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Review

Three-dimensional co-culture models to study prostate cancer growth, progression, and metastasis to bone

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Abstract

Cancer-stromal interaction results in the co-evolution of both the cancer cells and the surrounding host stromal cells. As a consequence of this interaction, cancer cells acquire increased malignant potential and stromal cells become more inductive. In this review we suggest that cancer-stromal interaction can best be investigated by three-dimensional (3D) co-culture models with the results validated by clinical specimens. We showed that 3D culture promoted bone formation in vitro, and explored for the first time, with the help of the astronauts of the Space Shuttle Columbia, the co-culture of human prostate cancer and bone cells to further understand the interactions between these cells. Continued exploration of cancer growth under 3D conditions will rapidly lead to new discoveries and ultimately to improvements in the treatment of men with hormonal refractory prostate cancer.

Keywords: Cancer-stromal interaction; Epithelial to mesenchymal transition; 3-Dimensional cell culture; Prostate cancer bone metastasis; Bone mineralization; Cancer metastasis

Contents

1. Introduction

Cancer cells reside histologically as three-dimensional (3D) organoids in an organotypic host microenvironment, which is now known to be pivotal to malignant progression of the cancer cells [1–8]. Most studies in the literature, however, were conducted with cancer cells grown in 2D on plastic surfaces. Cancer cells are highly variable and their gene expression and cellular behavior are easily modulated by extracellular microenvironment and culture conditions. It

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is not surprising that further insight into cancer biology and therapy requires 3D models with results validated both in vivo in animal models and by clinical specimens. Gene expression changes in cancer cells from 2D culture often cannot be validated in clinical specimens and the screening of drugs for cancer treatment using cell lines grown on plastic dishes has rarely resulted in the identification of a clinically successful chemotherapeutic agent. We review here our experience using 3D cell culture, co-culture methods to study prostate cancer growth and progression and the formation of bone in vitro.

In this article, we focus our discussion in three areas: (1) the use of rotary wall vessel (RWV) culture to study the behaviors of prostate cancer cells upon in vitro interaction with either prostate or bone stromal cells; (2) our preliminary experience in adapting RWV culture as a model to study bone formation in vitro; and (3) information learned from a space Shuttle Columbia flight, when prostate cancer and bone cells were cultured together for the first time in space. We provide rationales for the use of 3D models to study tumor–stroma interaction, in the belief that 3D systems are grossly underutilized in the investigation of molecular mechanisms and novel therapies for the prevention and treatment of cancer. The future development of 3D cell culture models will be significantly improved by validation of results in live tissues, integrating the disciplines of molecular bioinformatics and system biology.

1.1. Models facilitating active interactions between tumor cells and their microenvironment

Cancer is the malignant transformation of epithelial cells, and therefore is considered as a single-cell disease with heterogeneity among epithelial cells only. Convenience and ease of culturing single cell types made 2D cell culture one of the most commonly used methods in cancer research. Past research focusing on cancer largely assumed that tumor microenvironment was a passive and an inert entity. Recently, however, ample evidence has emerged that in vivo cancer progression is supported by active participation of both host (epigenetic) and tumor (genetic) factors. Cancer and host stroma are proposed to co-evolve with each other in a fashion that may be mechanically described as a "vicious cycle" between cancer and bone stroma [9,10].

It has long been proposed that cancer metastasis is based on a mechanism analogous to a "seed (cancer)" and "soil (microenvironment)" relationship [11]. This theory could explain the well-known organ specificity of breast cancer metastasis. Primary tumors contain heterogeneous cancer cells, and progression and metastasis of the tumor is the result of a non-random, sequential, and successive selection or evolution of these cancer cells linked to microenvironment. In this respect, the active involvement of the microenvironment may lead to divergent consequences, probably depending on the compatibility between the cancer and the neighboring stromal cells. For instance, certain embryonic epigenetic and microenvironmental factors could reverse cancer growth and tumor progression [12–15], while some "inductive" stromal cells have the potential to convert adjacent benign epithelia to malignant cells [16–18]. In these cases, stromal cells appear to play a dominant role, directing the growth or differentiation of the cancer cells. Interestingly, cancer cells often seem to be responsive and their growth and differentiation depend entirely on the microenvironmental cues. The host microenvironment participates actively in a rather inefficient but non-random process that is driven by either clonal selection or evolution, in which some cancer cells acquire growth and survival advantages and others may lose them. This selection or evolution of cancer cells via tumor–stroma interaction at the primary as well as the secondary site of growth could lead some cancer cells to exhibit more aggressive behaviors, such as increased migration, invasion, and metastasis.

Little progress has been made in elucidating the molecular basis of tumor–microenvironmental interaction. This is in part due to the lack of a well-defined system by which to study the cellular interaction between relevant cell types in vivo and in vitro. Our laboratory and others have examined several aspects of the molecular process associated with this interaction. First, we evaluated cancer-microenvironment communication in the context of its contribution to the development of "osteomimicry" and "vascular mimicry" by the cancer cells [19,20]. Cancer cells can mimic the phenotypes of their microenvironment by changing their gene expression profile to one similar to that of their surrounding stromal cells. Some prostate cancer cells, for instance, can express a series of bone cell-specific genes and display osteomimetic properties [19,21,22]. Melanoma cells can mimic vasculogenic features by expressing markers that are otherwise specific to vascular endothelial cells [20,23,24]. It seems that some tumor cells are highly plastic, with stem-cell-like properties and the ability to sense environmental cues in the extracellular milieu and to adapt to or co-evolve with the host microenvironment.

Second, we analyzed the ability of cancer cells to undergo morphologic transitions, such as the transition of cancer cells from columnar sheath-like cells to dominantly spindle-shaped cells. Prostate epithelial cells are terminally differentiated and in the glandular prostate they are tightly arranged in a layer to ensure the polarity of their secretion. Prostate cancer cells, however, would abandon this geometric bondage and acquire the ability to invade and migrate, which are features of spindle-shaped mesenchymal cells. This epithelial–mesenchymal transition (EMT) and the reciprocal mesenchymal–epithelial transition (MET) may well be the basis of prostate cancer cell invasion, migration, and metastasis [4,25–28] upon prostate cancer progression through different downstream signaling networks that are dictated by the tumor microenvironment.

Thirdly, we used 3D cell culture to study epithelial–stromal interaction. We found that the survival and proliferation of cancer cells, grown as 3D organoids, are intimately linked to the surrounding extracellular matrix and soluble factors in the microenvironment [29]. As a general rule, these

cells display coordinated rather than sporadic changes of gene expression. Changes in the extracellular matrix or soluble factors could alter such cancer cell behaviors as cell polarity, organization, migration, and invasion. The high dependence of these cell features on the microenvironment strongly indicates the possible reversibility or vulnerability of cancer cells under certain growth conditions, which could be exploited for therapeutic gain.

In view of the active participation of the microenvironment in cancer progression, the therapeutic co-targeting of both cancer and stroma must be considered in cancer therapy [17,30–32]. In terms of the plasticity and dependence of cancer cells on the stroma, certain stromal milieus may serve as a cancer microenvironment only transiently along the routes of cancer cell migration and metastasis, and the possibility that cancer cells and their adjacent stromal cells co-evolve like moving targets offers additional challenges for the development of effective targeted therapies for the management of cancer in patients.

1.2. Modeling prostate cancer progression and metastasis

Several cell lines are available for prostate cancer research, but only three methods are established and commonly used to study the malignant transition of these cells. First, stable transfection to prostate cancer cell lines of oncogenes, cell cycle regulators, and mediators for cell proliferation, survival, and anti-apoptosis are widely applied to evaluate their contribution to the progression and metastasis of prostate cancer. Second, prostate cancer cells are grown in media with androgens removed, or inoculated into surgically castrated hosts to select those that can survive and proliferate in an androgen-independent fashion. Third, prostate cancer cells are co-inoculated with relevant organ-specific stromal cells or tumor-derived extracellular matrices in intact or castrated hosts. There are limited models that can be used to specifically modify the prostate cancer microenvironment in experimental animals, but the available transgenic animals at best only partly recapitulate the metastatic profiles of human prostate cancer. This situation leaves us with a knowledge gap about the precise contribution of the tumor microenvironment to cancer progression and metastasis. Since co-inoculation of prostate cancer cells with stroma promotes tumor progression and metastasis, acquisition of additional tumorigenicity and metastatic potential must be due to both the genetic make-up of the cancer cells and the proper host microenvironment.

Consistent with the above assertion, it has been shown that orthotopically, rather than ectopically, inoculated prostate tumor cells can be promoted to acquire metastatic ability in soft tissues and bone [33–37]. In addition, we observed that cellular interaction with bone stromal cells in vivo, or under 3D culture in vitro, could permanently alter both the phenotype and genotype of the prostate cancer cell lines. Prostate cancer cells acquire increased metastatic potential and additional cytogenetic abnormalities after interaction with bone stromal cells [17]. Organ-specific stromal milieus, with complex cell types releasing specified growth factors, extracellular matrices, and metalloproteinases, must be responsible for the malignant evolution of the marginally tumorigenic and non-metastatic human LNCaP prostate cancer cell line into its genetically related bone metastatic variants, the C4-2 and C4-2B LNCaP sublines [38–42].

Direct evidence to support the assertion that host factors contribute to tumor formation and angiogenesis was provided by Huang et al. [43,44], who found that human ovarian cancer cells grew less in animals with MMP-9 deficiency, which led to decreased level of extracellular matrix remodeling and angiogenesis adjacent to the sites of tumor colonization. Macrophages were identified as the major source of the MMP-9 supporting the growth, angiogenesis, and metastasis of the ovarian tumors through enhanced extracellular matrix remodeling. As splenic macrophages isolated from wild-type animals supplemented the deficiency of MMP-9, the growth, angiogenesis and colonization of ovarian cancer in the MMP-9 deficient transgenic mice resumed. Such results support the concept that stroma is a potential target in ovarian cancer therapy.

Table 1 summarizes a number of well-characterized human prostate cancer models that can be used to study the interaction between tumor and the host microenvironment. These include the LNCaP prostate cancer progression model and the invasive ARCaP model established in our laboratory, both established from 3D tumor growth in xenografts under the influence of host microenvironment and through tumor–stromal interaction. These two models both encompass the two lethal phenotypes of human prostate cancer, androgen independence and bone metastasis. Both the LNCaP and ARCaP prostate cancer progression models consist of their respective lineage-related sublines evolved from the parental cells, which share common genetic backgrounds but display diverse tumorigenic potential. Cells in these models express androgen receptor and secrete prostate specific antigen (PSA). The most remarkable aspect of these models is how prostate cancer cells acquire additional tumorigenic and metastatic potentials merely through interaction with the host microenvironment, without introduction of any exogenous genetic materials. In contrast, control 2D cultures, either grown alone or with organ-specific stromal cells, failed to generate any invasive and metastatic sublines from the same parental cell lines. Our analyses revealed that cancer progression to metastasis would occur if tumor cells interacted with the host microenvironment in a contact-dependent manner. Proper alignment and organization of cancer cells with their surroundings in a 3D geometry is essential for tumor progression.

1.3. Establishing a 3D culture with the RWV module to study epithelial–stromal interaction

Throughout development, epithelial cells are found in close contact with interstitial mesenchymal tissues, which

Table 1
Human prostate cancer cell and xenograft models to study cancer-microenvironment interaction

Model	Commonly used cell lines	Comments (references)
Lineage-related models		
LNCaP	C4. C4-2, C4-2B	LNCaP subclones with increasing androgen-independence and bone metastatic potential [38,39,41]
ARCaP	ARCaP sublines	Clones selected from ARCaP bone metastasis [77,78]
PC-3	PC-3M, PC-3M-Pro4, PC-3M-LN4	Selected after orthotopic implantation of PC-3M cells [79,80]
CWR22	CWR22Rv1	Hormone relapsed CWR22 tumor grown in a castrated host [81,82]
RWPE-1	RWPE-1 sublines	Immortalized prostate epithelial cell line RWPE-1 gave rise sublines with increased tumorigenicity upon N-methyl-N-nitrourea exposure [83]
Xenograft models		
MDA-PCa	MDA-PCa2a, MDA-PCa-2b	Derived from specimens of a single prostate cancer patient with bone metastasis [84]
LuCaP	LuCaP35, LuCaP35v, LuCaP23-1, LuCaP23-12	Developed from multiple patients with prostate cancer metastasis to lymph node and liver [85,86]
LAPC	LAPC-4, LAPC-8, LAPC-9	Derived by implanting surgical specimens from patients into SCID mice [87]
PC	PC-82, PC-133, PC-135, PC-295, PCEW	The PC xenografts were established by growing primary prostate cancer or lymph node metastasis in nude mice [88]
DuCaP	DuCaP	Derived from the dura mater of a prostate cancer patient [89]
VCaP	VCaP	Derived from the vertebra of prostate cancer metastasis [90]

are composed of differentiated types of stromal cells of mesodermal origin [45–48]. Not only is an intimate relationship between epithelial and stromal cells a prerequisite to the maintenance of tissue architecture and organization, but also for the morphogenesis, cytodifferentiation, and expressed function of the epithelial cell compartment in all glandular organs including the prostate gland. Upon neoplastic transformation, disturbance of the epithelial–stromal interaction could work as the "driving force" for further malignant transformation of the epithelial cell compartment [30,49,50]. Recent studies, for example, uncovered "reactive stroma" in the vicinity of tumor nodules. These stroma produce a spectrum of growth factors and cytokines similar to those normally found in inflammatory stroma during wound healing [30,49,51,52]. This observation supports the early proposal that cancer is a wound that does not heal [53]. In addition, the whole process of cancer progression is accompanied by the cancer cells "recruiting" non-cancerous host stromal cells, including infiltrating inflammatory cells, the migration and attachment of new blood vessel cells, the differentiation of smooth muscle cells, and the accumulation of poorly characterized mesenchymal fibroblasts [54–58]. Through soluble and matrix-associated factors or cell–cell contact, these cells may direct the fate and guide the migration and invasion of the cancer cells by means of EMT and MET.

A number of 3D techniques have been designed to investigate the mechanisms of tumor–stromal interaction and determine its consequences. Table 2 summarizes the main models used in such studies in vitro. With regard to the interaction between prostate cancer cells and bone stroma, we will discuss our experience using the rotary wall vessel, a NASA-designed 3D cell culture model. With the RWV, cells are grown suspended in microgravity-simulating conditions in the presence of beads. The overall effect is a negative contribution of gravity on the cells through the mechanism of perpetual fluid rotation. Fluid rotation of the cells assist the experimental cells to form 3D conglomerates spontaneously, without any need for other manipulations.

We found that upon culture in the RWV 3D module, LNCaP prostate cancer cells form 3D organoids spontaneously. Importantly, prostate stromal cells became mandatory for the maintenance of androgen responsiveness and PSA production by the LNCaP cells [59]. LNCaP cells grown alone in 3D lost the ability to produce PSA, even in the presence of androgens. Only co-culturing LNCaP with prostate stromal cells could maintain androgen responsiveness and PSA production. These results suggested a critical role of prostate stroma in supporting the growth and differentiation status of the prostate epithelium, a result known to agree with the normal physiology of the prostate epithelium and its control by host endocrine factors. In contrast, in 2D cultures LNCaP cells can produce PSA in response to androgen stimulation regardless of the presence or absence of the stroma. This result indicates an uncoupling of the epithelial–stromal interaction while prostate epithelial cells are maintained as the 2D culture. If this holds true for other prostate cancer cells when cultured as 2D as opposed to 3D, we suggest that a large body of the current literature on androgen action in prostate cancer cell lines must be re-evaluated. Moreover, in view of the tight coupling of growth and PSA expression by prostate epithelium when co-cultured with prostate stroma as 3D organoids under RWV conditions, promising future opportunity of discovering new therapeutics could come from the use of this 3D model.

1.4. Forming large prostate organoids in space

One unique opportunity with RWV 3D studies is the possibility of transferring concepts and experimental designs in cancer biology from ground-based study to a space station

Table 2
Common models for 3D cell culture or tumor growth

Model	Method	Comment	Applications (references of examples)
Xenograft transplantation	Cells implanted to immune-compromised host	in vivo	Tumorigenicity [91,92]
		Microenvironment close to biological conditions	Tumor invasion and metastasis [93,94]
		Complex factors contribute to the microenvironment	Chemosensitivity [95,96]
			Angiogenesis [97,98]
Pellet culture	Cells cultured in spheroid formed by sedimentation	in vitro single or multiple cell types	Growth/differentiation [99,100]
Chorioallantoic membrane	Cells inoculated on chorioallantoic membrane of the chick embryo	in vivo	Vasculo-angiogenesis [101]
		Immunodeficient host Limited experimental time	Tumor invasion and metastasis [102,103]
Matrigel	Cells cultured in mixture with reconstituted basement membrane components extracted from EHS tumor	in vivo or in vitro	Growth/differentiation [92]
		The most popular assay for cell growth and invasion	Tumor invasion [104,105]
			Angiogenesis [106] Chemosensitivity [107]
Liquid-overlay	Cells cultured on the surface of agarose gel matrix, which blocks attachment of the cells	in vitro	Tumor growth [108,109]
		Most cells form spontaneous aggregate spheroids	Differentiation [110]
Soft agar colony formation	Single cell cultured in suspension in agarose gel matrix	in vitro	Tumor growth/survival [111]
		Anchorage-free growth The colony contains cells in 3D arrangement	Tumorigenicity [112] Chemosensitivity [113,114]
			Differentiation [115,116]
Synthetic matrix	Cells cultured in synthetic 3D-simulating matrices	in vivo or in vitro	Tissue/organ engineering [117–120]
		Scaffolds simulating mechanical conditions	Chemosensitivity [121]
Rotary cell culture	Cells/tissues/organs cultured in rotary wall vessel under micro-gravity simulated condition	in vitro	Tissue engineering [122]
		Spontaneous cell aggregation Single cell type or co-culture	Tissue reconstitution [123] Growth/differentiation [124] Tumor-stromal interaction [125] Metabolism/toxicology [126]

in the future, where reduced gravity conditions exists naturally. The reduced gravity system may mimic cancer cell growth and differentiation as would occur in patients more closely. We live in a $1 \times g$ terrain constantly subjected to unit gravity, but cancer cells carried in blood, lymphatic fluid, or even in bone marrow could be seen as existing in sub-gravity conditions, just as cellular aggregates or emboli exist in a weightless state [60]. To understand the effect of gravity on the 3D growth of chimeric prostate cancer organoids comprised of human prostate cancer cells (LNCaP) and human osteoblasts (MG63), we conducted the first study in space through a NASA-sponsored Shuttle Columbia flight. The experimental design included multiple runs in microgravity-simulated ground conditions to determine the optimal cell numbers for inoculation, cell feeding schedules, and duration (duration was a constraint of the mission length rather than culture due to the need of bring back the live cultures) of co-culture. A reciprocal cellular interaction model was established on the ground and in space to evaluate how human prostate cancer cells may interact with human osteoblasts under these co-culture conditions since prostate cancer bone metastasis occurs most frequently and is generally considered as lethal. The major focus of these ground and space-based studies is to test the hypothesis that reciprocity of cellular interaction between prostate cancer and bone stromal cells results in a permanently altered genotype and phenotype of these cells after exposure to a reduced gravity 3D culture conditions. The tragedy of the Shuttle

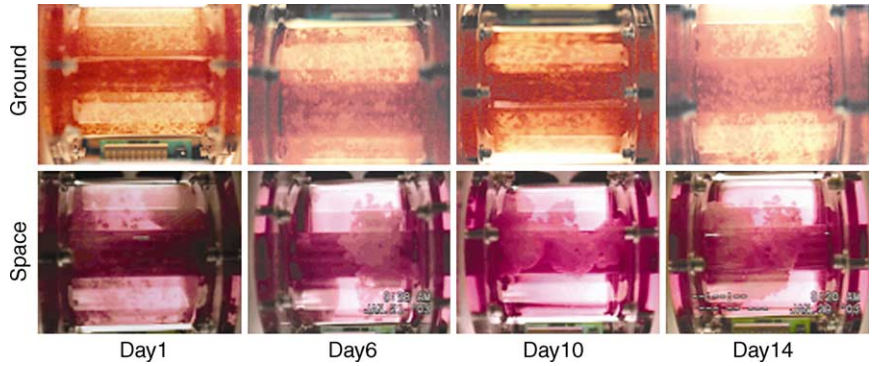

Fig. 1. Formation of large prostate cancer organoids with the 3D RWV culture in space. LNCaP and MG-63 cell lines were cultured in T-medium containing 10% FBS, 10 μg/ml Ciprofloxacin, 1 μg/ml Amphoterecin, and 90 mg/dl glucose. In preparation for flight readiness, the two cell lines were each determined to be free of bacteria, fungus, HIV-1, HIV-2, HTLV-1, HTLV-2, hepatitis B and C, and mycoplasma by microbiological laboratory testing, and thus did not pose any infectious risk to the Shuttle crew. In preparation for inoculating the bioreactor, one gram of Cultispher GL beads were hydrated in phosphate buffered saline (50 ml) and autoclaved (121 °C, 20 min). The beads were washed twice and resuspended in T-medium (50 ml). The beads (312 mg) were loaded into a 60 cc syringe and injected into the vessel. A total of 35×10^6 LNCaP cells and 7×10^6 MG-63 cells were each harvested and loaded into 5 cc syringes. Immediately after injecting the beads, each cell line was injected into the bioreactor. The rotation speed and medium feeding schedules of each bioreactor, two on ground and one in space, were each controlled by computers to assure both the near-zero gravity of the cultures and that the conditions of the media and thus the cultures were equivalent (not shown). A media sample from the cell culture was taken for archiving and monitored for pH, CO_2, O_2, HCO_3, Na, K, Cl, BUN, and glucose concentrations at regular intervals to determine real-time culture status. In parallel, a cell sample was archived for post experiment biochemical and molecular analyses. The pictures depicted were transmitted to NASA Mission Control during the indicated period of space flight. As shown in the pictures, much larger prostate organoids were formed in space as compared to the parallel ground study despite there was no difference in glucose utilization rate between ground and space studies (data not shown).

Columbia flight unfortunately resulted in the loss of flight crews and our ability to recover most of the valuable samples cultured in space. We obtained, however, electronically transmitted pictures from Shuttle Columbia describing the real-time growth and aggregation of the chimeric prostate cancer comprised of the LNCaP and MG63 osteoblast cell lines into growing prostate organoids (Fig. 1). These pictures are lined up on a real-time basis with the control study conducted on the ground, using identical bioreactor equipment and computer programs to control medium feeding, sampling and monitoring. There was a remarkable difference in the sizes of the aggregates. Prostate organoids in space reached golf-ball size within 6 days after co-culture, whereas in the same period prostate aggregates only 3–5 mm in diameter were formed on the ground (Fig. 1). This difference is most likely due to the lower shear forces applied to the cells in microgravity conditions as compared to the high shear forces applied on the cells cultured in the ground-based experiment. Higher rotation rates of the RWV are required to suspends the cells and simulate the microgravity conditions in space. Growth of coculture of cancer cells types in space offer several advantages: (1) it reduces the shear forces on the cells and (2) allows cancer cells to form natural interactions with other relevant cell types. The RWV 3D co-culture of cancer cells with stroma will likely offer new insights into the nature of the cellular interaction between cancer and its microenvironment and allow us to dissect the molecular steps underlying the co-evolution of cancer and stromal cells. These models can be carefully characterized and utilized to benefit future drug discovery programs.

1.5. Using RWV 3D co-culture to model prostate cancer–stromal interaction

We found a series of remarkably permanent genetic and behavioral changes in LNCaP cells co-cultured with either prostate or bone-derived stromal cells using both the 3D RWV module and chimeric xenografts in mice [41,61]. The observed changes included permanent chromosomal recombination as assessed by cytogenetics [41,61] and comparative genomic hybridization [62]. These changes were accompanied by behavioral changes in the prostate cancer cells, which exhibited both increased androgen independence and increased bone metastatic potential. That stromal cells were a prerequisite for these cellular interaction models involving human LNCaP cells was confirmed by control experiments using 2D co-culture, where no genetic and behavioral changes were noted in the LNCaP cells. Using the RWV co-culture system under 3D growth conditions, we were able to establish a simplified epithelial–stromal interaction model with only two partners, LNCaP and the prostate or bone stroma. This model can be expanded and applied to molecular analysis of prostate cancer progression by incorporating additional relevant cell types such as vascular endothelial, smooth muscle, neuroendocrine and inflammatory cells, in the construction of chimeric prostate cancer organoids.

Reciprocity of genetic and behavioral changes was also observed in 3D co-culture with reactive stromal cells. We recently determined that stroma isolated from prostate cancer specimens, unlike stroma from normal donors, preferentially induced tumor formation by LNCaP cells in xenograft (Sung, et al., unpublished results). To investigate this phenomenon

in vitro, we reactivated the prostate stromal and bone stromal cells by co-culturing them with C4-2, an androgen-independent subline of the LNCaP lineage, in the RWV 3D model [63]. The tumor-reactivated stromal cells were then purified and mixed with LNCaP cells in xenograft inoculation. While stromal cells were able to promote cancer growth, this series of studies revealed that stroma reactivated by cancer cells could promote further cancer progression (unpublished results). There was a clear difference between the androgen-dependent LNCaP and the androgen-independent C4-2 cells in terms of their capability to reactivate the stromal cells [63], with the C4-2 but not the LNCaP cells displaying significant greater potential to activate bone stromal cells (unpublished data). Importantly, none of these phenomena could be observed in 2D cultures. If the RWV 3D culture recapitulates the real microenvironment for prostate cancer growth, these results may indicate that in vivo prostate cancer cells are engaged in a reciprocal communication with the surrounding stroma. It can be speculated etiologically that in an organotypic microenvironment, stroma provide favorable factors facilitating cancer cell growth and survival while cancer cells return the favor by reactivating the stroma for further accommodation. This scenario will inevitably lead to a "vicious cycle" that is likely the foundation of the uncontrollable cancer growth observed in several metastatic models [9,10,17,51,64].

1.6. Regulating cell polarity, gene expression, and behavior in 3D organoids

The growth of cancer cells in situ is not the same as that of a 2D adherent cell array. Our experience and others showed that 3D culture forms a compacted organoid in close interaction with its microenvironment [65], a scenario that better mimics in vivo conditions. A number of 3D experimental models have been developed to assess how the microenvironment regulates cellular behavior, including cell polarity, gene expression, and cellular function. Malignant breast cancer cells growing on plastic dishes exhibited a similar phenotype to non-malignant breast epithelium. When placed in a 3D reconstituted extracellular scaffold, however, malignant breast cells formed amorphous structures with an enhanced rate of proliferation, partly due to a change in the expression of integrins and receptors for epidermal growth factors, as well as a disability to sense contextual cues in the surrounding microenvironment. In contrast, under the same conditions, non-malignant breast epithelial cells will undergo growth arrest and differentiate into a polarized alveolar structure that secretes milk [66]. In addition, developing kidney, cartilage, heart, pancreas, and ovary have been successfully cultured under similar conditions (Table 2). These results suggest a parallelism of growth condition requirement between organ development and carcinogenesis.

A 3D assembly of prostate cancer cells can be conveniently constructed by growing them with their relevant stromal cells using the 3D RWV technology [59,61,67]. Although the precise mechanism of cellular interaction between cancer cells and their in vivo environment remains speculative, the underlying molecular basis is most likely contributed by coordinated changes of gene expression in response to inductive cues from the host environment. In a series of reviews, Ingber [68–71] elegantly summarized the principle of mechanochemical transduction of signals controlling normal and malignant tissue differentiation. Mechanical stretch of cells can trigger a list of chemical signals, aimed at controlling the growth of the cell, expansion of basement membranes, and orderly progression of morphogenesis and cytodifferentiation of the developing tissues. In carcinogenesis, a deregulated epithelial–mesenchymal interaction could cause accelerated turnover of basement membrane and release of mechanical constraints on epithelium; and abnormal growth, differentiation, and migration may ensue. These properties have been validated with breast cancer and breast stroma co-cultured organoids grown as 3D tissues using the RWV technology.

1.7. Modeling prostate cancer bone metastasis with the RWV 3D co-culture system

Most prostate cancer metastases elicit an osteoblastic response in bone. It would be ideal if an in vitro model could be established specifically for the mechanistic study of prostate cancer bone metastasis. Because a 3D architecture and cellular interaction are essential for ex vivo formation of human bone [72], we initiated a project to develop an in vitro 3D culture model for bone formation, with a modified protocol from the 3D co-culture of prostate cancer cells and prostate or bone stroma. Most of the current 2D and 3D cultures resort mineralization conditions to induce differentiation of the bone-forming osteoblasts. The mineralization condition usually includes addition of crucial nutrients of ascorbic acid phosphate, vitamin D3, β-glycerol phosphate, and calcium gluconate to the culture media. In co-culture of the bone-forming osteoblasts and prostate cancer cells, however, some of these reagents may affect prostate cancer cells and complicate data interpretation. Our ultimate goal was to develop a simplified bone-forming culture condition, which would facilitate co-culture of prostate cancer cells with the bone.

We used the RWV 3D culture system to screen a panel of bone-derived primary cultures and established osteoblastic cell lines for their ability to form mineralized bones in the culture medium that accommodates prostate cancer cell growth (RPMI 1640 with 5% FBS). All the osteoblastic cells tested tolerated this culture condition well, and upon RWV 3D culture, they readily formed conglomerates, which were examined for bone formation with histological and biochemical methods. Through this screening, we were able to identify hFOB1.19, a conditionally immortalized human fetal osteoblastic cell line, that could spontaneously form mineralized bone without addition of any mineralizing components to the media, though adding the above reagents could

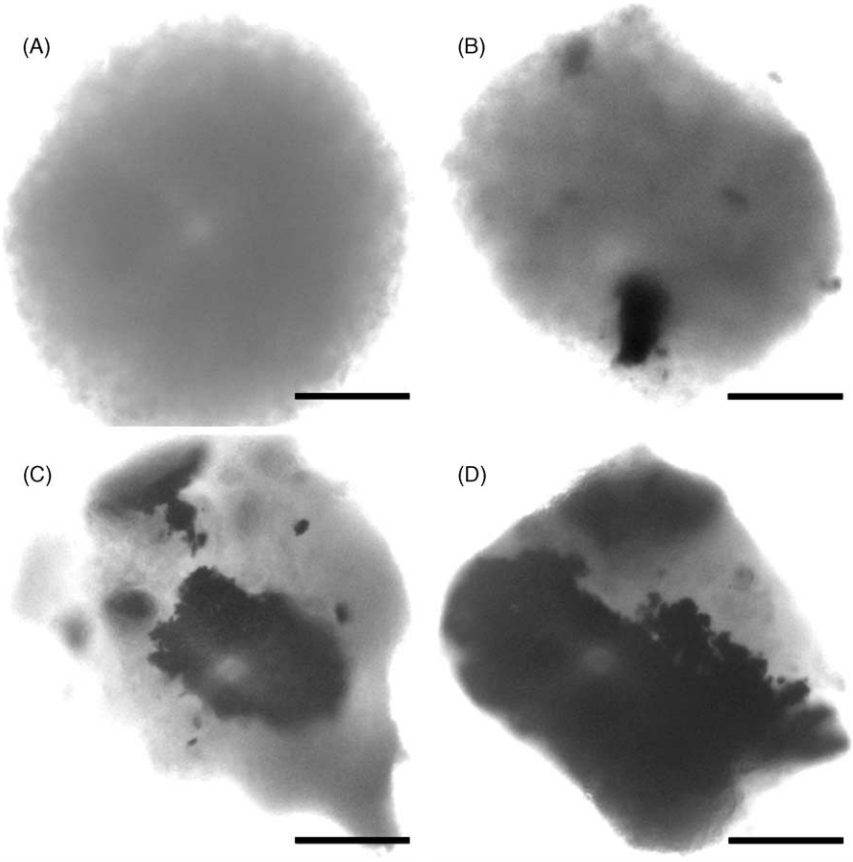

Fig. 2. Mineralization of the hFOB1.19 cells in 3D culture. Human osteosarcoma cell MG63 (5×10^6) and the immortalized human embryonic osteoblastic cell hFOB1.19 (5×10^6) were incubated separately in RPMI 1640 with 5% FBS, in the NASA RWV system for 9 days. Cell balls obtained from the 3D culture were placed onto a cell culture dish and photographed under transmitting light microscope at low magnification ($\times 10$). Panel A: After 9 days of culture, the MG63 cells aggregated into tightly packed cell balls, without visible mineralization. No mineralization was seen in MG63 cell balls even after extended 3D culturing (up to 14 days). On the other hand, the hFOB1.19 cells formed loose aggregates, with palpable and hard cores, which are opaque under transmitting light. The mineralization was confirmed with histological assays (not shown). Panels B, C, and D represent progressive mineralization within the hFOB1.19 cell balls after 3, 6, and 9 days of culture, respectively. Bar = 1 mm.

increase the subsequent mineralization in a quantitative manner (Fig. 2 and data not shown). We found that the hFOB1.19 conglomerates were visible at 24 h of RWV 3D culture, and mineralization within the conglomerates visible 48 h later (Fig. 2). In contrast, other osteoblastic cells had only marginal capability to form visible bone with mineralization under the same cell culture condition (data not shown). Interestingly, when cultured in 2D, the hFOB1.19 cells rarely form appreciable crystallized bone, even at full confluence. It seemed that the bone-forming ability of this cell line was genetically programmed but required the input from cell microenvironment under RWV 3D culture condition. These features can be explored in routine RWV 3D co-cultures without the use of additional mineralizing components.

Bone formation is currently a subject of intensive investigation. Most of the mineralization models reported so far used primary bone marrow mesenchymal precursor cells. Several murine osteoblastic cell lines are widely used for mineralization studies for their capability to form mineralized nodules in 2D culture. Most of these studies pursue gene expressions regulated by endocrine factors, rather than bone formation per se. A few human osteoblastic cell lines are available for the study of bone formation, but only two of them, MG63 and hFOB1.19, have been reported to form actual mineralized nodules in 2D cultures [73,74]. Traditionally, bone formation by human cell lines is of interest for the potential bioengineering as disease treatment. In this field, the 3D formation of mineralized bone nodules has been reported only within artificial matrices [75], with the experiments carried out under $1 \times g$ condition. Our screening thus provided a unique finding that certain osteoblastic cells could form mineralized bone spontaneously in RWV 3D culture, in the absence of key medium components necessary for mineralizing conditions. As for the mechanism, the differentiated bone formation between 2D and the RWV 3D culture indicates a fundamental requirement for 3D condition by bone cell biology. On the other hand, it may be due to differentiated production of extracellular matrix proteins in these cells under terrestrial gravity and zero gravity, because by altering mechanical sensing, gravity has a fundamental influence on gene expressions [76]. The finding of the spontaneous bone mineralization by hFOB1.19 cells in the RWV 3D module

will have a great impact on the study of bone mineralization beyond the field of prostate cancer research.

In relation to prostate cancer research, our study indicated that it is possible to carry out an in vitro molecular analysis of prostate cancer bone metastasis. By first establishing a unique bone formation model under RWV 3D culture conditions with a single specified osteoblastic cell line, we are now able to co-culture each prostate cancer cell line with the mineralized bone. Because this co-culture can be easily manipulated and the samples evaluated quantitatively, the interaction of the prostate cancer cells with bone can be carefully monitored. Integrating this model with genetic and therapeutic studies will yield valuable information on the mechanisms mediating the interaction between prostate cancer cells and bone.

2. Conclusion

In the last decade, detailed molecular genetic studies have led to realization of discrepancies of expression profiles between clinically obtained specimens and prostate cancer cell lines cultured as 2D cultures. In this article, we described our experience using the RWV 3D module to model the interaction between prostate cancer cells, prostate stroma, and bone. Our experimental data indicate that RWV 3D co-culture is a valuable model for meaningful molecular analysis of prostate cancer progression and metastasis. The RWV 3D co-culture model has advantages over conventional 2D culture, allowing reciprocal permanent genetic and behavioral changes to be observed in prostate cancer and bone or prostate stromal cells upon co-culture of these cells only under 3D but not 2D conditions. These results support the in vivo chimeric xenograft prostate cancer progression and metastasis model, in which the RWV 3D provides a much-simplified system for the detailed assessment of factors mediating prostate cancer progression and bone metastasis. Finally, our laboratory developed a unique in vitro bone formation model with human fetal osteoblasts grown under RWV 3D conditions. Remarkably, we observed that these cells when cultured in RWV 3D conditions could form mineralized bone in the absence of components usually required for bone formation in vitro. This study opens up new opportunities for future mechanistic studies and a new model recapitulating human prostate cancer bone metastasis. Further development of this system will accelerate the discovery of agents that can be applied to prevent and treat localized and disseminated prostate cancer.

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References

[1] Haslam SZ, Woodward TL. Host microenvironment in breast cancer development: epithelial cell–stromal cell interactions and steroid hormone action in normal and cancerous mammary gland. Breast Cancer Res 2003;5:208–15.
[2] Shekhar MP, Pauley R, Heppner G. Host microenvironment in breast cancer development: extracellular matrix-stromal cell contribution to neoplastic phenotype of epithelial cells in the breast. Breast Cancer Res 2003;5:130–5.
[3] Chung LWK, Baseman A, Assikis V, Zhau HE. Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. J Urol 2005;173:10–20.
[4] Vincent-Salomon A, Thiery JP. Host microenvironment in breast cancer development: epithelial–mesenchymal transition in breast cancer development. Breast Cancer Res 2003;5:101–6.
[5] Tlsty TD. Stromal cells can contribute oncogenic signals. Semin Cancer Biol 2001;11:97–104.
[6] Bissell MJ, Rizki A, Mian IS. Tissue architecture: the ultimate regulator of breast epithelial function. Curr Opin Cell Biol 2003;15:753–62.
[7] Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer 2003;3:453–8.
[8] Mueller MM, Fusenig NE. Friends or foes—bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 2004;4:839–49.
[9] Chirgwin JM, Guise TA. Molecular mechanisms of tumor–bone interactions in osteolytic metastases. Crit Rev Eukaryot Gene Expr 2000;10:159–78.
[10] Mundy GR. Mechanisms of bone metastasis. Cancer 1997;80:1546–56.
[11] Fidler IJ. The organ microenvironment and cancer metastasis. Differentiation 2002;70:498–505.
[12] Mintz B, Illmensee K. Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc Natl Acad Sci USA 1975;72:3585–9.
[13] Illmensee K, Mintz B. Totipotency and normal differentiation of single teratocarcinoma cells cloned by injection into blastocysts. Proc Natl Acad Sci USA 1976;73:549–53.
[14] Pierce GB. Relationship between differentiation and carcinogenesis. J Toxicol Environ Health 1977;2:1335–42.
[15] Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, et al. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol 1997;137:231–45.
[16] Barcellos-Hoff MH, Ravani SA. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. Cancer Res 2000;60:1254–60.
[17] Chung LW. Prostate carcinoma bone–stroma interaction and its biologic and therapeutic implications. Cancer 2003;97:772–8.
[18] Maffini MV, Soto AM, Calabro JM, Ucci AA, Sonnenschein C. The stroma as a crucial target in rat mammary gland carcinogenesis. J Cell Sci 2004;117:1495–502.
[19] Koeneman KS, Yeung F, Chung LW. Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of

prostate cancer metastasis and growth in the bone environment. Prostate 1999;39:246–61.
[20] Hendrix MJ, Seftor EA, Hess AR, Seftor RE. Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nat Rev Cancer 2003;3:411–21.
[21] Matsubara S, Wada Y, Gardner TA, Egawa M, Park MS, Hsieh CL, et al. A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. Cancer Res 2001;61:6012–9.
[22] Carlinfante G, Vassiliou D, Svensson O, Wendel M, Heinegard D, Andersson G. Differential expression of osteopontin and bone sialoprotein in bone metastasis of breast and prostate carcinoma. Clin Exp Metastasis 2003;20:437–44.
[23] Folberg R, Hendrix MJ, Maniotis AJ. Vasculogenic mimicry and tumor angiogenesis. Am J Pathol 2000;156:361–81.
[24] Bissell MJ. Tumor plasticity allows vasculogenic mimicry, a novel form of angiogenic switch. A rose by any other name? Am J Pathol 1999;155:675–9.
[25] Kang Y, Massague J. Epithelial–mesenchymal transitions: twist in development and metastasis. Cell 2004;118:277–9.
[26] Gotzmann J, Mikula M, Eger A, Schulte-Hermann R, Foisner R, Beug H, et al. Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. Mutat Res 2004;566:9–20.
[27] Thiery JP. Epithelial–mesenchymal transitions in development and pathologies. Curr Opin Cell Biol 2003;15:740–6.
[28] Hay ED. An overview of epithelio-mesenchymal transformation. Acta Anat (Basel) 1995;154:8–20.
[29] Bogdanos J, Karamanolakis D, Tenta R, Tsintavis A, Milathianakis C, Mitsiades C, et al. Endocrine/paracrine/autocrine survival factor activity of bone microenvironment participates in the development of androgen ablation and chemotherapy refractoriness of prostate cancer metastasis in skeleton. Endocr Relat Cancer 2003;10:279–89.
[30] Edlund M, Sung SY, Chung LW. Modulation of prostate cancer growth in bone microenvironments. J Cell Biochem 2004;91:686–705.
[31] Camirand A, Pollak M. Co-targeting IGF-1R and c-kit: synergistic inhibition of proliferation and induction of apoptosis in H 209 small cell lung cancer cells. Br J Cancer 2004;90:1825–9.
[32] Yeung F, Chung LW. Molecular basis of co-targeting prostate tumor and stroma. J Cell Biochem Suppl 2002;38:65–72.
[33] Bibby MC. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. Eur J Cancer 2004;40:852–7.
[34] Yamaura T, Doki Y, Murakami K, Saiki I. Model for mediastinal lymph node metastasis produced by orthotopic intrapulmonary implantation of lung cancer cells in mice. Hum Cell 1999;12:197–204.
[35] Killion JJ, Radinsky R, Fidler IJ. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. Cancer Metastasis Rev 1998;17:279–84.
[36] Hoffman RM. Orthotopic is orthodox: why are orthotopic-transplant metastatic models different from all other models? J Cell Biochem 1994;56:1–3.
[37] Miller FR, Heppner GH. Cellular interactions in metastasis. Cancer Metastasis Rev 1990;9:21–34.
[38] Thalmann GN, Sikes RA, Wu TT, Degeorges A, Chang SM, Ozen M, et al. LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. Prostate 2000;44:91–103. July 101;144(102).
[39] Wu TT, Sikes RA, Cui Q, Thalmann GN, Kao C, Murphy CF, et al. Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. Int J Cancer 1998;77:887–94.
[40] Gleave M, Hsieh JT, Gao CA, von Eschenbach AC, Chung LW. Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. Cancer Res 1991;51:3753–61.
[41] Thalmann GN, Anezinis PE, Chang SM, Zhau HE, Kim EE, Hopwood VL, et al. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. Cancer Res 1994;54:2577–81.
[42] Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S, Chung LW. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int J Cancer 1994;57:406–12.
[43] Huang S, Van Arsdall M, Tedjarati S, McCarty M, Wu W, Langley R, et al. Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. J Natl Cancer Inst 2002;94:1134–42.
[44] Huang LW, Garrett AP, Bell DA, Welch WR, Berkowitz RS, Mok SC. Differential expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 protein and mRNA in epithelial ovarian tumors. Gynecol Oncol 2000;77:369–76.
[45] Robinson GW, Karpf AB, Kratochwil K. Regulation of mammary gland development by tissue interaction. J Mammary Gland Biol Neoplasia 1999;4:9–19.
[46] Matsumoto A, Hashimoto K, Yoshioka T, Otani H. Occlusion and subsequent re-canalization in early duodenal development of human embryos: integrated organogenesis and histogenesis through a possible epithelial–mesenchymal interaction. Anat Embryol (Berl) 2002;205:53–65.
[47] Saxen L, Sariola H, Lehtonen E. Sequential cell and tissue interactions governing organogenesis of the kidney. Anat Embryol (Berl) 1986;175:1–6.
[48] Cunha GR, Donjacour AA, Sugimura Y. Stromal–epithelial interactions and heterogeneity of proliferative activity within the prostate. Biochem Cell Biol 1986;64:608–14.
[49] Tuxhorn JA, Ayala GE, Rowley DR. Reactive stroma in prostate cancer progression. J Urol 2001;166:2472–83.
[50] Singh H, Dang TD, Ayala GE, Rowley DR. Transforming growth factor-beta1 induced myofibroblasts regulate LNCaP cell death. J Urol 2004;172:2421–5.
[51] Sung SY, Chung LW. Prostate tumor–stroma interaction: molecular mechanisms and opportunities for therapeutic targeting. Differentiation 2002;70:506–21.
[52] Rowley DR. What might a stromal response mean to prostate cancer progression? Cancer Metastasis Rev 1998;17:411–9.
[53] Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986;315:1650–9.
[54] Lin EY, Pollard JW. Role of infiltrated leucocytes in tumour growth and spread. Br J Cancer 2004;90:2053–8.
[55] Labrousse AL, Ntayi C, Hornebeck W, Bernard P. Stromal reaction in cutaneous melanoma. Crit Rev Oncol Hematol 2004;49:269–75.
[56] Knowles H, Leek R, Harris AL. Macrophage infiltration and angiogenesis in human malignancy. Novartis Found Symp 2004;256:189–200, discussion 200–184, 259–169.
[57] Mantovani A, Sozzani S, Locati M, Schioppa T, Saccani A, Allavena P, et al. Infiltration of tumours by macrophages and dendritic cells: tumour-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Novartis Found Symp 2004;256:137–45, discussion 146–138, 259–169.
[58] Carlos TM. Leukocyte recruitment at sites of tumor: dissonant orchestration. J Leukoc Biol 2001;70:171–84.
[59] Zhau HE, Goodwin TJ, Chang SM, Baker TL, Chung LW. Establishment of a three-dimensional human prostate organoid coculture under microgravity-simulated conditions: evaluation of androgen-induced growth and PSA expression. In Vitro Cell Dev Biol Anim 1997;33:375–80.

[60] Freed LE, Vunjak-Novakovic G. Spaceflight bioreactor studies of cells and tissues. Adv Space Biol Med 2002;8:177–95.
[61] Rhee HW, Zhau HE, Pathak S, Multani AS, Pennanen S, Visakorpi T, et al. Permanent phenotypic and genotypic changes of prostate cancer cells cultured in a three-dimensional rotating-wall vessel. In Vitro Cell Dev Biol Anim 2001;37:127–40.
[62] Hyytinen ER, Thalmann GN, Zhau HE, Karhu R, Kallioniemi OP, Chung LW, et al. Genetic changes associated with the acquisition of androgen-independent growth, tumorigenicity and metastatic potential in a prostate cancer model. Br J Cancer 1997;75:190–5.
[63] Chung LW, Hsieh CL, Law A, Sung SY, Gardner TA, Egawa M, et al. New targets for therapy in prostate cancer: modulation of stromal–epithelial interactions. Urology 2003;62:44–54.
[64] Guise TA, Chirgwin JM. Transforming growth factor-beta in osteolytic breast cancer bone metastases. Clin Orthop 2003:S32–8.
[65] Abbott A. Cell culture: biology's new dimension. Nature 2003;424:870–2.
[66] Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW. The organizing principle: microenvironmental influences in the normal and malignant breast. Differentiation 2002;70:537–46.
[67] Margolis L, Hatfill S, Chuaqui R, Vocke C, Emmert-Buck M, Linehan WM, et al. Long term organ culture of human prostate tissue in a NASA-designed rotating wall bioreactor. J Urol 1999;161:290–7.
[68] Ingber DE. Mechanobiology and diseases of mechanotransduction. Ann Med 2003;35:564–77.
[69] Ingber DE. Tensegrity II. How structural networks influence cellular information processing networks. J Cell Sci 2003;116:1397–408.
[70] Ingber DE, Tensegrity I. Cell structure and hierarchical systems biology. J Cell Sci 2003;116:1157–73.
[71] Ingber DE. Cancer as a disease of epithelial–mesenchymal interactions and extracellular matrix regulation. Differentiation 2002;70:547–60.
[72] Kale S, Biermann S, Edwards C, Tarnowski C, Morris M, Long MW. Three-dimensional cellular development is essential for ex vivo formation of human bone. Nat Biotechnol 2000;18:954–8.
[73] Shih NR, Jo OD, Yanagawa N. Effects of PHEX antisense in human osteoblast cells. J Am Soc Nephrol 2002;13:394–9.
[74] Harris SA, Enger RJ, Riggs BL, Spelsberg TC. Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. J Bone Miner Res 1995;10:178–86.
[75] Qiu QQ, Ducheyne P, Ayyaswamy PS. Fabrication, characterization and evaluation of bioceramic hollow microspheres used as microcarriers for 3D bone tissue formation in rotating bioreactors. Biomaterials 1999;20:989–1001.
[76] Hughes-Fulford M. Function of the cytoskeleton in gravisensing during spaceflight. Adv Space Res 2003;32:1585–93.
[77] Zhau HY, Chang SM, Chen BQ, Wang Y, Zhang H, Kao C, et al. Androgen-repressed phenotype in human prostate cancer. Proc Natl Acad Sci USA 1996;93:15152–7.
[78] Xu JC, Xie ZH, Huang W, Kelly S, Phillips C, Cui YY, et al. Clonal interaction, not clonal selection, maintains the heterogeneity of prostte tumor cells. J Urol 2003;169(Suppl):81.
[79] Stephenson RA, Dinney CP, Gohji K, Ordonez NG, Killion JJ, Fidler IJ. Metastatic model for human prostate cancer using orthotopic implantation in nude mice. J Natl Cancer Inst 1992;84:951–7.
[80] Pettaway CA, Pathak S, Greene G, Ramirez E, Wilson MR, Killion JJ, et al. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. Clin Cancer Res 1996;2:1627–36.
[81] Wainstein MA, He F, Robinson D, Kung HJ, Schwartz S, Giaconia JM, et al. CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma. Cancer Res 1994;54:6049–52.
[82] Sramkoski RM, Pretlow II TG, Giaconia JM, Pretlow TP, Schwartz S, Sy MS, et al. A new human prostate carcinoma cell line, 22Rv1. In Vitro Cell Dev Biol Anim 1999;35:403–9.
[83] Bello-DeOcampo D, Kleinman HK, Webber MM. The role of alpha 6 beta 1 integrin and EGF in normal and malignant acinar morphogenesis of human prostatic epithelial cells. Mutat Res 2001;480–481:209–17.
[84] Navone NM, Olive M, Ozen M, Davis R, Troncoso P, Tu SM, et al. Establishment of two human prostate cancer cell lines derived from a single bone metastasis. Clin Cancer Res 1997;3:2493–500.
[85] Ellis WJ, Vessella RL, Buhler KR, Bladou F, True LD, Bigler SA, et al. Characterization of a novel androgen-sensitive, prostate-specific antigen-producing prostatic carcinoma xenograft: LuCaP 23. Clin Cancer Res 1996;2:1039–48.
[86] Corey E, Quinn JE, Buhler KR, Nelson PS, Macoska JA, True LD, et al. LuCaP 35: a new model of prostate cancer progression to androgen independence. Prostate 2003;55:239–46.
[87] Klein KA, Reiter RE, Redula J, Moradi H, Zhu XL, Brothman AR, et al. Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. Nat Med 1997;3:402–8.
[88] van Weerden WM, Romijn JC. Use of nude mouse xenograft models in prostate cancer research. Prostate 2000;43:263–71.
[89] Lee YG, Korenchuk S, Lehr J, Whitney S, Vessela R, Pienta KJ. Establishment and characterization of a new human prostatic cancer cell line: DuCaP. In Vivo 2001;15:157–62.
[90] Korenchuk, S, Lehr, JE, L, MC, Lee, YG, Whitney, S, Vessella, R, Lin, DL, Pienta, KJ. VCaP, a cell-based model system of human prostate cancer. In Vivo 2001;15:163–8.
[91] Miller FR. Xenograft models of premalignant breast disease. J Mammary Gland Biol Neoplasia 2000;5:379–91.
[92] Benelli R, Albini A. In vitro models of angiogenesis: the use of Matrigel. Int J Biol Markers 1999;14:243–6.
[93] Chung LW, Kao C, Sikes RA, Zhau HE. Human prostate cancer progression models and therapeutic intervention. Hinyokika Kiyo 1997;43:815–20.
[94] Chung LW. The role of stromal–epithelial interaction in normal and malignant growth. Cancer Surv 1995;23:33–42.
[95] Kerbel RS. Human tumor xenografts as predictive preclinical models for anticancer drug activity in humans: better than commonly perceived-but they can be improved. Cancer Biol Ther 2003;2:S134–9.
[96] Kerbel RS, Cornil I, Theodorescu D. Importance of orthotopic transplantation procedures in assessing the effects of transfected genes on human tumor growth and metastasis. Cancer Metastasis Rev 1991;10:201–15.
[97] Alani RM, Silverthorn CF, Orosz K. Tumor angiogenesis in mice and men. Cancer Biol Ther 2004;3:498–500.
[98] Goldbrunner RH, Wagner S, Roosen K, Tonn JC. Models for assessment of angiogenesis in gliomas. J Neurooncol 2000;50:53–62.
[99] Bai X, Xiao Z, Pan Y, Hu J, Pohl J, Wen J, et al. Cartilage-derived morphogenetic protein-1 promotes the differentiation of mesenchymal stem cells into chondrocytes. Biochem Biophys Res Commun 2004;325:453–60.
[100] Tallheden T, Karlsson C, Brunner A, Van Der Lee J, Hagg R, Tommasini R, et al. Gene expression during redifferentiation of human articular chondrocytes. Osteoarthritis Cartilage 2004;12:525–35.
[101] Ribatti D, Nico B, Vacca A, Roncali L, Burri PH, Djonov V. Chorioallantoic membrane capillary bed: a useful target for studying angiogenesis and anti-angiogenesis in vivo. Anat Rec 2001;264:317–24.
[102] Chen J, Rodriguez JA, Barnett B, Hashimoto N, Tang J, Yoneda T. Bone sialoprotein promotes tumor cell migration in both in vitro and in vivo models. Connect Tissue Res 2003;44(Suppl 1):279–84.
[103] Scher C, Haudenschild C, Klagsbrun M. The chick chorioallantoic membrane as a model system for the study of tissue invasion by viral transformed cells. Cell 1976;8:373–82.
[104] Sweeney TM, Kibbey MC, Zain M, Fridman R, Kleinman HK. Basement membrane and the SIKVAV laminin-derived peptide

promote tumor growth and metastases. Cancer Metastasis Rev 1991;10:245–54.
[105] Yamamura K, Kibbey MC, Jun SH, Kleinman HK. Effect of Matrigel and laminin peptide YIGSR on tumor growth and metastasis. Semin Cancer Biol 1993;4:259–65.
[106] Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N. Angiogenesis assays: a critical overview. Clin Chem 2003;49:32–40.
[107] Albini A, Benelli R, Noonan DM, Brigati C. The "chemoinvasion assay": a tool to study tumor and endothelial cell invasion of basement membranes. Int J Dev Biol 2004;48:563–71.
[108] Carlsson J, Yuhas JM. Liquid-overlay culture of cellular spheroids. Recent Results Cancer Res 1984;95:1–23.
[109] Hedlund TE, Duke RC, Miller GJ. Three-dimensional spheroid cultures of human prostate cancer cell lines. Prostate 1999;41:154–65.
[110] Nishimoto SK, Stryker WF, Nimni ME. Calcification of osteoblast-like rat osteosarcoma cells in agarose suspension cultures. Calcif Tissue Int 1987;41:274–80.
[111] Sarosdy MF, Einhorn LH, Donohue JP, Von Hoff DD. Correlation of clonogenic growth with histologic diagnosis after resection of residual tissue following chemotherapy for nonseminomatous testis cancer. Urology 1989;34:396–8.
[112] Hamburger AW. The human tumor clonogenic assay as a model system in cell biology. Int J Cell Cloning 1987;5:89–107.
[113] Agre P, Williams TE. The human tumor cloning assay in cancer drug development. A review Invest New Drugs 1983;1:33–45.
[114] Bertelsen CA, Sondak VK, Mann BD, Korn EL, Kern DH. Chemosensitivity testing of human solid tumors. A review of 1582 assays with 258 clinical correlations. Cancer 1984;53:1240–5.
[115] Shalit M. Growth and differentiation of eosinophils from human peripheral blood CD 34+ cells. Allerg Immunol (Paris) 1997;29:7–10.
[116] Bergwitz C, Wendlandt T, Kispert A, Brabant G. Wnts differentially regulate colony growth and differentiation of chondrogenic rat calvaria cells. Biochim Biophys Acta 2001;1538:129–40.
[117] Galois L, Freyria AM, Grossin L, Hubert P, Mainard D, Herbage D, et al. Cartilage repair: surgical techniques and tissue engineering using polysaccharide- and collagen-based biomaterials. Biorheology 2004;41:433–43.
[118] Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, et al. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials 2005;26:599–609.
[119] Tumia NS, Johnstone AJ. Promoting the proliferative and synthetic activity of knee meniscal fibrochondrocytes using basic fibroblast growth factor in vitro. Am J Sports Med 2004;32:915–20.
[120] Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues—state of the art and future perspectives. J Biomater Sci Polym Ed 2001;12:107–24.
[121] Dhiman HK, Ray AR, Panda AK. Characterization and evaluation of chitosan matrix for in vitro growth of MCF-7 breast cancer cell lines. Biomaterials 2004;25:5147–54.
[122] Su GN, Hidaka M, Kimura Y, Yamamoto G. In situ collagen gelation: a new method for constructing large tissue in rotary culture vessels. In Vitro Cell Dev Biol Anim 2003;39:368–74.
[123] Dutt K, Harris-Hooker S, Ellerson D, Layne D, Kumar R, Hunt R. Generation of 3D retina-like structures from a human retinal cell line in a NASA bioreactor. Cell Transplant 2003;12:717–31.
[124] Jessup JM, Frantz M, Sonmez-Alpan E, Locker J, Skena K, Waller H, et al. Microgravity culture reduces apoptosis and increases the differentiation of a human colorectal carcinoma cell line. In Vitro Cell Dev Biol Anim 2000;36:367–73.
[125] Chopra V, Dinh TV, Hannigan EV. Three-dimensional endothelial–tumor epithelial cell interactions in human cervical cancers. In Vitro Cell Dev Biol Anim 1997;33:432–42.
[126] Gonda SR, Wu H, Pingerelli PL, Glickman BW. Three-dimensional transgenic cell model to quantify genotoxic effects of space environment. Adv Space Res 2001;27:421–30.