms while being dispensable for embryonic development [48]. Further characterization of *Eda* signaling is necessary to understand each cell lineage differentiation. The function of additional signaling pathways should also be studied in LG postnatal differentiation, especially in epithelial cells.

Lessons from embryonic signaling in the lacrimal gland and other branched organs

Embryonic signaling in the LG and other branched organs can elucidate pathways of LG epithelial differentiation. Pathways for acinar cell differentiation possibly involve EGF signaling [11,30], and BMP7-induced MET with E-cadherin expression [10,34,35,39]. In the late embryonic salivary gland, neural-epithelial talk via NRG1-EGFR/ERBB3-mTORC2 is involved in acinar cell differentiation too [49]. Ductal differentiation in the embryonic salivary gland is also driven by the Hippo pathway in association with E-cadherin[50]. E-cadherin controls fibronectin and connective tissue growth factors required for ductal differentiation during duct formation in the salivary gland [50]. Thus, the salivary gland shows additional pathways that might be involved in LG ductal cell differentiation. The fetal and postnatal events described in this section, establish the dynamic cellular heterogeneity and cell lineages in adults. This demonstrates that both fetal and postnatal development should be better characterized as LG development stages.

In vitro modeling and LG development share similar limitations

Lastly, future efforts of understanding development might also benefit from recently developed in vitro LG models. Previously discussed gaps of knowledge in development overlap with the current limitations of in vitro modeling research. In vitro modeling studies lack representative markers of epithelial differentiation and progenitor cells. For example, a common acinar cell differentiation marker *AQP5* [7,28,41,51,52] is not exclusive to acinar cells. *AQP5* is also expressed in terminally differentiated duct cells [39]. This shows common differentiation markers do not accurately represent cell heterogeneity in adults, requiring further characterization. Furthermore, the implications of LG sexual dimorphism in gene expression and progenitor populations during development and in vitro modeling also require additional research [6]. Ultimately, both in vitro modeling and development are limited by the lack of markers defining the cellular heterogeneity of the adult LG.

Recent in vitro modeling of LG tissue relies on stem cell populations for turnover and differentiation. The lack of representative progenitor and differentiation markers shows understanding development is necessary for further advancing in vitro modeling. Adult stem cell-derived LG organoids have only been differentiated into acinar-like, ductal-like, or myoepithelial-like cells, and lack expression levels of the adult tissue[7,51,52]. On the other hand, iPSC-derived LG organoids differentiate in vitro into *AQP5*+ acinar-like and ductal-like cells forming branched structures[28,41]. However, these branched structures lack duct lumens and adult-like branching[28,41]. Therefore, stem cell-derived organoids require further optimization to recapitulate additional aspects of development. Conversely, further optimization of in vitro models can elucidate uncharacterized aspects of development.

In addition to in vitro models, knowledge of LG development is also relevant for disease research. For example, the alteration of LG postnatal development pathways causes dry eye disease symptoms[48]. This indicates pathways involved in LG disease could also be present in LG development, and vice versa.

Conclusion

This review originated from the lack of studies linking LG morphogenesis, cellular biology, and cell signaling during development. The known interactions between these aspects of LG development are reviewed here in each development stage: during presumptive bud formation, bud elongation, branching, and fetal and postnatal maturation. This review of LG development discusses gaps of knowledge in cellular and signaling dynamics, also in less well-characterized stages of development. The adult LG is a highly heterogeneous tissue that reflects these cellular dynamics. The cellular heterogeneity during development is evident in other branched organs that are similar to the LG. Limitations in the study of LG development, such as sexual dimorphisms and interspecies compatibility, should be considered carefully moving forward. Lastly, this review compiles an updated model of cell lineages during LG development and describes the potential of recent sequencing techniques and in vitro models to aid current research efforts. Understanding LG development is therefore beneficial for guiding in vitro modeling and dry eye disease research.

Figures

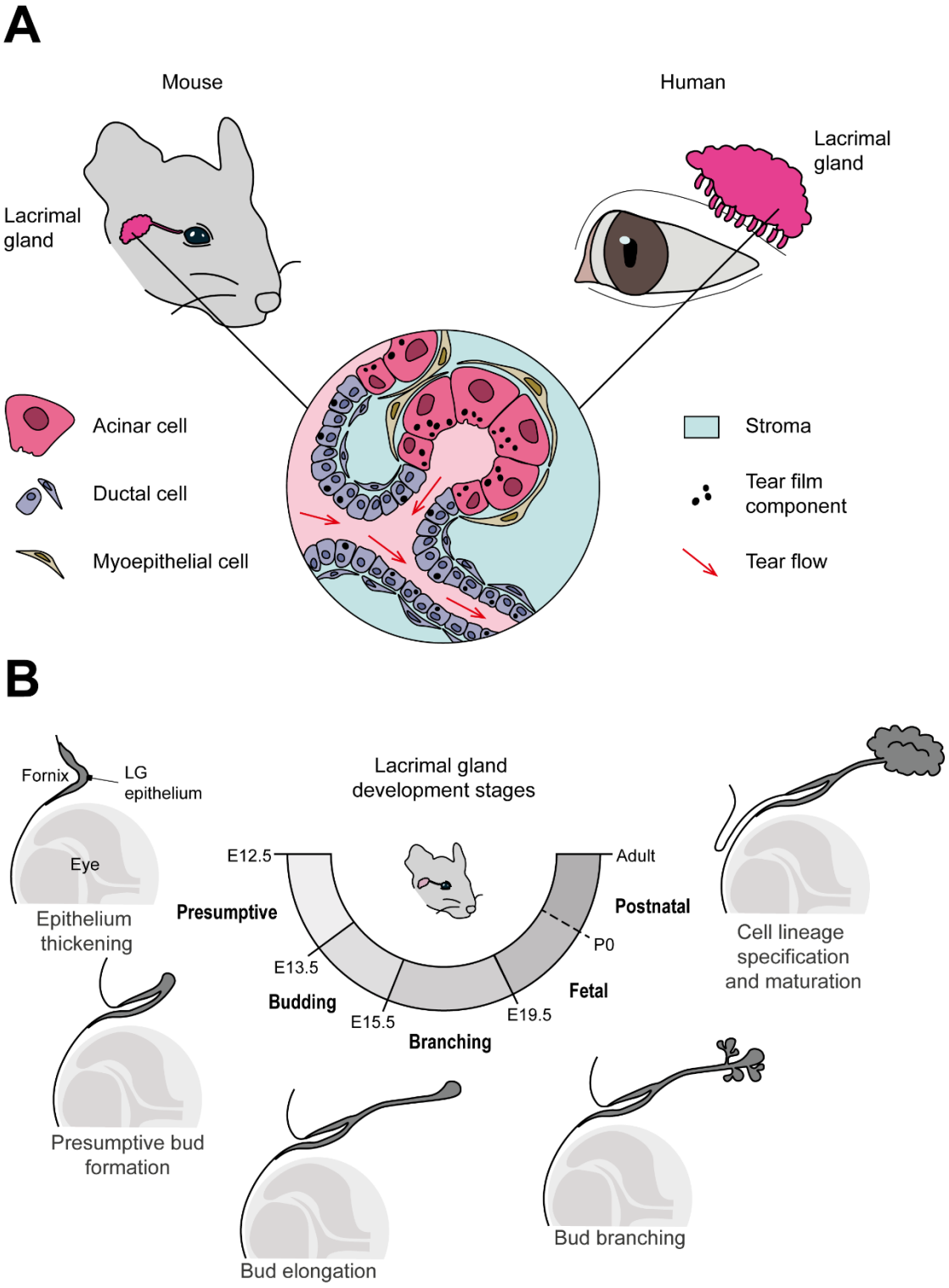
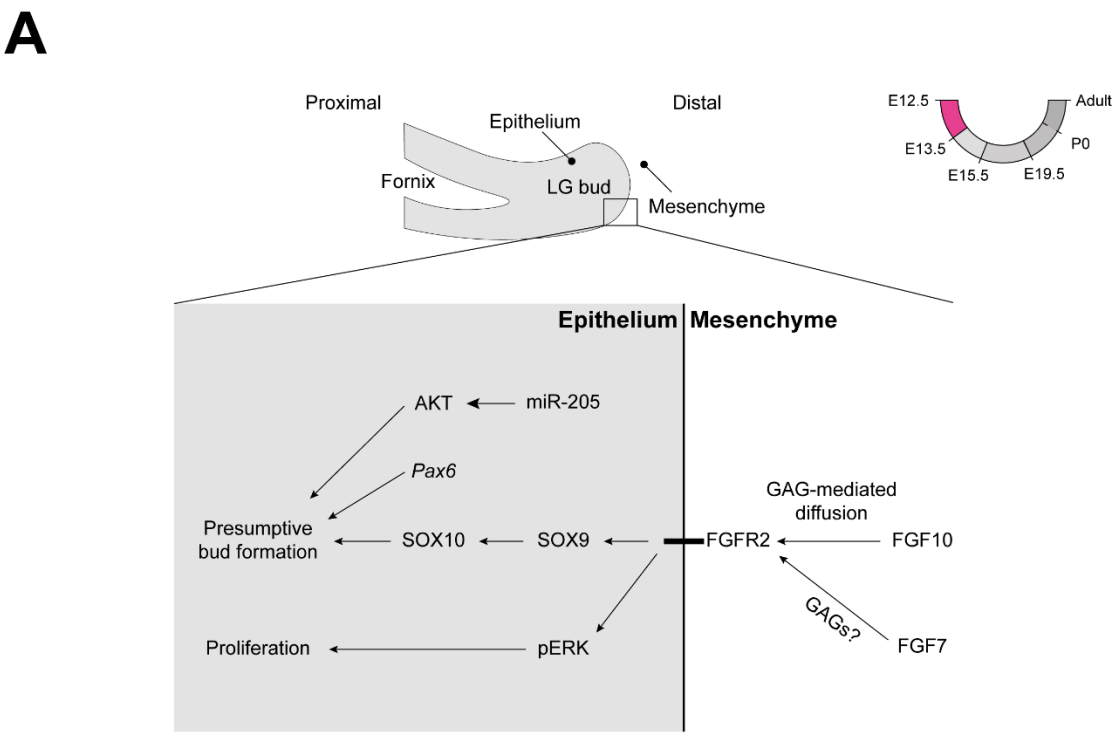

A
Mouse
Human
Lacrimal gland
Lacrimal gland
Acinar cell
Ductal cell
Myoepithelial cell
Stroma
Tear film component
Tear flow
B
Fornix
LG epithelium
Eye
Epithelium thickening
Lacrimal gland development stages
E12.5
Adult
Presumptive
Postnatal
P0
E13.5
Budding
Fetal
E15.5
E19.5
Branching
Cell lineage specification and maturation
Presumptive bud formation
Bud elongation
Bud branching

Figure 1. Illustration of LG histology and development. (A) Schematic representation of LG tissue organization. The LG is an exocrine gland located above each eye that secretes part of the tear film, important for maintaining ocular health [3,53]. The LG anatomy, histology, and expression patterns are significantly conserved between human and mouse [5,17]. Three epithelial cell types populate the murine LG: acinar, ductal, and myoepithelial cells [3,53]. Acinar cells are polarized cuboidal secretory epithelial cells [3,53]. Acinar cells form buds with luminal cavities where they secrete intracytoplasmic granules containing tear film components. Ductal cells are columnar epithelial cells that line the lacrimal gland ducts, which connect acinar cell structures to the ocular surface [3,53]. Ductal cells also secrete additional tear film components. Lastly, myoepithelial cells are flattened epithelial-smooth muscle cells that surround acinar and ductal cells in the LG [3,53]. Myoepithelial cells facilitate tear film secretion and support other cell functions. Other cell types of the LG play a role in homeostasis, including nerve cells, fibroblasts, tissue progenitors, stromal cells, and immune cells [3,39,53]. (B) Schematic representation of LG developmental stages and morphological hallmarks. Murine LG development can be chronologically divided into four stages: presumptive bud formation (E12.5-E13.5); LG budding and elongation (E13.5-E15.5); branching of additional buds (E15.5-P0); and prenatal and postnatal cell lineage specification and maturation. [8,9,39]. LG development starts at E12.5 with the epithelium thickening that forms the presumptive bud by E13.5 [8,9]. Further budding and bud elongation takes place until E15.5 when the bud branching begins [8,9]. The first LG branched structures are created by E19.5 but still develop until birth (P0) [8,9]. Cell lineage specification and maturation are completed in adulthood after prenatal and postnatal development of the LG [39].


A
Proximal
Distal
Epithelium
Fornix
LG bud
Mesenchyme
E12.5
Adult
E13.5
P0
E15.5
E19.5
Epithelium
Mesenchyme
AKT
miR-205
Pax6
Presumptive
bud formation
SOX10
SOX9
FGFR2
GAG-mediated
diffusion
FGF10
GAGs?
FGF7
Proliferation
pERK

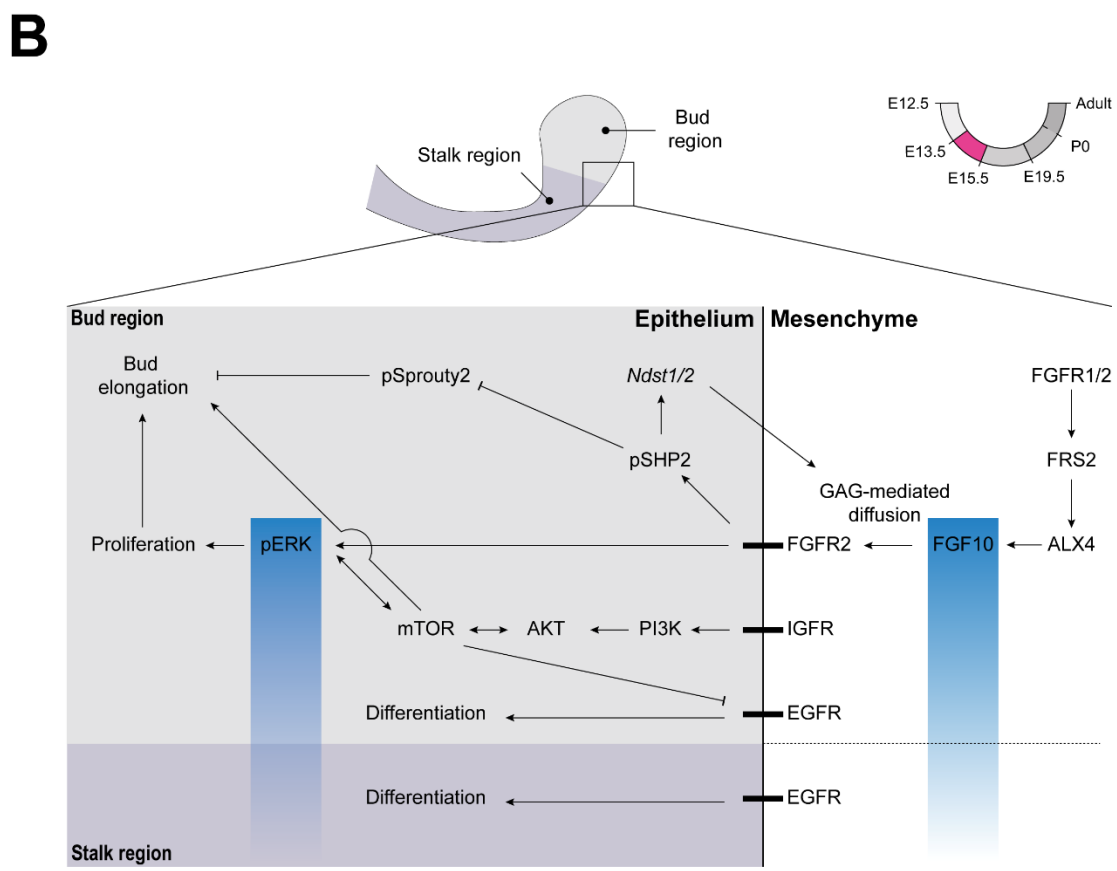
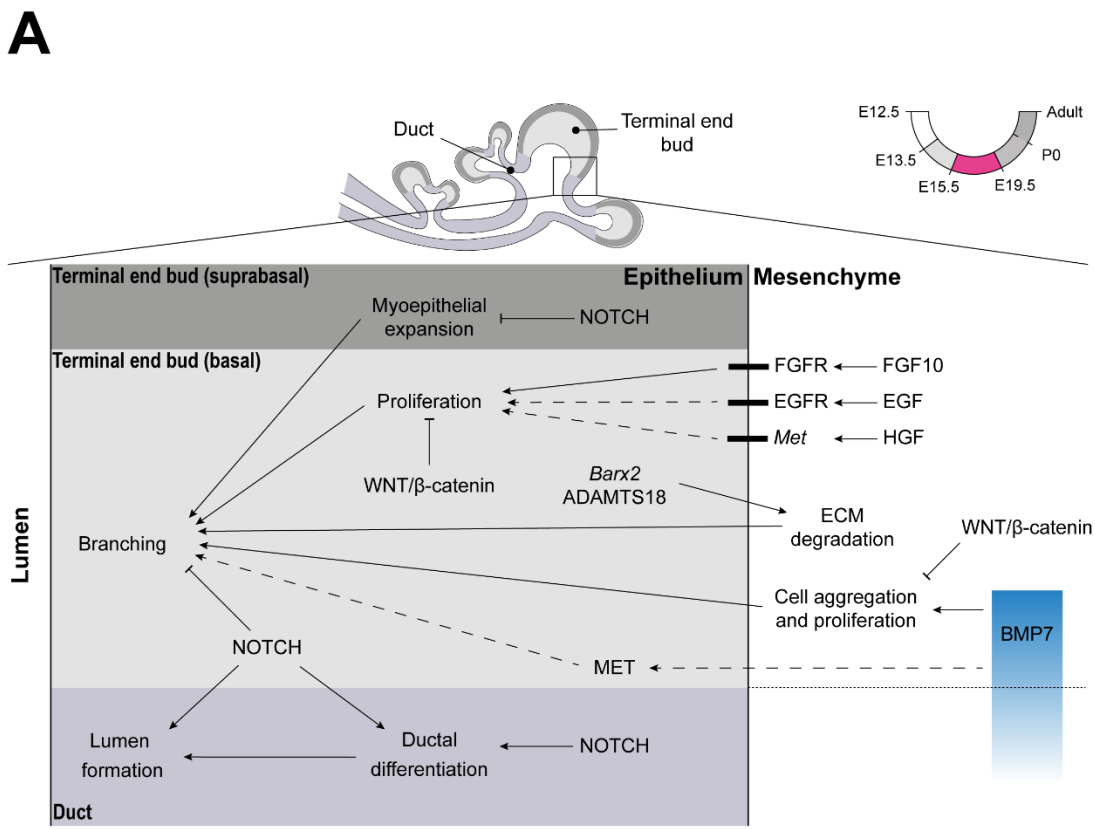

B
Stalk region
Bud
region
E12.5
Adult
E13.5
P0
E15.5
E19.5
Bud region
Epithelium
Mesenchyme
Bud
elongation
pSprouty2
Ndst1/2
FGFR1/2
pSHP2
FRS2
GAG-mediated
diffusion
Proliferation
pERK
FGFR2
FGF10
ALX4
mTOR
AKT
PI3K
IGFR
Differentiation
EGFR
Differentiation
EGFR
Stalk region

Figure 2. Signaling pathways and cell specification in the presumptive and budding stages of LG embryonic development. (A) Presumptive stage. Conclusions from previous studies reveal FGF10 and FGF7 in the mesenchyme drive the thickening and formation of the presumptive LG bud via FGFR2 epithelial expression[9]. GAGs produced by GAG synthesis enzymes in the mesenchyme limit FGF10 diffusion by serving as co-receptors [19]. FGFR2 further signals in the epithelium of the presumptive bud through Sox9, which in turn targets Sox10, ERK phosphorylation, Mia1, Dusp6, and Erm to induce bud formation [18,19]. miR-205 signaling via Cadm1 and Inppl1 also induces bud formation, and Pax6 signaling acts as a FGF10-FGFR2 competence facto [9,20]. (B) Budding stage. The budding stage starts at E13.5 after the presumptive bud is formed and ends with the bud elongation at E15.5 [9]. FGF10-FGFR2-*Sox9* drives LG bud elongation [18]. The sulfation of heparan sulfate by *Ndst* mediates this interaction [29]. In particular, FGF10 and ERK activation gradients drive proliferation in the distal bud and differentiation via EGFR in the proximal stalk of elongating LG buds [30]. Upstream, FGFR1/2-FRS-ALX4 induces the FGF10 gradient that drives budding [33]. Complex negative feedback loops of SHP2-RAS-SPYR2 signaling also regulate budding [31]. Similarly, crosstalk between the PI3K-Ras-ERK-AKT pathway inhibits EGF signaling to allow bud proliferation and extension[11].


A
Duct
Terminal end bud
E12.5
Adult
E13.5
P0
E15.5
E19.5
Terminal end bud (suprabasal)
Epithelium
Mesenchyme
Myoepithelial expansion
NOTCH
Terminal end bud (basal)
FGFR
FGF10
Proliferation
EGFR
EGF
Met
HGF
WNT/β-catenin
Barx2
ADAMTS18
Lumen
Branching
ECM degradation
WNT/β-catenin
Cell aggregation and proliferation
BMP7
NOTCH
MET
Lumen formation
Ductal differentiation
NOTCH
Duct

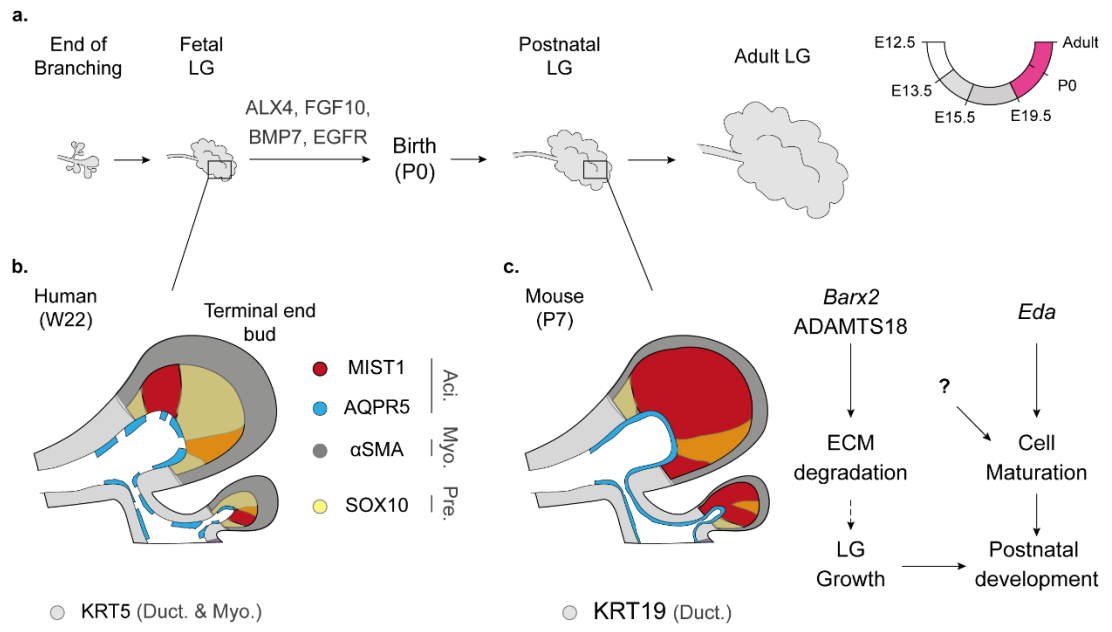
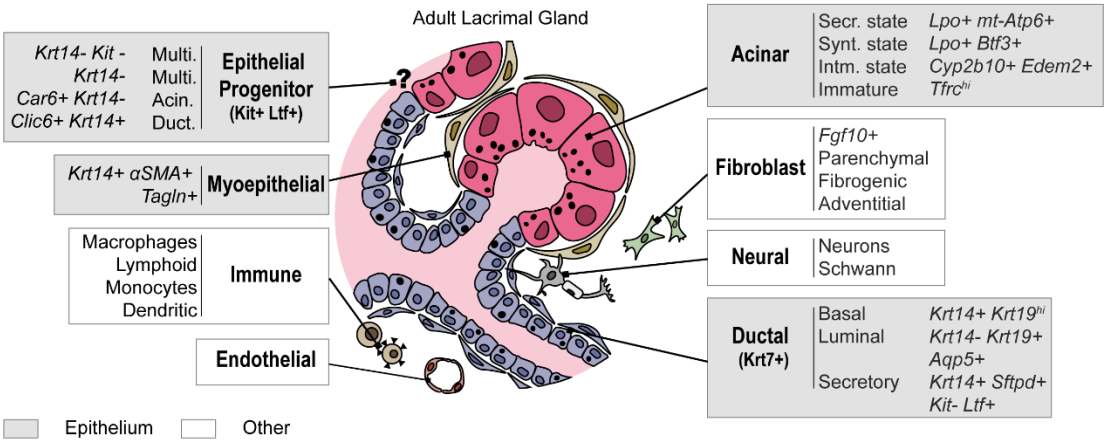

B
a.
End of Branching
Fetal LG
ALX4, FGF10, BMP7, EGFR
Birth (P0)
Postnatal LG
Adult LG
E12.5
Adult
E13.5
P0
E15.5
E19.5
b.
Human (W22)
Terminal end bud
MIST1
AQPR5
αSMA
SOX10
Aci.
Myo.
Pre.
KRT5 (Duct. & Myo.)
c.
Mouse (P7)
Barx2
ADAMTS18
Eda
?
ECM degradation
Cell Maturation
LG Growth
Postnatal development
KRT19 (Duct.)

Figure 3. The lacrimal gland undergoes major structural changes during branching, fetal and postnatal development. (A) Branching stage. The elongated bud at E15.5 forms an arborized structure with many acinar branches connected to a main duct by E19.5 [8–10]. Branching is created by cell proliferation of terminal end buds and apoptosis of ductal cells[10]. In addition, E-cadherin expression also indicates a role in MET during branching [10]. Key regulators of branching are the gradients of BMP7 and FGF10 that drive mesenchymal and epithelial cell proliferation and death, respectively [34]. Involved in BMP7 control, WNT/β-catenin signaling antagonizing BMP7 activity and branching [36]. Control of cell population expansion via NOTCH signaling draws the borders between LG ducts and terminal end bud regions during branching [10]. Other RTK signaling pathways than FGF, namely EGF and HGF signaling also likely drive proliferation during branching [15]. In addition, fibronectin cleavage via ADAMTS18 and interaction of *Barx2*-FGF10-MMPs are also required for branching [12,38]. (B) Schematic representation of fetal and postnatal development. (a.) Murine fetal development overlaps with branching while postnatal development exhibits cell maturation and an increase in LG size until adulthood [10]. Human fetal development shows similar features when compared to murine early postnatal development [8,39]. (b.) In human, expression of differentiation markers resembling the adult epithelium is present by W23 [39]. At W23, *SOX10* and *MIST1* mark different populations of acinar-like cells and *AQP5* marks both acini and small ducts[39]. Myoepithelial genes *KRT14* increase by W23, and colocalize with *aSMA* in suprabasal cells[39]. Basal ductal and myoepithelial marker *KRT5* is also present by W23 [39]. (c.) In comparison, differentiation marker distribution in postnatal P7 murine development resembles adult tissue [39]. S*OX10* expression decreases from P1 onwards, while *MIST1* is expressed in most acinar cells by P7 [39]. *AQP5* increases in expression in acinar and ductal cells by P4, localizing apically [39]. *KRT19* marked all luminal ductal cells and *aSMA* started acquiring cell shape maturation from P3 [39]. Only a few pathways have been identified in postnatal development. These are related to ECM remodeling via Barx2 and ADAMTS18, as well as the cell maturation pathway via *Eda* [12,38,48]. Abbreviations: Aci., acinar; Myo., myoepithelial; Pre., precursor.

A


Adult Lacrimal Gland
Krt14- Kit - Multi.
Krt14- Multi.
Car6+ Krt14- Acin.
Clic6+ Krt14+ Duct.
Epithelial Progenitor (Kit+ Ltf+)
Krt14+ αSMA+ Tagln+
Myoepithelial
Macrophages
Lymphoid
Monocytes
Dendritic
Immune
Endothelial
Acinar
Secr. state Lpo+ mt-Atp6+
Synt. state Lpo+ Btf3+
Intm. state Cyp2b10+ Edem2+
Immature Tfrc^hi
Fibroblast
Fgf10+
Parenchymal
Fibrogenic
Adventitial
Neural
Neurons
Schwann
Ductal (Krt7+)
Basal Krt14+ Krt19^hi
Luminal Krt14- Krt19+ Aqp5+
Secretory Krt14+ Sftpd+ Kit- Ltf+
Epithelium
Other

B

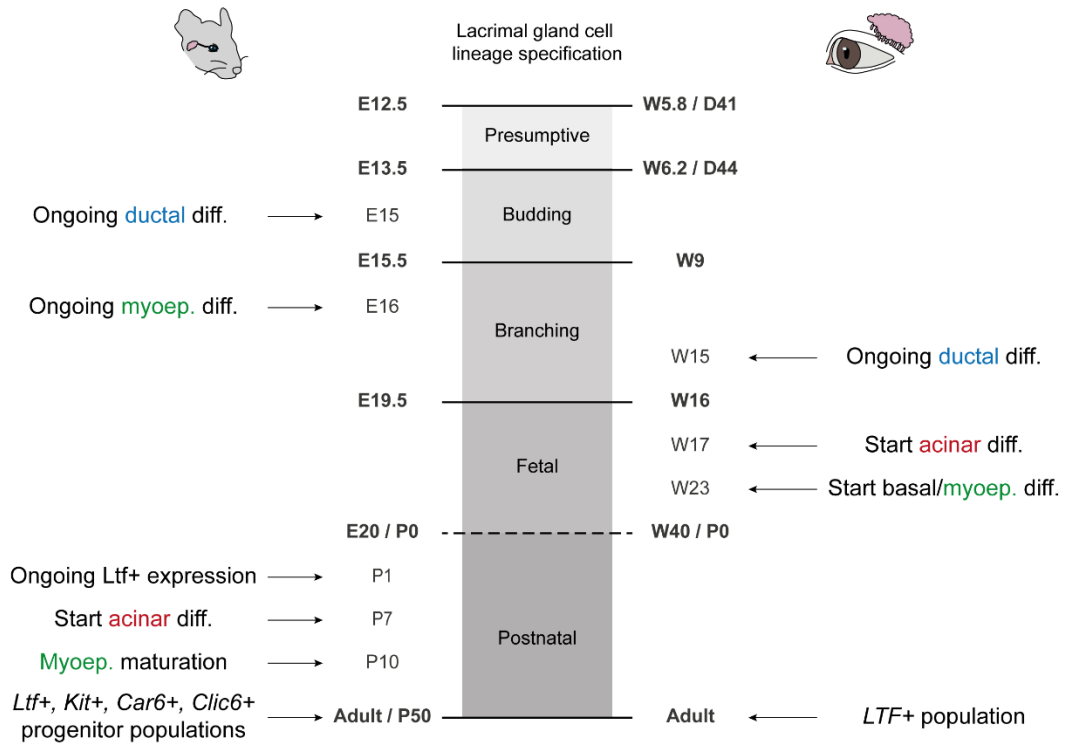

Lacrimal gland cell lineage specification
E12.5
W5.8 / D41
Presumptive
E13.5
W6.2 / D44
Ongoing ductal diff.
E15
Budding
E15.5
W9
Ongoing myoep. diff.
E16
Branching
W15
Ongoing ductal diff.
E19.5
W16
W17
Start acinar diff.
Fetal
W23
Start basal/myoep. diff.
E20 / P0
W40 / P0
Ongoing Ltf+ expression
P1
Start acinar diff.
P7
Myoep. maturation
P10
Postnatal
Ltf+, Kit+, Car6+, Clic6+ progenitor populations
Adult / P50
Adult
LTF+ population

Figure 4. Lacrimal gland adult cellular heterogeneity and cell lineages during development. (A) Cellular heterogeneity in the adult LG. The adult LG is composed of different cell types arising from highly dynamic developmental changes. These cell types include glandular epithelial cells, multiple fibroblasts subpopulations, endothelial cells, four major immune cell types, pericytes, neurons, and Schwann cells [6,13,44]. The glandular epithelial cells consist of multiple acinar cell types, secretory ductal cells, basal ductal cells, luminal/intercalated ductal cells, and a single myoepithelial cell type [6,13]. Subpopulations of acinar cells are classified based on their secretory state [6,13]. Epithelial progenitor cells are identified among *Kit*+ and *Ltf*+ cell subpopulations [6,13]. Abbreviations: Multi., multipotent; Acin., acinar; Duct., ductal; Secr., secretory; Synt., synthesizing; Intm., intermediate. (B) Cell lineage specification during LG development in mouse and human, compared based on acquisition of morphological features[8–10]. The first differentiation steps of the human LG epithelium occur during fetal development, except for ductal cells that are already specified during branching [39]. In addition, adult humans also have an *LTF*+ population[7,17]. Ductal cell differentiation already occurs during budding in mice, while myoepithelial expression is already reported in branching[10]. Unlike humans, acquisition of acinar differentiation markers takes place much later in postnatal development [39]. In mice, *Ltf*+ progenitors are already present by P1 [39]. Abbreviations: diff., differentiation; myoep., myoepithelial.

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