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Tissue cell differentiation and multicellular evolution via cytoskeletal stiffening in mechanically stressed microenvironments

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Abstract

Evolution of eukaryotes from simple cells to complex multicellular organisms remains a mystery. Our postulate is that cytoskeletal stiffening is a necessary condition for evolution of complex multicellular organisms from early simple eukaryotes. Recent findings show that embryonic stem cells are as soft as primitive eukaryotes-amoebae and that differentiated tissue cells can be two orders of magnitude stiffer than embryonic stem cells. Soft embryonic stem cells become stiff as they differentiate into tissue cells of the complex multicellular organisms to match their microenvironment stiffness. We perhaps see in differentiation of embryonic stem cells (derived from inner cell mass cells) the echo of those early evolutionary events. Early soft unicellular organisms might have evolved to stiffen their cytoskeleton to protect their structural integrity from external mechanical stresses while being able to maintain form, to change shape, and to move.

Keywords

Cytoskeleton; Force; Bacteria; Amoebae; Eukaryotes

How eukaryotes have originated is at the center of debate in evolutionary biology. Various models on the evolution of eukaryotes have been proposed [1–6]. Recent molecular phylogeny evidence appears to support the three-domain model of bacteria, archaea, and eucarya [1] and seems to deviate from the models of evolution from prokaryotes to eukaryotes [2,3,5,6]. However, these models are not well-suited to address late stages of evolution, i.e., how complex forms of multicellular organisms have evolved from simple eukaryotes. Life could have stayed simple eukaryotes like their single-celled ancestors or like amoebae. Contributions from genome evolution [7] and cell-cell adhesion molecule cadherins [8] have been proposed as some of the necessary conditions for the evolution of the complex multicellular animal organisms. The question then is: what are other conditions that must be met for the complex multicellular animals to evolve?

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We propose a cell stiffness postulate: for a complex multicellular animal to evolve, early soft unicellular organisms must evolve to stiffen their cytoskeleton to protect their structural integrity from being irreversibly damaged by external mechanical stresses while being able to maintain form, to change shape, and to move.

To provide support for this postulate, let us first examine stiffness values of various cell types. It is known that, due to the presence of a rigid cell wall of peptidoglycan, a bacterium has a cell stiffness of ~1000 kPa [9]. Archaea whose membrane consists of stiff protein surface layers (S-layers) possibly also have a stiffness of ~1000 kPa [10]. Although the cell stiffness of the very first eukaryotes is not known, simple primitive eukaryotes such as an amoeba has a stiffness of ~0.1 kPa [11] that is at least 4 orders of magnitude lower than that of bacteria or that of archaea. The stiffness of the plasma membrane likely contributes to less than 1% of the total cell stiffness [12] and hence the majority of the cell stiffness originates from that of the cytoskeleton. Do all modern-day cell types in a complex multicellular land animal have a similar stiffness as an amoeba? The answer appears to be no.

Now let us examine the stiffness of various cell types in an animal. An embryonic stem cell has a stiffness of ~0.5 kPa [13]. Neural cells generally have a stiffness of ~0.1–0.5 kPa [14]. A differentiated cell has a stiffness of ~5 kPa [13], similar to that of a mesenchymal stem cell [15]. A typical tissue cell (e.g., a smooth muscle cell, an endothelial cell, or a fibroblast) has a stiffness of ~1–5 kPa [16]. A skeletal muscle cell has a stiffness of ~12 kPa [17]. The stiffest cells in a modern-day animal appear to be the skeletal muscle cells that generate extremely high stresses themselves. It may not be a coincidence that these muscle cells have a very stiff cytoskeleton to withstand its own stresses, as the muscle cells need to generate high stresses to perform various essential functions of the organism. Similarly, besides closing a wound, one of fibroblasts' main tasks is to secrete matrix proteins to produce extracellular polymers and to pull on the matrix polymers to generate specific patterns and forms [18]. Therefore, it is not surprising that stiffness of the fibroblast is very high. In a similar vein, a smooth muscle cell needs to generate high stresses to regulate the caliber of a lumen, whether it is a blood vessel, a gastrointestinal lumen, or an airway in the lung. Therefore, smooth muscle cell stiffness would also be very high. It is probably true that most types of tissue cells that can remodel its own microenvironment matrix protein polymers have stiffness of similar magnitudes. It is interesting that the differentiated cells that generally have much lower stiffness are those that are either an amoeboid-like cell (e.g., a neutrophil) or a neural cell of the central nervous system. It is important to note that the process of cytoskeletal stiffening does not have to be irreversible during differentiation. For example, a stiff mesenchymal stem cell becomes softer when it differentiates into an adipocyte that typically has a stiffness of ~0.6–0.9 kPa [19], possibly after depositing a soft extracellular matrix (ECM).

Since archaea and bacteria are both rigid cells, the question raises whether the very first ancestor cells of all eukaryotes are soft or rigid cells. It is not clear at this time. Nevertheless, one thing appeared to be happening after penetration of its membrane by an aerobic aproteobacterium or after phagocytosis of the α-proteobacterium: a newly formed cell was a soft cell that did not have the rigid wall as the archaea and the bacteria did. This primitive eukaryote was probably as soft as today's amoebae.

How did then the primitive soft eukaryotes evolve to be stiffer to give rise to modern day different eukaryotes of various stiffnesses? One way to stiffen these soft cells was to cluster transmembrane cell-matrix adhesion molecule integrins [20,21] and to cluster cell-cell adhesion molecule cadherins [22]. Another way was to express crosslinking/bundling proteins to stiffen the cytoskeleton [23,24]. Moreover, myosin-II (both a crosslinking protein and a force generator) might have evolved [25] to form actomyosin bundles to generate large forces for power generation and for prestress-dependent cell shape stability [14,15,26–28]. The stiffening responses of the primitive eukaryotes were also likely to be triggered when they were pulled by nearby cells via cell-cell adhesion molecule cadherins [22] and when they pulled on the stiff ECM via cell-matrix adhesion molecule integrins [14]. The nuclear lamina is a fibrillar network that lies underneath the nuclear envelope in eukaryotes. The nuclear lamina consists of lamins (e.g., lamin A/C and lamin B) that are a stiff polymer structure [29] to provide protection to the genome from being damaged by mechanical stress [30]. It is increasingly evident that the nucleus can be viewed as a mechanosensor [31] in which lamins are an important component responsible for differentiation [32] and for regulation of transcription factors [33]. It is recently revealed that the nuclear envelope and lamina are physically tethered to the cytoskeleton via the **l**inker of neucleoskeleton and cytoskeleton (LINC) complex to propagate force signals [34] for the chromatin to sense forces to activate genes [35]. Most differentiated eukaryotes have a nucleus that is several folds stiffer than its cytoskeleton [29,36]. Therefore, as cells begin to differentiate during embryonic development [37,38], their cytoskeleton and nucleus should become stiffened to accommodate the need to withstand high mechanical stresses in their microenvironment. However, it is important that the cytoskeleton and the nucleus should not have stiffened too much. Because if all modern day eukaryotes were still as stiff as those bacteria or archaea, we might not have a modern-day complex animal whose cells are able to readily change shapes during development and under physiological conditions. Although the 1000 kPa stiffness of the bacteria is beneficial for the bacteria to survive in the presence of large external mechanical stresses or osmotic challenges, it is just too rigid for shape change. It is so stiff that they cannot change their shape easily to perform spreading, to migrate through porous extracellular matrices, and/or to contract to change cell shape since it is energetically too costly. This is probably why the modern-day stiffest animal cell (the skeletal muscle cell) has a modulus of only ~10 kPa and not 1000 kPa. In contrast, simple cells like amoeba are too soft (0.1 kPa) to sustain any large mechanical stresses to form a complex multicellular organism, although they do form a colony of cells under certain conditions. It is well known that these cells have a very soft and dynamic cytoskeleton [11]. Therefore, although the dynamic nature of the cytoskeleton alone cannot explain the evolution of the complex multicellular organisms, it facilitates cell shape change. The first principle for survival is probably to protect the integrity of the cell (i.e., without structural failure in the presence of changes in mechanical stresses or osmotic pressures) while still being able to perform necessary physiological functions. Neurons appear to be specialized cells whose primary function is to conduct electrical/chemical signaling. That is probably why they are soft (~0.1–0.5 kPa) and reside deep inside other tissues in the animal body to be protected against mechanical insults.

Cell shape is determined by the organization of the cytoskeleton whose stiffness, in turn, is determined by the clustering of adhesion molecules like integrins and cadherins, crosslinking and bundling of the cytoskeletal filament systems, and the prestress generated by myosin-II motor proteins. The final shape of the cell is the result of the balances of those external and internal forces, the overall stiffness of the cell, the cell-matrix and/or cell-cell adhesion, the stiffness of the ECM, and the shape and organization of the cytoskeleton. It is known that a normal cell cannot survive if its plasma membrane is detached from the underneath cytoskeleton for long. Therefore, cell shape per se, while being an easily measurable parameter, is not a mechanistic factor in a complex multicellular organism.

One open question that could be asked is the following: is it the cytoskeleton stiffening or the changed mechanical microenvironment that drives organ evolution? Of all the organs and tissues in an organism, only the cells are the “live” components that can actively “sense” changes in their microenvironments, including mechanical microenvironments. The ECM that is the main component of the mechanical microenvironments that can be remodeled by the living cells and by the ambient environment such as radiation [39]. Part of the cytoskeletal stiffening of the cell is through the generation of active tension in the cytoskeleton that is utilized by the cells to mechanically remodel their microenvironment [18]. In addition, for the cells to secrete enzymes to remodel it enzymatically. Therefore, it is possible that both the cytoskeletal stiffening and the changed mechanical microenvironment contributes to organ evolution but the living cells dictate organ evolution by actively sensing changes in their microenvironments. It is reported recently that cell stiffness increases with intracellular crowding as a result of water efflux induced cell volume reduction [40], although the experiments are generally performed in short time scales (e.g., tens of seconds to minutes via osmotic challenges). Consistent with this notion is the fact that an animal egg that is very soft (the cytoplasm of an unfertilized frog egg has a stiffness of ~0.01 kPa [41]) and is generally much bigger in volume than a stiff differentiated cell. It remains to be determined in the future, however, how much intracellular crowding has to increase to account for the cell volume reduction and the cell stiffness elevation to impact on multicellular evolution.

It has been proposed that the principles of tension-dependent integrity (tensegrity) of the cytoskeleton might have guided the evolution of the first cells on the earth [42]. A different hypothesis is that fluidization of the cytoskeleton during invasion might manifest early evolutionary adaptations of the eukaryotic cell to material properties of a soft inert microenvironment [43]. It is possible that the capacity to maintaining the cell shape stability in the presence of mechanical challenges and the dynamic malleability of the cytoskeleton both are necessary for animal cells to evolve. However, for a complex multicellular land animal to evolve and to perform challenging mechanical tasks (e.g., movement, running, jumping, climbing, etc.), it also needs to develop stiff tissue cells such as skeletal muscle cells. These cells are generally as stiff as their substrates. The evolution of stiff ECM polymers of cartilage and of rigid bones provided the animal with additional mechanical strength and stiffness to sustain impacts of the gravitational forces and/or external mechanical insults by predators. In addition to changes in mechanical environments, changes in chemical environments such as estrogen deficiency induce osteoporosis in the rigid bone and result in lower stiffness and higher plastic deformation in the bone than the

control bones [44], influencing the overall capacity of the organism to sustain large compressive stresses. It is interesting that differentiation of stem cell like cells leading to cell stiffening has been demonstrated in tumor. Melanoma tumor-repopulating cells, which express high levels of self-renewal gene Sox2, are extremely soft (cell stiffness is ~0.15 kPa, 4-fold lower than their differentiated counterpart cells) [45]. These cells are highly tumorigenic and metastatic, enabling them to change cell shape easily to extravasate and to invade the 3D ECM *in vivo* in a zebrafish model [46]. The cell stiffness postulate is testable: if one finds that a complex land animal that can generate huge mechanical stresses and can sustain great external stresses but is still composed of only very soft cells (~0.1 kPa) in the absence of the stiff ECM and rigid bones, then this postulate is disproven.

It has been reported that matching cell stiffness with that of its substrate is critical in forming striation in skeletal muscle cells [17] and in optimizing cardiomyocyte beating [47]. However, stiffness matching may not be limited to skeletal muscle cells and cardiomyocytes. The adaptability of the cell stiffness to the stiffness of the ECM could be a critical feature for a multicellular life to evolve. Embryonic cells and fibroblasts adapt and change their stiffness to match their mechanical microenvironments [48–53]. For a complex multicellular land animal to evolve, it must adapt its different tissue cells' stiffness to their local micromechanical environment. If the earliest unicellular eukaryotes had a cell stiffness of ~0.1 kPa, their stiffness might match that of nutrient-rich uncompacted soft ocean sediments deposited ~2 billion years ago. These cells would be favored to evolve because they would be better able to perform mechanical functions optimally when material properties of the cell match those of very soft paste-like microenvironments. Evidence suggests that archaea are the ancestor of eukaryotic cells [54–56]. High stiffness of archaea (~1000 kPa) that is similar to that of bacteria endows their ability to cope with the challenges of changing environmental mechanical stresses and osmotic pressures. It appears that soft embryonic stem cells become stiffer as they differentiate into tissue cells of the complex multicellular organisms to match their microenvironment stiffness. We perhaps see in the differentiation of embryonic stem cells (derived from inner cell mass cells of the blastocyst) the echo of those early evolutionary events of complex multicellular animals (Fig. 1).

The evolution of the genome and of the cadherins has been proposed as some of the necessary conditions for a multicellular animal to evolve [7,8]. In this report, we propose, at the functional level, a necessary condition of cytoskeletal stiffening for the evolution of the complex land animals. The postulate of the cytoskeletal stiffening evolution complements that of the genome evolution and of the cadherin evolution. If all the cells in a multicellular land animal organism were still as soft as those primitive organisms like amoebae, there probably would be no emergence of complex multicellular animals that are able to exhibit various forms and patterns, and to generate and sustain large mechanical stresses. The evolution of the cytoskeleton based on the strategy of crosslinking/bundling and tension-dependent prestress stiffening might be a key determinant whether a present-day animal can evolve. These are likely achieved by the selective pressures of evolution. If these were not the case, we might have seen a planet that was still filled with rigid-walled bacteria, archaea, and soft simple organisms like amoebae.

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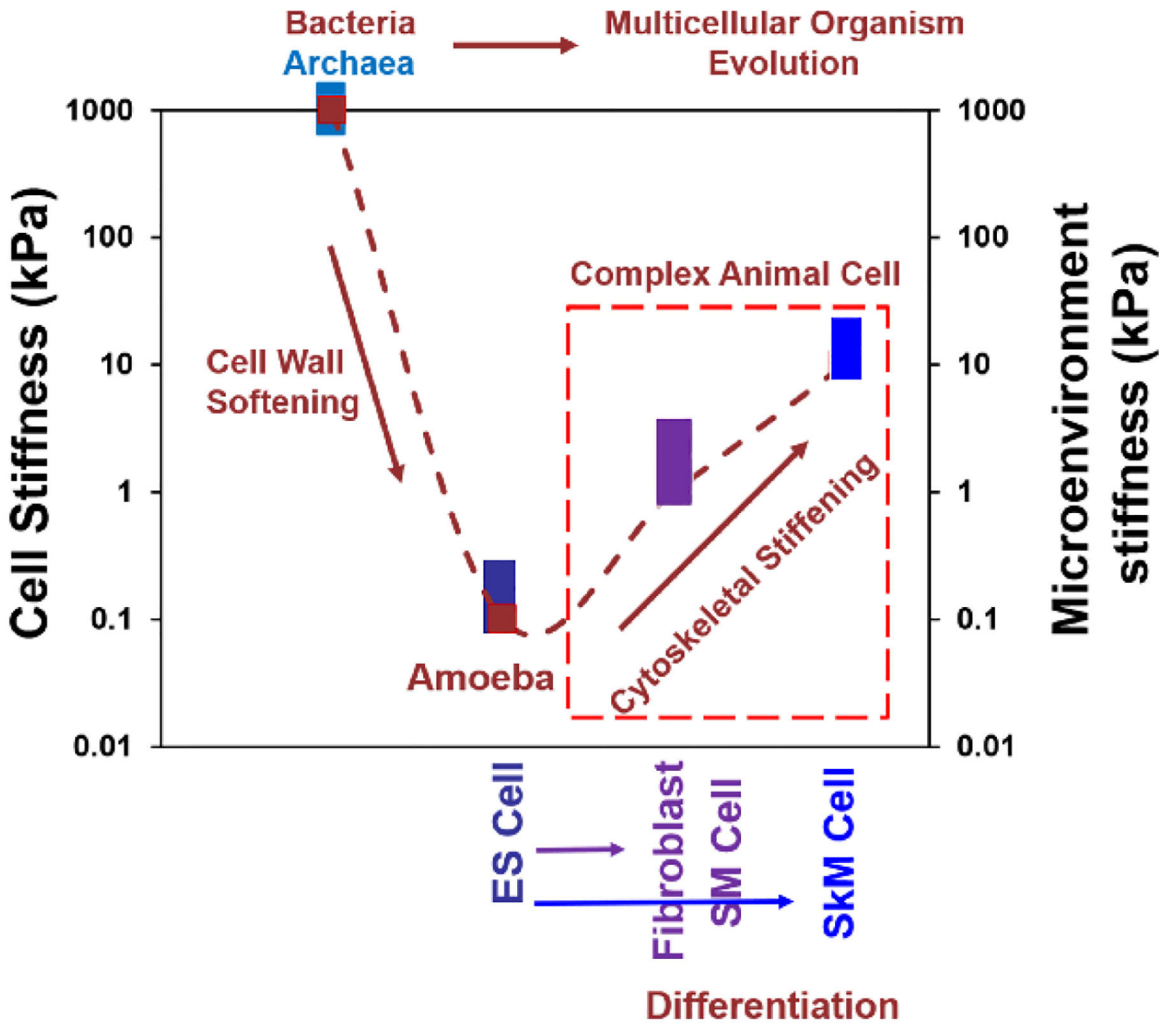

Fig. 1.

Cytoskeletal stiffening during differentiation might echo evolution events from simple eukaryotes to complex animal cells. When an embryonic stem (ES) cell (derived from inner cell mass cells of the blastocyst) differentiates into germ layer cells and then into fibroblasts, smooth muscle (SM) cells, or skeletal muscle (SKM) cells, the cell stiffness increases by ~2 orders of magnitude: from ~0.1–0.5 kPa to 12 kPa [13,16,17]. Stiffness of an amoeba is ~0.1 kPa [11], which is ~4 orders of magnitude lower than that of a bacterium [9] or that of an archaeon [10]. The microenvironmental stiffness of ES cells is 0.1–1 kPa [37,48]; that of vascular smooth muscle cells is ~20 kPa [49]; that of skeletal muscle cells is 20–50 kPa [17,47]; that of fibroblasts is 1–10 kPa [32,51–53]; that of amoebae is probably ~0.1 kPa; those of bacteria and archaea are assumed to vary from low (0.01 kPa) to high (thousands of kPa). We hypothesize that differentiation of embryonic stem cells (days to weeks) echoes those early evolutionary events (millions of years). We propose a model that cytoskeletal stiffening is necessary for evolution from early simple eukaryotes to complex multicellular land animal cells. This working model for evolution and survival of multicellular land

animals in mechanically stressed environments complements the models of genome evolution [7] and cadherin evolution [8].