CELLS IN SPACEFLIGHT

Past, Present and Future

A workshop sponsored by
the Center for Advanced
Studies in the Space Life Sciences
at the Marine Biological Laboratory

March 7 & 8, 1999
Marine Resources Center, Room 210

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The Marine Biological Laboratory and the National Aeronautics and Space Administration have established a cooperative agreement with the formation of a Center for Advanced Studies in the Space Life Sciences (CASSLS) at the MBL. This Center serves as an interface between NASA and the basic science community, addressing issues of mutual interest.

The Center for Advanced Studies in the Space Life Sciences provides a forum for scientists to think and discuss, often for the first time, the role that gravity and aspects of spaceflight may play in fundamental cellular and physiologic processes. In addition the Center will sponsor discussions on evolutionary biology. These interactions will inform the community of research opportunities that are of interest to NASA.

This workshop is one of a series of symposia, workshops and seminars that will be held at the MBL to advise NASA on a wide variety of topics in the life sciences, including cell biology, developmental biology, evolutionary biology, molecular biology, neurobiology, plant biology and systems biology.

For additional information about the Center please visit our website
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Cells in Spaceflight: Past, Present and Future

March 7 & 8, 1999
Room 210, Marine Resources Center
Marine Biological Laboratory, Woods Hole, MA

Sunday, 7 March 1999

7:00 - 8:30 am	BREAKFAST available	*Private Dining Room, Swope*
9:30 am	COFFEE	*Marine Resources Center, outside Room 210*
9:50 am	**Lenny Dawidowicz** — Opening Remarks	*MRC 210*
10:00 am	**Randy Wayne** — The Plasma Membrane-Extracellular Matrix Junction is the Site of Gravity Sensing in Chara	
11:00 am	**Jan Monzer** — Graviperception in Basidiomycete Fungi - Implications for Cellular Gravisensing	
12:00 noon	Discussion	
12:30 pm	LUNCH	*PDR, Swope*
2:00 pm	**Johannes Boonstra** — Growth Factor-Induced Signal Transduction in Mammalian Cells is Sensitive to Gravity	
3:00 pm	COFFEE	
3:30 pm	**Jason Hatton** — The Effect of Gravity on Mammalian Cell Intracellular Signal Transduction: A "Key" to Understanding the Mechanisms of Eukaryotic Cell Gravisensitivity	
4:30 pm	Discussion	
5:30 pm	MIXER	*outside PDR, Swope*
6:00 pm	DINNER	*PDR, Swope*

Monday, 8 March 1999

7:00 - 8:30 am	BREAKFAST available	*PDR, Swope*
	Marine Resources Center, Room 210	
9:30 am	**Herman Vandenburgh** — Tissue Engineered Skeletal Myofibers can Directly 'Sense' Gravitational Force Changes	
10:30 am	**Peter Lee** — Poster presentation: The Effects of Microgravity on the Expression of a Foreign Gene in Tissue Engineered Skeletal Muscle	
10:45 am	COFFEE	
11:00 am	**Millie Hughes-Fulford** — The Effect of Gravity Fields on Cellular Gene Expression	
12:00 noon	Discussion	
12:30 pm	LUNCH	*PDR, Swope*
2:00 pm	**Arthur J. Sytkowski** — Erythroid Cell Growth and Differentiation in Simulated Microgravity	
3:00 pm	COFFEE	
3:30 pm	**Dilip Kondepudi** — Summary	
4:30 pm	Discussion	
5:30 pm	MIXER	*outside PDR, Swope*
6:00 pm	DINNER	*PDR, Swope*

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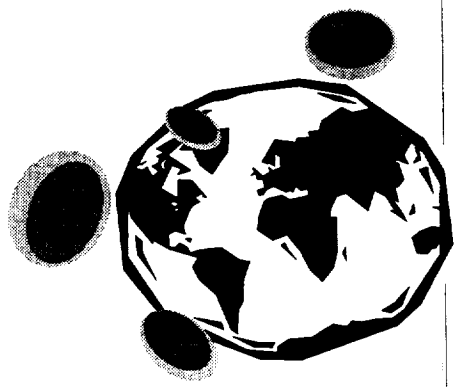

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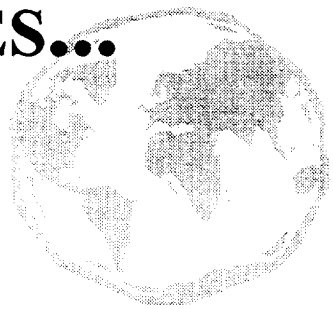

The Plasma Membrane-Extracellular Matrix Junction is the Site of Gravity Sensing in Chara

5/-51

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It is fascinating to consider the mechanisms involved in the perception of gravity in living organisms since the force of gravity is the weakest of the fundamental forces. Yet gravity influences many biological processes. In 1806, Thomas Knight (1806) wrote, "It can scarcely have escaped the notice of the most inattentive observer of vegetation, that in whatever position a seed is placed to germinate, its radical invariably makes an effort to descend towards the centre of the earth, whilst the elongated germen takes a precisely opposite direction." Likewise, gravity effects the orientation of animals.

Sedimenting *extracellular* conglomerates of sand or limestone, known as statoliths are involved in the uprighting response of many but not all animals (Prentiss, 1901). For example, when a horseshoe crab or a lobster is tilted, the statoliths fall through the *low viscosity extracellular* medium, and compress the extracellular matrix (ECM)-plasma membrane junction of some hair cells while relieving the compression on the hair cells on the opposite side. The cells that experience an increased compression send a signal to the brain that causes the animal to right itself. Once the animal is upright again, the response is terminated.

Bertold (1886) and Noll (1892) suggested that plants may also respond to gravity by sensing the falling of heavy bodies. Subsequently, Nemec (1900) observed that starch grains were prevalent in the cap cells of gravitropic roots, and these starch grains sedimented through the *viscous intracellular* medium. Moreover, he found that the roots did not bend in response to gravity after he removed the root cap. Haberlandt (1900) performed similar surgical experiments on shoots. From these experiments, Nemec and Haberlandt independently concluded that starch grains were the gravisensor in plants, named them statoliths and enunciated what has come to be known as the starch-statolith hypothesis of gravity sensing (Darwin, 1903, 1904).

What's in a name? Molière [1673] in his play "Le Malade Imaginaire" insinuated that it sounds like we understand a subject when we discuss it with Greek or Latin terms. For example, in the last act of this play, a medical student was asked at his qualifying exam, "Why does opium put people to sleep?" He answered, "Opium puts you to sleep because it is a soporific", and passed the exam.

We are not immune from this terminology problem in cell biology. We are reminded of Otto Bütschli's response to Hanstein's (1882) suggestion to call the granules in the cytoplasm, "microsomes". After Hanstein christened these granules with a Greek name, Bütschli (1894) bewailed that they had now "obtained the right of entry among the privileged and recognized units of cytoplasmic structure, for anything that

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is called by a Greek name at once seems to many people to be much better known, and as something which must be definitely reckoned with". To this day, microsomes have neither a clear structure nor function in the cell. Once the falling starch grains were given the Greek name "statolith", which means the rock that is involved in standing or orientation, it appeared that their function was understood, or at least these organelles would have to be reckoned with in any discussion on gravity sensing. Consequently experiments were carried out, not so much to test the hypothesis that statoliths were involved in orientation responses to gravity, but with the notion that they were the gravity sensors. Thus any experiments that characterized the statoliths themselves were considered to be experiments that characterized the mechanism of gravisensing.

In order to act as a mechanical signal, a sedimenting statolith must transmit energy to a gravireceptor, and the kinetic or potential energy must be greater than the energy of thermal noise. However, due to the high viscosity of the cytoplasm, the kinetic energy of a falling statolith is approximately ten million times less than the energy of thermal noise. By contrast, the potential energy of an amyloplast falling a distance of just 100 nanometers is approximately twice as large as thermal noise; and the greater the distance the amyloplast sediments, the greater is the probability that a gravireceptor will be activated by the amyloplast. Thus it is possible for a falling amyloplast to function as a statolith by transferring its potential energy to a receptor, as long as a structure is available to provide a nexus between the amyloplast and the object to be activated by the amyloplast. Sievers *et al.* (1995) proposed that the falling amyloplasts pull on actin microfilaments which in turn activate a receptor in the plasma membrane or cortical endoplasmic reticulum. However, while there is evidence that cytochalasin D disturbs the polarity of cells involved in the sensing of gravity (Hilaire *et al.*, 1995); increases the sedimentation velocity of plastids (Sievers *et al.*, 1989); and affects the membrane potential in gravistimulated roots (Sievers *et al.*, 1995); the researchers that conducted these experiments never performed the critical experiment to test whether or not cytochalasin D affects gravitropism itself. We find that cytochalasin D has no effect on gravitropism in corn, rice or Lepidium roots (Staves *et al.*, 1997), indicating that actin microfilaments do not transmit the potential energy of falling amyloplasts to any receptor.

Another difficulty with the statolith-microfilament theory of gravisensing comes from the observation that mutants that lack starch in their plastids still respond to gravity, although with a reduced sensitivity (Casper and Pickard, 1989; Kiss *et al.*, 1989). In the cells of these mutants, the starchless plastids are smaller, less dense and sediment

imperceptively at 1g. Thus the potential energy available to activate a receptor would be lower than in the wild type; and the proposal that utilization of the potential energy of sedimenting plastids is sufficient to activate the gravireceptor, is not free from doubt.

The evidence underlying the classical statolith theory rests on the *correlation* between the presence of sedimenting starch grains and the ability of an organ to respond to gravity. However there are other gravity responses, such as the gravitropic responses of Phycomyces and Physcomitrella, the differentiation of vascular tissue and the polarity of cytoplasmic streaming in characean internodal cells where the ability to respond to gravity is *not correlated* with the presence of sedimenting statoliths. In these cells, it is possible that the plasma membrane-extracellular matrix junction acts as the gravireceptor, and the settling of the mass of the whole protoplast is important for the realization of the graviresponse, as originally suggested by Wilhelm Hofmeister (1867), Wilhelm Pfeffer (1881) and Frederick Czapek (1898). It is possible that the plasma membrane-extracellular matrix junction acts as the gravireceptor in *all* cells, and the starch grains function merely as *ballast* to make the cell more sensitive to gravity. In my talk, I will describe to you the experiments that led us to support the conclusion that the protoplast as a whole settles in response to gravity, and the gravireceptor is present in the plasma membrane-extracellular matrix junction.

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GRAVIPERCEPTION IN BASIDIOMYCETE FUNGI - IMPLICATIONS FOR CELLULAR GRAVISENSING

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Cellular gravity sensing was found responsible for the gravitropism of fruiting bodies of the basidiomycete fungus *Flammulina velutipes*. In basidiomycetes, the vertical orientation of the hymenium, or gills, is a necessary prerequisite for successful spore distribution. In *Flammulina*, the gravitropic response is governed by a small gravisensitive zone between stipe and cap, which laterally controls cell elongation to align the fruiting body to the vertical (Monzer *et al.* 1994, Moore *et al.* 1996). These observations established striking parallels to plant gravitropism. In plants, confined gravisensitive areas control directional cell elongation to align whole organs. The gravitational stimulus is perceived by specialized statocytes, sensing the dislocation of cellular statoliths. To current knowledge, the actin cytoskeleton plays a central role in the translation of the physical stimulus to physiological responses (Sievers *et al.* 1991).

Actin filaments were identified to be involved in *Flammulina* graviperception, too (Monzer 1995). Cytochalasin treatment both impaired the gravitropic response and disrupted the close spatial correlation between the actin cytoskeleton and the nuclei. Fungal hyphae lack plastids, which are commonly attributed the statolith role in plant cells. However, our calculations showed that the nuclei have sufficient mass to meet the physical requirements for a statolith. Studies on nuclear motion dynamics also supported the assumption that the nuclei are likely candidates for a statolith function in fungal hyphae (Monzer 1996).

Our results and theoretical approximations bear general implications for cellular gravisensing. They suggest that nuclei, among other organelles, can fit the requirement to serve as a cellular statolith, a view that also gains support from certain observations on plant cell gravisensing (Lorenzi and Perbal 1990, Ridge and Sack 1992). This concept would imply that, technically, most eukaryotic cells should be capable to suscept a gravitational stimulus, since the nuclei are subject to significant forces. Whether they can perceive it, and hence become gravisensitive, would depend on whether they are equipped with the cellular machinery to translate the physical force to a physiological response.

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Growth Factor-Induced Signal Transduction in Mammalian Cells is Sensitive to Gravity

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Polypeptide growth factors, amongst them epidermal growth factor (EGF), have been demonstrated to play an essential role in the regulation of mammalian cell proliferation and differentiation. These growth factors activate well-characterised signal transduction cascades, and this activation leads usually to increased cell proliferation in most cell types. Among the early effects evoked by EGF are receptor clustering, cell rounding and early gene expression. The influence of gravity on EGF-induced EGF receptor clustering and gene expression as well as on actin polymerization and cell rounding has been investigated in A431 epithelial cells using sounding rockets to perform microgravity conditions.

EGF-activated signal transduction in A431 cells results within five minutes in the rapid induction of the proto-oncogenes *c-fos* and *c-jun*. Experiments performed during sounding rocket flights demonstrated clearly the EGF-induced expression of *c-fos* and *c-jun* decreased under microgravity conditions. This was caused by alteration of the EGF receptor and protein kinase C mediated signal transduction pathways. In contrast, neither the binding of EGF to the receptor nor the receptor clustering were changed under microgravity conditions, indicating that the observed effects are due to a gravity-sensitive cellular component.. Since cell morphology was also modulated under microgravity conditions, and the growth factor-induced signal transduction cascades have been demonstrated to be linked to the actin microfilament system, it is tempting to suggest that the actin microfilament system constitutes the gravity sensitive cell component. Preliminary experiments indeed suggest that the actin microfilament system is modulated under microgravity conditions. Furthermore we have demonstrated that the actin microfilament system plays a prominent role in protein kinase C-mediated feed-back regulation of EGF-induced signal transduction. In combination, we suggest that the effects of microgravity on EGF-induced signal transduction are due to modulations in actin-mediated regul;ation of protein kinase C activity.

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The Effect of Gravity on Mammalian Cell Intracellular Signal Transduction: A "Key" to Understanding the Mechanisms of Eukaryotic Cell Gravisensitivity

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Early spaceflight experiments showed that the behaviour of some mammalian cell types appeared to be modified under microgravity conditions. Observed alterations in cells exposed to cells exposed to microgravity compared to cells under 1.g conditions include decreased proliferation and sensitivity to mitogens in peripheral human lymphocytes, reduced cytokine synthesis in human T-cell and monocyte cell lines and reduced early immediate gene expression in a human epidermal carcinoma cell line. The results of these experiments were suggestive of microgravity induced changes occuring in the early events associated with cell activation, namely intracellular signal transduction. Subsequent experiments, most notably performed using the ESA Biorack facility, have provide evidence for gravity dependant changes in selected signal transduction pathways. In particular the translocation and sub-cellular distribution of some protein kinase C (PKC) isoforms in human monocytes and T-cells varies in proportion to the applied g-level. In parallel significant changes in the actin and tubulin cytoskeleton have been observed in diverse cell types. There is a close association between some protein kinases and the cytoskeleton suggesting that there may be a link between the gravity dependant changes which have been observed in signal transduction and the cytoskeleton.

There are many potential mechanisms by which a cell may sense gravity, either through the cell sensing gravidependant effects on the cell culture environment (indirect gravisensing) or direct sensing through intracellular structures. Elucidating the mechanisms of graviperception by non specialised mammalian cells will require a concerted programme of ground and flight experiments to isolate which physical gravidependant changes in the environment the cell is detecting, exclude artefactual effects and determine the response characterstics of the system. The demonstrated gravisensitivity of selected signal transduction processes provides a useful means of assaying the effect of altered g-levels on early cellular activation events.

Tissue Engineered Skeletal Myofibers can Directly 'Sense' Gravitational Force Changes

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Long-term manned space flight requires a better understanding of skeletal muscle atrophy resulting from microgravity. Atrophy most likely results from changes at both the systemic level (e.g. decreased circulating growth hormone, increased circulating glucocorticoids) and locally (e.g. decreased myofiber resting tension). Differentiated skeletal myofibers in tissue culture have provided a model system over the last decade for gaining a better understanding of the interactions of exogenous growth factors, endogenous growth factors, and muscle fiber tension in regulating protein turnover rates and muscle cell growth. Tissue engineering these cells into three dimensional bioartificial muscle (BAM) constructs has allowed us to extend their use to Space flight studies for the potential future development of countermeasures.

Embryonic avian muscle cells were isolated and BAMs tissue engineered as described previously (Vandenburgh *et al.,* 1998b; Shansky *et al.,* 1997). The myoblasts proliferate and fuse into aligned postmitotic myofibers after ten to fourteen days *in vitro.* A cylindrical muscle-like structure containing several thousand myofibers is formed which is approximately 30 mm in length, 2-3 mm in diameter, and attached at each end. For the Space Shuttle experiments, the BAMs were transferred to 55 mL bioreactor cartridges (6 BAMs/cartridge). At Kennedy Space Center, the cartridges were mounted in two Space Tissue Loss (STL) Modules (three to four cartridges per Module) and either maintained as ground controls or loaded in a Mid-Deck locker of the Space Shuttle. The BAM cartridges were continuously perfused during the experiment at 1.5 mL/ min with tissue culture medium. Eighteen BAMs were flown for nine days on Mission STS66 while eighteen BAMs served as ground controls. The complete experiment was repeated on Mission STS77 with twenty four BAMs in each group.

BAMs could be maintained in a healthy state for at least 30 days in the perfusion bioreactor cartridges (Chromiak *et al.,* 1998). The BAM muscle fibers directly detected both the loss of gravity and the reloading effects of 1 x g. While total cellular metabolism and total protein degradation rates were not altered during 9 to 10 days in Space, protein synthesis rates were significantly reduced and resulted in significant myofiber atrophy compared to ground controls. One g reloading of the flight muscle cells post-flight significantly increased protein synthesis rates and the synthesis rates of myosin heavy chain, fibronectin, and collagen.

Tissue cultured muscle cells can directly "sense" changes in gravity and provide a valid model to begin the study of countermeasures. Based

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on our ground-based experiments (Vandenburgh *et al.,* 1998a), and the experiments of others, growth hormone and/or insulin-like growth factors are attractive protein therapeutics which may assist in attenuating skeletal muscle wasting in Space. Our laboratory is developing a new cell-based delivery system for this and other potential therapeutic factors for attenuating muscle and bone wasting.

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The Effect of Gravity Fields on Cellular Gene Expression

Millie Hughes-Fulford

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Early theoretical analysis predicted that microgravity effects on the isolated cell would be minuscule at the subcellular level; however, these speculations have not proven true in the real world. Astronauts experience a significant bone and muscle loss in as little as 2 weeks of spaceflight and changes are seen at the cellular level soon after exposure to microgravity. Changes in biological systems may be primarily due to the lack of gravity and the resulting loss of mechanical stress on tissues and cells.

Recent ground and flight studies examining the effects of gravity or mechanical stress on cells demonstrate marked changes in gene expression when relatively small changes in mechanical forces or gravity fields were made. Several immediate early genes (IEG) like *c-fos* and *c-myc* are induced by mechanical stimulation within minutes. In contrast, several investigators report that the absence of mechanical forces during space flight result in decreased sera response element (SRE) activity and attenuation of expression of IEGs such as *c-fos, c-jun* and *cox-2* mRNAs. Clearly, these early changes in gene expression may have long term consequences on mechanically sensitive cells.

In our early studies on STS-56, we reported four major changes in the osteoblast; 1) prostaglandin synthesis in flight, 2) changes in cellular morphology, 3) altered actin cytoskeleton and 4) reduced osteoblast growth after four days exposure to microgravity. Initially, it was believed that changes in fibronectin (FN) RNA, FN protein synthesis or subsequent FN matrix formation might account for the changes in cytoskeleton and/or reduction of growth. However our recent studies on Biorack (STS-76, STS-81 and STS-84), using ground and in-flight 1-G controls, demonstrated that fibronectin synthesis and matrix formation were normal in microgravity.

In addition, in our most recent Biorack paper, our laboratory has documented that relative protein synthesis and mRNA synthesis are not changed after 24 hours exposure to microgravity. We did, however, find significant changes in osteoblast gene expression of IEGs, *c-fos* and *cox-2* in microgravity exposure as compared to ground and in-flight 1-G controls. Subsequent ground studies suggest that the molecular mechanism underlying these changes may involve prostaglandin c-AMP receptors (EPs) and/or subsequent alteration of intracellular signaling in the absence of gravity.

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Erythroid Cell Growth and Differentiation in Simulated Microgravity

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INTRODUCTION

The microgravity conditions experienced in space flight have been show to have adverse effects on hematopoietic cells leading to anemia and reduced immune responsiveness. The cellular basis for these effects is unknown. We have now begun to investigate potential mechanisms responsible for the reduced erythropoiesis encountered in microgravity.

METHODS

We used the erythropoietin responsive Rauscher murine erythroleukemia cell line. We compared the growth and hormone responsiveness of these cells in unit gravity with the unique simulated microgravity environment of the NASA rotating wall vessel (RWV) bioreactor. Cells were inoculated into tissue culture flasks or dishes at 1xg or into the RWV in the absence or presence of erythropoietin. At specified times thereafter ranging over a 4 day period, cell densities were determined and erythroid differentiation was quantified by determining the number of hemoglobin containing cells.

RESULTS

We found a profound inhibitory effect of simulated microgravity on erythroid cell growth and differentiation. At both 1xg and in the simulated microgravity of the RWV, the cells grew at log phase for 72 hours. However, the growth rate in the RWV was significantly less than that at 1xg. The cells were equally viable under both conditions, and no increase in apoptotic cells in the RWV was detected. Erythropoietin induced differentiation under both culture conditions. However, the number of hemoglobin containing cells in the RWV was only half that observed at 1xg. Importantly, when cells were grown in simulated microgravity for 24 hours before addition of erythropoietin, differentiation was inhibited virtually completely.

CONCLUSION

Our results suggest a profound effect of microgravity on erythropoiesis at the cellular level. This effect may be responsible in part for the anemia of space flight. We propose that one or more aspects of erythropoietin receptor binding and/or signal transduction are inhibited under conditions of reduced gravity.

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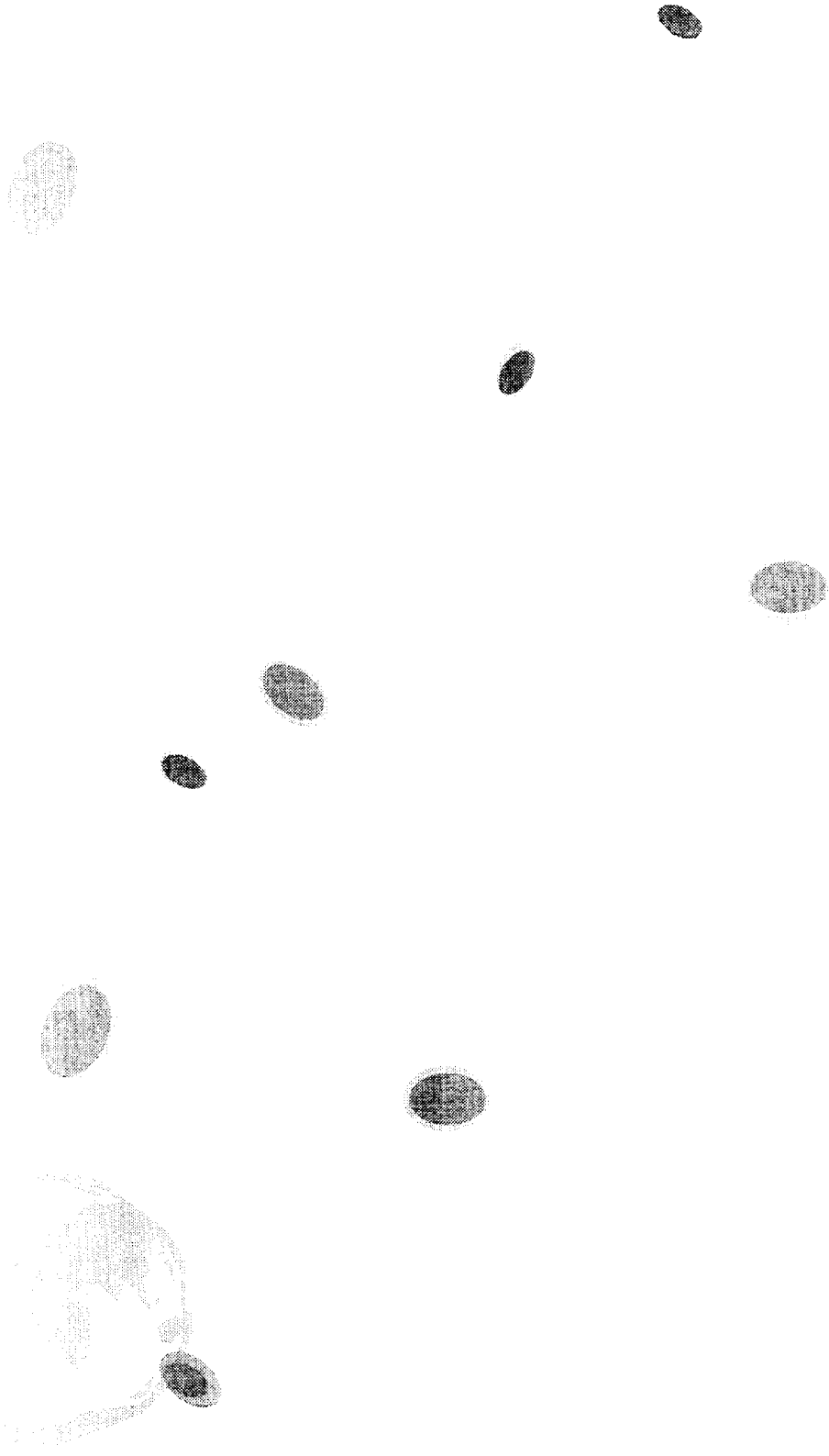

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