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Concise Review: Primary Cilia: Control Centers for Stem Cell Lineage Specification and Potential Targets for Cell-Based Therapies

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ABSTRACT

Directing stem cell lineage commitment prevails as the holy grail of translational stem cell research, particularly to those interested in the application of mesenchymal stem cells and adipose-derived stem cells in tissue engineering. However, elucidating the mechanisms underlying their phenotypic specification persists as an active area of research. In recent studies, the primary cilium structure has been intimately associated with defining cell phenotype, maintaining stemness, as well as functioning in a chemo, electro, and mechanosensory capacity in progenitor and committed cell types. Many hypothesize that the primary cilium may indeed be another important player in defining and controlling cell phenotype, concomitant with lineage-dictated cytoskeletal dynamics. Many of the studies on the primary cilium have emerged from disparate areas of biological research, and crosstalk amongst these areas of research is just beginning. To date, there has not been a thorough review of how primary cilia fit into the current paradigm of stem cell differentiation and this review aims to summarize the current cilia work in this context. The goal of this review is to highlight the cilium's function and integrate this knowledge into the working knowledge of stem cell biologists and tissue engineers developing regenerative medicine technologies. STEM CELLS *2016; 00:000—000*

SIGNIFICANCE STATEMENT

This review is important and timely in the context of Stem Cells readership. It is focused on primary cilia, which are emerging as a novel and important structure in stem cell differentiation. Their importance has been demonstrated fairly extensively in the context of developmental biology and through the study of ciliopathies. Now that we have a better understanding of the signaling pathways requiring the presence of the cilia structure, we are on the cusp of understanding its lineage-dependent function in stem cells. We predict the primary cilium will likely emerge as a novel therapeutic target in controlling stemcell phenotype. Its expression can be controlled through modulating the chemical and physical culture environment and thus can inform culture procedures for stem cells in tissue engineering. Our review synthesizes information from in vivo and in vitro cilia studies and stem cell work and we integrate these findings in the context of tissue homeostasis and tissue pathologies, something that has not been extensively discussed to date.

INTRODUCTION

As of March 16, 2015, there were 475 clinical trials utilizing mesenchymal stem cells (MSCs) and 157 clinical trials utilizing adipose-derived stem cells (ASCs) to treat a wide variety of ailments ranging from neurological disorders, blood disorders, liver disease to orthopedic pathologies. The clinical interest in adult stem cells derived from bone marrow and fat tissue continues to grow each year, but elucidating the mechanisms underlying these processes remains a very active area of research, critical to the success of their clinical application. Stem cell and tissue engineering researchers have actively pursued an assortment of culture approaches to directing cell phenotype: including stimulation via chemical factors, application of mechanical, through tensile strain, fluid shear or compression and modulating the rigidity, architectural and chemical composition and/or dimensionality of the culture environment. Changes in stem cell morphology, gene expression, cytoskeletal organization, and

end-product expression are the typical metrics used to gauge stem cell differentiation, however, the primary cilium has emerged as a new structure of interest in the context of cell phenotype, which may be another important piece to the stem cell puzzle.

Primary Cilia Basics

The primary cilium is an organelle present on most vertebrate cell types at some point during the cell cycle. It is thought to be an important sensory organelle, coordinating a multitude of critical cell processes, and it has garnered much research attention over the last decade. Primary cilia were first described by Zimmerman in 1898 in his observations on the lumen of kidney tubules [1]. However, the structure was largely ignored for the following century, with many researchers deeming it a vestigial organelle. The primary cilium is now known to modulate many cell processes, including cell proliferation, differentiation and cell migration [1–5]. Primary cilia are composed of nine microtubule doublets arranged concentrically in a 9 + 0 configuration, and they are generally considered nonmotile, with the exception of specialized nodal cilia [6] (Fig. 1A, 1B). In contrast, motile cilia structures present in epithelial mucociliary systems such as the airway express a 9 + 2 microtubule configuration, with a central pair of microtubules in the center of the ciliary axoneme. In addition to structural disparities between the two classes of cilia, nonmotile primary cilia are typically thought to lack the axonemal dynein motor proteins that facilitate the beating motion of motile cilia [7]. In the context of this review, we examine how the structure and function of the primary cilium relates to cell phenotype, particularly focusing on cilium's mechanistic contribution to the development, maintenance and degeneration of specific progenitor and/or stem cell phenotypes.

Primary cilia typically localize to the apical cell surface of epithelial cell types and cells grown in monolayer culture. Their structure is contained within a ciliary membrane contiguous with the cell membrane [8]. However, they have also been observed within a membrane infolding just below the membrane surface of the cell [8]. Primary cilia emanate from the mother centriole, anchoring the basal body and docking just below the surface of the cell membrane [9]. The presence of the primary cilium is intimately associated with the cell cycle, as they are most frequently expressed during the G_0 phase, but they can be observed any time during interphase and are normally assembled during the G_1 phase [10].

In addition to their cell cycle link, many researchers have identified the primary cilium as a chemo- and/or mechanosensory organelle in a variety of cell types, including those derived from bone [11–13], cartilage [14–16], kidney [17, 18], cardiovascular [19–21], and neural tissue [22, 23]. These studies collectively allude to the idea that the primary cilium may be the physical and chemical balance point for cell lineage specification, fulfilling the paradigm that cell proliferation is at odds with cell differentiation; as proliferative activity slows, cells can then differentiate [24, 25].

The function of the primary cilium is not limited to basic cell physiology, and its dysfunction has been implicated in a number of diseases. Clinical presentations of motile or nonmotile cilia-associated genetic diseases and disorders have recently been classified as "ciliopathies" [26]. Ciliopathies can arise at any time from development through adulthood, and

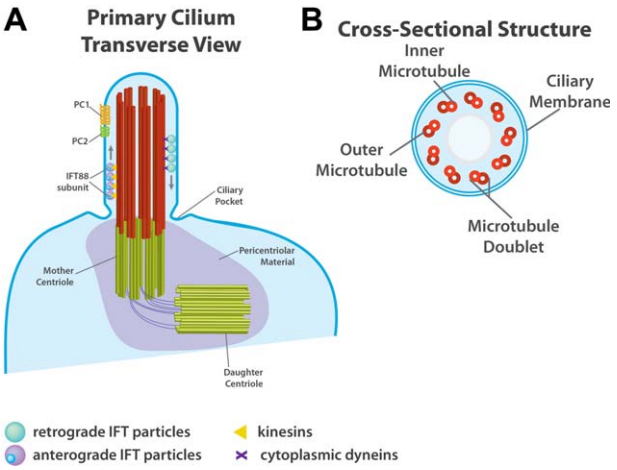

Figure 1. Schematic of the primary cilium. Transverse view of the cilium structure showing the ciliary organization along the ciliary axoneme with the cilium emanating from the mother centriole (**A**). Cross-sectional view of the cilium showing the microtubule doublet arrangement in the 9 + 0 configuration (**B**). Abbreviations: IFT, intraflagellar transport; PC1, polycystin-1; PC2, polycystin-2.

they can affect virtually any tissue in the body. Ciliopathies frequently result in neurosensory or respiratory system disorders or abnormal patterning in bone development. They can also cause tissue degeneration in the liver or kidney due to aberrant cell proliferation and cyst development [26]. Particular cilia-associated genes have been linked to a number of ciliopathies including polycystin-1 (PKD1), polycystin-2 (PKD2), intraflagellar transport protein-88 (IFT88 or Polaris), kinesin-like protein (KIF3a), and inversin (INV).

Research at the convergence of clinical ciliopathies and cilia biology has the potential to uncover the fundamental cell proliferation and differentiation processes that are mediated by the cilium, a once-overlooked vestige of evolution. Investigators estimate that the primary cilium is associated with the activity of over 300 proteins and mediates the signaling processes of many critical signaling pathways [27, 28]. This review aims to highlight the major studies elucidating the functions of primary cilia in cell phenotype, with a focus on stem cells and tissue morphogenesis. We will begin with a summary of the methodologies used to direct and define cell phenotype, introduce the major signaling pathways relevant to primary cilia discussed in this paper, and describe recent work investigating the role of the primary cilium in regulating adult stem cell fate. We will then follow with a brief background on the physiological function of the primary cilium as shown through landmark studies performed in the kidney. Subsequently, we will explore the role of the primary cilium during environmentally induced changes in cell phenotype, modulating the chemical, mechanical and substrate microenvironment. Each section describes induction factors and processes of cell lineage specification, maintenance of cell lineage and degeneration into a pathological cell phenotype, with primary emphasis on connective and musculoskeletal cell and tissue phenotypes.

In Vitro Approaches to Define Cell Phenotype

In the context of tissue engineering and regenerative medicine, defining cell phenotype has been a critical barrier to the

widespread clinical application of stem cell-based tissue replacement therapies. In both stem cell and committed cell types, culture conditions such as chemical factors, mechanical stimulation, and substrate microenvironment modulation all affect cell phenotype. A variety of cellular attributes can be examined to determine a committed cell phenotype: cellular morphology, cytoskeletal organization, focal adhesion formation, gene expression profiles, protein expression profiles, and end-product expression [29–32]. Emerging evidence suggests that the primary cilium may be another indicator of cell phenotype and is likely linked to phenotypic observations in cytoskeletal reorganization; however, its distinct function in determining/predicting cell phenotype is a relatively new area of study.

Cilia-Relevant Signaling Pathways

A variety of signaling pathways have been associated with the primary cilium, particularly pathways that are critical in processes related to development and cell differentiation [1, 33]. Most prominently these include the hedgehog (Hh) and Wnt signaling pathways along with recent reports including the platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) signaling pathways. All these pathways have been implicated in localizing at least one portion of their signaling proteins to the primary cilium [2, 27, 34–37]. In this section, we provide a brief overview of the pathways relevant to cell differentiation in the context of this review. However, the reader should note that a variety of other signaling pathways and proteins not mentioned here have been associated with the primary cilia.

The hedgehog signaling pathway is critical in the developing embryo across animal species [38]. Spatial distribution of hedgehog proteins and specific concentration gradients dictate tissue morphogenesis and cellular differentiation particularly in the context of limb and digit development [39, 40]. Recent studies have identified Patched (PTCH) and Smoothened (SMO), both important proteins in the hedgehog pathway to localize their activity to the primary cilium during Hh signaling [41–43]. PTCH is thought to block the SMO from being trafficked through the ciliary axonmeme in the absence of Sonic hedgehog (SHH), however, when SHH ligand is present, it binds PTCH, allowing SMO to move into the cilium and activate the GLI transcription factor proteins (Fig. 2) [44]. Thorough reviews by Nozawa et al. and Oro summarize the current knowledge in Hh signaling and ciliary localization and trafficking of Hh proteins [42, 44].

Similar to the Hh pathway, Wnt signaling is another highly conserved pathway across the animal kingdom and is involved in tissue patterning and cell fate determination in the developing embryo [45]. Of the three branches of Wnt signaling, noncanonical Wnt signaling associated with planar cell polarity has been most frequently implicated to the primary cilium structure [46]. In noncanonical Wnt signaling the Frizzled protein, a receptor for Wnt ligands, is thought to localize to the ciliary membrane [27]. Downstream Wnt proteins such as Inversin and disheveled (Dsh) also localize their activity to the cilium axoneme and the base of the cilium, though the details of ciliary regulation of Wnt signaling are still somewhat elusive (Fig. 2) [1, 47]. There is some evidence that canonical Wnt signaling may in some way interact with the primary cilium, however, there is a dearth of data in this area [48].

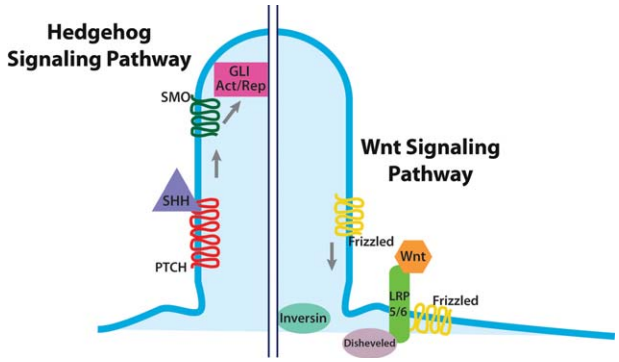

Figure 2. Proposed model of Hedgehog (left side) and Wnt signaling (right side) in the primary cilium. In the vertebrate hedgehog pathway, Patched (PTCH) is thought to block trafficking of Smoothened (SMO) through the ciliary axoneme in the absence of Sonic hedgehog ligand (SHH). When SHH ligand is released, it binds to PTCH which then allows the transport of SMO into the cilium where it can recruit GLI activator and repressor transcription factor proteins. In the noncanonical Wnt signaling pathway, Frizzled is hypothesized to reside in the cilium, in the absence of Wnt ligand, however, when Wnt ligands are expressed, they are thought to bind the LRP/Frizzled/Disheveled complex, initiating downstream Wnt signaling activity which involves localization of Inversin at the base of the primary cilium.

The PDGF signaling pathway is generally associated with processes of angiogenesis and cellular migration. As such this pathway is frequently implicated in cancer development and wound healing as well as cellular migration and differentiation [49]. In the context of primary cilia, there is evidence that PDGFRα, a receptor for PDGF-AA ligand, localizes to the primary cilium during mouse embryonic fibroblast chemotaxis to bind to extracellular PDGF-AA, suggesting that the cilium structure is acting in part as a chemical antenna [50]. The suggested link between PDGF signaling and the primary cilium structure is particularly compelling as this data suggests that cilium structure may orient in response to a chemical gradient, likely due to the concentration of PDGFRα at the ciliary membrane.

TGF-β signaling is broadly associated with a variety of cell processes including cellular proliferation, homeostasis, and differentiation. This signaling pathway is particularly important in the context of mesenchymal tissue homeostasis, inflammation, the epithelial-mesenchymal transition (EMT) and the development of cancer [51]. In mouse embryonic endothelial cells, shear stress activates TGF-β signaling which localizes to the primary cilium during the EMT [21, 52]. Further, TGF-β activity has also been associated with the endocytosis processes at the ciliary pocket as evidenced by accumulation of SMAD 2/3 and ERK1/2, downstream TGF-β signaling proteins, at the base of the cilium [36]. The link between primary cilia TGF-β signaling is a newer discovery in the realm of cilia biology, though recent literature posits that the cilium is an important site of TGF-β activity.

There is a very large body of work that has been done to elucidate the variety of signaling mechanisms of Hh, Wnt, PDGF, and TGF-β signaling that is beyond the scope of this review, however, the link between these pathways and the primary cilium remains an important area of study. Specific studies that address the ciliary link to these signaling pathways are highlighted in this review. For a more in depth

review of the involvement of the cilium structure and protein localization in these signaling pathways we invite the reader to reference reviews by Christensen et al. [37], Satir et al. [27], Simera and Kelley [48], and Nozawa et al. [42].

Primary Cilia in Stem and Progenitor Cell Types

To date, primary cilia have been observed on a variety of stem and progenitor cell types under in vitro cell culture and alterations in their length, expression frequency and protein colocalization to the cilium structure has hinted at their function in stem cells [53–58]. One of the earliest studies to look at the function of primary cilia in a progenitor cell type characterized Wnt signaling in mouse embryonic fibroblasts [53], cells which are known to have comparable differentiation potential to MSCs [59]. Corbit et al. found that knockdown of cilia protein Kif3a and thus abrogation of the cilium structure enhanced canonical Wnt signaling. This suggested that the primary cilium somehow suppresses canonical Wnt signaling, an important signaling pathway in osteogenic and chondrogenic differentiation processes [53].

An early study by Plaisant et al. describes the effect of osteogenic differentiation media on human adipose stem cell (hASC) cilia expression, reporting that 92% of undifferentiated hASCs expressed primary cilia; however, following 5 days of culture in osteogenic media, only 48% of the differentiating hASCs expressed cilia [54]. The authors also noted that this level of expression was closer to that observed in osteoblastic cell types [54]. Primary cilia on MSCs have also displayed a level of chemosensitivity. When cilia protein IFT88 is knocked down in MSCs, they exhibit a significantly reduced differentiation response to chemical induction media for osteogenic, adipogenic, and chondrogenic cell types, as measured by gene expression markers Runx2, PPARγ, and Sox-9, respectively [55]. This study suggested that a functional cilium structure was required for chemical induction of lineage specification.

Following this work, another study in MSCs demonstrated a more global function of primary cilia-associated protein IFT88 in lineage commitment processes [55]. With transient siRNA knockdown of IFT88, Tummala et al. reported a disruption of early osteogenic, chondrogenic and adipogenic differentiation signals with chemical induction media, as determined by evaluating hallmark mRNA markers for gene expression of each lineage [55]. Moreover, a subsequent study emphasized that this function was conserved in IFT88 knockdown in hASCs as well, and further implicated the function of cilia-associated protein of polycystin-1 (PC1) in modulating osteogenic end-product expression [60]. PC1 knockdown in hASCs conferred a downregulation of osteocalcin gene expression, as well as diminished calcium accretion and reduced alkaline phosphatase activity after two weeks of culture in osteogenic induction media [60]. Additionally, IFT88 knockdown was found to increase hASC proliferation activity, suggesting that the abrogation of the cilium structure allows the hASCs to maintain a more proliferative phenotype.

MSC and ASC phenotype can be directed based on the architecture of the substrate environment through effecting changes in cytoskeletal tension [31]. The primary cilium organelle has been observed to be sensitive to substrate architecture and is emerging as an organelle of interest in addition to morphological and cytoskeletal changes [61]. MSCs cultured on nanotopographical grooved surfaces exhibited distinct changes in ciliary and orientation in relation to the long axis of cell body with the more elongated, aligned cilia expressed on the MSCs cultured on grooved surfaces [61]. The authors concluded that the grooved surface, enhanced ciliogenesis and in turn, suppressed canonical Wnt activity and thus suppressed proliferation. The authors also found that knockdown of IFT88 led to a reduction in cilia length and expression, and restored proliferative activity in spite of culture on the grooved nanotopography [61]. These data further support that the cilium structure delicately regulates the balance between a proliferative "stem-like" phenotype and a differentiated cell type. This link between IFT88 knockdown conferring an increase in proliferative capacity is consistent with findings in hASCs [60].

Another study out of the same group analyzed changes in ciliary length in MSCs undergoing adipogenic differentiation and found the differentiating MSCs expressed lineage-dependent chemosensitivity in the presence of dexamethasone and insulin [62]. Culture in adipogenic differentiation medium conferred an elongation in MSC primary cilia with a subsequent upregulation of nuclear PPARγ levels and recruitment of IGF-1Rβ to localize to the cilium [62]. Interestingly, these findings are consistent with an earlier study by Marion et al. which identified that Bardet-Biedl syndrome proteins, BBS10 and BBS 12 localized to the basal body at the base of the cilium, and regulate ciliogenesis [63]. Additionally, they found that the primary cilium was transiently expressed in differentiating preadipocytes, but not on those undergoing expansion or those which had already committed to a mature adipocyte phenotype [63]. This finding is consistent with unpublished results in hASCs from our group and others [62]. This study further corroborates the previous work suggesting that both the mere expression of the cilium structure and activity of its associated proteins may dictate stem cell phenotype, and in part mediates differentiation processes.

Concomitantly supporting the idea that the primary cilium plays a role in phenotypic determination, loss of cell phenotype and dysregulation of tissue homeostasis is observed in ciliopathies such as autosomal polycystic kidney disease, and these observations form the basis for much of the current work in primary cilia today.

Role of Primary Cilia in Kidney Organ Function

The physiological function of the primary cilium was discovered when investigators identified its link with polycystic kidney disease (PKD). That work was the first prominent study to recognize the vertebrate primary cilium as a mechanosensory organelle [64, 65]. PKD is an autosomal genetic disorder characterized by a loss of primary cilia in the epithelial cells of the kidney distal tubule. Absence of the primary cilia leads to a loss of mechanosensitivity followed by hyperproliferation of the epithelial cells, which eventually form cysts in the kidney [17, 66]. The process of cilia loss is associated with a loss of phenotype or de-differentiation of the epithelial layer. Frequently in autosomal PKD, the genes PKD1 and PKD2, respectively encoding functional ciliary polycystin-1 (PC1) and polycystin-2 (PC2), are mutated [66]. The resulting loss of cilia in PKD leads to the deregulation of typical kidney tissue homeostasis, suggesting that PC1 and PC2 serve a functional mechanosensitivity role.

In a study by Nauli et al., murine cells lacking functional PC1 still formed cilia, but demonstrated a loss of fluid

sensitivity as measured by fluid-induced calcium fluxes [65]. Other polycystic kidney models have identified KIF3A and IFT88 as critical cilia proteins required for healthy kidney homeostasis, motivating the work behind a large majority of studies on primary cilia in musculoskeletal function [66].

Role of Primary Cilia in the Musculoskeletal System

As the story with primary cilia has unfolded, a retrospective look at the literature reveals that the ciliary structure was observed on chondrocytes and osteogenic cell types approximately 30 years prior to their in-depth study in the kidney. However, much of the work studying the cilium structure and its associated proteins in musculoskeletal cell types have more recently been identified to play critical chemo- and mechanosensory roles in osteocytes [12, 13], osteoblasts [11], chondrocytes [8, 67], and tenocytes [68], among other connective tissue cell types [55, 60]. Though the expression patterns of these musculoskeletal cell types still remain something of an enigma, they are clearly an important locale for the signaling pathways that modulate musculoskeletal cell lineages, such as Wnt [69], Hedgehog [54], and TGF-β signaling pathways [36].

Skeletogenesis

On the heels of the foundational kidney studies, Xiao et al. observed primary cilia structures on bone cells in culture and expressed on osteoblasts and osteocytes in extracted bone tissue [70]. Not only were primary cilia structures present, Xiao et al. reported the link between a functional PKD1 gene encoding polycystin-1 and the expression of osteogenic gene markers. Using heterozygous and homozygous *Pkd1*^{m1Bei} mouse models that possessed an inactivating point mutation within the first transmembrane domain of the PC1 protein structure, they demonstrated that this mutation led to osteopenia and skeletal abnormalities in the developing embryo. Additionally, embryonic mice with the homozygous *Pkd1*^{m1Bei} mutation expressed a more severely disrupted skeletal phenotype [70]. Osteoblasts isolated from these mice exhibited reduced osteogenic *Runx2* expression at both the gene and protein level. Further, they exhibited a reduced capacity for differentiation ex vivo, suggesting their osteogenic phenotype was impaired. Xiao et al. also showed that an overexpression of PC1 in MC3T3E1 osteoblasts conferred an upregulation of osteoblastic gene markers. Further, in osteoblasts derived from the *Pkd1*^{m1Bei} mice, co-expression of PC1 restored *Runx2* P1 promoter activity and osteogenic gene expression levels [70]. This study was the first major study to functionally link primary cilia with the osteogenic phenotype.

Following the Xiao et al. report, a wide range of in vivo and in vitro studies implicated primary cilia as a part of the osteogenic and chondrogenic phenotype. Intraflagellar transport is a critical aspect of primary cilia biology because their proteins must be synthesized outside of the ciliary body and subsequently transported into the ciliary pocket or membrane [3, 28]. In addition to PKD1 and functional cilia-localized PC1 protein, a group of kinesin, intraflagellar transport (IFT) and dynein proteins are responsible for antero- and retrograde protein transport through the axoneme of the primary cilium and have been found to modify ciliary structure and function [11, 71].

Mouse Models of Primary Cilia Defects

Mouse Models—Kif3a Knockout. Kif3a is a protein subunit of the Kinesin II Motor complex, which is active in the process of ciliary transport. Kif3a has been of particular interest in the bone and cartilage development of the postnatal growth plate and in the endochondral ossification of developing/growing subchondral tissue. One of the first reports to implicate Kif3a in ciliary activity and skeletogenesis described impaired cilia formation in mouse chondrocytes modified by a Col2a-Cre-mediated Kif3a deletion [72]. Mice possessing the Kif3a deletion exhibited postnatal dwarfism and premature loss of the endochondral growth plate in long bones. Additionally, this deletion resulted in aberrant chondrocyte rotation; its healthy function is necessary to maintain the columnar organization of the long bone epiphyseal growth plate. This misorientation was concomitant with disruption of the actin cytoskeleton and focal adhesion formation, likely affecting the differentiation activity in the growth plate [72].

The role of Kif3a in cartilage was further confirmed in a study by Koyama et al., describing *Kif3a*-deficient mice that by postnatal day 7 lacked ordered zones of proliferative and hypertrophic regions of chondrocytes in the cranial synchondrosis growth plate [73]. Unusual intramembranous ossification coupled with ectopic cartilage development was also observed. The resulting *Kif3a*-deficient cranial phenotype somewhat coincided with that of mice deficient in the *Indian Hedgehog* gene (*Ihh)* and led Koyama et al. to investigate the distribution of Hedgehog signaling within the Kif3a mutant mice. They reported a differential distribution of topographical Hedgehog signaling activity in the synchondroses of the *Kif3a*-deficient mice relative to the controls. Further, they found that *Ihh*-deficient mice developed abnormal synchondroses. However, the phenotype was distinct from that of the *Kif3a*-deficient mice [73], indicating that the cilium uniquely contributes to the localization of Hedgehog signaling in the cranial growth plate.

Mouse Models—IFT88 Knockout. The *Tg737*^{orpk} mouse model for autosomal polycystic kidney disease expressed epiphysis and growth plate morphology comparable to the *Kif3a*-deficient mouse [74]. *Tg737*^{orpk} mice express a global mutation in the *Tg737* gene resulting in a disruption of its encoded protein IFT88, also known as Polaris. Disruption of IFT88/Polaris leads to a lack and/or shortening of cilia expressed in the kidney cells as well as cells of other tissues [75]. Chondrocytes in the growth plate of *Tg737*^{orpk} mice were reported to express a dearth of ciliary structures and to lack the characteristic cilia orientation in those chondrocytes within the surface layers of the cartilage tissue [74]. Consistent with the Kif3a epiphyseal growth plate findings, the organization of the actin cytoskeleton was affected by the lack of cilia.

To more closely examine the specific effects of IFT88 on endochondral bone formation, Haycraft et al. developed a conditional mutant mouse using a Cre-*lox* system targeting only the mesenchymal tissues, avoiding the lethality of a global IFT88 mutation [76]. Mice at postnatal day 11 expressing the conditional *prx1cre;Ift88*$^{fl/n}$ mutation exhibited forelimbs stunted along the proximodistal axis with severe polydactylism [76]. Associated with the disrupted

endochondral bone formation in the mutant mice, Haycraft et al. identified irregular Sonic Hedgehog (SHH) signaling in conjunction with abnormal digit patterning and reduced Ihh signaling [76]. Ectopic deposition of chondrocytes and perichondral organization were abnormal in this mutant model [76]. These abnormalities were similar to endochondral phenotypes observed in the growth plate irregularities of the Kif3a and Tg737^{orpk} studies [72, 74].

Though IFT88 is a different protein involved in intraflagellar transport within the cilium, like Kif3a it is a critical ciliary protein required for functional cilia expression. This supports the idea that the cilium is required for appropriate cell specification and tissue morphogenesis processes. Taken together, these studies suggest that the primary cilium, and more specifically the intraflagellar transport activity of the cilium, plays an important role in cartilage tissue morphogenesis, with the primary cilium structure likely mediating distinct protein activity in the Hedgehog signaling pathway.

Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease leading to the degradation of articular cartilage and subchondral bone. It can be genetically inherited and/or caused or exacerbated by acute or chronic injury. In OA patients, primary cilia have been observed on localized subpopulations of human chondrocytes undergoing degeneration of fibrillated and nonfibrillated cartilage, both in the superficial and middle zones [14]. In this early study, cilia were most frequently observed in the middle zone of cartilage from OA patients, while few were observed in the cartilage of non-OA control patients [14]. A subsequent study published nearly 12 years later quantified the frequency of cilia observed on OA tissue in a bovine model [77]. Those authors reported increased cilia expression and length in OA chondrocytes at the degenerating articular surface as compared with healthy cartilage; further, they found an increase in ciliated cells corresponding to the degree of tissue degeneration [77]. These observations allude to the function of primary cilia in phenotype, corresponding to a change in the degenerating extracellular environment.

Cilia seem to serve an important function in developing endochondral tissue and diseased subchondral tissue of the skeletal system, suggesting that the cilium may indiscriminately coordinate processes of cell differentiation in both tissue homeostasis and tissue degeneration. The primary cilium's multifaceted functionality in directing chondrogenic and osteogenic phenotypes in the subchondral tissue suggests that it may be a potential therapeutic target in treating OA.

Chondrogenesis

Primary cilia have not only been observed in the endochondral regions of ossifying bone and in cartilage degeneration in OA; they are also present in mature cartilage and likely serve a mechano-active function in cartilage tissue. In 1997, primary cilia were identified in situ on canine articular cartilage of the tibial plateau and femoral condyle [78]. Cilia were also observed on agarose-cultured canine chondrocytes isolated from the same regions, but the ciliary structure projected out farther from the cytoplasmic surface than the in situ cilia, which were observed within a membranous ciliary pocket [78]. These early reports on cilia in cartilage highlight the differences in cilia structure between chondrocytes in vivo and those cultured in vitro, pointing to the relationship between the primary cilium and chondrogenic phenotype.

Following the 1997 work, transmission electron microscopy (TEM) and confocal microscopy analyses by Poole et al. and Jensen et al., respectively, described the ultrastructure and orientation of the primary cilium in hyaline cartilage derived from the sterna of chick embryos [79, 80]. Those studies determined that the chondrocyte primary cilium ranges in length from 1-4 μm, and it was categorically observed by Poole et al. to express three patterns of orientation in situ: (1) fully extended into the extracellular matrix (ECM), (2) partially extending with bending along the axoneme, (3) folded along the surface of the cytoplasm with minimal matrix contact [79]. Further, the authors frequently observed the Golgi apparatus to be anchored between the nucleus and the base of the cilium. They hypothesized that the cilium may act as a conduit collecting chemical and/or mechanical cues from the ECM to direct Golgi activity [79]. These studies demonstrated that the primary cilium structure can be deflected or bent within the cartilage matrix; the mechanical force applied to the tissue is likely transduced to the cilium from the surrounding matrix of collagen fibers and proteoglycans [79, 80]. This idea was supported by TEM images illustrating potential attachment points between collagen fibers and the ciliary membrane. Confocal images also illustrated the differential bending profiles expressed by the chondrocyte primary cilia [80].

A recent study published in 2014 explored the relationship between the presence of primary cilia and the mechanical properties of cartilage tissue. It was observed that loss of primary cilia in the *Col2aCre;ift88*$^{fl/fl}$ transgenic mouse model results in upregulation of OA markers. This occurs in conjunction with reduced structural integrity and a thickening of the cartilage tissue [15]. Somewhat in contrast to the frequency of ciliated cells in typical OA models, in this model the chondrocytes devoid of cilia were associated with an OA phenotype. However, this study points to the importance of primary cilia in maintaining a healthy chondrogenic phenotype and its role in cartilage homeostasis [15].

Primary Cilia as Chemosensors

Chemical stimulation of cells in vitro is known to affect the frequency of expression, length and orientation of primary cilia in culture, demonstrating their sensitivity to the surround chemical environment. Through loss of function animal models, investigators have been able to show the importance of cilia in tissue development in the embryo; however, in vitro studies aim to clarify their specific cellular function. The expression patterns of primary cilia on cultured cell types remain an enigma due to the high variation observed across cell types. However, their expression is thought be largely linked to quiescence within the cell cycle [10]. Under in vitro culture conditions, primary cilia exhibit chemosensitivity to soluble factors in the surrounding culture environment in a broad range of cell types. This is frequently demonstrated through serum starvation to induce cilia formation and elongation [2, 28]. Their structure and function has been implicated in cell fate determination processes as well as in cellular chemotactic migration [47, 50].

Purportedly, cilia elaboration or resorption modulates Hedgehog and Wnt signaling, and this modulation is likely in response to changes in the chemical environment. Below we

describe a number of studies illustrating the chemosensory properties of the cilium and the implications for defining cellular behavior.

Chondrogenic Cell Types

As described above, cilia expression is intimately linked to developing chondrogenic tissue and healthy and degenerating cartilage tissue; however, the specific mechanisms dictating these changes in cartilage cilia expression remain unclear. Changes in chondrocyte cilia expression patterns have been observed in response to the chemical environment, and these changes have been proposed to detect to inflammatory signaling [67]. IL-1 secretion is a characteristic inflammatory signal present in osteoarthritic tissue [67]. When stimulated with IL-1β, cultured primary bovine chondrocytes increase both their length and expression frequency, demonstrating chemosensitivity in the chondrogenic phenotype [67]. Interestingly, the authors also tested this effect against fibroblasts and noted a similar increase in cilia length in response to IL-1β, suggesting that this may be a chemosensing property of cilia across cell types [67]. To further support that this response was specific to the cilia acting as mediators of IL-1 stimulation, the authors measured nitric oxide (NO) and prostaglandin release in response to proinflammatory cytokine IL-1, utilizing chondrocytes derived from IFT88 knockout mice ($Tg737^{ORPK}$). They found that cells devoid of cilia did not characteristically upregulate NO and PGE_2 in response to IL-1 stimulation, whereas wild type cells exhibited the prototypical response described in bovine chondrocytes and fibroblasts [67]. This study suggests a mechanistic explanation for the typical cilia length increase seen in the inflammatory signaling of osteoarthritic tissue [77].

Another study by Rich and Clark described the impact of an osmotic environment on chondrocyte primary cilia [81]. Interestingly, the authors reported that the primary cilia on explanted, intact femoral condyles derived from mice exhibited changes in the length of the ciliary axoneme within minutes of exposure to both a hypo–osmotic and hyperosmotic environment (200-400 mOsm) in the surrounding culture medium [81]. The authors did note that they did not distinguish between intracellular and extracellular length and therefore did not account for cilia retraction into the ciliary pocket, so length changes were direct measurements of ciliary resorption [81]. Osteoarthritic (OA) tissue is generally thought to produce a hypo–osmotic environment for chondrocytes with a degenerated cartilage matrix devoid of glycosaminoglycans (GAGs); this study illustrates contrasting results to those described in OA chondrocytes with elongated cilia by McGlashan et al. [77]. Transient receptor potential vanilloid 4 (TRPV4) is a calcium-permeable ion channel which colocalizes with the primary cilium and has been proposed to be involved in chondrocyte response to hypoosmotic stress [82]. The TRPV4 channel is gated by changes in osmolarity and mechanical stimuli and when the primary cilia of porcine chondrocytes were abrogated with the use of chloral hydrate, the cells exhibited a diminished TRPV4 calcium signaling response to hypo–osmotic stimulation [82]. These observations taken together with the IL-1 study by Wann et al. suggest a differential response in primary cilia chemosensitivity, highlighting its complex function in integrating a variety of chemical signals controlling chondrogenic cell phenotypes.

Primary Cilia Mechanosensitivity in Osteogenic Cell Types

An interesting study by Hoey et al. proposed a paracrine signaling function of primary cilia MSCs [13]. Their findings indicated that when MSCs were cultured in conditioned media from mechanically stimulated osteocytes (MLO-Y4), the MSCs upregulated osteopontin (OPN) and cyclcooxygenase-2 (COX-2) osteogenic gene expression. MSCs cultured in conditioned media derived from osteocytes that were not mechanically stimulated did not upregulate osteogenic genes [13]. In contrast, conditioned media derived from mechanically stimulated osteoblasts (MC3T3-E1) did not have this effect on MSCs, suggesting unique paracrine signaling activity between mechanically stimulated osteocytes and MSCs.

In osteogenic cell lines, primary cilia exhibit sensitivity to oscillatory fluid flow (OFF), and their frequency of expression is related to osteogenic differentiation [11]. In MC3T3-E1 osteoblast-like cells and MLO-Y4 cells, both siRNA knockdown of ciliary protein IFT88 and physical abrogation of primary cilia using chloral hydrate resulted in a loss of mechanosensitivity. MC3T3E1 osteoblasts characteristically upregulate osteopontin gene expression and release prostaglandin E2 (PGE2) in response to fluid shear. However, cilia disruption diminished this behavior following exposure to OFF for 1 hour at 1 Hz. Further, in MLO-Y4 osteocytes/osteoclast-like cells, characteristic flow-induced increases in cyclooxygenase-2 (COX2) gene expression and the ratio of osteprotegerin (OPG) to RANKL gene expression were suppressed under both chloral hydrate exposure and siRNA knockdown. That study also explored the role of primary cilia in the flow-induced release of intracellular calcium, as osteogenic cells tend to exhibit calcium fluxes in response to fluid shear and kidney cells exhibit cilia dependent calcium release via polycystin-2 (PC2), a stretch activated calcium channel. However, they found that the flow-induced release of intracellular calcium occurred independently from primary cilia expression in osteogenic cell types [11].

A subsequent report demonstrated that primary cilia mediated fluid induced osteogenesis in MLO-A5 osteoblasts as measured by calcium accretion following exposure to 2 hours of OFF/day for 12 days, applied with the use of a rocker platform [83]. Through physical disruption of the primary cilia with chloral hydrate, MLO-A5 cells exhibited a reduced capacity for calcium accretion in response to OFF, suggesting that cilia were required for this process. The authors further reported a reduction in the number of primary cilia in response to OFF stimulation.

Other evidence suggests cilia-specific protein polycystin-1 (PC1) may play an integral role in osteoblastic mechanosensing of the surrounding mechanical environment. When cultured under cyclic tensile strain (2% strain at 0.5 Hz), MC3T3-E1 osteoblasts show PC1-dependent mechanically induced osteogenesis [84]. PC1 shRNA lentiviral knockdown diminishes characteristically upregulated osteogenic gene markers such as Runx2, osteocalcin, osterix and osteonectin in response to osteogenic induction via chemical or mechanical stimulation. This was observed as early as one hour following mechanical stimulation [84]. Though this study did not specifically focus on the cilium structure as the mechanosensor in this case, PC1 localizes to the primary cilium, and its mechanosensitivity likely functions in conjunction with the cilium structure.

Primary Cilia in Neural Cell Types

In the developing nervous system, primary cilia have been identified as a critical structure in proliferation and maintenance of the neural progenitor cell populations [85–87]. The primary cilium structure is required for proper left-right signaling in the developing embryo. Chicken and mouse models with disrupted cilia proteins often express deficient neural patterning [40, 87, 88]. In addition, functional cellular migration is necessary for healthy tissue development and a recent study by Higginbotham et al. showed that primary cilia regulated migration and targeted localization of interneurons in the developing mouse cerebral cortex [89]. A recent study from the same group further indicated that expression is also required for normal radial glial scaffold configuration, orientation and polarity [88].

Ciliary components in neural progenitor cells have also hinted at the relationship between the cilia structure, stemness and tissue homeostasis [90]. Paridaen et al. demonstrated that the ciliary membrane is asymmetrically inherited during neural cell division in the brain tissue of mouse embryos [91]. Their data suggested that this asymmetrical inheritance marks the daughter cell which retains stem-like character. It is in these neural stem cell studies that the cilium's association with stemness and its role in determination of cell phenotype has been most clearly demonstrated [23, 91–93]. In addition to the motile cilia which move cerebrospinal fluid in the nervous system, primary cilia act as sensors, detecting signals from the cerebrospinal fluid [94]. Cilia have also been identified in many neural cell types in both the developing and adult brain, and found to be expressed on induced pluripotent stem cells [95].

There is a large and growing body of work supporting the critical and highly specific function of the primary cilium in both healthy and diseased neural tissue, much of which is outside the scope of this review. For a comprehensive review of primary cilia and neural development see the recent articles published by Louvi and Grove [85], Ruat et al. [96], and Jimenez et al. [97].

Conclusions

Overall, primary cilia functionally contribute to the chemo- and mechanosensing activities of a variety of cell types both in vivo and in vitro. They are critical in defining the proliferative and differentiation capacity of stem cells and in part mediate their lineage specification processes. Primary cilia are differentially expressed in a lineage-dependent fashion in differentiating ASCs and MSCs, and thus appear to serve a specific function in mediating specific cell signaling pathways such as Wnt and Hedgehog. They appear to play a crucial role in tissue homeostasis and are the site of critical signaling pathways involved in the phenotypic development of cells and tissues. Within the musculoskeletal system, Kif3a, IFT88, and PC1 emerge as critical cilia proteins required for both their form and function. Clearly, primary cilia are no longer relegated to the vestiges of evolution as they were once considered to be; however, their apparent multifunctional physiological purpose combined with their often elusive expression frequency, has contributed to their broad study in a wide array of cell and tissue types. The emerging evidence surrounding primary cilia research in the context of progenitor and stem cell types suggests that the cilium may be a novel therapeutic target in controlling stem cell differentiation towards developing tissue engineered therapies.

Author Contributions

J.B.: conception and design, collection and/or assembly of data, data analysis and interpretation, Manuscript writing; E.L.: conception and design, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors have no potential conflict of interest.

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