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Human 3-D tissue models in radiation biology: current status and future perspectives

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ABSTRACT

In this review, we discuss the use of a variety of 3-D models (particularly 3-D skin, lung, breast and endothelial) in radiobiological research and highlight the differences in responses compared to 2-D culturing conditions (monolayers). We review the characteristics of existing 3-D models and aim to point out the substantial advantages 3-D cultures provide for modern radiobiology. In particular, they may facilitate the shift from the classical DNA damage and repair studies mainly carried out in monolayer cultures to the investigation of more generalized responses through pathway analysis and a system biology approach. 3-D models are expected to be very informative for investigations on radiotherapy responses in addressing the low dose risk. However, the 3-D model systems are not as easy to propagate and standardize as monolayer cultures. Therefore, we discuss the problems and limitations of 3-D models and propose ways to overcome some of the problems.

Keywords: *3-D tissue models, radiation biology.*

Definitions, history and development of various 3-D models used in radiation biology

There are numerous studies of the targeted and non-targeted effects of ionizing radiation (IR) in two-dimensional (2-D) cell cultures. As a result, most of the dogmas in radiation biology have been postulated after experiments on monolayer cultures in-vitro. However, 2-D culture models lack the normal structural organization of tissues in an organism. More complex models containing different cell types enabling the intercellular interactions characteristic for a tissue *in-vivo* are therefore required to validate the extent and relevance of the effects in relation to human radiation exposures. Models of either monotypic three-dimensional (3-D) cultures or more sophisticated organotypic co-cultures including multiple cell types have been developed. As a definition the 3-D cell culture should incorporate both the special organization and differentiated function of the tissue *in-vivo* [(1)]. 3-D *in-vitro* models allow the study of cell-to-cell and cell-extracellular matrix interactions, as well as the influence of the microenvironment on cellular differentiation, proliferation, apoptosis and gene expression. The monotypic cultures include a single cellular type. However, they still retain the capacity to differentiate into minimal units of the tissue type they originate from [(1)]. Despite this, in some research aspects e.g. studies on stroma-epithelial cell interactions and in general the interactions and paracrine signaling between the different cell types in a tissue, there is a need of more-sophisticated

model systems. First developments in this direction have been the use of reconstituted substitutes of the extracellular matrix (ECM). As cells *in-vivo* encounter dissimilar growth conditions and cell shape as compared to 2-D cultured monolayer cell cultures, it is not astonishing that the cellular behavior upon stress is different. For example, cells in-vivo are surrounded by proteins of the ECM. To better mimic physiological conditions and to maintain the normal tissue microenvironment, 3-D ECM-based models were developed, in which cells grew embedded in ECM proteins [2-8]. For these purposes different compounds have been employed, such as laminin-rich Matrigel™ and collagen [1-3].

In addition to the environmental factors, morphology seems to play an important role for cellular behavior. While 3-D extracellular matrix -grown tumor cells of different origin exhibit a round cell shape similar to tumor cells from cancer biopsies [2, 3], 2-D grown cells are flat. Several studies exist about the impact of morphology on DNA replication. It was concluded that contact of cells to ECM is essential for both normal cell cycle transition and damage-induced cell cycle arrest [2, 3].

One of the first 3-D monotypic models has been derived from the mammary gland epithelial cell line MCF-10A, grown on reconstituted basal membrane (BD Biosciences, Matrigel™). Analysis of the acinar structure revealed that they consisted of a well polarized outer layer in contact with the basal membrane, while the inner layer showed poor polarization [3, 4]. By day eight the cells in the middle area started to die via apoptosis and a hollow lumen was formed in this area. The whole process was paralleled with changes in the expression of numerous genes related to cell-cell adhesion, basement membrane depolarization and polarity. Later the cells showed properties to form polarized acini-like structures with hollow center, similarly to the normal glandular organization in breast.

There are three major groups of 3-D systems, those propagated in the absence of solid substrate and those that are grown in the presence of ECM-resembling components. The first group is maintained in rotating bioreactors which are constantly moving the cell media and this way providing optimal conditions for cell assembly. Such 3-D cultures were established from cartilage, lymphoid cells, bone marrow, endothelial and also from prostate cells [1, 2]. A more recent second group includes tissues such as corneal, skin, lung bronchial epithelium, mammary gland, liver endothelia, pancreas, kidney, bladder, intestine, salivary gland, thyroid gland, and cardiovascular endothelium [1] that were built using a substrate of ECM-resembling components. In addition, a large variety of substrates have been tested for 3-D culturing including collagen I, amniotic basement membrane or reconstituted basement membrane [3, 9]. Finally, co-cultures with stromal cells (fibroblasts) have also been used for functional studies with main focus on adhesion, migration, mechanisms of polarity and branching morphogenesis. A third group of 3-D models deals with cells which form 3-D structures during cultivation in coated ultra-low attachment surface equipment without use of ECM-resembling components like Matrigel™. Due to the coating with a covalently bound hydrogel layer, attachment of cells on the surface of the cell culture container is inhibited. Instead cells form spheroids by clustering together. This kind of 3-D modeling is used for organ culture applications, studies of monocytes, lymphocytes, macrophages and other phagocytic cells, and for stem cell research, e.g. for investigation of neurospheres or mammospheres [10-13].

Advantages of 3-D over 2-D models

From 2-D monocultures to 2-D multiculturing: a step forward

Moving from monotypic 2-D culturing to multitypic 2-D co-culturing is a step towards closer resemblance to the *in-vivo* situation. Cellular communication and interaction between different cell types are known to be important determinants in cellular and tissue functioning *in-vivo* [14]. For example, surrounding cells such as fibroblasts, smooth muscle cells and pericytes will control together with endothelial cells the formation and growth of new blood vessels

[15, 16]. Indeed, co-cultures of fibroblasts and endothelial cells have been developed to mimic angiogenesis, the formation of new blood vessels, *in-vitro* [16, 17]. Vascular wall cells also interact in other processes such as inflammation. Therefore, co-cultures of endothelial cells and smooth muscle cells have been used to investigate the influence of the muscle cells on the inflammatory response of endothelial cells to TNF-α [18, 19]. Another example are 2-D co-cultures of pericytes with endothelial cells which have been used to investigate capillary dysfunction in amongst others, diabetes (diabetic retinal microvascular environment) [20] and the blood-brain barrier [21, 22]. Two dimensional co-culture systems are also of interest in the research field of diseases where outcome is determined by more than one specific cell type [23]. With regard to the response after irradiation, immunomodulatory effects of irradiation have been investigated by co-cultivation of dendritic cells and *T-cells* [24, 25].

Despite extensive efforts in preclinical studies, many novel drugs fail to be translated from bench to bedside. Therefore, new models better reflecting the conditions *in-vivo* are needed to generate results, which transfer more reliably into the clinic. One substantial progress in this regard is the development of 3-D cell culture models. Specific cell functionality, for example, is lost in 2-D breast epithelial cell cultures with regard to beta-casein expression but can be regained in 3-D through formation of physiological acini-like structures [3].

3-D has advantages over the animal models

Whenever tissue specific reactions have been of interest for biological studies and that treatment has not been acceptable to be applied on volunteers, *in-vivo* animal studies have often been used. Even though there may be plenty of robust data that have been collected on many topics as skin carcinogenesis, toxicology and also radiosensitivity, one always needs to keep in mind that direct extrapolation from animals to humans is difficult. In this regard pharmaceutical industry can offer a wide range of examples on promising new pharmaceutical agents that successfully passed the pre-clinical trials on 2-D systems and later on animal models, but fail at the final stages when the drugs have been given to patients [26]. Therefore, 3-D *in-vitro* models using human cells could be more relevant and also much more cost effective.

3-D provides a way forward to systems biology

The 3-D cultures, especially the multitypic ones, allow studying the specific cell signaling mechanisms and networks, characteristic for the *in-vivo* conditions. These models could be used together with the approaches of the systems biology to reveal the mechanisms of radiation-induced biological phenomena such as genomic instability, bystander effect, low dose hypersensitivity, will aid in bridging the gap between initial events and the effect outcomes for the local tissue, the organs and the whole organism [27, 28].

Tissue specificity: 3-D organotypic cultures as a model system for normal tissue

Skin models

3-D organotypic cultures including co-culturing of different cell types have been very successfully applied, initially as a test model for irritability and toxicity studies, in the epidermal biology. Those models were based on co-culturing of keratinocytes on de-epidermized dermis or fibroblast-embedded collagen gels [29-31]. The cultures have the typical differentiation pattern and functional features of the in-vivo epidermis. Despite the fact that 3-D skin cultures are in-vivo simplified models, they still can be used as a tool for tissue-specific differentiation studies *in-vitro* [29].

Lung models

Various lung epithelial tissue models have also been created [26-28]. The majority of the 3-D structures were monotypic, consisting of primary bronchial cells, grown at the air-liquid interface (ALI) in media containing hormones, vitamines and growth factors, facilitating the cell differentiation process [32, 33]. Even though these models have been reported to include well-differentiated bronchial cell components

like ciliated, goblet and basal [32, 33], the creation of these models have the drawback of constant requirements of primary lung tissue supply. Other studies reported differentiation of primary bronchial cells into acinar structures when grown onto a reconstituted basal membrane (Matrigel™) [34]. Later, there was a need of implementing the *in-vivo* conditions, where the tissue has a microenvironment of ECM and stromal cells, and development of more sophisticated systems has taken place. For example, the group of Prof. Jerry Shay from University of Texas Southwestern described that by ectopically expressing human telomerase enzyme (hTERT) and cyclin-dependant kinase 4 (CDK4) the Human Bronchial Epithelial Cells (HBEC) can still develop into the normally differentiated 3-D model. This model has the advantage of almost unlimited replicative capacity without oncogenic transformation [34, 35]. Depending on the chosen culturing system, the HBEC cells in co-culture with normal fetal (IMR90) fibroblasts were able to form either 3-D structure of differentiated ciliated and goblet cells when plated onto fibroblasts-embedded collagen gels, cyst-like structures when embedded in Matrigel™, or tubular structures able to form branches when overlaid onto the Matrigel™ [34]. The expression analysis of differentiation markers further revealed that depending on the microenvironment and *in-vitro* culture conditions these cells are able to differentiate into different compartments of the lung – bronchial, alveolar or bronchiolar. These characteristics make them a suitable model for the introduction of oncogenes or gene mutations, characteristic for lung cancer and application of possible treatments on the 3-D system.

Importance of the microenvironment for the development and characteristics of 3-D lung cultures was investigated by Pageau and coworkers [6]. His group tested both IMR90 or cancer-associated fibroblasts (LuCAF) embedded in collagen I as matrix for human bronchial epithelial cells (NHBE) 3-D cultures. The cultures that contained LuCAFs were invading the collagen and were expressing genes characteristic for immune response, apoptosis and cancer. These data again show the applicability of the 3-D lung tissue studies in the lung tumorigenesis studies, this time with respect to the role of microenvironmental signals - in the development of cancer.

Breast models

Also for breast tissue numerous different 3-D culture models exist [4, 36-38]. Both monotypic 3-D culture models, including only breast epithelial cells, and multitypic 3-D breast models were developed. *In-vivo* normal epithelial cells are characterized by a defined apico-basal polarity, which is established by cell-cell and cell -ECM adhesions and which contributes to induction and maintenance of tissue specificity [39]. In the mammary gland, myoepithelial and luminal epithelial cells form polarized and bilayered acini, which secrete milk into the lumen of the acini during lactation. When mammary cells are cultured on 2-D plastic substrates, crucial signals for cell proliferation, metabolism, differentiation and cell death responsible for the formation of accurate tissue-specific architecture and function, are lost [40]. 3-D cultivation of mammary epithelial cells in laminin-rich ECM gels and treated with lactogenic hormones provides reconstruction of the acinar structures known from *in-vivo* conditions and restore a number of mammary-specific functions [41]. So, in contrast to cultivation of mammary epithelial cells within conventional 2-D techniques, in 3-D cultures the cells are able to form acinar-like structures and express tissue-specific milk proteins if treated with lactogenic hormones (figure 1).

Beside differences in morphology and functional performance for mammary cells it was also shown that in 3-D cultures tissue-specific gene expression and signaling pathways are regulated in a fundamentally different way than in cells cultured in 2-D [40].

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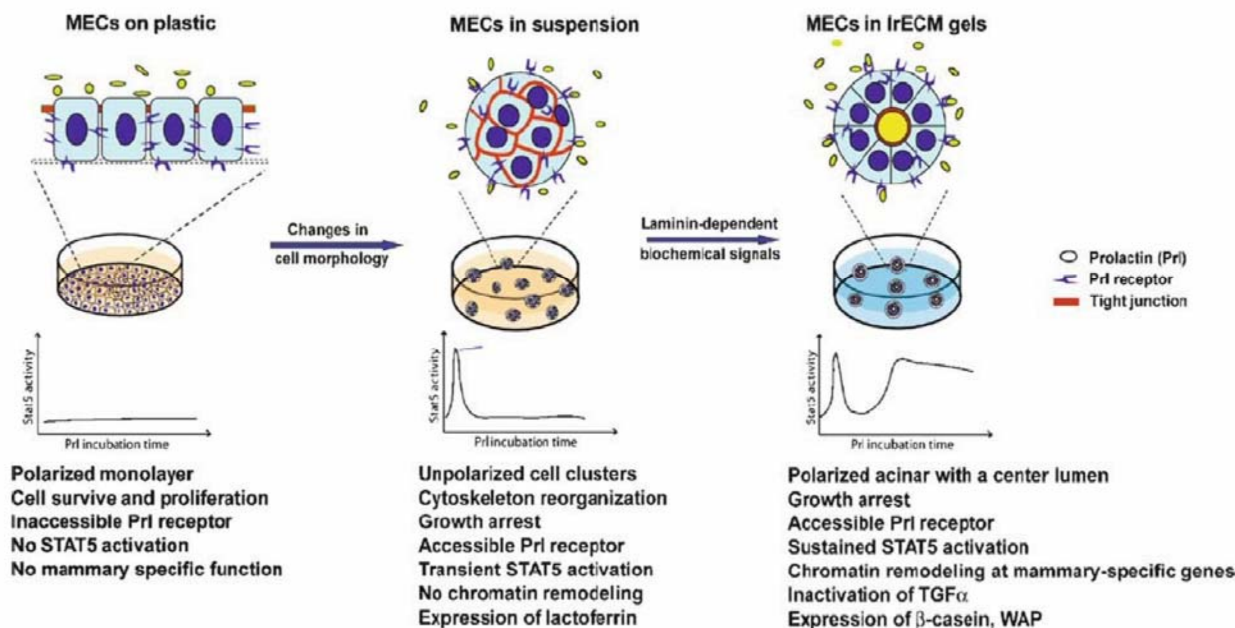

Figure 1. Characteristics of mammary epithelial cells (MECs) growing in 2-D or 3-D culture systems respectively, and the importance of ECM for breast tissue-specific functions. lrECM: laminin-rich ECM; STAT5: signal transducers and activators of transcription protein 5; TGFα: transforming growth factor-α; WAP: whey acidic protein, with permission from (41).

An important role in normal embryonic development and carcinogenesis of the human breast is played by interactions between the epithelium and stroma (e.g. adipocytes, fibroblasts). However, the underlying mechanisms of these events are only fragmentarily understood at the moment. Therefore, beside monotypic 3-D cultures for breast models also multitypic 3-D models using different co-culture techniques were created. Primary human mammary epithelial cells and pre-adipocytes, both derived from mammoplasty reduction in healthy women, were co-cultured in 3-D matrices to investigate the possibility to regenerate human autologous breast tissue as tissue engineering method [42]. It was shown that the cells maintained normal intercellular distribution and growth-pattern when co-cultured in a 3-D collagen gel. Also cell lines were utilised for 3-D co-cultivation for breast tissue models. Co-cultivation of normal human mammary epithelial MCF10A cells with pre-differentiated human adipose-derived stem cells in a mixture of Matrigel™ and collagen in 3-D porous silk scaffolds resulted in inhibition of MCF10A cell proliferation as well as induction of both, alveolar and ductal morphogenesis, and enhancement of their functional differentiation [43]. Campbell and co-workers [44] developed a 3-D model of mammary gland that causes a defined, porous collagen/hyaluronic acid scaffold to form a physiologically relevant foundation for co-cultivation of epithelial and adipocyte cells without tumor-derived reconstituted basement membrane hydrogel. Further investigations of epithelial-stromal interactions in the mammary gland were performed using 3-D co-cultures of fibroblasts together with the normal human mammary epithelial cell line MCF10A [45] or with mammary tumor cells of different metastatic potential (MDA-MB-231, MCF-7 cells) [44]. Wang and co-workers [47] constructed a complex tri-culture system with MCF10A cells, human fibroblasts and adipocytes based on a Matrigel™/collagen mixture on porous silk protein scaffolds for modelling breast morphogenesis and function.

As mentioned above, mammary epithelial

cells cultivated in 3-D cultures recapitulate numerous characteristics of the glandular epithelia *in-vivo*, such as the formation of cyst-like spheroids with a hollow lumen, apico-basal polarization of the cells comprising these structures, strong cell growth and proliferation control as well as the establishment of a basement membrane [36, 37]. Therefore 3-D breast models provide an important tool to study fundamental biological questions of breast tissue under physiologically relevant conditions, including investigation of intracellular organization as well as the role of cell–cell and cell–substrate interactions in regulation of breast epithelium response to hormones. They also provide cell signalling information, and are utilized to study mammary stem cells, the influence of tissue architecture on tumor development and how apico-basal polarity is established, maintained, and compromised in the mammary epithelia.

Endothelium models

Sprouting, migration and the formation of tube-like structures of endothelial cells can be evaluated in 3-D using gel matrices that consist e.g. of the ECM proteins, fibrin or collagen. In addition, endothelial tube formation can already be evoked within 24 h in Matrigel™. This is a complex mixture containing laminin as a major, collagen, heparan sulfate proteoglycans, entactin/nidogen and growth factors [47].

Co-culture of endothelial cells with fibroblasts in a 2-D assay is also used to assess angiogenesis [16, 17]. The further addition of growth factors or matrix proteins is not required since the fibroblasts secrete the necessary matrix components needed for the formation of capillary-like structures from endothelial cells. Usually, capillary-like structure formation develops between day 7 and 14 [17].

By seeding endothelial cells first onto gelatin-coated micro carrier beads, the confluent monolayer of endothelial cells that is present at the beginning of angiogenic sprouting *in-vivo* is mimicked [5]. Moreover, the presence of the beads facilitates the quantitative analysis of cell migration and sprout formation. In an attempt to quantify the degree of angiogenesis, usually branching or sprouting combined to tube length is measured [5, 7, 48, 49].

Alternatively, Korff and Augustin [50] described that endothelial cell spheroids form spontaneously within 4 h when seeded in nonadhesive round bottom plates in medium that prevents cell adhesion. Spheroids could be maintained in suspension cultures for several weeks. After 7 days, the unorganized center cells have disappeared to form spheroids with an almost acellular core.

Studying the effects of microgravity, it was observed that endothelial cells form tube-like structures when they are exposed to microgravity simulated by a random position machine (clinostat) [51]. These tube-like structures consisted of several cell layers surrounding a central lumen. At the inner surface of the tube-like structures, endothelial cells were lined up and incorporated into abundant extracellular matrix so that the innermost layer of the tubes resembled the intima of blood vessels. This result was an important progress from earlier studies on tissue formation of bovine aortic endothelial cells in which the cells cultured in the microgravity-based rotating wall vessel bioreactor were observed to form several layers around aggregates of beads [52]. It became clear that endothelial cells do not need a scaffold under microgravity to form cell layers surrounding a column-like cavity [53].

Exploitation of 3-D models in radiation biology studies

Ionizing radiation (IR) is a well described genotoxic agent, which causes early and late tissue damage depending on the specific tissue characteristics as cell turnover, oxygenation, repopulation abilities and cell cycle phase [54] and on radiation characteristics. Several studies on skin, lung epithelium and mammary gland models have concluded that the responses between 2-D and 3-D cellular systems to IR can be different [35, 45, 56]. As the 3-D microenvironment models are more characteristic for a tissue in vivo, their use is recommended in order to

reduce uncertainties related to the extrapolation of data from in vitro to in vivo conditions. On figure 2 we have summarized the main 3-D human tissue systems applied in the radiation biology studies by now.

IR-induced DNA damage and apoptosis in 3-D tissue cultures

There are increasing numbers of studies focusing on evaluation of the effects of IR in 3-D tissue models. The data are concerning double strand breaks (DSB) formation, micronucleation and apoptosis in the directly irradiated and bystander cells following exposure of 3-D models to high and low LET radiation (table 1).

Sedelnikova and coworkers [57] used two types of commercially available 3-D models: EpiAirway™, which mimics the epithelial tissue of the respiratory tract, and the full-thickness model of the human skin EpiDerm™FT (both from MatTek™, Ashland, MA, USA) and irradiated them with 7 MeV $^{4}He^{2+}$ ions at 3.2 Gy (1.9 particles at 5x5 µm area). They observed a peak of the γ-H2AX foci formation at 30 min post -irradiation for the directly irradiated samples and delayed formation of the foci in the bystander cells up to 12 to 48 h after irradiation [57, 58]. This effect was observed at distance up to 2.5 mm from the irradiated cell plane. The increase in γ-H2AX foci levels was observed in 40-60% of the bystander cells and was as high as 4-6-fold over the controls [57].

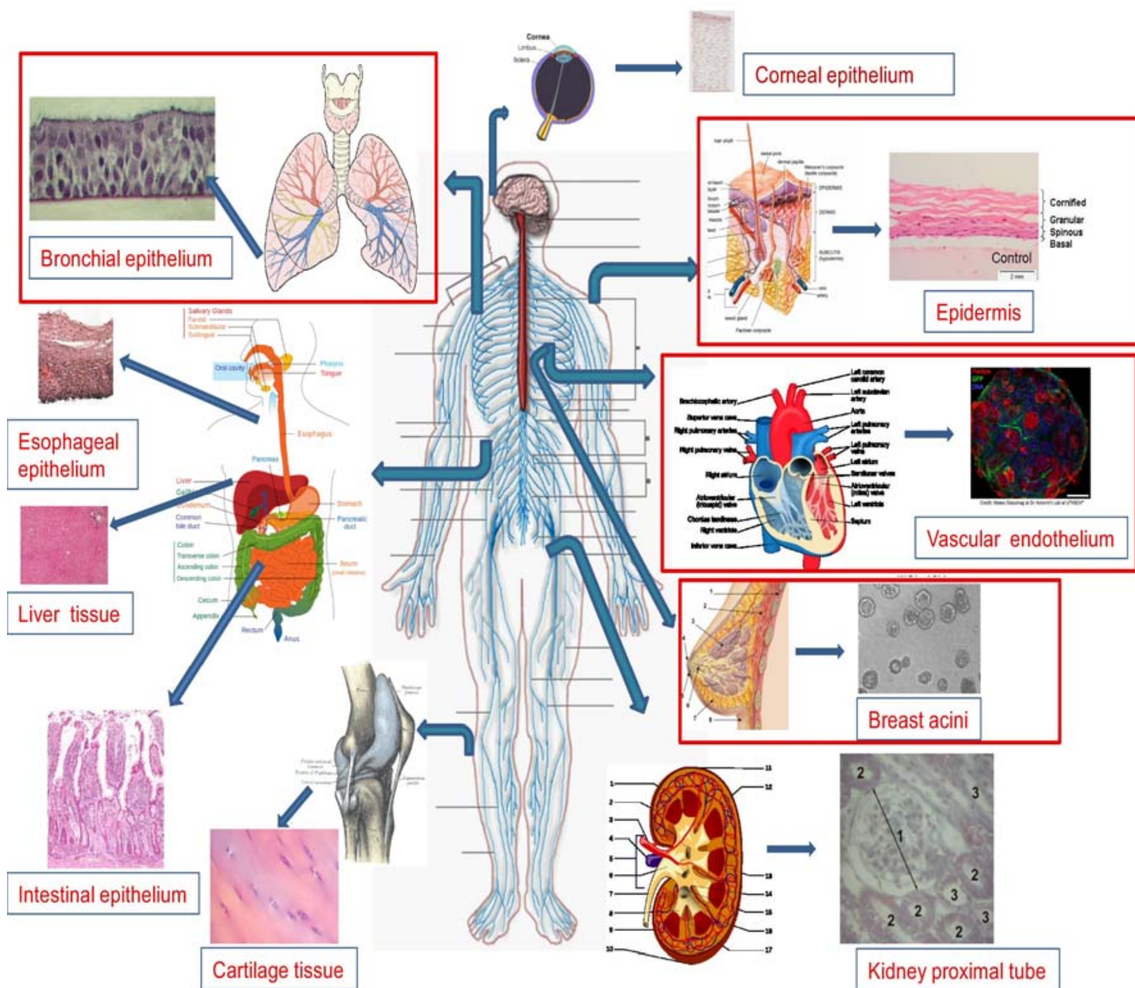

Figure 2. Representative scheme of most commonly used 3-D monotypic and organotypic models in the radiation biology studies. Red rectangulars mark 3-D models studied by the authors of the review.

Table 1. Main 3-D tissue models in radiation biology.

3-D tissue models in radiation biology studies			
Tissue	**Model**	**Radiation quality**	**Main findings**
Skin	EpiDerm MatTek™	High LET	DNA damage foci in directly irradiated areas in 3D cultures peak at 30 min; by-stander damage up to 2.5 mm from irradiated cells with delayed formation (12-48 h). Additionally, induction of apoptosis, micronuclei and senescence markers (β-gal), in both irradiated and bystander areas [57].
		Low LET	Dose-dependent DNA damage foci, delayed accumulation of repair signals [55].
		High LET	Gene expression changes: substantial up-regulation of MMP1 and COX-2 at 4h post irradiation and down regulation of DMBT1; the effect observed up to 1000 µm from irradiated cells. Genes are involved in inflammation, embryonic development and tissue remodeling [59].
		High LET	Low vs. high doses: low doses stimulate expression of repair genes, while high doses induce genes connected to loss of integrity and terminal differentiation; the differentiation pattern of the same samples showed low doses trigger increased proliferation in the basal layer, suggesting recovery, and high doses – hypercornification, suggested as a protective terminal differentiation mechanism [60].
Endothelium	3-D on Matrigel™ or collagen gel	Low LET	IR-induced formation of tube-like structures and cellular migration of HUVEC cells plated on Matrigel™; also stimulation of migration and wound closure in lung microvascular cells after low doses (<1 Gy) [70, 71].
		High and Low LET	A 3-D capillary-like model from HUVEC cells on collagen gels showed larger sensitivity to high LET compared to low LET; high LET caused more complex and persistent 53BP1 DNA damage foci [74].
Lung	EpiAirway MatTek™	High LET	3-D cells had more complex and persistent DNA damage than 2-D HBEC3-KT lung epithelial line, probably due to down-regulation of repair genes in 3-D. The outcome was chromosomal instability in 3-D [34, 66].
Breast	3-D acini	High and Low LET	EMT transition in irradiated acini as TGF-β treatment enhances the effects of radiation; no dose or radiation quality dependence in responses [66].
	“hanging drops”	Low LET	Presence of endothelial cells in the 3-D spheroid tumor cells protected the tumor cells from radiation [79].
	3-D acini on Matrigel	Low LET	Cell survival of human mammary epithelial cell line 184A1 was increased 4-fold following doses of 2.5 and 5 Gy when cells were in 3D. The protection was not directly attributable to the presence of ECM during or after IR. Amount of apoptosis following IR significantly decreased in 3-D culture relative to the 2-D monolayer after the same IR dose[56].

Using low LET radiations (X-rays), recent studies from Suzuki and coworkers [55] showed induction of 53BP1 DNA DSB foci in the basal and partly in the spinous layer of a 3-D skin model (containing rapidly dividing and undifferentiated cells). The 53BP1 foci induced in the 3-D skin after 1 Gy X-rays followed a dose-dependent induction and biphasic decrease with time after irradiation. The authors also reported persistent foci size growth up to a few hours post irradiation which was suggested to be related with G1 arrest and especially with amplifying the G1 checkpoints signaling sufficiently to induce p53 phosphorylation in cells with very low number of remaining 53BP1 foci. This process has been regarded as a tumor suppressor mechanism preventing cells with remaining DNA damage from division [8]. On the other hand, there have been suggestions that the foci diameter growth is actually related with chromatin remodeling in order to facilitate DNA repair [55, 58].

The group of Sedelnikova has also evaluated the downstream effects of DSB in epidermal and bronchial tissue models as induction of apoptosis, micronuclei formation and senescence [57]. Cleaved caspase 3-positive apoptotic cells were present in both tissue models used and increase

in apoptosis was observed at day 1 after irradiation. In EpiAirway™ the apoptotic levels continued to increase up to day 4 after irradiation and the effect was 6.6–fold higher than the control levels. The EpiDerm™ model showed smaller response: 2-fold increase in apoptosis for the keratinocytes and 2.7-fold for the fibroblasts (57). They also observed increased levels of micronucleated cells, senescence markers (such as SA-β-galactosidase) and DNA hypomethylation. Accumulation of DSB probably led to the hypomethylation of DNA, which is usually observed in various cancer types. Apoptotic response as well as senescence in the cells surrounding irradiated areas was suggested to be a local protective response to potentially carcinogenic cellular changes (57).

Using a 3-D human mammary epithelial tissue model, Sowa *et al.* (56) found a protective effect of 3-D cell cultures on cell survival after low LET (X-rays) irradiation. The initial state of the cells (2-D versus 3-D culture) at the time of irradiation did not alter cell survival but long-term culture in 3-D offered a significant reduction in cytotoxicity at a given dose between 2 and 5 Gy. They concluded that a likely mechanism for the cytoprotective effect provided by the 3-D culture conditions is down-regulation of radiation-induced apoptosis in 3-D structures.

Studies of Asaithamby and coworkers (35) showed differences in the 2-D and 3-D cellular model repair mechanisms using high LET ^{56}Fe ions for their experiments and bronchial epithelial cells (HBEC3-KT) either in 2-D cultures or in 3-D organotypic set-ups. The irradiated 3-D cells showed persistent complex DNA damage that has been repaired with slow kinetics (approx. 71% of the damage) while in 2-D cells only 20% of complex damage were repaired slowly. Also the number of persistent foci was 3-4 times higher in 3-D cultures 5 days post irradiation with 0.1-1 Gy, than in 2-D cultures [35]. Further studies showed that the cells in 3-D had downregulation in DNA repair genes and the later consequences from the accumulation of DNA damage was chromosomal instability manifested as an increased level of chromosome aberrations. This study emphasized the specific differences and repair abilities of differentiated 3-D epithelial lung cells in comparison to the frequently used for radiobiological experiments monolayer cultures.

In this context, it was shown that the distribution of radiation-induced residual γH2AX/53BP1 foci clearly demonstrated a significant difference in irradiated 2-D monolayers as compared to irradiated 3-D cultures or xenografts (2, 80). Lung carcinoma cells grown in 3-D or *in vivo* showed a similar distribution of eu- and heterochromatin associated γH2AX/53BP1 foci after irradiation. The authors have found a dissemination of one euchromatin focus to one heterochromatin focus. In 2-D, this pattern changed from two euchromatin foci to one heterochromatin focus. These results suggest that 3-D cultures better reflect the physiological conditions *in vivo* for examination of the cellular radiation response (2, 80).

Cell signaling and gene expression in directly irradiated and bystander cells in 3-D skin cultures.

In order to reveal the mechanistic differences between 2-D and 3-D radiation responses, experiments have been performed on signaling pathways and gene expression analysis. A research project of Amundson's group in Columbia University, was aiming to elucidate the expression profile of EPI-200 EpiDerm™ (MatTek, Ashland, MA) 3-D skin model after exposure of a small area of the culture to irradiation with X-rays or α-particles. The experimental set-up included a shielding mask, which allowed only a narrow strip (≈40 μm) of the 3-D skin to be irradiated (59). They performed gene microarrays on the irradiated and bystander regions of the cultures as the cultures were divided in 250 μm stripes and the time points of the study were 0, 4, 6 and 24 h after irradiation. From the 500 genes studied, 2 were substantially up regulated 4 h post irradiation (6-fold for Cyclooxygenase 2 PTGS2 (COX-2) and 3-fold for Matrix Metallopeptidase 1 (MMP1). Only one (Deleted in Malignant Brain Tumors 1 (DMBT1)) was downregulated 4 h post irradiation. The distance for the up- or downregulation of the genes was spreading up to 1000 μm from the

irradiated cells. These genes play important functions in inflammation, embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. This highlights the main effects that the bystander signaling could cause in the surrounding tissue.

A more recent study from the same group evaluated the gene expression profiles of low (0.1 Gy) and high (2.5 Gy) doses of 4.5 MeV protons exposed 3-D EpiDerm™ skin cultures [60]. The gene expression profiles were investigated only in directly exposed cultures, without investigating the bystander effect. Although there was similar ontology in the regulated genes, there were significant differences between the high and low dose exposed samples. In general, low doses upregulated genes responsible for the tissue recovery and repair, while high doses were triggering up regulation of genes associated with loss of structural integrity and terminal differentiation. The network analysis of the genes with significant changes in regulation was indicating that high dose response is ruled by TP53 and low dose effects by a novel transcription factor HNF4A. The detailed ontology analysis was revealing the different processes that could be affected by low as well as high dose exposure in 3-D skin model.

Yunis and co-workers [61] studied genomic responses to low (0.1 Gy) versus moderate doses (1 Gy) of X-ray exposures over 24 hour periods using EpiDerm™FT 3-D full thickness skin model. They showed that acute low dose exposure activated pathways associated to tissue protection and survival, whilst exposure to moderate doses promotes activation of apoptotic pathways leading to elimination of cells with DNA damage.

Differentiation studies

Earlier attempts to maintain lung structure *in vitro* have been performed as explants of human bronchial epithelium or large airways mucosa have been cultured on agar-coated plates. The cultures showed differentiated epithelium underlayed with basal membrane. It consisted of secretory, ciliated and basal cells. The cells were producing mucosa and maintaining their structural integrity including beating cilia for more than three weeks [56]. Both reported studies were focused on high dose rate brachytherapy effects on the pulmonary tissue. The earlier study of Kotsianos and coworkers [62] investigated response of bronchial epithelium miniorgans to doses from 10 to 75 Gy of iridium-192. The tissues showed high radioresistance, as morphological changes were induced from doses higher than 30 Gy and for cell viability significant results for reduction were evident only after 75 Gy. The cultures were either analysed immediately or cultivated for 4 or 18 days post irradiation. The 3-D miniorgans had very high radioresistance, explained with the very slow turnover of the bronchial epithelium (8 weeks). Comparison of the radiation response of tissue explants, monolayers of bronchial cells (BEAS-2A) and also *in vivo* animal irradiation (Göttingen minipigs) have been performed after doses of 10 and 30 Gy [63]. For the cell monolayers severe diminishment of the cell number 96 h post irradiation have been observed, while the tissues had smaller reduction in cell number 21 days post irradiation. Morphological changes had been reported 3 weeks after the *in vivo* irradiation of Göttingen pigs. However, the epithelium recovered its normal structure within 8 weeks. These data suggests that the 2-D cell models and the organotypic cultures as well as the *in vivo* tissue have different radiosensitivity and repair capacity.

Recent studies from Mezentsev and Amundson [60] on EPI-200 EpiDerm™ (MatTek, Ashland, MA) skin model after exposure to 4.5 MeV protons at doses 0.1 and 2.5 Gy, analysed the differentiation pattern of the model. At both doses, disrupted differentiation was induced in the directly irradiated samples, which eventually resulted in hypercornification. The higher dose also induced abnormalities in the development of the basal layer. This was found to be increased at 48 and 72 h after low doses and in contrast, decreased at 72 h in the higher dose irradiated samples. As an overall observation of the high dose proton irradiated 3 -D skin cultures were lacking the normal integrity of the basal layer and the cells were

showing discontinued pattern. In contrast, the low dose irradiated 3-D cultures had increased proliferation, which was pointing out initiation of recovery. The hypercornification of the low dose irradiated cultures was also milder, suggesting dose-dependent induction of terminal differentiation in the epidermal model. Moreover, the cornified layer of the irradiated samples was containing nucleated cells, which is a sign for premature differentiation of the irradiated samples. During the disrupted process of terminal differentiation the cells are failing to commit nuclear disintegration during the transition from the squamous to the cornified layer. There were also abnormalities in the cornified layer appearance – it was sharply delineated and tightly condensed - and probably this resulted in disrupted barrier function of the 3-D epidermal model (60) .

Epithelial to mesenchymal transition after IR of 3-D structures

There are several studies from the group of Barcellos-Hoff (3, 64), where they used 3-D mammospheres to evaluate the role of local pre-irradiation and TGF-β signaling for the epithelial to mesenchymal transition of the mammary epithelial cells. The process of reduction in epithelial traits and markers and induction of mesenchymal ones, known as epithelial-to-mesenchymal transition (EMT), has been suggested to be indicator for invasive breast cancer (58, 59). The work by Park and coworkers (64) suggested that pre-treatment with even low doses (25 cGy) of radiation (X-rays) sensitizes the breast epithelial cells to TGF-β and facilitates the EMT. Same group's work (65, 66) showed loss of epithelial and gain of mesenchymal markers after 2 Gy irradiation which could be visualized in the 3-D mammary acinar model and could correspond to changes in cellular polarity. The studies of the Barcellos-Hoff group with different doses 1 Gy high LET (^{56}Fe ions) and 2 Gy low LET (^{137}Cs γ-rays) radiation on 3-D mammary acini, revealed lack of radiation dose and quality dependence in the TGF-β induced EMT, similar to other non-targeted effects of IR (66). The mammary gland tissue has been one of the successful 3-D models used in the radiation biology, since later *in vivo* mouse experiments (58, 67) showed similar results.

Angiogenesis studies in 3-D endothelium models after IR

Some reports exist regarding endothelial cells in 3-D culture and the induction of angiogenesis upon irradiation. The effects of radiation on the process of angiogenesis are mostly investigated in the context of radiotherapy. Since the development of new blood vessels in a tumor is required for tumor growth and metastasis, knowledge about the influence of radiotherapy on this process is needed (68). Sonveaux and coworkers (69) showed that human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells, irradiated with 6 and 20 Gy of X -rays and plated after 24 h on Matrigel, rapidly form tube-like structures (in contrary to non-irradiated cells). They also showed an increased capacity of migration with increasing doses (2 to 20 Gy) of X-rays (69). Likewise, by another research group, 15 h after exposure to 3 and 10 Gy a dose-dependent increase in cellular migration was observed using modified Boyden chambers with gelatin-coated filters. In addition, when 3 and 10 Gy irradiated HUVECs were plated on Matrigel™, capillary-like structures were formed (70). Another study showed a stimulation of lung human microvascular endothelial cell migration and wound closure at doses between 0.5 and 0.8 Gy (X-ray) (71). These findings emphasize the need for associating radiotherapy with antiangiogenic approaches to enhance its overall therapeutic efficacy. However it has also been suggested that exposure of the vasculature to ionizing radiation suppresses the process of angiogenesis (72). Indeed, Imaizumi and coworkers (73) have revealed suppression of VEGF-induced sprouting of high dose X-rays irradiated endothelial cells. Using an *in vivo* Matrigel™ plug angiogenesis assay in mice, they have observed that radiation exposure (20 Gy) locally suppresses VEGF and FGF-2 induced angiogenesis. Next, they used the *ex vivo* aortic ring endothelial cell sprouting assay where aorta is removed from mice, cut in segments and embedded in collagen gel to evaluate VEGF-induced sprouting. By this assay

they revealed suppression of VEGF-induced sprouting of aortae originating from both mice irradiated with 15 Gy single or fractionated (5 x 3 Gy) dose compared to non-irradiated mice. Also, *in vitro* VEGF-induced sprouting of HUVECs coated on spheroids that are embedded in collagen gel is suppressed by high dose irradiation (8 Gy). Finally, using the *in vitro* scratch wound closure assay they observed decreased migration of 15 Gy irradiated HUVECs compared to non-irradiated HUVECs [73]. Overall, their findings support radiation-induced suppression of angiogenesis in contrast to the previous mentioned studies.

The influence of ionizing radiation (high and low LET) on angiogenesis has also been studied in the context of space radiation. Grabham and coworkers [74] have used an established 3-D vessel model, where non-proliferating differentiated HUVECs are arranged in capillary-like tubes in a collagen gel, to assess the effect of three different radiation qualities (^{56}Fe-ions, protons and gamma-rays) on vessel formation. Comparing the different radiation qualities they observed a differential effect on vessel formation and breakdown. ^{56}Fe-ions (high LET) were the most damaging to both developing and mature vessels followed by protons (high LET) and gamma rays (low LET) [74]. Using the same vessel model they have shown that high LET radiation induce more complex and persistent DNA repair foci (53BP1) compared to low LET radiation. It should be noted that they observed essentially the same kinetics of radiation-induced 53BP1 foci in the 3-D vessel model compared to 2-D monolayers. Overall, their findings point to a larger sensitivity of the 3-D vessel model to high LET radiation compared to low LET radiation [74].

Importance of 3-D systems for bystander effect

Bystander effect in the radiation biology is classically explained as a response observed in cells that have not been directly exposed to IR, but have been in contact with or received conditioned media from irradiated cells [54, 57, 59]. During the 20 years of bystander effect studies numerous 2-D experiments have been performed. However, when the importance of the observed effects (endpoints as DNA damage, micronucleation, apoptosis, and gene mutation) for example in radiotherapy or for imaging diagnostics exposures needs to be evaluated, the use of 3-D tissues became very important. Such studies have been performed by several groups [57, 59, 75] and have already been discussed in this review. The major questions in the bystander effects area are: what is the initial signal, how far could it be transmitted at tissue level and what non-tissue damaging agents could be applied to inhibit or modify this bystander signal. These questions have been addressed in many studies mainly performed with artificial 3-D epidermis [1, 51, 53, 69].

Limitations of 3-D models: Technical and methodological issues and troubleshooting

One of the main problems for the generation of 3-D models is the need to use non-transformed primary cells. These cells usually differentiate well into functional tissue at the appropriate conditions, but have the disadvantage of very short replicative capacity - for HFK (human foreskin keratinocytes) about 50, for NHBE approximately 15 population doublings. Respectively, the need of constant primary cell supply from human tissues creates an obstacle for the broad use of these models. Important breakthrough in this direction is the transfection of the cDNA encoding the catalytic subunit of human telomerase enzyme (hTERT) in the cells and in this way creating immortalized cell lines [76]. Cells with hTERT have typically normal features and ability to differentiate, but overcome senescence and have population doublings of about 100 (over three months in cell culture) [77]. Such models had been developed for bronchial epithelium [34] and also for epidermis (NOTE project, STUK's unpublished data) and have been successfully implemented into the radiation biology studies. Commercially available 3-D models (MatTek™, Ashland, USA; Epithelix, Geneva, Switzerland) seem to facilitate the use of 3-D models in radiation biology. Although the good differentiation characteristics of most of the models and their easy handling, some of them showed poor

reproducibility and morphology and did not express similar levels of differentiation markers (STUK, unpublished data). Experiments with EpiAirwayFT™ organotypic lung epithelial model were performed in STUK. Two separate batches of tissues in triplicates were irradiated with α-particles (from the STUK's ^{238}Pu source, 5.499 MeV) with average dose of 0.2 Gy or 1 Gy. Irradiations were performed on the apical site of the tissue. As a control 2-D cultures of human bronchial epithelial cell line BEAS-2B were exposed with the same doses. Following irradiation, cells were cultured for 24 hours and tissues for 1, 3, 5, 24 hour(s) and 3, 5 and 7 days. Cells and tissues were immunostained for DNA damage marker 53BP1. The tissues were subjected to morphological analysis, which included H&E staining and also immunofluorescence staining of differentiation markers cytokeratin 5/6 and cytokeratin 14 of formalin-fixed paraffin-embedded sections. Most of the tissues showed abnormal gland-like morphology in the central areas. The edges on the other hand had normal pseudo-stratified morphology. The gland-like structure was more prominent in the later time points of incubation (7 days). Although the BEAS-2B cells showed radiation-induced 53BP1 DNA-damage related foci, the EpiAirway™FT tissues revealed only few positive nuclei with one or two foci per nucleus in analyzed sections. No clear difference was noticed between irradiated and control tissues in any of the analyzed time points or doses (0.2 Gy, 1 Gy). Previous studies with human skin 3-D tissue models have shown that diminished focus induction has been associated with differentiated, non-proliferative cell types of the tissue constructs [55]. This may partially explain why we could not detect clear 53BP1 foci induction in the EpiAirway™FT tissues after particle exposure. Moreover, the analyzed two separate batches of tissues did not fulfill the morphological criteria for differentiated lung tissue. This suggests substantial problem with the initial set-up of the model, which additionally complicates the analysis and interpretation of results.

Another challenge shown from the 3-D models is the difficulty in precisely measuring the distance between irradiated and non-irradiated cells and areas, which is of crucial importance for the bystander experiments. STUK group's experience with bronchial 3-D model (EpiAir100, MatTek™) showed that it is very difficult to mark exactly the line of microbeam irradiation due to the plasticity of the tissues and uncertainties at the cutting procedure, while preparing the formalin-fixed paraffin embedded tissue blocks for further analyses. This could be overcome by the use of fluorescent markers as beads for labeling positions [5, 7, 48] in the tissue or implementation of more precise cutting and embedding procedures. Such example is the very precise customer designed microtome created for tissue experiments at the RARAF of Columbia University [75]. The blade of the microtome had 5 µm resolution and allowed cutting of 50 µm wide tissue stripes. The device had been used to study micronuclei formation in directly targeted and bystander cells. Another advantage in this experimental design has been precise marking of the irradiation point during the irradiation using 50 µm slot of the irradiation set-up. Further possibility is to divide the tissue sections into fields and since we know the length of the image under the microscope magnification to analyze only the ones that contain pre-defined number of fields. This approach has been used by STUK group in the NOTE project during tissue irradiation experiments performed at the LIPSION ion beam facility in Leipzig University. Irradiated area consisted of 121 irradiation positions creating a square shaped area 2x2 mm^2 (figure 3) and therefore, the side areas of the tissue contain un-irradiated cells e.g. bystander cells. Each point was irradiated with 2 α-particles ($^3He^{2+}$). The nearest analyzed area next to irradiated cells ranging from 5 to 290 mm (differs from sample to sample) away from the cutting line was considered to contain both irradiated and bystander cells. From each tissue, an area covering approximately 0.6 mm x 12 mm in the middle of each tissue was analyzed from full-length tissue sections (figure 3).

The whole tissue is about 12 mm in width and corresponds to 40 fields under the microscope (magnification 63X) (figure 3, bottom part). During the sample handling and analysis

process, it became evident that only part of the slides consists in full-length sections. To avoid the possibility that irradiated cells were analyzed instead of bystander cells, data were restricted to sections that contained at least 25 fields. Sections were divided into horizontal parts, i.e., to a center and borders. The border areas were considered to contain bystander cells. The borders were defined on both sides of a center area of either 10, 16 or 20 fields. Apoptosis was chosen as an endpoint for radio-sensitivity. The distance between irradiated and bystander area was managed to handle by this approach, however, the results from three experiments showed wide over dispersion. The basic level of apoptosis seemed to vary between the samples possible due to differences in the cell viability of the tissues even though commercially available samples generated from pooled donor cells have been used. As similar

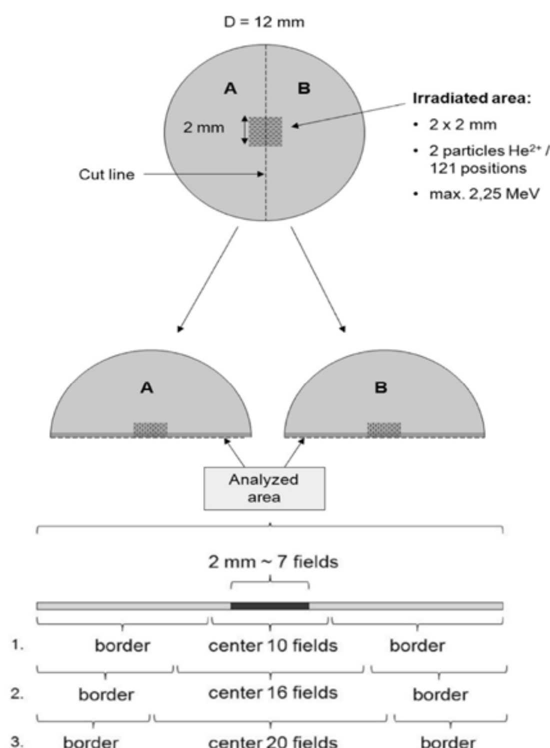

Figure 3. Experimental design of irradiations and schematic presentation of the analyzed areas. Tissues were cut into two halves as shown creating final tissue blocks A and B. Apoptotic cells were analyzed on both halves covering cells approximately 290 µm away from the cutting line (dark grey area). The irradiated area formed a 2 mm x 2 mm square in the middle of the tissue (black box). The border areas represent bystander cells.

variability of results have been encountered with other 3-D tissue models (e.g. epidermis), it is of high importance to identify the reasons for this divergence. Possible method for testing is to prepare 3-D models from several pooled samples and compare the results of the tissues to both radiation exposure and viability tests like MTT.

An additional point that should be mentioned regarding the 3-D models, especially the ones that develop at the air-liquid interface, is that they could have cells oxygenation levels that vary and also deviate considerably from the biological levels. The oxygen levels are very important factor that enhances the radiation damage [54]. A little could be done regarding modulation of this factor without affecting the differentiation abilities of the tissue. However, this problem could be potentially overcome by use of mathematical models for the oxygen distribution where normalization factors could be calculated and applied to the 3-D systems.

In the attempts to make the 3-D models maximally resemble the natural tissues, different co-culturing systems have been used such as collagen gels embedded with fibroblasts and epithelial cells plated on the gels [9, 3, 34]. These co-cultures, however, could be challenging if there is need to determine the separate responses of the different cells to irradiation, as the cultures are very plastic and it is not easy to precisely disintegrate them mechanically. Due to this fact, some of the widely used analysis techniques such as clonogenic survival or Western blotting may not be applicable to the multi-typic 3-D systems differential response studies.

Interpretation of results is also complex in some cases. For example, the role of angiogenesis in cardiovascular disease is a controversial issue. Stimulation of angiogenesis is regarded as a promising therapy for vascular diseases such as ischemic heart disease and peripheral arterial disease [78]. On the other hand, it has been suggested that angiogenesis of microvessels in an atherosclerotic plaque might contribute to plaque instability and thus rupture [72]. Therefore it is difficult to determine to which extent angiogenesis plays a role in radiation-induced cardiovascular disease and what the

effect of radiation on this process is. Moreover, also other radiation-induced effects as inflammatory responses have to be taken into account when looking at radiation-induced cardiovascular disease.

CONCLUSIONS AND PERSPECTIVES

In conclusion, we summarize that the 3-D models can be applied in radiobiology as suitable tissue *in vitro* models and that they have several advantages over the 2-D monolayer cultures traditionally applied in radiobiology. They allow studying the radiation response at tissue level on humanized models, which could give significant benefits over animal studies that face problems such as differences in genetics and radiosensitivity between animals used and humans. The recent 3-D models can be maintained in laboratory conditions for up to several months, which is of considerable advantage for studying long term or late effects of ionizing radiation. However, there still remains a wide variety of challenges on the standardization and reproducibility of the 3-D cultures, which limits their application. As for the standardization efforts, it is important to keep in mind that there is natural biological variability at individual and tissue level and the 3-D tissues are also derived from different donors and different tissue types.

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