Cell mechanotransduction: cytoskeleton and related signaling pathways

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Abstract

Mechanical stimuli regulate a variety of cell physiological functions including gene induction, protein synthesis, proliferation and/or differentiation; understanding mechanotransduction at the cellular level is key to understanding basic biology. Here on Earth, signal transduction affects a wide array of receptors and ligands that signal induction of gene expression. The most common signaling pathways include receptor tyrosine kinase (RTK), G-Protein coupled receptors (GPCR) and extracellular matrix components (integrins). The cytoskeleton functions to maintain cell shape and to move cellular components, separate chromosomes during mitosis and provides sensing networks for mechanotransduction. Mechanotransduction is the process of translating mechanical force on a cell into a biological response. Over the last few decades, mechanotransduction has been shown to occur via extracellular matrix, integrins, cytoskeleton signals, GTPases, adenylate cyclase, PLC and MAP kinases (MAPK), all of which play significant roles in early mechanical signaling. During the last decades a wide variety of space flight experiments have demonstrated that gravity has profound effects on whole organisms, organs and tissues, resulting for example in bone and muscle resorption as well as in the occurrence of cardiovascular malfunctioning, immuno-suppression and many other aspects of clinical medicine. Interestingly, the virtual absence of gravity also has profound effects on the cellular and molecular level, including changes in cell morphology, collapse of the actin cytoskeleton, modification of gene expression, changes in signal transduction cascades and even changes in the polymerization of tubulin. The effects of mechanical stress (e.g. gravity) or lack of stress (microgravity) on cell and molecular properties is discussed with an emphasis on the involvement of signal transduction cascades of RTK, integrins and FasR as well as their role in cytoskeleton perception of gravity in mammalian cells.

Introduction

Mechanical forces have been known for long time to influence cell behaviour. The mechanism by which mechanical forces are translated by cells into a biological response has been described as mechanotransduction. During the last decades a wide variety of studies have demonstrated that mechanotransduction involves the

components of the extracellular matrix and several plasma membrane associated proteins (Fig.1). These proteins play a central role in the transmission of a mechanical force to a biological response; the most central proteins in this process include the integrins and cadherins. Subsequently the cytoskeleton has also been demonstrated to play an important role in transmission of the signals inside the cells. The eukaryotic cytoskeleton is composed of three basic types of filaments and their associated proteins. Cytoskeletal filaments are interconnected and their functions are coordinated by hundreds of associated cytoskeletal accessory proteins. The cytoskeleton has been demonstrated to be involved in cell adhesion through integrins

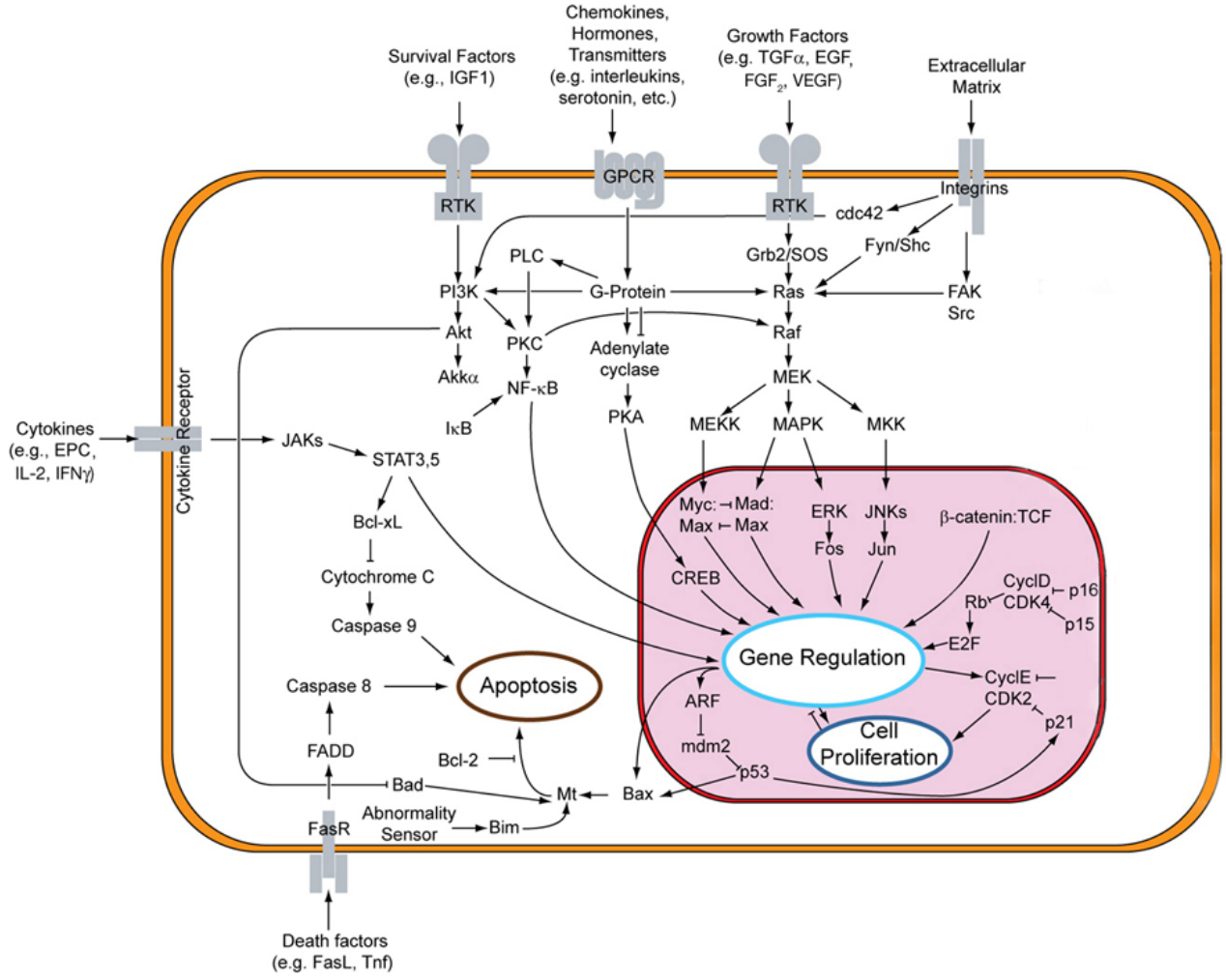

Figure 1: Major signaling pathways and transcription factors in cells.

In addition, the cytoskeleton appears to be involved in signal transduction cascades induced by growth factors. Altogether the interactions between integrins, cadherins, growth factor receptors, signal transduction molecules and the cytoskeleton constitute a network through which mechanical forces influence gene expression (Fig. 1). In this contribution we will briefly describe the effects of mechanical stress (e.g. gravity) or lack of stress (microgravity) on cell and molecular properties with emphasis on the involvement of signal transduction cascades induced by receptor tyrosine kinases and extracellular matrix, as well as their role in cytoskeleton perception of gravity in mammalian cells.

Role of integrins in mechanotransduction

The cytoskeleton not only functions to maintain cell shape, it is also important in the movement of cellular components, segregation of chromosomes during mitosis and in forming a sensing network for mechano-transduction. The eukaryotic cytoskeleton (CSK) is composed of three basic types of filaments; actin microfilaments, intermediate filaments and microtubules. CSKs are interconnected and their functions are coordinated by associated cytoskeletal accessory proteins including integrins. The binding of these proteins with cooperative groups to cytoskeletal filaments is dynamic and causes rapid polymerization and depolymerization of filaments. Integrins comprise a large family of transmembrane glycoproteins that bind to extracellular matrix components at the extracellular side of the plasma membrane and to the cytoskeleton at the cytoplasmic side. Integrins are heterodimers having a α and a β subunit. Each subunit has a large extracellular domain, a single transmembrane domain and a relatively small cytoplasmic domain. Integrins usually reside in complexes in the cell membrane, called focal adhesion complexes. The focal adhesion complexes also constitute the end points of the actin stress fibers. In addition, to being involved in cell attachment to the ECM, integrins have been demonstrated to be able to directly activate several intracellular signal transduction cascades. One of the best-known cascades is the MAP kinase pathway. Upon binding of integrin to the ECM, the focal adhesion kinase (FAK) is phosphorylated and activated. FAK is a tyrosine kinase and activates subsequently

the small G-protein RAS. RAS in its turn activates the serine/threonine kinase, RAF and then activated RAF phosphorylates and activates the dual specificity kinase MEK. MEK phosphorylates and activates MAP kinase leading to activation of transcription factors. The MAP kinase pathway has been demonstrated to play an essential role in cell cycle regulation by the induction of cyclin D, the essential cyclin for progression through the G1 phase of the cell cycle. In addition to RAS, FAK is also able to activate other signal transduction proteins, including PI3 kinase, c-SRC, GRAF (a Rho-GAP) and structural proteins such as talin and paxillin. Consequently, the integrins play a prominent role in several important processes such as cell cycle regulation and apoptosis [1-3]. The direct activation of the Rho-family GTPases by integrin is especially of interest. The Rho-family GTPases influence many cellular processes, but are of particular importance in the regulation of the actin microfilament system [3].

In addition to signal transduction, integrins have also been demonstrated to act as mechanotransducing components. Increasing tension on integrins leads to the rapid recruitment of vinculin, zyxin and probably other focal adhesion proteins to the focal adhesion site, thereby increasing the size of the focal contact. Moreover, this tension leads also to an induced binding of "free" integrins to ECM components, and these latter events have been demonstrated to result in a modified gene expression through the activation of Jun kinase. Accordingly, this pathway tension may affect cell cycle progression. The general idea is that tension leads to conformational changes of the integrin, which then leads to downstream modifications such as the activation of p130Cas. P130Cas mediated the activation of Rap1 [4] upon application of force.

Interestingly, the integrins present in the focal adhesions are linked to the actin microfilaments through a cluster of proteins. This suggests that force induced modifications of the integrins may subsequently lead to modifications of the actin microfilaments [5-7]. Indeed it has been demonstrated that actomyosin-based contractile forces are transmitted from cells to the ECM at the focal adhesions sites [8, 9]. Inhibition of the contractile forces leads to a disassembly of focal adhesions [10, 11]. The amount of force acting on the focal adhesions has been shown to determine its size and the application of force on the cells was shown to enlarge focal

adhesions complexes [11, 12]. Furthermore, it has been demonstrated that mechanical forces induce an accumulation of F-actin at the focal adhesions in a zyxin-dependent manner, involving the strengthening of the ECM-integrin-actin linkage [9, 13, 14].

Actin microfilaments have also been demonstrated to regulate integrins. Treatment of cells with cytochalasin D to cap actin filaments inhibits cell adhesion. In other cells it was demonstrated by Bennett et al. that inhibition of actin polymerization resulted in an induction of ligand binding to integrins [15]. The platelet cytoskeleton regulates the affinity of the integrin $\alpha_1\beta_3$ for fibrinogen [15]. Activation of Cdc42 and Rac is associated with the formation of focal complexes in fibroblasts [16, 17] and inhibition of Rho results in a decrease of integrin-mediated aggregation of leukocytes and platelets [18].

In conclusion, mechanical force accelerates integrin activation, both through extracellular and intracellular rearrangements, which induce protein recruitment leading to integrin clustering [19]. These observations suggest that the ECM-integrin-actin complex in the focal adhesion complexes may also constitute a gravity sensitive component. Indeed, it has been demonstrated that exposure of the epidermoid human A431 cells to real and simulated microgravity conditions leads to a rapid (within minutes) rounding of the cells [20]. Similar results were obtained in fibroblasts incubated in a random positioning machine [Moes et al. unpublished observations].

Role of cadherins in mechanotransduction

Cadherins are transmembrane glycoproteins playing an important role in cell-cell adhesion. The extracellular domains are responsible for adhesive recognition due to their interaction with the extracellular domains of cadherins of neighboring cells. The cytosolic domains of cadherins interact with a wide variety of proteins including actin microfilaments and intermediate filaments. One of the best known cadherin-associated proteins concerns α-catenin. The cadherin-catenin complexes associate to actin filaments to form the adherens junctions and the association with intermediate filaments result in the formation of desmosomes. The role and biochemistry of both cadherins and catenins have been described recently in several review papers [21-24].

Cadherins have been demonstrated to be involved in mechanotransduction, particularly in specialized systems such as the inner ear hair cells [25]. In addition, it has been demonstrated in fibroblasts that mechanical forces applied to intercellular junctions induced intracellular responses mediated by cadherins, suggesting that cadherins function as intercellular mechanotransducers [26]. Furthermore cadherin engagement was shown to modulate RhoA signaling and contractility in endothelial cells [27]. These findings strongly suggest a close cross talk between signal transduction cascades induced by cadherins and integrins [28, 29]. As described above, cadherins play a prominent role in cellular junctions and as such are essential in the establishment of the endothelium. It is well known that the endothelium responds to mechanical deformations, although the mechanisms by which endothelial cells recognize mechanical stimuli are not as yet understood. Many potential mechano-sensing systems have been suggested including the cytoskeleton [30], G-proteins [31] and junction proteins [32, 33].

Involvement of the actin cytoskeleton in growth factor and extracellular matrix signalling

Actin is an extremely abundant protein in virtually all eukaryotic cells, and is involved in many cellular functions including migration, endocytosis, intracellular transport, docking of proteins and mRNA, attachment, signal transduction, membrane ruffling, neuronal path finding and cytokinesis. Moreover, it largely determines the cell shape and the position and shape of organelles within the cytoplasm.

The actin family consists of α-, β- and γ-isoforms. The α-isoform is mostly present in muscle cells whereas the β- and γ-isoforms are present in all cells. Actin is present in cells in an unassembled, globular form and a polymerized, filamentous form, called G-actin and F-actin, respectively. The F-actin filaments are composed of two linear strands of polymerized G-actin wound around each other in a helix. Within these filaments the actin monomers are oriented in the same direction resulting in inherent polarity of the filaments resulting in the barbed or plus end and the pointed or minus end. The barbed ends are characterized by a rapid polymerization and a slow de-polymerization and the pointed ends exhibit the opposite features. In the

cells actin continuously cycles between the polymer and monomer state, a process called treadmilling.

The actin filaments constitute a highly dynamic network in the cells, the dynamics being regulated by a large number of actin binding proteins (ABPs) [34, 35]. The ABPs are characterized by their function, such as cross-linking proteins, actin severing, capping and de-polymerizing proteins, monomer binding proteins, membrane-associated proteins and actin-regulatory proteins. Several conserved domains of actin have been identified that act as binding domains for the ABPs, including the myosin motor domain, the gelsolin homology domain, the calpain homology (CH) domain, the actin depolymerizing factor/cofilin (ADF/cofilin) domain and the Wiskott-Aldrich syndrome protein (WASP)-homology domain-2 (WH2) [36-40]. These observations clearly demonstrate that actin metabolism is

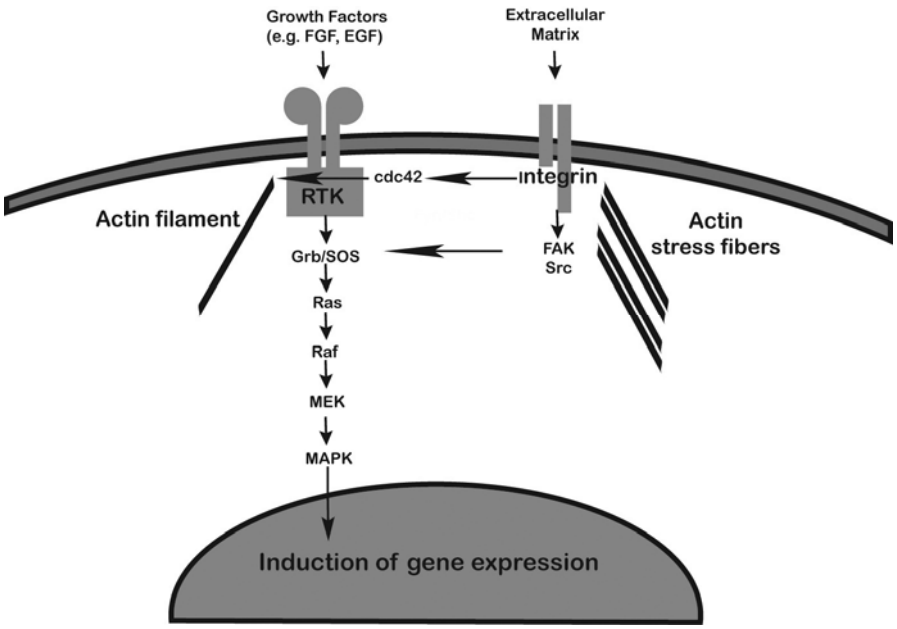

Figure 2: Involvement of the actin cytoskeleton in growth factor and extracellular matrix signalling. The cytoskeleton is directly involved in the signal transduction of many of the RTK receptors. These signaling pathways includes the involvement of integrins, ECM, actin stress fibers and cdc42.

regulated by a large number of proteins, which on their turn are subject of regulation as well. This complicated network of actin and the ABPs play an essential role in cell metabolism and consequently also in cell cycle regulation [41].

The role of actin in ECM-induced signalling is especially apparent from the structural role of actin in focal adhesions. Actin binds to integrins indirectly through several proteins like vinculin and α-actinin and disruption of this interaction has large consequences on the complex structure and function of focal adhesions (Fig.2). In addition the actin filaments constitute a highly dynamic network, with the dynamics being regulated by a large number of actin binding proteins, for review see [41]. The first indications for the relationship between actin and signal transduction were obtained by studies on the effects of growth factors on cell morphology. For example, addition of EGF or PDGF cause the formation of membrane ruffles within minutes after addition of the growth factor [41, 42] this suggests modulation of actin metabolism through a TRK cascade (Fig. 2). It was demonstrated that exposure of cells to EGF caused a rapid actin polymerization, the formation of membrane ruffles and the translocation of several of the down stream signaling molecules to these newly formed membrane ruffles. This suggests the formation of signaling complexes at the plasma membrane in the membrane ruffles [43, 44].

Interestingly, treatment of the cells with F-actin disrupting agents like cytochalasin caused a severe reduction in growth factor induced signaling [45], demonstrating the mutual interaction between signaling cascades and the actin microfilaments. Finally, actin has also been reported to be localized in the nucleus [46, 47]. There is evidence that nuclear actin is involved in chromatin remodeling, transport of proteins and mRNA transcription. In this latter case, it was demonstrated that actin acts as a regular component of all RNA polymerases and is probably related to actin-dependent chromatin remodeling previously reviewed [47-49]. In summary, all these observations indicate that actin plays a dominant role in cells, not only as a structural protein, but also as a protein involved in dynamic processes like signal transduction and transcription.

Taken together, the data shows that actin plays an important role in growth factor- and in integrin-induced signal transduction. In addition, both signal

transduction pathways are interacting as exemplified by the MAP kinase pathway (Fig. 2). MAPK is recruited to focal adhesions in response to several stimuli such as integrin activation, activation of v-SRC, activation of PKCε and activation of the FGF receptor. PDGF and EGF induce cell migration and cause localized cell de-adhesion requiring MAPK signaling. The effect of growth factors on cell adhesion required the activation of calpain 2 [50]. The observation that calpain activity was decreased in FAK-deficient cells are of particular interest [51]. In addition, it was demonstrated that FAK induces the formation of a complex constituting calpain 2, FAK and MAP kinase [52]. These data suggest FAK is critical in the integration of migratory signals from growth factor receptors and integrins via the MAPK pathway to the calpain proteolytic system, resulting in focal adhesion turnover and cell migration [53].

Involvement of microtubules in growth factor and extracellular matrix signalling

Microtubules constitute one of the major components of the cytoskeleton and have been demonstrated to be involved in cell division by segregation of the chromosomes during mitosis, intracellular transport and cell morphology [54]. The major component of microtubules is the heterodimeric protein tubulin. Tubulin polymerization is dependent upon the GTP/GDP. GTP binding is required for polymerization, while GTP hydrolysis, most likely through intrinsic tubulin GTPase activity, results in depolymerization [55]. The functioning of the microtubules depends largely on the dynamics of polymerization and depolymerization. In addition to the dynamic behaviour of microtubules, an important role of microtubules in cells is also realized by the action of motorproteins, like dynein and kinesin, which allow the transport of cargo's along the microtubules. Although much effort has been made to elucidate the cellular mechanisms that underlie microtubule dynamics, the precise spatial and temporal control of this process is not fully understood yet. However, a wide variety of signal transduction proteins appear to be associated with microtubules, suggesting also a role of microtubules in signal transduction. Amongst others, MAP kinase interacts with microtubules [56] and from these studies it was

concluded that microtubules retained MAP kinase in the cytoplasm to regulate cytoplasmic events. A transcription factor that may be regulated by microtubules is NFκB. The inhibitor of NFκB, IκB, has been shown to interact with the motorprotein dynein, and this interaction may sequester NFκB, while on the other hand depolymerization of microtubules leads to IκB breakdown and consequently to NFκB activation [57]. Furthermore a close interaction has been demonstrated between microtubule dynamics and heterotrimeric G proteins [for review see [58]]. These observations suggest an intimate interaction between microtubules and signal transduction cascades activated by growth factors and possibly even by the ECM, which may result in modification of signal transduction by the microtubules.

Microtubules in normal gravity and microgravity

Microtubules have been implicated in cell organization and are required for separation of chromosomes during mitosis [59-61]. During mitosis, the precise timing of key cellular processes such as microtubule organizing centers (MTOC), and cytokinesis is essential for high fidelity chromosome segregation. Temporal organization of these events is coordinated by a group of proteins collectively termed cell cycle regulators. Many regulators are kinases or phosphatases that respond to cellular cues and orchestrate cell cycle progression by altering the phosphorylation and activity of other downstream regulatory proteins. In recent years studies in yeast have revealed that many regulators localize near CSKs [62].

Over the past few years, Tabony's laboratory has shown that microtubule self-organization in a cell free system is dependent on gravity, suggesting that gravity is required for normal self-assembly of microtubules in animal cells and that the microtubule system may be disrupted in microgravity in a living cell [63-65]. Lewis et al. reported that Jurkat cells flown in space had disrupted microtubules and increased apoptosis. The increased apoptosis was accompanied with a time dependent elevation of Fas/APO-1 suggesting an increase in Fas Receptor (FasR) signal transduction in microgravity. Postflight confocal microscopy of the Jurkat cells revealed diffuse shortened microtubules extending from poorly defined microtubule organizing centers (MTOCs) [66, 67]. These observations were confirmed in later

microgravity studies with Jurkat and Drosophilia melanogaster (Schneider S-1) cells that showed cytoskeletal and mitochondrial alterations after exposure to spaceflight and in insect cells of Drosophila melanogaster (Schneider S-1) after exposure to conditions created by clinostat rotation [68]. The effects of both treatments were similar in the different cell types. Fifty percent of the cells displayed effects on the microtubule network in both cell lines. Under these experimental conditions, mitochondria clustering and morphological alterations of mitochondrial cristae were observed to various degrees after 4 and 48 hours of culture. Jurkat cells underwent cell divisions during exposure to spaceflight but a large number of apoptotic cells were also observed. Similar results were obtained in Schneider S-1 cells cultured under clinostat rotation. Both cell lines displayed mitochondrial abnormalities and mitochondria clustering toward one side of the cells which could be interpreted to be the result of microtubule disruption and failure of mitochondria transport along microtubules. Studies by Meloni et al. have also noted altered CSK and motility in J-111 monocytes during exposure to altered gravity in a Random Positioning Machine (RPM) [69].

Ground-based experiments revealed a similar enhancement of the spontaneous and evoked lamellar protrusive activity when the cells were kept at 2g hypergravity for at least 6 min. This gravity response was independent of the direction of the acceleration vector in respect to the cells [70]. Exposure of the cells to "simulated weightlessness" (clinorotation) had no obvious influence on this type of lamellar actin cytoskeleton dynamics. A 20 min exposure of the cells to simulated weightlessness or to changing gravity (6 to 31 parabolas) - but not to 2g (hypergravity, centrifugation) - resulted in an altered arrangement of microtubules indicated by bending, turning, and loop formation. A similar altered arrangement was shown by microtubules, which had polymerized into lamellipodia after release from a taxol block at simulated weightlessness (clinorotation) or during changing gravity (5 parabolas). Data suggest that in human SH-SY5Y neuroblastoma cells, microgravity affects the dynamics and spatial arrangement of microtubules but has no influence on the Rac-controlled lamellar actin cytoskeleton dynamics and cell spreading. The latter, however, seems to be promoted at hypergravity [70].

The actin cytoskeleton in growth factors and extracellular matrix signalling in microgravity

Early experiments in sounding rockets under real microgravity conditions demonstrated not only a rapid cell rounding, but also modified actin polymerization [20]. The changed actin polymerization may represent the basis of other gravity-induced changes as well. It has been demonstrated, under both real and simulated microgravity conditions, that EGF-induced expression of the early genes *c-fos* and *c-jun* was severely inhibited. Interestingly, the inhibition was also observed if *c-fos* and *c-jun* expression were induced under microgravity conditions by the phorbol ester (PMA), but no effect was observed by *c-fos* and *c-jun* induction by the Ca-ionophore A23187 or the cyclic AMP inducing forskolin [71, 72]. Changes in cytoskeleton were also noted by Guignandon et al. [73] when they examined cells in parabolic flight microgravity and found cytoplasmic retraction and membrane ruffling in ROS/17/2.8 cells. Increased PGE_2 was found in flight medium accompanied by significant flight-induced changes that included a decrease in cell area and irregular shape in some cells. These observations demonstrate clearly the specificity of the effect of microgravity on signal transduction. Notably, both EGF and PMA are known to stimulate protein kinase C (PKC) activity and therefore PKC may represent a downstream microgravity sensitive target in the cells. PKC activity has been also related to actin dynamics. During the past decades numerous studies demonstrated that microgravity conditions result in dramatic changes in the actin cytoskeleton as reviewed by Crawford-Young [74]. To date, the cytoskeleton appears to play an essential role in gravity sensing of the cells and the actin microfilament system also plays an essential role in growth factor and integrin-induced signal transduction, consequently causing changes in cell proliferation, differentiation and apoptosis.

Microgravity (10^{-3}-10^{-9}g) includes other variables characteristic of orbital phase of spaceflight which include: launch effects, altered electromagnetic fields, pressure changes, changed content of cabin atmospheric gases, mechanical vibrations from motors and crew activities, cosmic radiation and absence of sedimentation-induced convection [75, 76]. Because of these conditions, many of the recent spaceflight

experiments have included onboard 1g samples to control the effects of these spaceflight conditions. Alterations in cytoskeleton actin, intermediate filaments and microtubules have been noted when there is a significant load reduction on the cell in microgravity [20, 73, 77-80]. Since multiple investigators have observed actin and microtubule cytoskeletal modifications in microgravity, this suggests a common root cause in the microgravity environment which alters cell architecture. Since the cell cycle is dependent on the cytoskeleton, alterations in cytoskeletal structure can block cell growth either in G1 (F-actin microfilament collapse), or in G2/M (inhibition of microtubule polymerization during G2/M-phase). It is then possible that microgravity may inhibit growth in either G1, or G2/M phases of the cell cycle.

The absence of mechanical stress (microgravity) can cause change in cell shape and signal transduction when exposed to as little as 20 seconds of microgravity in parabolic flight [73, 77]. When quiescent osteoblasts are activated by sera under microgravity conditions there is a 60% reduction in growth ($p<0.001$) when compared to ground controls. Moreover, a collapse of the osteoblast actin cytoskeleton and loss of focal adhesions have been noted after several days in microgravity. The changes seen in the cytoskeleton are probably not due to alterations in fibronectin message or protein synthesis since no differences have been noted in microgravity [81]. The altered ability of cells to respond to stimuli like growth factors and sera suggests that there is a major alteration in anabolic signal transduction under microgravity conditions, most probably through the growth factor receptors and/or the RTK pathways that are connected to the cytoskeleton. The fact that several investigators have noted that changes in specific gene expression are associated with microgravity exposure [71, 72, 78, 81-90] reinforces the concept that microgravity is interfering with signal transduction from the cell membrane receptors to internal signaling pathways.

Studies on STS-56/IML-2 examined sera activation of quiescent osteoblast-like cells in orbit and demonstrated that microgravity caused a decrease in cell proliferation within days of exposure to microgravity. In the 1g flight cells, the sera activated cells had activated Rho activity as evidenced by stress fiber formation. The collapse of the actin cytoskeleton [78] and the elongation of the nuclear shape [78,

89] of osteoblast-like cells were noted in spaceflight while glucose metabolism per cell was unchanged [78]. The expression of *cox*-2 mRNA was not induced by sera in microgravity, but paradoxically, media PGE_2 content 24 hours after activation was significantly increased in flight in both the static (μg) and 1g onboard controls [78, 88, 89, 91]. In normal cells and tissues, the presence of PGE_2 causes an induction of the *cox-2* message. This lack of induction of the *cox*-2 message in the presence of elevated levels of PGE_2 suggests a malfunction of the PGE_2 feed-forward-regulation. This lack of feedback in microgravity may be caused by reduced signaling at the level of the GCPR signal cascade.

In-flight studies by Stein et al. in astronauts have demonstrated a reduction in sera levels of PGE_2 in flight [92, 93]. Normally in the human, PGE_2 is cleared by the kidneys within seconds, and must be made continually to maintain high sera/urine levels. In contrast, in the isolated osteoblasts the PGE_2 is degraded by the enzyme 15-hydroxyprostaglandin dehydrogenase; it is therefore possible that the activity of the degrading enzyme or alterations in the degradation process may be inhibited in the isolated cell in microgravity allowing for higher levels of PGE_2.

It was demonstrated that exposure of cells to EGF caused a rapid actin polymerization, the formation of membrane ruffles and the translocation of several of the down stream signaling molecules to these newly formed membrane ruffles, suggesting the formation of signaling complexes at the plasma membrane in the membrane ruffles [43, 44]. These initial observations of changes in EGF and PDGF signaling were followed by studies in which it was demonstrated that a wide variety of signal transduction proteins associated with actin, amongst these the EGF receptor [94], PI3 kinase, and phospholipase C (PLC) [43, 94].

Fibroblast Growth Factor-2 (FGF-2) is the ligand for another actin associated RTK receptor, FGFR. SRC kinase activity has a crucial role in the regulation of FGFR1 signaling dynamics. Following receptor activation by ligand binding, activated SRC is colocalized with activated FGFR1 at the plasma membrane. This localization requires both active SRC and FGFR1 receptor tyrosine kinases, which are inter-dependent. Src-mediated transport and subsequent activation of FGFR1 require both RhoB endosomes and an intact actin cytoskeleton for full activity [95].

RTK receptors like FGFR are implicated in bone cell growth and bone cells synthesize the FGF-2 growth factor endogenously. Normal bone remodeling is characterized by a series of cellular events, cell proliferation, sequential activation and up regulation of osteoblast-characteristic genes, and matrix mineralization.

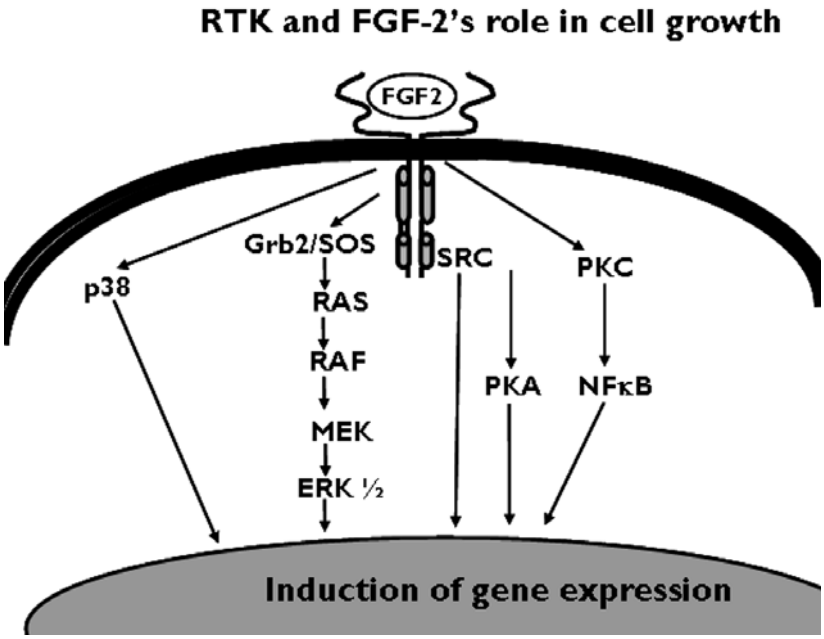

Figure 3: FGF-2 signal transduction FGF-2 causes induction of several pathways including p38, Ras/MAPK, SRC, PKA (exact mechanism unknown) and PKC through its interaction with the FGFR1, FGFR-2 and FGFR-3 receptors in bone.

These events are tightly controlled and coordinated by a number of regulatory molecules, such as growth factors (GFs), and their downstream transcription factors ensuring normal growth and development of the skeleton [96]. As seen in Fig. 3, fibroblast growth factors (FGFs) have important regulatory functions in bone formation [97-99]. FGFs belong to a gene family currently comprised of 23 members in mammal evolution. They are secreted peptides with molecular size of approximately 20–35 kDa and expressed in many different types of tissues during various stages of development. In addition to their mitogenic effects, FGFs are

involved in diverse biological processes, including cell motility [100], and migration [101]. FGF signaling is triggered by the binding of FGFs to their high affinity receptors, fibroblast growth factor receptors (FgfRs), followed by dimerization and auto-/trans-phosphorylation of FgfRs [102, 103].

The phosphorylated FgfR kinases selectively activate intracellular signaling intermediates, eliciting specific cellular responses. Four FgfRs (FgfR1– FgfR4) have been identified to date. Among them, the isoforms FgfR1a, FgfR1b, FgfR2 and FgfR3 are the major receptor isoforms expressed in bone. FGF-2 activates FgfR1 (b and c), FgfR2 (c), and FgfR3 (c) receptors. Of these receptors, 1c, 2c, and 3c result in mitogenic responses to FGF-2 in osteoblasts [104]. FgfR knockout mice have helped elucidate the roles of the individual receptors in skeletal development. The FgfR1 and FgfR2 genes appear to regulate formation and elongation of the limbs in the developing embryo [105-107]. During the development of the mouse skull, FgfR2 is expressed only in proliferating osteoblasts. Once these cells start differentiating, FgfR2 is downregulated and FgfR1 is upregulated [108]. Furthermore, disruption of the FgfR2 gene results in increased osteoblast differentiation, suggesting its role in the switch between proliferation and differentiation [109].

When osteoblast-like cells and bone cells are put under increased mechanical stress in a centrifuge (max. of 12g), they synthesize *fgf-2* message and protein which in turns stimulates bone growth [110]. When stem cells from $Fgf2^{-/-}$ mice (Fgf-2 knockout mice) are subjected to stress of 120 μstrain by centrifugation, no Fgf-2 is synthesized and bone cells do not grow in response to stress [99]. Mechanical stress promotes Fgf-2 mediated growth via both PKA and a MAPK pathways [99] .

A recent report discovered a lack of *fgf-2* mRNA and protein synthesis in osteoblast-like cells grown in microgravity [89]. The lowering of Fgf-2 content was associated with a significant change in nuclear shape of the μg flight cells. The cells under 1g-flight environment had normal nuclear cell shapes. Since nuclear shape is maintained by the nuclear lamins this might implicate a change in conformation of two intermediate filaments; nuclear Lamin A and nuclear Lamin C under microgravity conditions. Since Fgf-2 growth factor increases in *cox*-2 mRNA through the FGF-2/RTK pathway, the lowered synthesis of *cox*-2 message may be

associated with lowered RTK activity as was seen in the EGF experiments mentioned earlier in this chapter. *Fgf*-2 mRNA levels and *cox*-2 mRNA levels return to normal values in the 1g flight controls [89]. Lack of signaling from the RTK class is suggested by both results from A432 and MC3T3-E1 and preosteoblast stem cell experiments. At this time it is unknown why RTK cascades are affected by μg, but this phenomenon is under investigation by several laboratories.

There are other downstream pathways from the cell surface receptors that have been shown to be affected by the absence of gravity during flight or in simulated microgravity, they include PKC [111-115] and Protein Kinase A (PKA) [116, 117]. The Hughes-Fulford Lab had previously reported that PKA and PKC are key early regulators in T-cell activation. In other studies of human T-cells grown on the RPM, there was a significant loss of CREB message (PKA pathway). In addition, there was a loss in NFκB and ten other key regulators in the T-cells grown in the RPM. The group analyzed differential gene expression to find gravity-dependent genes and pathways (n=3) independent samples for each condition using Affymetrix full genome gene arrays. There was an inhibited induction of 91 genes in the simulated freefall environment of the RPM. Altered induction of the ten genes regulated by key signaling pathways was verified using real-time RT-PCR [116]. It was discovered that impaired induction of early genes were regulated primarily by transcription factors NF-κB, CREB, ELK, AP-1, and STAT in the altered gravity environment. Since the majority of the genes were regulated by NF-κB, CREB, ELK and AP-1, the pathways that regulated these transcription factors were studied on the RPM. Boonyaratanakornkit et al. found that the PKA pathway was down-regulated in simulated μg using the RPM. In contrast, PI3K, PKC, and its upstream regulator pLAT were not significantly down-regulated by vectorless gravity [116]. Earlier studies demonstrated that PKA was an essential part of early T-cell activation since inhibition of that pathway inhibited production of IL-2 and IL-2Ra, two key steps in T-cell activation [117]. Since NF-κB, AP-1, and CREB are all regulated by PKA and are transcription factors predicted by microarray analysis to be involved in the altered gene expression in vectorless gravity, the data suggest that PKA may be a key early player in the loss of T-cell activation in altered gravity [116]. The same changes in

NFκB and CREB were recently discovered in the Leukin studies flown on an experiment in the International Space Station, ISS. Human T-cells were activated in spaceflight with and without gravity. This preliminary data suggests a similar mechanism of downregulation both in the RPM and in true microgravity (manuscript in preparation)

A considerable amount of experimental evidence support the fact that changes in mechanotransduction in microgravity occur at the level of RTK signal transduction [20, 73, 77-80, 89]. It is possible that the disruption of the actin CSK in microgravity renders the receptor inactive, or that alterations in the cell membrane itself alters the activity of the RTK receptor response to its growth factor ligand. In a similar way, a blunting of the self-organization of the microtubules in microgravity and hence altering the structure of the MTOC could alter cellular processes related to response in much the same way as disruption of the kinases at the growth factor receptors. As opportunities to conduct spaceflight experiments using modern technology become available, the exact molecular causes of change in cell function, mechanotransduction and downstream signaling in microgravity will become understood.

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