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INVITED REVIEW

Thymic Mimetic Cells: Evolutionarily Ancient Mirrors of the Periphery

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1 | Introduction

T cells display a diversity of T cell receptors (TCRs), enabling them to recognize and respond to the vast range of threats they encounter. Since TCR diversity is generated by essentially random processes, self-reactive receptors are sometimes generated and can result in autoimmunity. Self-tolerance is imposed in the thymus: differentiating T cells encounter antigens, and self-reactive cells are either deleted or diverted to the regulatory T (Treg) cell lineage (reviewed [1]). Medullary thymic epithelial cells (mTECs) are responsible for purging many of the self-reactive T cells, through their expression of transcripts encoding peripheral tissue antigens (PTAs), also referred to as tissue-specific antigens (TSAs). mTECs express PTAs by two mechanisms: Aire-induced transcription and thymic mimetic cells. The transcription factor, Aire, induces expression of a large repertoire of PTA transcripts via a complex mechanism that has been the subject of a great deal of interest since its function was first elucidated [2, 3]. In contrast, thymic mimetic cells express PTAs in a biologically logical fashion. As transcriptional hybrids of mTECs and peripheral cell types, mimetic cells imitate peripheral cells in their chromatin landscape, transcriptional profiles, and dependence on lineage-defining transcription factors (TFs), while still maintaining their mTEC identity [4]. "Misplaced" thymic stromal cells were first observed histologically, as early as the mid-1800s [5], with no real understanding of their function(s). Interest in them was reignited by their sporadic observation in single-cell RNA sequencing (scRNA-seq) data, but their underlying biological rationale awaited the discovery that sharing of lineage-defining transcription factors with their peripheral counterparts drove their development [4].

Many adaptive immune processes are evolutionarily ancient, including the appearance of the thymus (reviewed recently [6]). Animals in the lineage of jawed vertebrates, which emerged approximately 530 million years ago, exhibit RAG-recombinase-mediated recombination and have a thymus. In contrast, the more evolutionarily ancient jawless vertebrates have functionally similar adaptive immune systems but rely on different recombination mechanisms and lack a thymus, although thymus-like "thymoid" structures have been reported in lamprey [7]. Though mimetic cells were first characterized in depth in mice, studies in zebrafish and humans highlight their evolutionary conservation. Here we review what is known about mimetic cells and their identities in diverse species, provide perspectives on evolutionary pressures shaping the mimetic cell repertoire, and discuss future applications and implications for human health.

2 | Early Histological Observations of Mimetic Cells Across Various Species

Before modern descriptions of mimetic cells, "misplaced" stromal cells were observed in thymi of evolutionarily divergent organisms. In 1846, Arthur Hill Hassall described cornified, skin-like structures in the human thymus [5]. These "concentrischen Körper der Thymus" ("concentric bodies of the thymus"), named Hassall's corpuscles, were also found in several other species, including rabbits and birds [8, 9], dogs [10], frogs [11], and pigs [12].

Also in the late nineteenth century, striated cells—akin to skeletal muscle—were observed within the thymus by microscopy. Thymic myoid cells were reported in the frog thymus in 1888 [13] and later in other species, including chickens, buzzards, snakes, and lizards [14]. Myoid cells were described in the human thymus several years later [15]. The morphology and location of thymic myoid cells varied (reviewed [16]), including round or elongated morphologies occurring singly or in groups and occasionally at the edge of Hassall's corpuscles [15, 17].

Misplaced stromal cells were observed before the function of the thymus was determined, and these enigmatic cells confounded understanding of the nature and function of the thymus. For instance, the presence of cell types like thymic myoid cells, dissimilar to other lymphatic tissue, was cited as "arguments against the lymphatic nature of the thymus" [15]. Indeed, studies on frogs revealed seasonal thymic involution (so-called "winter involution"), including diminished Hassall's corpuscles [11]. It was postulated that these changes were due to a nutritive function of the thymus, resulting in depletion of the thymus akin to reserves in the muscle and fatty tissue [11]. These changes may reflect what we know today as stress-induced TEC degeneration [1].

3 | Identification and Unification of Mouse Mimetic Cells in the 21st Century

At the start of the 21st century, the function of the thymus had been elucidated and a great deal of interest was focused on the transcriptional regulator, AIRE, whose function in upregulating PTA gene expression had recently been ascribed [2]. Modern interest in misplaced thymic stromal cells was rekindled in the last several years by the advent of scRNA-seq.

To characterize thymic stromal cell heterogeneity, two groups concurrently profiled mouse thymic stromal cells and reported tuft mTECs [18, 19]. These cells were akin to peripheral tuft cells, chemosensory first-responders found in the intestine, trachea, and other epithelial barriers [20, 21]. Thymic tuft cells expressed classical tuft cell marker genes such as *Trpm5*, *Dclk1*, *IL25*, and *Pou2f3* [18, 19]. Imaging analyses revealed DCLK1$^+$ thymic tuft cells with brush-like projections, akin to peripheral tuft cells [18], and similar to those previously observed histologically in the thymus [22]. Peripheral tuft cells are reliant on the TF, Pou2f3, for their differentiation [20], and thymic tuft cells proved to be similarly reliant on this TF, being absent in *Pou2f3*$^{-/-}$ mice [18].

The Miller et al. and Bornstein et al. studies also demonstrated functional roles for tuft mimetic cells in the thymus: *Pou2f3*$^{-/-}$ mice had diminished numbers of NKT2 cells [18] and a larger population of Innate Lymphoid Cells type-2 (ILC2s) [19]. Subsequent studies confirmed their roles in iNKT1 cell differentiation [23] and highlighted their regulation of thymus regeneration through IL-25-mediated ILC2 activation [24] (Figure 1).

Alongside thymic tuft cells, additional heterogeneity among thymic stromal cells was reported in other scRNA-seq studies, including ciliated, neural, *Gp2*-expressing, and *Tspan8*-expressing thymic epithelial cells [25, 26]. But, at this point, the derivation, regulation, and functions of these cells were unclear; in particular, there was no unifying concept that elucidated their role in T-cell tolerance induction.

An important advance came from the investigation of the chromatin landscape of mTECs at the single-cell level: scATAC-seq uncovered an unexpectedly rich set of "misplaced" stromal cells, and TF-motif-enrichment analysis revealed their expression of diverse TF families—including Pou2f3, FoxA, Grhl, Hnf4, and Sox—important for the development of tissues such as the gut, skin, and neuroendocrine tissues [4]. These cells constituted a "post-Aire" compartment characterized by down-regulation of Aire and MHCII molecule expression. Specific enrichment of the post-Aire compartment coupled with scRNA-seq was revelatory for understanding the true diversity of mimetic cells populating the murine thymus, which included tuft, microfold, ciliated, keratinocyte, ionocyte, muscle, neuroendocrine, goblet, basal (skin), basal (lung), entero/hepato, and Ptf1a$^+$ pancreatic mimetic cells [4]. Similar to the lack of tuft mimetic cells in *Pou2f3*$^{-/-}$ mice [18], deletions of the genes encoding the lineage-defining TFs, Spib or Sox8, diminished the population of microfold mimetic cells [4]. Likewise, in subsequent studies, mice with *Hnf4a*$^{-/-}$ and *Hnf4g*$^{-/-}$ TECs (and especially doubly deficient mice) had reduced numbers of entero/hepato mimetic cells [27], and thymic *Insm1* deletion resulted in reduced numbers of neuroendocrine mimetic cells and diminished expression of neuroendocrine antigens in the mouse thymus [28, 29]. These studies underscored the dependence of mimetic cells on lineage-defining TFs (Figure 1).

The unification of "misplaced" stromal cells as "mimetic" cells that constitute a second mechanism, complementary to Aire-mediated PTA expression, of previewing peripheral antigens raises several questions about the nature and function of mimetic cells. First, what role do mimetic cells play in tolerance induction? And do they have additional functions in the thymus? The sufficiency of mimetic cells to induce tolerance was demonstrated when a model antigen was expressed in ciliated or muscle mTECs, and reductions in CD4$^+$ T cells recognizing the antigen were observed [4]. In complementary experiments, mice lacking Insm1 in TECs were missing particular endocrine mimetics and had autoantibodies against stomach proteins [28], while mice lacking Hnf4a and Hnf4g in TECs had a defective entero/hepato-mimetic compartment, which resulted in more severe disease in an induced colitis model, though with mild evidence of spontaneous disease [27]. Mice lacking Pou2f3 in TECs were missing tuft mimetic cells; when immunized with IL-25 protein, these mice generated an anti-IL-25 antibody response that was not generated in wild-type mice [18]. These studies indicated that mimetic cells are important sources of PTAs for T cell education, although the exact interplay between Aire-mediated PTA expression and mimetic cell PTA expression is unclear.

Mimetic cells also have functions beyond the imposition of self-tolerance (Figure 1). In addition to tuft mTEC cross-talk with NKT and ILC2 populations [18, 19, 23, 24], endocrine and microfold mimetic cells show additional functions [28]. Knockout of ghrelin-expressing endocrine TECs resulted in

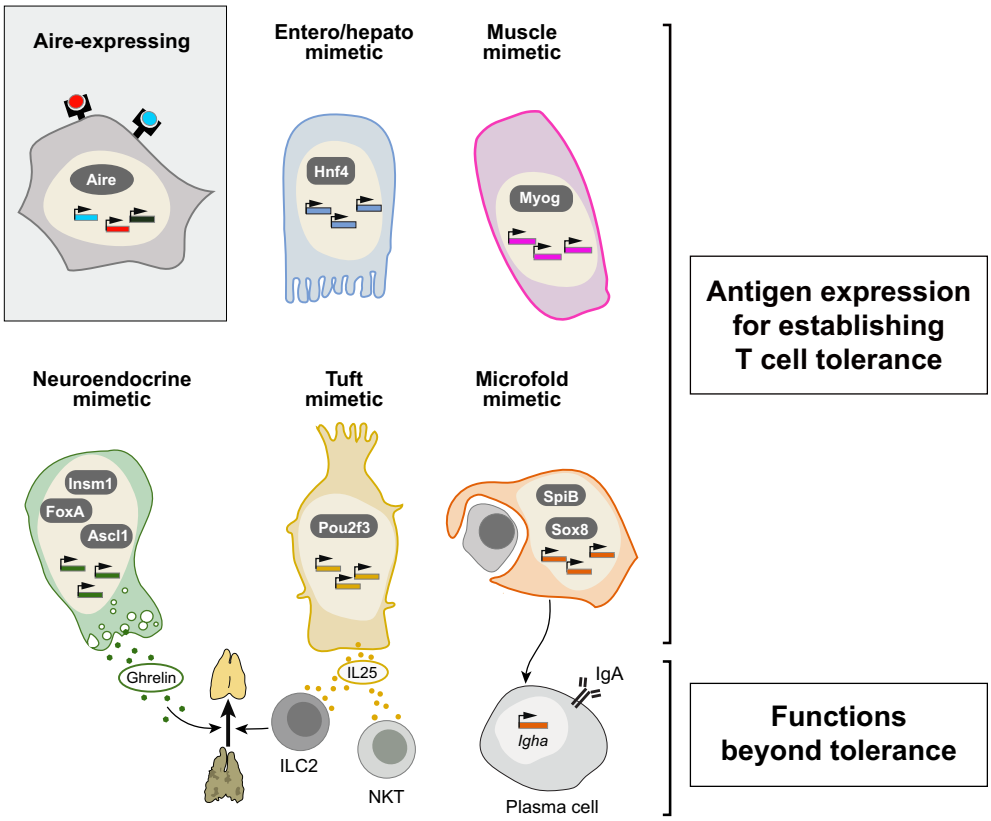

FIGURE 1 | Mimetic cell characteristics and roles. Like Aire-expressing mTECs, mimetic cells are involved in establishing central T cell tolerance. Self-reactive T cells are clonally deleted or diverted to become T regulatory cells. Mimetic cells can also perform functions reflecting their extra-thymic counterparts: Tuft mimetic cells express IL25 and interact with ILC2s and NKT cells, and the ILC2-mediated response promotes thymus regeneration; neuroendocrine mimetic cells secrete ghrelin, regulating thymic cellularity; and microfold mimetic cells are essential for IgA^+ plasma cell induction. Mimetic cells are also dependent on lineage-defining transcription factors. Enterocyte-hepatocyte mTECs depend on Hnf4, tuft mTECs on Pou2f3, neuroendocrine mTECs on Insm1, and microfold mTECs on SpiB and Sox8.

lower thymic cellularity and weight in older mice, which was rescued by intrathymic ghrelin injection [28]. Microfold mimetic cells induced thymic IgA^+ plasma cells, apparently engaging in crosstalk with APRIL-secreting macrophages and APRIL-receptor-bearing B cells and plasma cells [28], and Spib- and Sox8-deficient mice had increased numbers of thymic B cells [4].

A second question—how do mimetic cells arise?—remains only superficially elucidated, although there are hints to their ontogeny. Aire-lineage-tracing data revealed that a substantial fraction of cells from most mimetic cell subtypes went through an Aire stage [4]. However, those mimetic cell types that passed through an Aire stage showed a differential dependence on Aire. Aire-deficient mice had variable but diminished numbers of most mimetic cell subtypes, particularly neuroendocrine, ciliated, lung, and microfold mTECs [4]. But a few mimetic cell populations appeared to be independent of Aire: muscle mimetics and one of two tuft mimetic populations were not diminished in Aire-deficient mice [4]. Elucidating the differentiation pathways and regulatory networks underlying the emergence of diverse mimetics of peripheral tissues—and what prevents their continued differentiation—will undoubtedly be informative in understanding thymic development and may even be informative beyond the thymus in understanding differentiation pathways in the corresponding peripheral cell types, as was already demonstrated for the entero/hepato lineage [27].

In sum, modern tools for profiling gene expression and chromatin accessibility at the single-cell level enabled the identification and characterization of a constellation of mimetic cell types within the mouse thymus. These cells have roles in inducing T cell tolerance as well as additional functions mimicking their peripheral counterparts.

4 | Thymic Mimetic Cells in Various Mouse Strains

Understanding strain-to-strain variability in mouse mimetic cell identity and abundance may inform our understanding of the genetic regulation of mimetic cell differentiation and also presents new opportunities for the study of mimetic cells in specialized strains or in F1 models. Prior studies explored differences in mTEC composition between mouse strains, though without specific enrichment or attention toward mimetic cells [26, 30].

To explore the conservation of mimetic cell subtypes across mouse strains, we performed scRNA-seq on purified mimetic cell compartments (as per Michelson et al. [4]) from NOD, BALB/c, and C57BL/6 (B6) mice (Figure 2). Contaminating

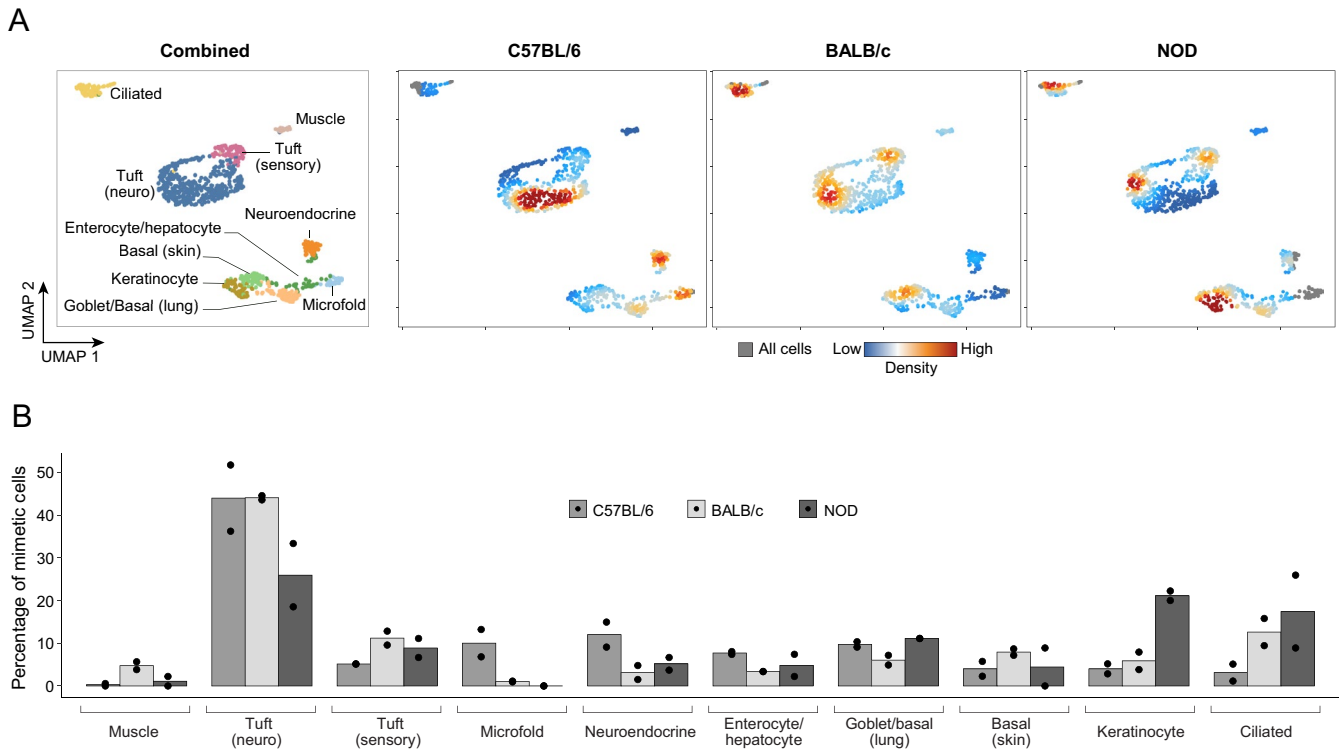

FIGURE 2 | Analysis of the mimetic cell repertoires in three mouse strains. (A) scRNA-seq characterization of the post-Aire compartment of B6, BALB/c, and NOD mice, shown in UMAP space. Cells from the three mouse strains are combined (left) and separated, shown as local cell densities (right). (B) Frequency of mimetic cell subtypes in each species. Bar plots show the average percentage of each subtype, and dots show the values for each mouse. UMAP, uniform manifold approximation and projection.

hematopoietic cells and immature mTECs were removed, and clusters were annotated based on the expression of cell-type markers, lineage-defining transcription factors, and overlap with published mimetic-cell subtype signatures [4]. We observed ciliated, muscle, keratinocyte, entero/hepato, microfold, neuroendocrine, two tuft, basal (skin), and basal (lung)/goblet mimetic cell subtypes (Figure 2A). Replicate mice from a single strain showed similar frequencies for most mimetic cell types (Figure 2B). There was a high degree of conservation across the three strains. All but one of the annotated mimetic cell subtypes appeared in all three strains (Figure 2A,B); although microfold cells were not detected in NOD mice, sequencing of more cells might uncover them. The relative abundances of mimetic cell subtypes did vary between strains: most prominently, keratinocyte mimetic cells were highest in NOD mice; microfold and neuroendocrine mimetic cells were most abundant in B6 mice; and muscle mimetic cells were the most frequent in BALB/c mice.

5 | Mimetic Cells in the Human Thymus

Molecular characterization of mimetic cells in mice prompted questions about the presence, identity, and function of mimetic cells in the human thymus. Interest was particularly acute given the potential relevance in human autoimmune disease. Initial observations of human thymic mimetic cells using scRNA-seq were published before misplaced thymic stromal cells were unified as "thymic mimetic cells" following their in-depth molecular characterization in mice. Studies variably reported the presence of muscle, neuro, tuft, ionocyte, ciliated, and corneocyte-like TECs [31–33].

An important advance for our understanding of human mimetic cells came when the repertoire was defined in depth, exploiting selective enrichment of the compartment with scRNA-seq, as was revelatory in mice [34]. Flow cytometric analysis and population-level RNA-seq of human mTECs revealed that the human thymus harbors an mTEC^{lo} PDPN^{-} CD104^{-} population analogous to that in mouse [34]. Subsequent scRNA-seq on cytofluorimetrically enriched mimetic cells provided an in-depth view of the human mimetic cell compartment, revealing a massively expanded muscle mimetic population and more diversified ionocyte and neuroendocrine mimetic populations in comparison with those of mice [34]. The composition of the human mimetic cell repertoire and frequency of each subtype was surprisingly conserved across individuals in early life [34], suggesting that mimetic cells arise in an orchestrated manner.

Several features of the human data provided additional insights into mimetic cells. Muscle mimetic cells were 25-fold more frequent in humans than in mice, mirroring the differentiation trajectory of peripheral skeletal muscle [34]. Mature muscle mimetic cells downregulated immature mTEC marker genes like *CCL19* and upregulated mature marker genes like *DMD* and *DLK1* [34]. Skeletal muscle developmental dynamics are well-characterized. TF gene expression suggested that human muscle mimetics relied on skeletal muscle TFs, although there was not a strict recapitulation of peripheral ontogeny. Namely, the genes encoding TFs that initiate skeletal muscle differentiation,

such as PAX3 and PAX7, were not expressed in muscle mTECs. Instead, immature and mature muscle mTECs alike expressed terminal muscle-cell differentiation TF genes such as *MYOG* and *MEF2A*, likely important for the maintenance of muscle identity among mTECs. This observation highlighted the reliance of mimetic cells in the human thymus on lineage-defining TFs, as in mice, but suggested that initiation pathways may differ from their peripheral counterparts. Immature and mature muscle mTECs could be visualized in the human thymus, and some mature muscle mTECs had elongated striated microstructures [34], similar to those previously reported in the human thymus in the early 1900s [15].

Compared with mice, the human thymus also harbored a more diverse complement of neuroendocrine mTEC, mirroring peripheral sensory and motor neurons, interneurons, and neurons of the brain [34]. Intriguingly, one subtype mimicked cochlear hair cells, found in the inner ear and important for mechanotransduction of sound. These cells expressed the gene encoding the TF ATOH1 along with other TFs (i.e., BARHL1, POU4F3, LHX3, PAX2) that constitute a network important for inner-ear hair-cell differentiation and maintenance. Also expressed were the *OTOF* and *USH2A* genes, whose mutations are associated with human auditory disease [34]. Cochlear hair cells are transcriptionally similar to Merkel cells, a mechanosensory cell type in the skin, and both cell types rely on the TFs ATOH1 and POU4F3 for differentiation [35]. An alternative interpretation of this mimetic cell type is that it represents a precursor to these two mechanosensory cell types, as suggested by Ragazzini et al. [36].

Another highly specialized cell subtype in the human thymus mimicked type B intercalated cells in the kidney, expressing the gene specifying the TF HMX2, as well as INSRR, SLC26A4, and SLC4A9 [34]. These highly specialized cells expressed *IL18*, which is thought to be important in defense against urinary tract infections [37]. In studies using analogous isolation and characterization approaches, ionocytes comprised less than 1% of mimetic cells in mice and approximately 22% in humans [4, 34]. It is unclear whether the observed specialization is unique to the human thymus or whether its capture was enabled only because of its abundance in the human thymus.

The data on human mimetic cells may also clarify some controversial reports of the past. Namely, one subset of human neuroendocrine mimetic cells expressed the gene encoding the TF FEZF2 [34]. Fezf2 has been reported to drive the expression of certain PTA transcripts in the murine thymus. Thymic deletion of *Fezf2* resulted in autoimmunity, although the mechanism was unclear [38]. Further complicating the picture, the ablation of mouse Fezf2 was associated with diminished numbers of tuft mimetic cells [39], potentially through Fezf2-mediated thymic regulation of some tuft-cell-associated genes [40]. The presence of *FEZF2*$^{+}$ neuroendocrine mimetic cells in humans, and the potential presence of a low abundance analog in mice, may contribute to the transcriptional changes in *Fezf2*$^{-/-}$ mice [38], although the relationship with tuft-cell-related effects is unclear.

Spatial localization of mimetic cells hinted at additional potential functions. By immunofluorescence imaging, there were incidences of muscle and neuroendocrine marker colocalization, as well as rare colocalization of muscle and tuft markers with intervening alpha-bungarotoxin, an alpha neurotoxin that binds to the nicotinic acetylcholine receptor (AChR) [34, 41]. These findings raised the possibility that muscle mimetic cells form structures akin to neuromuscular junctions in the thymus. This pattern of colocalization may be relevant in myasthenia gravis (MG), an autoimmune disease affecting the neuromuscular junction, in which 80% of patients have detectable antibodies against the AChR [42].

High-dimensional assessments of mimetic cell organization in the human thymus hold promise for elucidating interactions among mimetic cells, immunocytes, and other stromal cells. Yayon et al. applied 10X Genomics Visium spatial transcriptomics and the highly multiplexed IBEX ("iterative bleaching extends multiplexity") study protocol [43] to human pediatric and fetal thymi [44], reporting the presence of keratinocyte, muscle, neuro, ciliated, ionocyte, and tuft mimetic cells. They described a cortico-medullary axis system for characterizing thymic localization along with quantitation of distances from landmarks, namely from Hassall's corpuscles [44]. Mimetic cell subtypes had variable distances from Hassall's corpuscles, and the nearest mimetic cell types were ciliated mTECs and keratinocyte mTECs, consistent with historic and more current descriptions [5, 44, 45]. This work provides a technical framework for investigating mimetic cells spatially and illuminating potential interaction partners.

6 | Mimetic Cells in Zebrafish

Zebrafish, which diverged from humans and mice approximately 450 million years ago, provide an evolutionarily divergent comparator for humans and mice [46, 47]. Like these two species, zebrafish are jawed vertebrates, with a thymus that has demarcated medullary and cortical regions (reviewed [48]). The zebrafish thymus is distinguished by its bilateral nature, such that thymic lobes are anatomically separated [48]. Several mimetic cell types, including tuft and neural mTECs, were observed in zebrafish in the course of studying lymphocyte development [49], and were subsequently described in greater depth following a relative enrichment of mimetic cells and scRNA-seq [34]. Given the paucity of zebrafish antibody reagents, relative enrichment was performed via transgenic reporter-based depletion of T and B cells.

The zebrafish mimetic cells identified so far include tuft, muscle, ionocyte, neuroendocrine, periderm, ciliated, metaphocyte, macrophage-like, structural, and ear non-sensory cells [34]. Several of these subtypes represented specialized, fish-specific mimetic cells. For example, fish harbor specialized peripheral ionocytes, such as a subtype important for pH regulation in fluctuating aquatic environments, and another implicated in environmental calcium uptake [50–53], and these specialized subtypes were mirrored by zebrafish mimetic cells [34].

Zebrafish also had specialized neuro and sensory mimetic cells. Among these, neuromast mTECs mimicked the fish neuromast, which detects water displacement on the surface of fish via mechanosensory hair cells, and neuromast mTECs expressed the genes encoding the TFs atoh1a, sox2, prox1a, and drgx [34].

FIGURE 3 | Conservation of mimetic cell subtypes between humans, mice, and zebrafish. The frequency of each mimetic cell subtype was calculated based on its abundance among mimetic cells in published scRNA-seq datasets [4, 34].

Furthermore, fish had ciliated olfactory sensory neuron mimetic cells, which expressed marker genes such as *foxj1b* and *or132-5* [34]. In contrast, a more generic ciliated mimetic cell type was found in the mouse thymus [4].

The zebrafish thymus also harbored several unique cell types that appeared to be mimetic cells, although their epithelial identities could not be validated given the lack of zebrafish-specific antibodies. Notably, a subset of cells seemed to mimic zebrafish metaphocytes, an ectoderm-derived, macrophage-like cell type that is found in zebrafish barrier tissues [54, 55]. Metaphocytes are not known to be localized in the thymus, which supported their identity as metaphocyte mimetic cells. A related second population expressed *spi1a* and *spi1b* alongside conventional macrophage markers [34]. These cells also expressed the epithelial marker *epcam*, supporting their mimetic cell identity. The antigen-transferring properties of these zebrafish subtypes suggested that they may be akin to antigen-presenting microfold mimetic cells in the mouse thymus [4]. Additional tools, like relevant zebrafish-reactive antibodies or genetic reporters, would be useful for resolving their identities.

In sum, the zebrafish thymus harbored many of the same subtypes as humans and mice, despite around 450 million years of evolutionary divergence. Zebrafish mimetic cells also reflected fish-specific specialization of peripheral tissues.

7 | Factors Shaping Mimetic-Cell Composition

The mimetic-cell repertoires of evolutionarily divergent species have many of the same cell types, as summarized in Figure 3, although their specialization and frequencies can vary considerably. Muscle, ionocyte, neuroendocrine, tuft, skin, and ciliated mimetic cells are found in humans, mice, and zebrafish. But within these cell types, species-specialization is evident, such as the human-specific or fish-specific ionocyte cell types described above or the presence of neuromast mimetic cells in fish. Notably, several subtypes seem to be absent from certain organisms, such as the apparent absence of microfold and other gut-associated mimetic cells—found in mice—from the human thymus. What accounts for such differences in representation? First, it is important to keep in mind that a true absence is difficult to prove, as sequencing an order of magnitude more mimetic cells could potentially uncover them. Beyond that, several factors could contribute to the differences, ranging from technical factors—like variability in isolation protocols, compartment markers, and species age—to true biological disparities like divergent evolutionary pressures and gene-regulatory mechanisms.

Two studies, enriching for mimetic cells in humans and mice, utilized equivalent isolation protocols yet still reported differences in the repertoires annotated [4, 34]. As such, it is less likely that the thymic digestion and isolation protocols are solely responsible for observed mouse-human differences. In these two studies, the cell-surface markers for enriching mimetic cells were also equivalent. It is possible, though, that a subset of mimetic cells resides in different compartments in the two species. For example, perhaps microfold mimetic cells have elevated HLA class II expression in humans and were thereby missed by the flow cytometry protocol used to isolate the post-Aire compartment in the Michelson et al. and Huisman et al. reports. However, several human studies have included the entire mTEC stromal compartment in their assessment, and their scRNA-seq data did not reveal microfold or other types of mimetic cells [31, 36]. Thus, while these "missing" subtypes might be present at levels below our detection, their absence is unlikely to result from their residence in different thymic compartments.

The effects of aging on the mimetic cell repertoire have not been well characterized, nor whether any such effects differ across species. Perinatal and adult mice have the same mimetic cell types, although the younger mice exhibit fewer tuft mTECs and a greater fraction of muscle, entero/hepato, and ciliated mTECs [4]. Similarly, the distribution of human mimetic cells is similar across early life [34] as well as in fetal, postnatal, and adult thymi [31].

Mimetic cells are variably specialized within and across species. For example, neuroendocrine mTECs reflect a more generalized cell type in mice, while human neuroendocrine mimetics reflect a series of specialized subtypes, such as *ATOH1*$^{+}$ cochlear hair or *DRGX*$^{+}$ sensory neurons [4, 34]. One possibility is that such specialization of neuroendocrine mTECs does exist in mice, but is masked by the capture of relatively few cells. Variability in specialization is also evident within a given species. For example, in mice, closely related keratinized skin and basal skin cells are each represented in the thymus by separate mimetic-cell subtypes. Simultaneously, mouse gut enterocytes and liver hepatocytes are mimicked in the thymus by a single, composite entero/hepato mimetic-cell subtype [4, 56]. This variability

raises the question: why do some cells appear to abort their differentiation process early, while others develop into specialized mimetic cell subtypes? Potentially, mimetic cells rely on some of the same environmental cues as their peripheral counterparts to advance their differentiation processes, and variable availability of these signals may account for differences in specialization. Variable specialization may also reflect the propensities of TFs themselves to drive cellular differentiation to a late stage, which varies for individual TFs [57].

The evolutionary pressures shaping the composition of the mimetic cell repertoire appear to be independent of the abundance or relevance of a given tissue. For example, the presence of cochlear hair mimetic cells in the human thymus does not appear to reflect a need for enhanced tolerogenic protection, compared with a cell type such as a pancreatic beta cell, a cell type whose absence is lethal at an early age. Likewise, the presence of cochlear hair mimetic cells does not reflect their peripheral abundance—humans are estimated to have approximately 15,000 cochlear hair cells, a diminutive population compared with pancreatic beta cells, which are around 60,000-fold more abundant [58, 59]. As such, it is unclear if pressures shaping the mimetic cell composition are varied across organisms.

One could imagine other pressures, besides tolerance, that could potentially shape the appearance and abundance of mimetic cells in the thymus. Mimetic cells can perform functions akin to those of their peripheral counterparts, such as tuft cells interacting with NKT cells and ILC2s, microfold cells shaping thymic IgA production, and endocrine mTECs controlling thymic cellularity and regeneration [18, 19, 23, 24, 28]. Other mimetic cell types could possess additional, as-yet-undescribed extra-tolerance functions akin to their peripheral counterparts. Muscle mimetic cells, for instance, may have contractile capabilities. Studies in the 1970s-80s report contraction of myoid cells in thymic stromal cultures, although the exact identity of these cells is unclear [60–62]. Evolutionary pressures promoting or retaining such non-tolerance functions could shape the mimetic cell repertoire. Modern examination of muscle mimetic cells using advanced imaging techniques or careful molecular characterization of thymic stromal cultures may clarify if muscle mimetic cells can actually contract.

Recent findings concerning the target choices of Aire may inform our understanding of mimetic cell differentiation across species. A recent study identified Z-DNA-forming and NEF2-MAF-binding motifs as preferential targets of Aire [63]. Clues linking these Aire-target motifs with mimetic cells were provided in the same study: injection of an agent that stabilizes Z-DNA-formation resulted in upregulation of several mimetic cell signature genes in mTECs, including those for ciliated and tuft cells, and knock-out of NFE2L2 resulted in the downregulation of tuft and microfold signature genes in mTECs [63]. Features of the promoters and enhancers of lineage-defining transcription factors, such as the abundance and placement of Z-DNA, may differ between species and account for variable frequencies of a mimetic cell types between species.

In sum, there is a substantial degree of conservation of mimetic cell types across species. However, species-specific mimetic cell subtypes reflecting species-specialized peripheral analogs are also present. Additionally, some mimetic cells, such as microfold mTECs, have so far been identified only in a given species, despite the peripheral counterpart being present across species. The pressures and mechanisms underlying these differences are unclear, but might reflect roles beyond tolerance or might be a consequence of gene-regulatory mechanisms, such as the placement or abundance of Z-DNA.

8 | Implications for Human Health

The thymus is critical for the imposition of self-tolerance, and defects in its normal function can have detrimental health consequences, exemplified by autoimmunity resulting from mutations in the *AIRE* gene. The specific contributions of mimetic cells vis-à-vis Aire-mediated PTA expression to immunological tolerance are not fully elucidated, although studies in mice demonstrate that mimetic cells are important for establishing self-tolerance and regulating thymic regeneration and cellularity. Furthermore, features of human mimetic cells hint at their involvement in MG and thymic epithelial cell cancers. Mimetic cells also show an intriguing capacity to differentiate into diverse cell types while maintaining their TEC identity, which has promising applications in regenerative medicine and immune engineering. Here we discuss the implications of mimetic cells in human disease: for establishing or ameliorating central tolerance, in the context of MG and thymic epithelial cell tumors, and in regenerative medicine and transplantation.

8.1 | Relationship to Diseases of Altered Aire and PTA Expression

Understanding the potential implications of thymic mimetic cells for human health can be viewed in the context of other thymic alterations, abnormalities, and associated diseases. The autoimmune disorder APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) results from loss-of-function *AIRE* mutations (reviewed by [64, 65]). APECED is classically characterized by chronic mucocutaneous candidiasis, hypoparathyroidism, and primary adrenal insufficiency, although symptoms are broad and of variable severity, including type 1 diabetes, hepatitis, hypergonadotropic hypogonadism, alopecia, and anemia [64, 65]. Mimetic cells show a variable dependence on Aire expression in mice [4], though the repertoire of mimetic cells in patients with APECED has not been established. Assuming that human mimetic cells show a variable dependence on AIRE similar to that of mice, some mimetic cells may be present in the thymi of patients with APECED, and their expression of PTAs could potentially protect patients from some organ-directed autoimmunity.

Evidence on the role of mimetic cells in human immunological tolerance and potential roles in autoimmune disease is lacking. However, loss of Aire-driven PTA expression in APECED patients is a reference point for understanding the potential repercussions of the loss of mimetic-cell-mediated PTA expression. Deletion of mimetic cells in mice resulted in autoimmune effects of variable severity [18, 27–29]. One could imagine, then, that a loss of a particular subtype of mimetic cells could underlie certain cases of tissue-specific autoimmunity in humans. For

instance, loss of neuro mimetic cells might increase susceptibility to neuro-associated autoimmunity, or loss of muscle mimetic cells might augment the risk of myositis.

In contrast to APECED, patients with Down Syndrome (Trisomy 21) have an extra copy of the AIRE gene. Down Syndrome has been associated with autoimmunity and thymic abnormalities, including enlarged Hassall's corpuscles [66, 67], potentially due to altered AIRE expression [68]. In a study of the mimetic cell repertoire in pediatric thymi, a patient with Down Syndrome had the most substantially altered mimetic cell repertoire, with an increase in neuroendocrine mTECs and relative depletion of muscle and ionocyte mTECs [34]. Though only a single donor with Down Syndrome was profiled, the altered AIRE expression and mimetic cell repertoire could potentially underlie autoimmune pathogenesis, although the relative contributions of the two tolerization arms have not been explored.

8.2 | Engineering Mimetic Cells to Modulate Central Tolerance

Augmenting the numbers, identities, or gene-expression profiles of mimetic cells holds therapeutic promise. The establishment of additional mimetic cell types in the thymus might protect the corresponding peripheral tissue from autoimmune infiltration. For example, a beta cell mimetic cell might offer protection against type 1 diabetes. Indeed, studies inducing the expression of individual antigens in TECs have shown decreased incidence of diabetes in NOD mice [69]. Coordinated expression of tissue antigens via mimetic cells might promote tolerance to a general tissue program of antigens, rather than a single antigen, and thereby better protect against autoimmunity.

Intentionally diminishing mimetic cell numbers or gene expression has the potential to disrupt central tolerance to a cancer neoantigen and unleash anti-cancer immunity. The thymus establishes central tolerance to self-derived, tumor-associated antigens such as MAGE-A1 and MART1 [70], and mTEC depletion with RANKL blockade for depleting Aire-expressing cells increased anti-tumor T cells [71]. While there is much work to be done, CRISPR- or antibody-mediated targeting of mimetic cell subtypes has the potential to engineer central tolerance to cancer neoantigens.

8.3 | Myasthenia Gravis and Thymic Epithelial Cell Tumors

The potential implication of thymic mimetic cells in MG is of particular interest because of the expanded muscle mimetic cell population in the human thymus [34]. MG is an autoimmune disease that affects the neuromuscular junction and results in muscle weakness (reviewed by [42]). Approximately 80% of patients have autoantibodies against AChR, and a smaller fraction have antibodies against muscle-specific kinase (MUSK) or lipoprotein-receptor-related protein 4 (LRP4) [72]. MG is also characterized by the appearance of ectopic germinal centers in the thymus [73], as well as the development of thymoma, present in approximately 10% of MG patients [42]. In many patients, thymectomy results in improved myasthenic symptoms [42]. Imaging studies of the human thymus have shown colocalized muscle and neuroendocrine mimetic cell markers, such as colocalization of myosin heavy chain and calcium-dependent secretion activator (CADPS) or SRY-box transcription factor 2 (SOX2), alongside rare occurrence of colocalized muscle and tuft markers with intervening staining with alpha-bungarotoxin, which binds to the AChR and lights up neuromuscular junctions [34]. We hypothesize that mimetic cells might be autoimmunizing in MG. B cells reactive to one or more neuro-muscular junction proteins might arise in ectopic germinal centers in the thymus, potentially reacting against mimetic-cell-expressed antigen(s), and removal of this tissue would abort this process. Future studies could explore this possibility; in mice, deletion of AChR on muscle mimetic cells could reveal whether functional neuromuscular-junction-like structures are forming within the thymus, as well as their contribution to tolerance.

On the other hand, single-cell sequencing holds promise for understanding the relationship between MG and thymic epithelial tumor development [74]. This approach has been used to characterize thymic tumor and peripheral blood mononuclear cell (PBMC) samples [75–77], though few studies enriched for or captured substantial numbers of thymic stromal cells [78, 79]. A recent study of thymic epithelial tumors described mTEC clusters characterized by expression of *GNB3*, *MYOG*, or *CHGA* transcripts [79], akin to mimetic-cell subtypes described elsewhere as tuft, muscle, or neuroendocrine mimetic cells [34]. Xin et al. also described a nebulous *CHI3L1*$^{+}$ mTEC type enriched in a subset of thymic epithelial tumors [79]. Two additional studies explored the possibility that altered thymic stromal subtypes participate in thymomas. Yasumizu et al. described a neuromuscular TEC subtype in thymoma, though their study captured relatively few stromal cells for scRNA-seq analysis and the resolution of the spatial transcriptomic procedure did not reach the single-cell level [78, 80]. More precise isolation and characterization of thymic mimetic cells could yield new insights into their contributions to thymic tumors and MG pathogenesis.

Cross-species investigation of thymic mimetic cells might further inform our understanding of MG in humans. While MG can be induced in mice and rats, these models of disease do not recapitulate the etiology of spontaneous disease, limiting insights on the initiation of disease and thymic involvement [81, 82]. However, MG spontaneously develops in dogs and cats. It is a rare disease in dogs, often presenting as exercise-associated weakness and esophageal dilation (megaesophagus); dogs also show symptoms observed in humans like bilateral eyelid droop (ptosis) [83, 84]. Cats with MG often present with generalized weakness [84]. The autoimmune nature of the disease is similar to that of humans: in fact, detection of autoantibodies against AChR is a gold standard for diagnosis of canine MG [84]. As in humans, both canine and feline MG is associated with thymic tumors, particularly thymoma, which is particularly common in cats [83]. State-of-the-art molecular characterization of the canine and feline thymus is lacking, unfortunate because understanding the composition of the thymic stromal compartments of these two species might well shed light on the conserved elements of disease etiology and pathogenesis in humans. If the hypothesis that mimetic cells are an autoimmunizing agent in human MG is correct, the nature and abundances of mimetic cells in dogs and cats may be informative. Remarkably, MG can

spontaneously resolve in dogs, although seemingly only in dogs without thymic neoplasia [85]. Indeed, in a longitudinal study of dogs with MG, 47 of 53 dogs achieved immunological remission in an average of 6.4 months from the time of diagnosis [85]. What, then, underlies spontaneous remission in canine disease? One possibility is that the immunogen triggering the disease differs between dogs and humans, the immunizing agent being currently unknown. For example, if the hypothesized mimetic-cell and AChR-associated interfaces contribute to disease, we might expect alterations to the mimetic cell repertoire and/or thymic AChR expression in dogs over the course of disease.

It is also worth noting that a second thymic epithelial tumor type, thymic carcinoma, has many of the hallmarks of tuft mimetic cells. Thymic carcinoma expresses the tuft genes *POU2F3*, *CHAT*, and *TRPM5* [86]. Additional heterogeneity has been noted, including expression of genes found in other mimetic cell subtypes, like the ionocyte markers *FOXI1* and *CFTR* [87, 88]. The lineage of these cells is unclear, perhaps representing malignantly transformed tuft mimetic cells or altered TEC progenitors that follow a differentiation trajectory biased towards a tuft phenotype. Characterization of mimetic cell lineage definition in healthy individuals might unveil therapeutic vulnerabilities for treating this and other thymic neoplasms.

8.4 | Transplantation and Regenerative Medicine

Xenotransplantation is becoming an increasingly tractable option for organ transplantation. Combined organ-thymus transplantation has been explored to establish immunological tolerance to the donor organ (reviewed by [89]). The "thymokidney" is a prominent example, where the porcine thymus is implanted under the porcine kidney prior to transplantation of the joint organ into another species, including human recipients [90–92]. Hassall's corpuscles have been reported to persist in pig-to-baboon thymokidney xenografts for over 100 days [92]. The contribution of thymic mimetic cells in this context, both for establishing tolerance and for extra-thymic functions, is unclear. First, establishing the relative contributions of Aire-driven versus mimetic-cell-mediated PTA expression within a designated species would be logical, followed by establishing whether this balance differs between species, and whether it differs in the context of a thymus transplant.

Thymic mimetic cells may provide insights and/or materials relevant to regenerative medicine. Mimetic cells display a remarkable ability to differentiate from an epithelial lineage to diverse peripheral-cell lineages, reflecting both molecular and gross morphological features, such as striated muscle microstructures or brush-like tuft cell morphology. The molecular pathways enabling mTECs to follow a particular differentiation trajectory may inform cell reprogramming efforts. Likewise, this distinguishing feature of mimetic mTECs may render them a valuable source of cells. As an example, cochlear hair mimetic cells are present in the human thymus [34]. In the periphery, damage to cochlear hair cells is a common source of hearing loss; cochlear hair cells do not regenerate, though strategies to induce their regeneration are of interest [93, 94]. In the thymus, epithelial cells naturally undergo a reprogramming process to become a chimeric epithelial/cochlear-hair cell. What would happen if a mimetic cell type were transferred into the context of its peripheral counterpart? Would peripheral environment cues enable it to take on its peripheral function? Would it lose its mTEC transcriptional component? Even before the description of mimetic cells, it was reported that TECs could integrate into skin grafts when exposed to skin morphogenic signals [95]. Such an integration crosses embryonic lineages: endodermal TECs integrated into ectodermal skin grafts [95]. It remains to be seen whether the reprogrammable nature of mTECs is limited to skin and to what extent mimetic cells have the potential to integrate into the niches of their peripheral counterparts.

9 | Opportunities for Understanding Mimetic Cells via Study of Other Species

Single-cell characterization of mimetic cells in humans, mice, and zebrafish has been informative for unveiling the nature of mimetic cells, revealing physiologic specialization, and a substantial degree of conservation across evolutionary time. Interrogation of additional species might offer further insights. First, other species might serve as reference points or models for disease. As discussed previously, a dearth of spontaneous mouse or rat MG models could be offset by understanding gathered from dogs and cats. Second, species-specific characteristics present opportunities for new avenues of study. The naturally transparent nature of larval zebrafish and of certain adult variants [96] allows in vivo imaging, which could be a rich way to examine mimetic cell functions in their native context. For example, zebrafish could be used to image muscle mimetic cells to determine whether they form functional contractile units. The bilateral nature of the zebrafish thymus also permits the study of mimetic cell differentiation in spatially distinct niches. To distinguish deterministic vs. stochastic contributions to mimetic cell maturation, one could explore whether mimetic cells differentiate symmetrically in the two lobes.

Other alterations, like localized tissue injury or antigen delivery, could be informative in the study of mimetic cells or T cell differentiation more broadly. The residence time or trafficking patterns of differentiating T cells might also be naturally adapted for a given species' thymus morphology, as postulated by Boehm [6]. Cross-species studies on organisms with thymi of different sizes and anatomical connectivity could elucidate the effects on thymus residency time and antigen sampling.

Investigation of species that diverged before the development of RAG-dependent adaptive immunity may also be interesting. Lamprey, which do not have RAG-dependent immunity, have a thymoid structure [7]. It is unclear if hagfish, an agnathan like lamprey, also have a thymoid. The presence or absence of the thymoid in hagfish might shed light on the evolutionary emergence of the thymus. Likewise, further studies on the thymoid could elucidate whether it has misplaced stromal cells like the thymus.

However, many of these experimental extensions present technical hurdles. The limited availability of species-reactive antibodies or of genome assemblies for a given organism diminishes opportunities for studying that organism; for example, targeted profiling of zebrafish thymic stromal cells was limited to

depletion of T and B cells based on genetic reporters, rather than selective enrichment for mimetic cells [34]. Zebrafish-reactive antibodies or transgenic fish marking mimetic cell subtypes would allow for more fine-tuned interrogation of zebrafish mimetic cells.

10 | Conclusion

Thymic mimetic cells are intriguing transcriptional chimeras of diverse peripheral cell types and thymic epithelial cells expressing PTAs in an organized manner. As with Aire-driven PTA expression, mimetic cells are important for the induction of self-tolerance. They can also exhibit other functions, such as tuft and endocrine mimetic-cell-mediated regulation of thymic regeneration or microfold-mTEC-mediated induction of plasma-cell IgA. Future study might eventually unveil other unanticipated functions beyond promoting immunological tolerance.

Mimetic cells were reported in diverse species, ranging from frogs to birds to humans, from the mid-1800s. These prescient early observations foreshadowed a high degree of conservation across divergent species, eventually characterized molecularly in humans, mice, and fish via scRNA-seq. Many of the same mimetic cell subtypes occur in humans, mice, and fish, including those mimicking muscle, tuft, ionocyte, and neuroendocrine cells. Some cell types appear not to be conserved, though, such as microfold or enterocyte/hepatocyte mimetic cells which are found in mice but not humans, despite the presence in the periphery of both organisms. However, it cannot be ruled out that sequencing substantially more cells would uncover them. The frequencies, specialization, and subtype identities also vary across species. Exciting areas for future studies include elucidating the regulation of mimetic cell differentiation and identity-choice, and how these factors differ across species.

The human thymus harbors diverse mimetic cell types that could be relevant to human disease. Enriched muscle mimetic cells in the human thymus are of potential relevance in the development of MG, and the expression of tuft markers in thymic carcinoma is noteworthy, raising the possibility that tuft mimetic cells are malignantly transformed in this cancer type. The heterogeneous differentiation potential of mTECs to form mimetic cells may also offer new opportunities in regenerative medicine.

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Conflicts of Interest

D.M. is a cofounder of and advisor to Zag Bio Inc.

Data Availability Statement

The scRNA-seq dataset from three mouse strains is available at the Gene Expression Omnibus (GEO) with accession number GSE288941. Scripts for analyses of these data are available on GitHub: https://github.com/huismanb/mouse-cross-strain.

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