SPECIAL ISSUE REVIEWS–A PEER REVIEWED FORUM

The Primary Cilium as a Gravitational Force Transducer and a Regulator of Transcriptional Noise

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Circumstantial evidence has suggested that the primary cilium might function as a gravity sensor. Direct evidence of its gravity-sensing function has recently been provided by studies of rohon beard neurons. These neurons showed changes in the variability of gene expression levels that are linked to the cyclic changes in the Earth's gravitational field due to the Sun and Moon. These cyclic changes also cause the tides. Rohon beard neurons, after the primary cilia have been selectively destroyed, no longer show changes in gene expression variability linked to the cyclic changes in Earth's gravitational field. After the neurons regrow their primary cilia, the link between variability in gene expression levels and the Earth's changing gravitational field returns. This suggests two new functions for the primary cilia, detecting the cyclical changes in the Earth's gravitational field and transducing those changes into changes in the variability (stochastic nature) of gene expression. *Developmental Dynamics 237:1955–1959, 2008.*

Key words: gravity; vertebrate cells; zebrafish; rohon beard neuron; primary cilium; transcriptional noise

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INTRODUCTION

For the past decade, we have been studying the effects of simulated-microgravity on zebrafish embryos. The emphasis of this research for the past several years has been the effects of simulated-microgravity on gene expression in live zebrafish embryos. The results of these experiments were so varied and unpredictable for different developmental stages and tissues that we began to look for a new hypothesis. The primary cilium functioning as a gravity-sensor presented a potential unifying hypothesis to explain most if not all of the results we have accumulated to date. The primary cilium is a phylogenically old, recently rediscovered, nearly ubiquitous cell organelle that serves specific sensory functions on specific cell types. The primary cilium is a flow sensor on kidney tubule epithelial cells (see Pan et al., 2005) and a key modifier of the hedgehog signaling pathway (Corbit et al., 2005). The primary cilium has also been suggested to act as a strain gauge for osteocytes in vivo (Whitfield, 2003) and a molecular switch between the canonical and non-canonical WNT pathways (Otto et al., 2003). In addition, modified primary cilia are key determinants for left-right asymmetry (Ferrante et al., 2006; Tamakoshi et al., 2006). As a phylogenically very old cell organelle, it would be reasonable to predict that the primary cilium might have a common function in all or most cell types possessing them. Since we now have data that support the idea that the primary cilium can regulate the stochastic nature of gene expression, we suggest that regulating the stochastic nature of gene expression might be a common function for the primary cilium. Because all cells need to regulate the stochastic nature of gene expression, the Earth's gravitational field could provide the stimulus to the primary cilium in those cells where

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the primary cilium has not been co-opted for some other function such as sensing shear forces associated with fluid flow. In this review, we summarize the work that supports these two new functions for the primary cilium.

HEART AND BLOOD VESSEL PRIMARY CILIA

One of the initial observations we made was that simulated-microgravity caused significant increases in gene expression in the developing heart and blood vessels (Shimada et al., 2005). Primary cilia are found on endocardial cells in the developing chick heart, predominantly on cells in areas of low shear forces (Van der Heiden et al., 2006). This localization is probably due to the active remodeling of the heart that takes place in regions of high shear forces. In order for the heart to add endocardial cells as the chambers remodel, the primary cilia would have to be resorbed for the cells to actively divide (Alieva and Vorobjev, 2004). If the zebrafish heart is similar to the chick heart in this regard, then the primary cilia on the endocardial cells would be resorbed as the tubular heart begins to remodel into definitive atrium and ventricle around 48 hr after fertilization. The time frame of susceptibility to simulated-microgravity-induced changes in gene expression coincides with the developmental period when primary cilia would be present prior to the period of heart tube remodeling in the zebrafish embryo.

We recently investigated the effects of simulated-microgravity on expression of *fli1* in zebrafish embryos. *Fli1* is the earliest known endothelial cell marker in zebrafish (Kidd and Weinstein, 2003). Simulated-microgravity causes dramatic changes in *fli1:gfp* expression in endothelial cells, but only prior to the initiation of blood flow through the developing vessels (Shimada et al., 2005). This suggests that either endothelial cells lose their susceptibility to simulated-microgravity or that the effects of blood flow through the blood vessel outweigh the effects of simulated-microgravity. Since endothelial cells in vitro and in vivo have primary cilia (Iomini et al., 2004; Van der Heiden et al., 2007), the primary cilium on the endothelial cell might begin to function as a flow sensor as blood begins to flow through the blood vessel coinciding with the change in susceptibility to simulated-microgravity.

NOTOCHORD PRIMARY CILIA

The developing zebrafish notochord has a period of susceptibility to simulated-microgravity that peaks between 24–72 hr post fertilization (Shimada et al., 2005). This is the period of peak spontaneous contraction of the developing somites. In the zebrafish embryo, the notochord is composed of an arrangement of cells that has been compared to a stack of coins lying on its side. Each cell has a single primary cilium projecting from the center of one surface of the "coin" (unpublished observation). Since the primary cilia on many cell types function as a type of strain gauge (Pan et al., 2005; Whitfield, 2003), the primary cilium on each notochord cell would be ideally situated to monitor bending of the notochord in response to forces such as gravity or muscle contractions. The primary cilium is directly linked to the cytoskeleton through the mother centriole (see Satir and Christensen, 2006). If the primary cilia are acting as gravity sensors in the notochord, then unloading the notochord during exposure to simulated-microgravity might have a more dramatic effect on the cytoskeleton during those periods when the notochord is affected by other forces such as muscle contractions. This idea is supported by the observation that induced increases in β-*actin:gfp* expression in the notochord are more dramatic in simulated-microgravity during the developmental time periods when the embryo is more "active."

HSP70 AND PRIMARY CILIA

The cilia proteome was recently made available on-line (http://www.ciliaproteome.org/). This database represents a compendium of the repertoire of proteins required for ciliary biogenesis and function in eukaryotes (Gherman et al., 2006). Of the genes that we have assayed in zebrafish, only *hsp70* is present in the cilia proteome. The only tissue in zebrafish embryos that expresses *hsp70* in the absence of any environmental stressor is the developing lens. HSP70 probably functions in the lens to prevent the lens fiber cells from dying when their nuclei enter an apoptotic pathway (Ribeil et al., 2007). Since simulated-microgravity does not induce expression in any tissue of zebrafish embryos other than the lens, simulated-microgravity cannot be considered an *hsp70*-related environmental stressor. However, simulated-microgravity does cause an increase in *hsp70* expression in the lens and a coincident decrease in TUNEL-positive cells in the lens (Shimada and Moorman, 2006). This supports the idea that simulated-microgravity induced changes in gene expression could be mediated through effects on the primary cilium.

ROHON BEARD NEURON PRIMARY CILIA

When we initially began to suspect the involvement of primary cilia in mediating the effects of simulated-microgravity on gene expression, we were hard pressed to explain how primary cilia could be involved in the changes seen in rohon beard neurons. Rohon beard neurons are the primary sensory neurons of the zebrafish prior to the development of the dorsal root ganglia cells. Rohon beard neurons are a single rostro-caudal row of large neurons in the dorsal spinal cord on each side. Each rohon beard neuron has a single axon that projects peripherally to segmentally innervate the tissue that will ultimately be innervated by dorsal root ganglia cells. Our previous data indicated that there was a pronounced increase in β-*actin:gfp* expression in rohon beard neurons exposed to simulated-microgravity between 24 and 72 hr (Shimada et al., 2005). As the dorsal root ganglia develop, the rohon beard neurons degenerate. This process of degeneration begins at 72–80 hr post fertilization. This time frame coincides with the end of the period of susceptibility to simulated-microgravity induced changes in gene expression in Rohon Beard neurons, but does not implicate primary cilia.

The rohon beard neuron data consisted of measurments of β-*actin:gfp*

expression level in one rohon beard neuron in each of 4 spinal cord segments in 24 embryos in each group. As part of the original analysis, the coefficient of variation for each group of pooled (96) individual rohon beard neuron expression levels was calculated. There was no significant difference between any of the coefficients of variation for any of the groups. The data were reanalyzed separately calculating the coefficient of variation for the 4 rohon beard neurons in each embryo. When analyzed in this manner, the coefficients of variation for the simulated-microgravity embryos were significantly higher than for the other embryos. This indicates that β-*actin:gfp* expression levels in rohon beard neurons are much more variable when embryos are exposed to simulated-microgravity. This reanalysis of the rohon beard neuron data begins to make more sense if the primary cilium can be linked to pathways that modulate transcriptional noise.

All in all, the data discussed so far are consistent with the primary cilium playing a role in mediating the simulated-microgravity–induced changes in gene expression seen in zebrafish embryos and support the hypothesis that the primary cilium can act as a gravitational force transducer in vertebrate cells. It should be emphasized that these data do not prove that the primary cilium is involved. It should also be pointed out that the effects of microgravity or simulated-microgravity have little if any relevance to life on Earth. Just because the primary cilium might detect dramatic changes in gravitational force associated with the transition from normal gravity to microgravity, does not mean that the primary cilium is involved in monitoring Earth's gravitational field.

However, Earth's gravitational field is not constant, it undergoes very small cyclic changes in magnitude due to the Sun and Moon. These changes typically have a magnitude of 2×10^{-6} m/s^2 everywhere on the surface of the Earth. These changes only have an observable effect as the tides. The Earth's gravitational field is at its weakest at high tide and the Earth's gravitational field is strongest at low tide with a period for one tide cycle of about 12 hr and 25 min. If we assume that a typical cell has a diameter of 10×10^{-6} m, we can calculate the approximate shear force that would be applied to a cell's primary cilium by the cyclic change in Earth's gravitational field. typical cell – 10-μm diameter: $4/3\ \Pi\ r^3 = 523.6\ \mu m^3 = 523.6\ \mu g$ mass (assumes the cell has the same density as water): 523.6×10^{-6} g = 523.6×10^{-9} kg mass: Force = mass ∗ acceleration, Force = 523.6×10^{-9} kg ∗ 2×10^{-6} m/s^2 = $1{,}047.2 \times 10^{-15}$ (kg ∗ m^2)/s^2

A newton is the amount of force required to accelerate a body with a mass of one kilogram at a rate of one meter per second squared. Algebraically: $1N = 1(kg * m)/s^2$

Thus, a typical cell could exert up to $1{,}047 \times 10^{-15}$ N on its primary cilium due to the relative positions of the Earth, Sun, and Moon, depending on the orientation of the cell in the gravitational field with respect to its primary cilium. Interestingly, a single primary cilium is capable of detecting a shear force of 5.2×10^{-15} N (Resnick and Hopfer, 2007). This suggests that the primary cilium is sensitive enough to detect the cyclic change in Earth's gravitational field.

PRIMARY CILIA AS GRAVITY SENSORS

Recently, we were able to demonstrate that rohon beard neurons in the developing zebrafish spinal cord show a change in the stochastic nature of their gene expression linked to the small cyclic changes in Earth's gravitational field (Shorr and Moorman; unpublished data). Briefly, we measured neurogenin-3.1:*gfp* (ngn3.1:*gfp*) expression in rohon beard neurons in the same embryos at the time of day that high tide occurs on the Raritan River in New Brunswick, NJ (a site 3 miles from the lab) and again at the time of day that low tide occurred, on two successive days. The average coefficient of variation of ngn3.1:*gfp* expression in rohon beard neurons imaged at high tide was significantly higher than the average coefficient of variation for the same rohon beard neurons imaged at low tide, while their mean expression levels were the same at high and low tides. This suggests that expression of ngn3.1:*gfp* in rohon beard neurons is more variable when the Earth's gravitational field is at its weakest (high tide) than when the Earth's gravitational field is at its strongest (low tide). "Gravity clamping" the embryos at 1.1 g at high tide resulted in gene expression variability being the same at high tide as it was at low tide, supporting the idea that it is a change in gravitational force that is causing the normal change in gene expression variability at high and low tides.

To determine whether the primary cilium mediates the change in gene expression variability, we selectively destroyed the primary cilia by incubating the embryos in chloral hydrate (Praetorius and Spring, 2003; Praetorius et al., 2004). After pretreatment with chloral hydrate, ngn3.1:*gfp* expression variability was uniformly high at both high and low tide. When cells are removed from chloral hydrate, they regrow their primary cilia. The embryos were removed from the chloral hydrate and imaged again at high and low tide the next day. At that time, ngn3.1:*gfp* expression variability was again significantly higher at high tide than at low tide. These data suggest that the primary cilium is responsible for transducing cyclic changes in Earth's gravitational field into changes in the variability of gene expression levels in rohon beard neurons in the zebrafish spinal cord.

Why would rohon beard neurons need a mechanism to regulate the variability of gene expression levels and are these results unique to rohon beard neurons in the zebrafish? During development, rohon beard neurons are specified and maintained as a single rostro-caudal row of cells on each side of the dorsal spinal cord in zebrafish embryos. In order for this specification and maintenance to occur, presumptive rohon beard neurons need to be able to accurately and reproducibly respond to a specific combination of environmental factors. If presumptive rohon beard neurons could not tightly regulate the variability of gene expression levels during specification, they might not develop as a single row of cells but might develop as a more diffuse population in the dorsal spinal cord.

Being able to tightly regulate the stochastic nature of gene expression is not unique to rohon beard neurons. All cells need to regulate the variabil-

ity of gene expression levels to be able to accurately and reproducibly respond to their extracellular environment. There is a cost to increased accuracy, and some cell groups may be able to successfully tolerate transcriptional noise (variability), while others may have a lower tolerance for noise. For example, keeping transcriptional noise low is particularly important during development when combinations of extracellular cues are specifying cell, tissue, and organ phenotypes throughout the embryo. For instance, the gradient of Shh released by the notochord and floorplate in combination with BMP released by the roofplate and retinoic acid from the somites, specify the dorsal-ventral identity of neurons in the developing spinal cord (Wilson and Maden, 2005). In the absence of being able to tightly regulate gene expression levels in response to different morphogen concentration thresholds, the cell's response would be like a simple on-off switch making it significantly more difficult to specify the more than 8 cell types found along the dorsal-ventral axis of the spinal cord. The ubiquitous nature of the primary cilium suggests that using the primary cilium to regulate the stochastic nature of gene expression might be common to all vertebrate cells.

WNT PATHWAYS MIGHT LINK PRIMARY CILIA TO TRANSCRIPTIONAL NOISE

At the single-cell level, the transcription of individual genes is associated with noise, random molecular fluctuations that create variability in the levels of gene expression within a cell population. The sources of noise that are associated with gene expression include: the small number of molecules that are involved in a particular biochemical reaction in the cellular space, the stochastic nature of the molecular interactions that underlie the chemical reaction, and the built-in efficiency of the transcriptional and translational processes. Most studies of the noise that is associated with gene expression have focused on transcription, and have distinguished between noise that is derived from the interaction between polymerases and DNA (intrinsic noise) and noise produced by other influences such as random fluctuations in nutrients, cell division, or regulatory inputs to the transcriptional machinery (extrinsic noise) (Elowitz et al., 2002; Paulsson, 2004). In mammalian cells, the major source of transcriptional noise is due to random fluctuations between bursts of active transcription and transcriptional inactivity (Raj et al., 2006). These periods are thought to be due to chromatin remodeling, where active transcription coincides with chromatin de-condensation and transcriptional inactivity coincides with chromatin condensation (Raj et al., 2006).

It has recently been suggested that canonical WNT signaling plays a key role in stabilization and maintenance of target-gene expression (Arias and Hayward, 2006). It has been proposed that the Wnt effector, β-catenin, carries out a qualitatively different function on gene expression from that of the effectors of other signaling pathways. Unlike epidermal growth factor (EGF), Hedgehog proteins, or bone-morphogenetic protein (BMP) signaling, which target the transcriptional machinery and establish rates of transcription, WNT signaling might preferentially target chromatin remodeling and the stabilization of the expression rates and patterns that are set by transcription factors.

Several links between the primary cilium and the WNT pathways have been established. For instance, inversin, a protein localized to the primary cilium (Hou et al., 2002; Watanabe et al., 2003), inhibits the canonical WNT pathway and activates the non-canonical WNT pathway (Simons et al., 2005). In this way, inversin functions as a molecular switch between the two pathways by targeting the WNT pathway protein disheveled (DVL) for degradation. Fluid flow increases the level of inversin in the cilia (Otto et al., 2003), supporting the conclusion that the canonical WNT signal transduction pathways are down-regulated by the primary cilium. *Tg737*, the gene mutated in the *orpk* mouse model of PKD, encodes polaris, a protein required for proper ciliary assembly (Pazour et al., 2000; Taulman et al., 2001; Yoder et al., 2002b). There is an increase in β-catenin expression and localization to dilated ducts in pancreas and increased expression levels of WNT signaling transcription factors (Cano et al., 2004) in the *Tg737* mouse.

Both polycystin-1 and polycystin-2, the proteins encoded by *Pkd1* and *Pkd2*, respectively, localize to the cilium in mouse and human kidney cells (Yoder et al., 2002a). Polycystin-1 is a G protein–coupled receptor and polycystin-2 is a calcium channel. Since polycystin-2 is a calcium channel, it is possible that the primary cilium might also be involved in modulating the WNT/calcium pathway. This pathway, through increases in calcium concentration, is also thought to influence the canonical WNT pathways, which in turn could influence gene expression variability.

CONCLUDING REMARKS

The data presented are consistent with the primary cilium being able to play a role as a gravitational force transducer in vertebrate cells. The degree to which the expression level of the same gene varies in different cells is an important factor that influences phenotypes within cell populations. The large number of cell states and types that are present in the lifetime of an organism and the reproducibility with which they are generated predicts the existence of developmental programs and mechanisms that ensure their reliable execution. In the context of development, variability in gene expression opposes the precise and reproducible phenotypes observed in cell populations. If noise is an intrinsic property of biological systems, it needs to be controlled during the construction of an organism. For instance, cells in many different regions of the developing vertebrate embryo adopt different fates based on the concentration of morphogen detected in the extracellular environment. For this concentration-dependent specification to work, cells must be able to tightly regulate their gene expression levels, our hypothesized function of the primary cilium. Otherwise, the response to many different concentrations of morphogen would be similar and the morphogen would function more like a molecular on-off switch. The unchanging cyclic nature of the Earth's gravitational field over the

last 3–4 billion years, could have provided an evolutionary-selective advantage to cells that could use gravity as a stabilizing influence on gene expression levels, providing the control necessary to stabilize the phenotype of a cell population. The phylogenic age and ubiquitous nature of primary cilia suggest that regulating the stochastic nature of gene expression might be a function of primary cilia in most if not all vertebrate cells that possess them. In addition, for those cell types that do not use other stimuli such as shear forces associated with fluid flow to stimulate the primary cilia, the cyclic nature of Earth's gravitational field might provide the stimulus.

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