# Development of a Spheroid Model of Prostate Cancer to Test the Activity of pPPE-CD

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**Introduction**

Tumour cell culture typically involves the growth of cells as a monolayer on the surface of a flask. Analysis of these cells increases our understanding of tumour biology and can be used to test the potential of various treatment strategies. However, it is now established that although informative, this artificial environment is not an ideal representation of an *in vivo* tumour.[1-3] The growth of tumour cell lines in this manner results in the loss of some characteristics and the exaggeration of others. For example, cell-cell communication, polarization and differentiation are reduced while proliferation and inflammatory responses are enhanced.[4] Crucially, this could have a significant impact on the drugs and treatments that are effective in two-dimensional (2D) cell culture; those that target proliferation and mitosis will be effective but not those affect cell-cell interaction or maturation, for example. Their efficacy *in vitro* may not reflect how these drugs will behave *in vivo*.

For many years it has been recognised that a useful alternative to the typical culture of cancer cells as an adherent monolayer is their growth in a three-dimensional (3D) cell system as multicellular tumour spheroids, which are spheres of densely packed cells.[5] This system has advantages over 2D cell culture and has potential to bridge the gap between in vitro cell growth and animal models. Any improvement in the artificial representation of tumour behaviour *in vitro* would be advantageous and would improve the ability to assess the potential of a new drug or treatment strategy early in its development. The capacity to rule out potential drugs using cell culture reduces costly, time-consuming research using *in vivo* tumour models. It would also be advantageous to drug screening experiments if the 3D cell culture technique was suitable for high throughput techniques and compatible with automated liquid handling systems.[3]

Various groups have compared the gene expression profiles of spheroids and the equivalent cells grown as a monolayer culture in different tumour types, for example, in ovarian[6], colon[7] and hepatocellular carcinoma cell lines[8]. There are differences between cells grown in 2D and 3D culture in genes that regulate proliferation, differentiation and treatment survival and resistance. Importantly, gene expression in the spheroid models has been observed to be closer than monolayer cell culture to that seen *in vivo*. In the case of prostate cancer, Harma *et al*. grew a range of cell lines in Matrigel and found that approximately 3400 mRNAs were differentially regulated in spheroids compared to monolayer cultures.[4] Genes involved in mRNA processing, mitochondrial and ribosomal activities and metabolic processes were downregulated reflecting the reduction in growth and proliferation in spheroids.

There are a variety of different techniques available for spheroid formation. For large scale production of numerous spheroids a spinner flask can be used to prevent adherence to the flask surface and maintain an even distribution of nutrients. Similar approaches involves roller flasks or gyratory shakers.[2] A disadvantage of this type of rotating cell culture is the use of large amounts of media which would necessitate high levels of the drug of interest to achieve the desired concentration. In the liquid overlay method, growth of spheroids on a smaller scale can be achieved by coating the bottom of a plate with agar before seeding the cells. This method has been

shown to result in spheroids of very consistent size but a disadvantage is the labour burden associated with individually coating each well before use.[2] The hanging-drop method prevents cell-plate interaction by pipetting a drop of cell suspension on to a plate surface which is then turned upside down. This is only suitable for short-tem culture of spheroids although Tung et al. recently developed a 384-well culture plate for hanging-drop spheroid formation which allows for the use of liquid-handling machines and long-term growth of spheroids.[3]

At a sufficient diameter (200 – 500 μm) spheroids are large enough to reduce the diffusion of glucose and oxygen resulting in a gradient in concentration from the edge to the centre.[1, 5] Morphological characteristics of spheroids typically involve a rim of healthy, proliferating cells (modelling cells in a tumour which have access to a blood supply) surrounding a core of quiescent cells which will become necrotic over time.[2] Spheroids can act as an in vitro model of micrometastases or regions of solid tumour lacking adequate vascularisation. [9]

The efficacy of various drugs has been compared between cancer cell lines grown as adherent cells and as spheroids. For example, Mellor *et al.* examined a range of chemotherapeutic drugs in a study of drug resistance in a colon adenocarcinoma cell line.[10] Growth of these cells as spheroids reduced proliferation and increased levels of the quiescence marker p27(kip1). They found that in comparison to the 2D model, in 3D, cell death after doxorubicin or cisplatin treatment was reduced and was lost for 5-FU and vinblastine. Similar results were observed by Friedrich *et al.* testing 5-FU, Irinotecan, and C-1311 using the acid phosphatase assay in HT29 and HCT-116 cells.[11] Variation in the efficacy of 5-FU was observed between 2D and 3D cultures of A431.H9 cells.[3] At a concentration of 10 μM, viability was reduced by 95% in monolayers but only by 25% in spheroids. Tung *et al.* suggest that because 5-FU targets proliferating cells, it will cause less damage to the slower growing cells within spheroids and may also have difficulty penetrating the spheroid. Spheroids have also been used to test combination treatments involving radiation and drugs and/or radiosensitizers, which is of interest for this research. For example, in HepG2 spheroids (a hepatocellular carcinoma cell line), supra-additive effects on spheroid outgrowth and toxicity were measured when 10 Gy radiation was combined with cisplatin, gemcitabin or 5-FU.[12]

**Aims and Objectives**

This research involves the use of a novel technique for 22Rv1 spheroid formation. It is a result of collaboration with Dr. Anthony Davies, Head of Irish National Centre for High Content Screening and Analysis in Trinity College Dublin and Scientific Director of Biocroí Ltd, where an advanced suspension media, Happy Cell (HC) has been developed. It was hypothesised that this would allow for the generation of spheroids and this chapter discusses the optimization of this technique and subsequent spheroid characterization. This model may reflect better the range of environmental conditions observed within an *in vivo* tumour and affords the opportunity to explore the effects of gene therapy in an *in vitro* system closer to the *in vivo* comparison.

**Aim:** Optimise formation of 22Rv1 spheroids using HC system.

**Aim:** Confirm formation of spheroids and characterise morphology.

**Aim:** Use the spheroids as a model in which to test pPPE-CD activity under conditions investigated in 2D culture chapters 4 and 5.

## Materials & Methods

### *Cell culture*

The HC media contained Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) FBS and 1% Penicillin/streptomycin and an additional additive. Following counting, the cells were added to the HC media at the desired concentration and resuspended gently to get an even distribution throughout the media. An incubation time of 48-72 hours resulted in spheroid formation. As a control, adherent 22Rv1 cells were grown in normal DMEM without the extra additive.

### *Confocal microscopy*

22Rv1 cells were seeded in an 8-chambered Lab-Tek chamber slide (Thermo Scientific) at a concentration of $1 \times 10^5$ cells/well in 500 µl HC media and incubated at 37ºC for 2 days to allow spheroid formation. 25 µl Hoechst 33342 dye (1 mg/ml, Sigma Aldrich) was added to each well. Propidium iodide (PI) was diluted 1:100 with PBS and 50 µl was added to the wells. A confocal microscope was used to image the spheroids.

### *Automated liquid handling*

Two different automatic liquid handling systems were used to dispense cell-containing media.

*Labcyte Echo 550*

22Rv1 cells were counted and a 300 µl cell suspension of $4 \times 10^6$ cells/ml was prepared in HC media and DMEM media. 5 µl of Hoechst dye was added to each. The Echo Liquid Handling System (Labcyte) uses sound waves to dispense set amounts (2.5 to 10,000 nl) of liquid onto a plate surface. The cell-containing media was gently mixed before 40 µl was placed in the source well of the Echo and used to dispense 100 nl into 24 wells of a 384-well plate at various time points (1, 5, 10 and 20 minutes). The number of cells in each well was counted using the IN Cell Analyzer 1000 high-content screening system and compared between HC media and normal DMEM media.

*Labcyte Equator*

A Deerac Fluidics Equator Eight-Tip Pipetting System (Promega) was used to dispense 200 nl of cell-containing HC media onto 'Nanowell' slides (plastic slides with wells that hold up to 400 nl of liquid). The wells were imaged after seeding and then again on subsequent days using the Cellavista Analyzer (Roche)

***PSA EIA***

22Rv1 cells were seeded in 24-well plates, $0.9 \times 10^5$ cells/well in 1 ml of media. Normal plates and DMEM media were used for adherent cells; suspension plates and HC media were used for spheroids. 3 days later, the media in half of the wells containing adherent cells was replaced with HC media. 200 µl of media was added to the wells, either containing DHT to give a final concentration of 1000 nM or with no DHT. After a further 3 days, the spheroid containing media was transferred to 1.5 ml tubes, and spun down to pellet the cells. The media was removed and stored for later PSA EIA. 100 µl of trypsin was added to the spheroids to break them up and allow MTT analysis of cell number for normalisation of PSA EIA results. 400 µl of DMEM media was added to the cells and they were transferred to a 24-well plate. As a control, the adherent cells were also trypsinised before the MTT assay. For these cells, the media was removed and stored in 1.5 ml tubes. The cells were washed with PBS, then 100 µl of trypsin was added to the cells and subsequently deactivated with DMEM media. 37.5 ul of MTT dye was added to all the wells and the assay was carried out as previously described.

***GFP transfection***

22Rv1 cells were seeded at a concentration of 1 ×cells in a T25 flask in DMEM media. The following day the cells were transfected with a GFP-expressing vector. After a further 24 hours, the cells were counted and seeded in 96-well plates (normal and suspension), $0.9 \times 10^4$ cells/well. Over the next week, the adherent cells and spheroids were imaged using an Olympus CKX41 fluorescent microscope and DP70 camera to track GFP expression.

***pPPE-CD/5-FC toxicity***

22Rv1 cells were seeded in T25 flasks and transfected with pPPE-CD or pCMV-CD, or mock transfected. The following day the cells were seeded in DMEM media in four normal 96-well plates and in HC media in four suspension plates at a concentration of $0.9 \times 10^4$ cells/well. The cells were incubated for 72 hours to allow spheroid formation. DHT or ethanol (negative control) was added to the wells by adding 20 µl of the appropriate media containing DHT or ethanol to give a final concentration of 1000 nM. The plates were placed in the incubator or hypoxia chamber and half the plates were treated with 2 Gy radiation two hours later. After a further 3 days the cells were treated with 5-FC by adding a further 20 µl of media to obtain a concentration of 1mM 5-FC and then incubated in air for 72 hours. The cell toxicity was analysed by staining with Hoechst and PI and imaging on a IN Cell Analyzer.

***High content screening***

*IN Cell Analyzer*

The IN Cell Analyzer 1000 (GE Healthcare, NJ, USA) was used for high-throughput automated image capture of the spheroids and adherent cells. Cells were imaged as live cells. For the spheroids, 20 µl Hoechst 33342 dye (1 mg/ml) and 10 µl PI (1 mg/ml) were added to each well. For the adherent 22Rv1 cells, 1 µl of each dye was used. Both cell cultures were incubated with the stains for 30 minutes.

***Cellavista Analyzer***

The nanowell plates, as well as some 96-well plates were imaged using the Cellavista Analyzer (Roche), an automated imaging system with brightfield and fluorescence imaging capabilities.

**Results**

***Optimisation of spheroid formation***

Following optimisation of cell number, initial experiments led to the formation of spheroids in both 96- and 24-well plates. However, a percentage of the 22Rv1 cells did not remain in suspension and stuck to the plate surface (Figure). To try to remedy this, a layering technique was attempted during cell seeding. 100 µl of HC media was placed in the wells of a 96-well plate, followed by another 50 µl of cell containing HC media, which was gently layered on top. As a control, in another set of wells, the second lot of media was mixed with the first. Concentrations of $4 \times 10^4 - 8 \times 10^4$ cells/well were found to be optimal. This layering technique greatly reduced the number of adherent cells and therefore resulted in increased spheroid number (Figure 1). However, it did not eliminate the problem. Subsequently, spheroids were grown in Hydrocell low cell binding plates (Nunc) and this overcame the problem of concurrent spheroid and adherent cell growth (Figure 2).

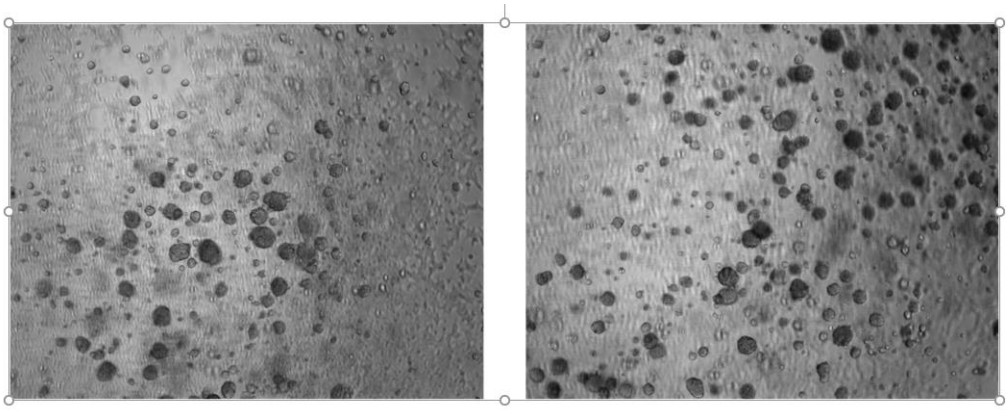

**Figure 1:** 22Rv1 cells seeded in HC media demonstrating the combination of spheroid formation and adherence of cells. Bright field images taken using an IN cell Analyzer 1000 at 4 × magnification.

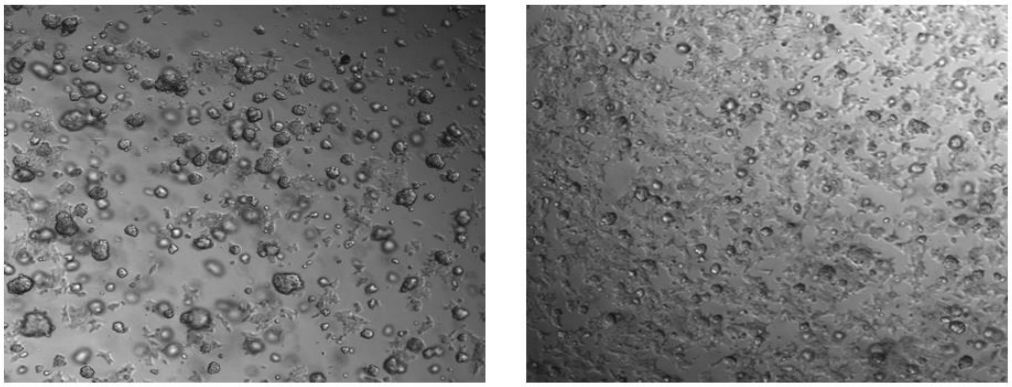

**Figure 1:** Left: 22Rv1 cells seeded in HC media using a 'layering' technique to reduce cell adherence. Right: a well in which the cells were mixed upon addition to HC media. Bright field images taken using an IN cell Analyzer 1000 at 4 × magnification.

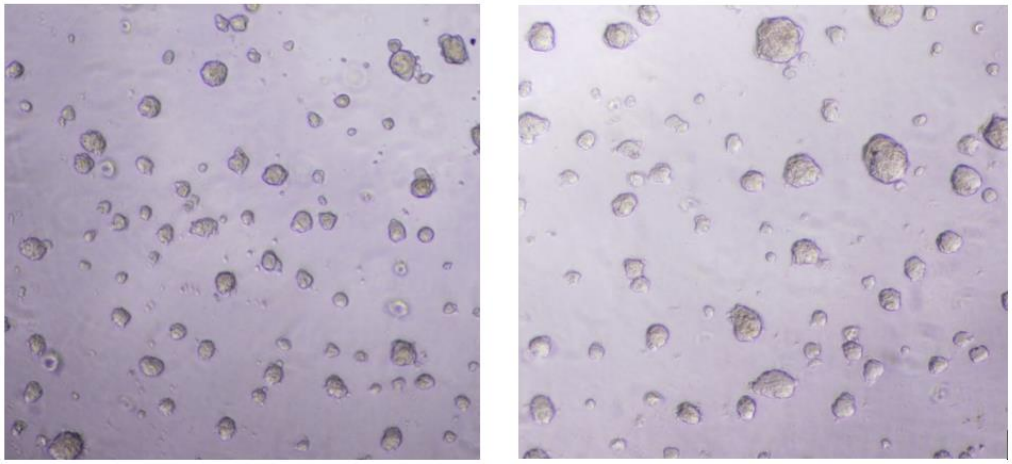

**Figure 2:** 22Rv1 cells seeded in HC media in low attachment 96-well plates. Bright field images taken using an Olympus CKX41 fluorescent microscope at 4 × magnification.

***Spheroids vs. aggregates***

One distinguishing feature of spheroids as opposed to cell 'aggregates' (loose bundles of cells) is their robustness.[1] 22Rv1 cells were transfected with a GFP-expressing vector before being seeded in a 24-well plate in HC media. 3 days later, a p1000 pipette was used to transfer 100 µl of spheroid containing media to the wells of a fresh 96-well plate which was then imaged with the IN Cell Analyzer (

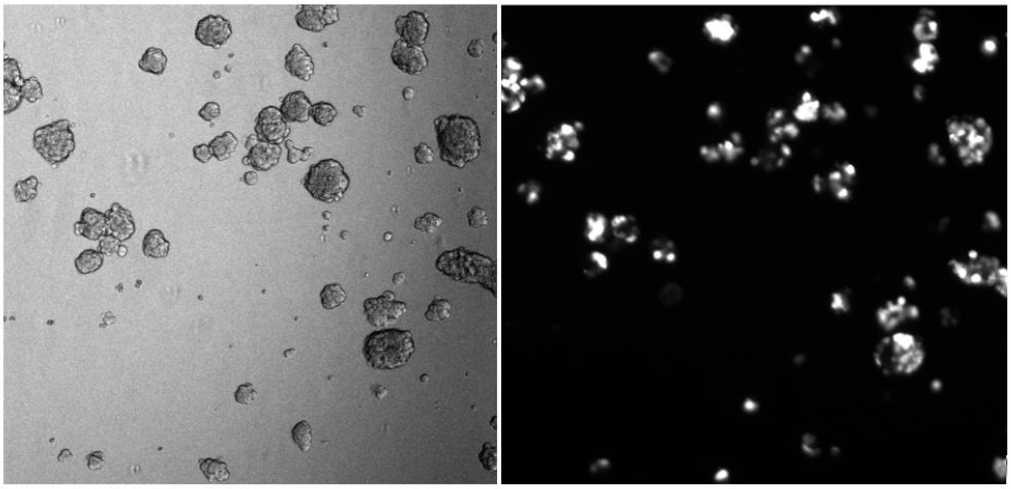

Figure 3). This demonstrated that the spheroids can be manipulated and remain intact when transferred from well-to-well.

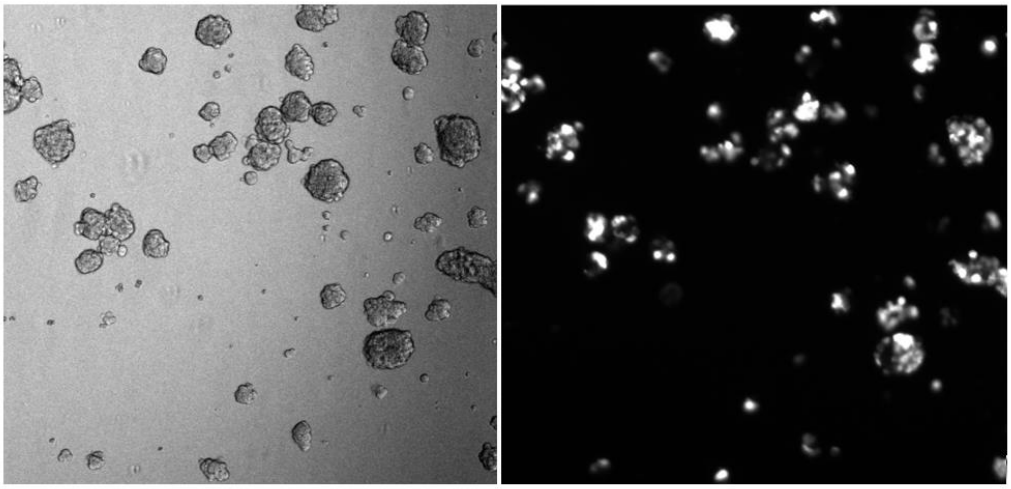

**Figure 3:** GFP-transfected 22Rv1 spheroids transferred from a 24-well to 96-well plate before imaging using high-content screening an IN cell Analyzer 1000 at 10 × magnification on the bright field setting (left) and fluorescent setting (right)

***Morphological assessment of spheroids***

Following optimisation, and successful spheroid formation, confocal microscopy was used to examine the spheroids using the fluorescent dyes Hoechst and PI. Hoechst, a nuclear stain, showed intact nuclei and confirmed that the cells were healthy and not clumps of dead cells (Figure 4). PI is a membrane impermeant dye that binds to DNA and can be used to detect dead cells. PI staining was mainly restricted to the spheroid core (Figure 5).

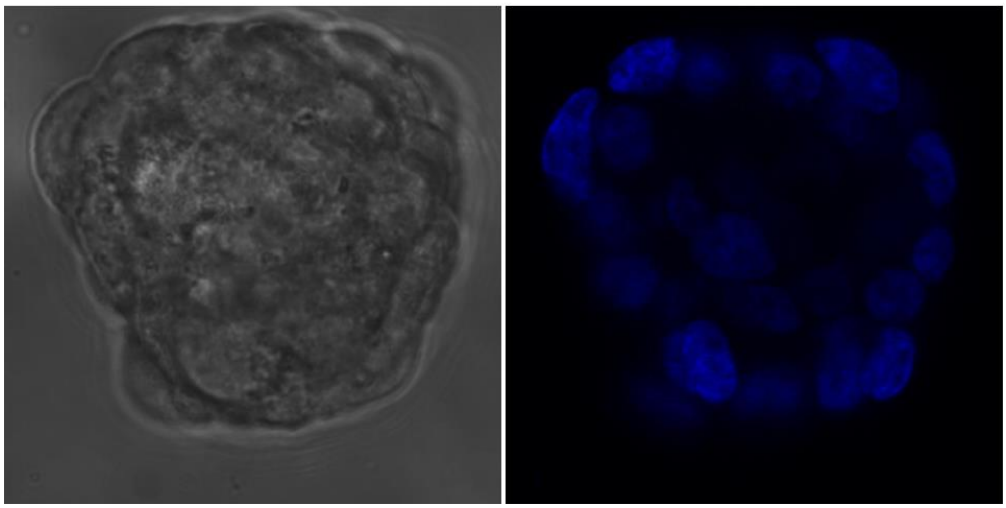

**Figure 4**: A representative spheroid stained with Hoechst and imaged at 40 × magnification using confocal microscopy on the bright field setting (left) and fluorescent setting (right).

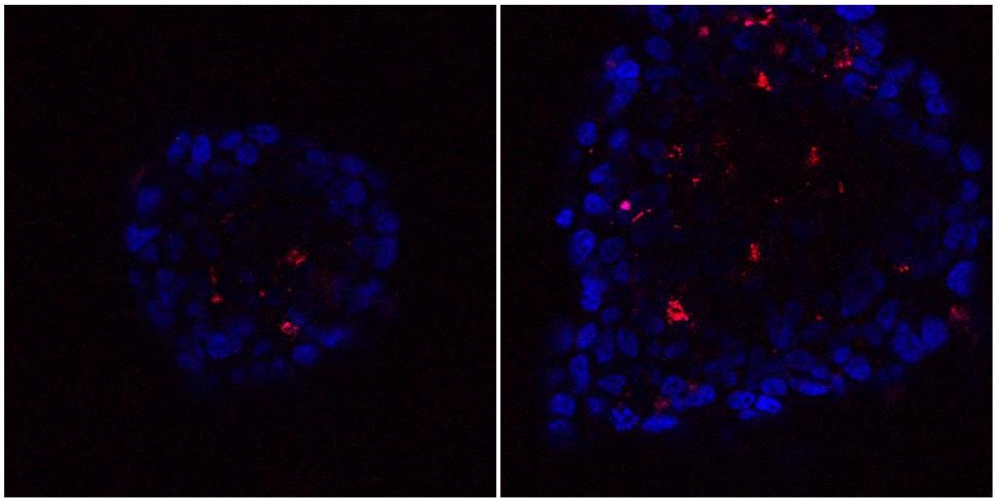

**Figure 5:** Representative spheroids stained with Hoechst and PI at 40 × magnification using fluorescent confocal microscopy.

***Mechanism of spheroid formation***

To try to elucidate whether spheroid formation is a result of cell division or aggregation, the 22Rv1 cells were seeded in HC media in 96-well plates, imaged immediately (Day 0) and then tracked over a number of days using the Innovatis Cellavista imaging machine (Figure 7). From these results it appears that initially, between time of seeding and 24 hours later (Day 1), the cells aggregate to form clumps. However the resulting spheroids increase in size between day 2 and 5, suggesting there is subsequent cell division within spheroids.

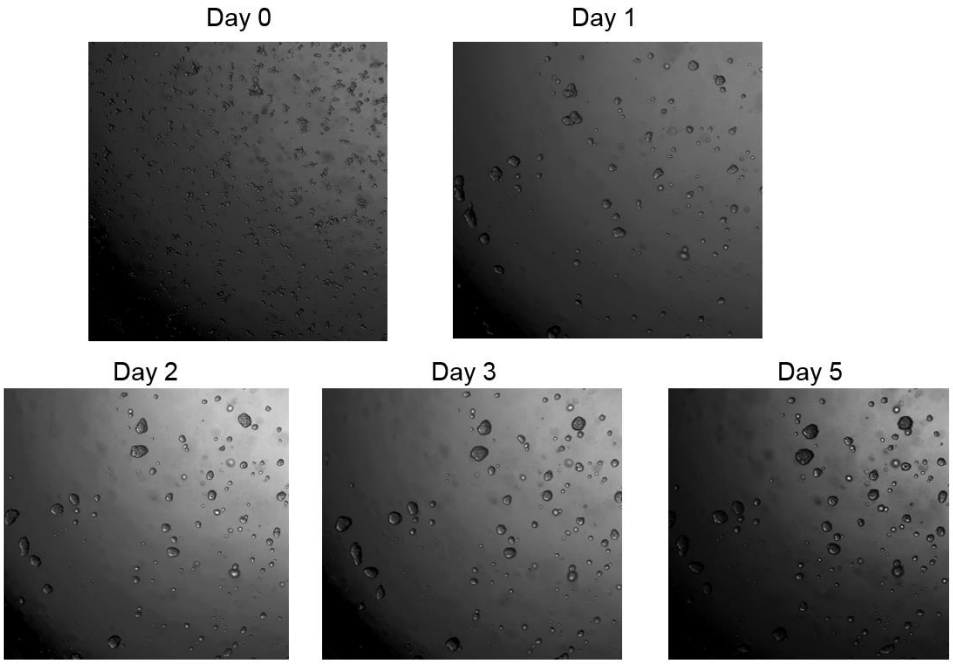


**Figure** Error! No text of specified style in document.**:** 22Rv1 cells seeded in HC media in a 96-well plate and imaged at 4 × magnification over 5 days using the bright field setting on the Cellavista Analyzer.

**Potential for use in high-throughput technology**

An experiment was carried out using the Echo Liquid Handling System to examine the potential for the use of HC media with automatic liquid handling robots. An equal number of Hoechst-stained cells in HC media or normal DMEM media was placed in the source well of the Echo and seeded in a 384-well plate at different time points before cell counting using high-content screening . This was carried out to compare the consistency in cell numbers over time, which can change due to sinking of the cells in the source well. For HC media there was a steady high level of cell seeding at 1, 5 and 10 minutes with a slight drop off at 20 minutes (

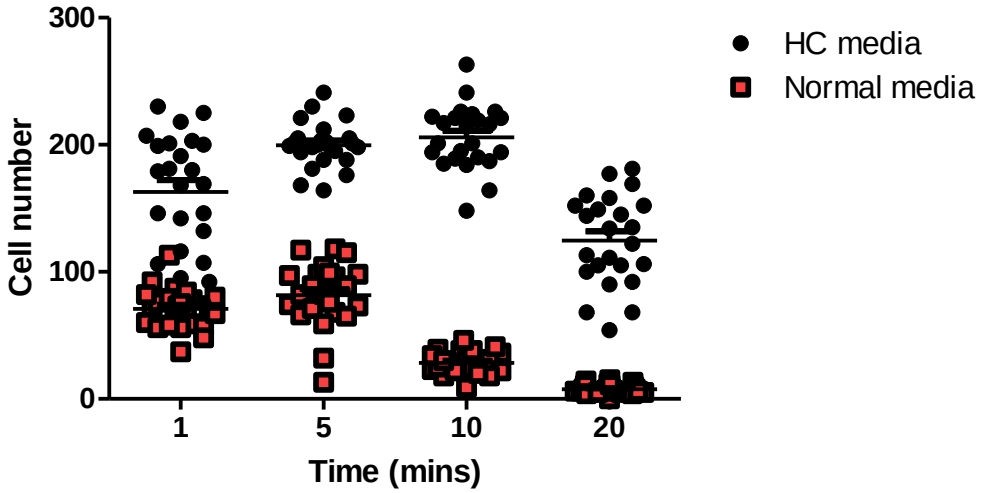


Figure 6 ). The levels of cell seeded for DMEM media was much lower and decreased with successive time points so that there were very few cells in the wells seeded after 20 minutes. These results suggest HC media could help avoid the problem of cells sinking in the original cell suspension, which can affect cell seeding density over time when automated liquid handling systems are used.

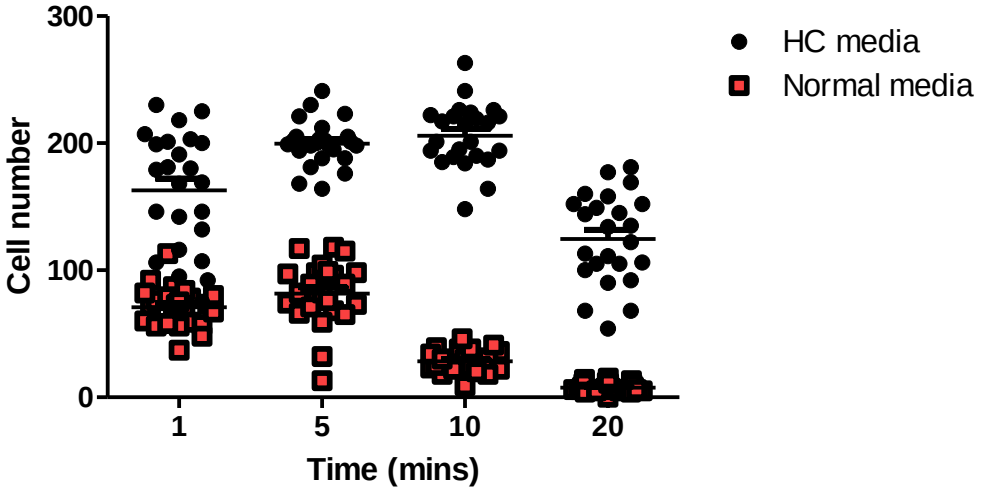


**Figure 6:** Cell number per well after automatic liquid dispensing at different time points using a cell suspension of HC media and normal DMEM media.

The possibility of high-throughput testing using the spheroid model was further explored. 'Nanowells' were used and the cells were dispensed in 200 nl of HC media using the Deerac Fluidics Equator Eight-Tip Pipetting System (Figure 9). The technique was optimised for cell number as well as various other parameters. Nanowells show the potential of this technique to be used in high-throughput

setting and demonstrate spheroid formation using cells dispensed by liquid handling robots.

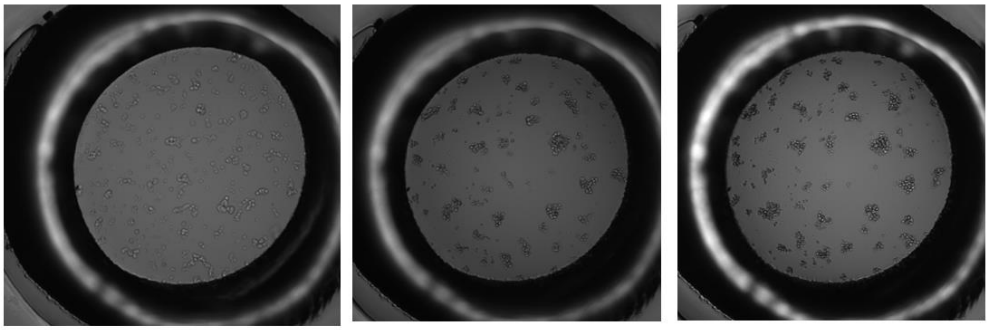


**Figure 7:** 22Rv1 cells in a well of a nanowell slide, imaged over the course of 3 days using the bright field setting on a Cellavista Analyzer at 4 × magnification.

***PSA EIA***

A PSA EIA was use to compare the levels of PSA secretion from the adherent 22Rv1 cells and those grown as spheroids. A third group that was tested was 22Rv1 cells that were allowed to adhere to the plate before the normal DMEM media was replaced with HC media. This was to control for any possible influence the HC media itself may have on PSA secretion. The results of the PSA EIA showed that the level of PSA secreted from the spheroids is significantly higher than for the adherent cells (Figure 8). In the absence of DHT there are detectable amounts of PSA in the media which are significantly higher for the spheroids ($p < 0.05$). When the cells are stimulated with 1000 nM DHT, the levels of PSA increase for all cell types but, again, are significantly higher for the 22Rv1 spheroids ($p < 0.05$).

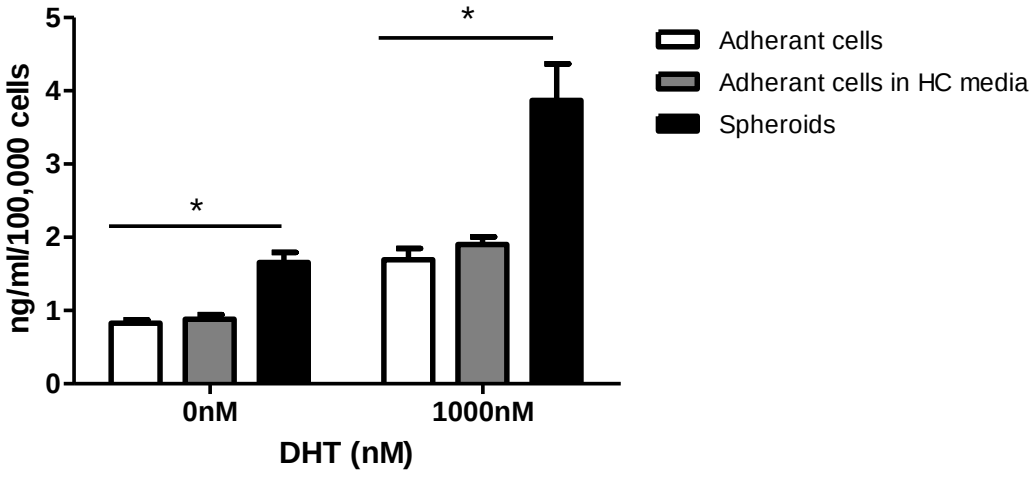

**Figure 8:** PSA protein levels following DHT stimulation in 22Rv1 adherent cells and spheroids. Values represent the mean of three replicates ± standard error of the mean. *, $p < 0.05$.

***GFP transfection***

Before pPPE-CD transfection of 22Rv1 and the growth of these cells as spheroids, a GFP-expressing vector was used to test whether the transfected cells would form spheroids and how long the expression would last. 22Rv1 cells were transfected with the vector and the next day were seeded in a 96-well plate (normal or suspension). The cells were tracked over the course of a week. The transfection rate was approximately 50%. Cells grown in HC formed spheroids and the GFP expression remained strong throughout the course of the experiment (Figure).

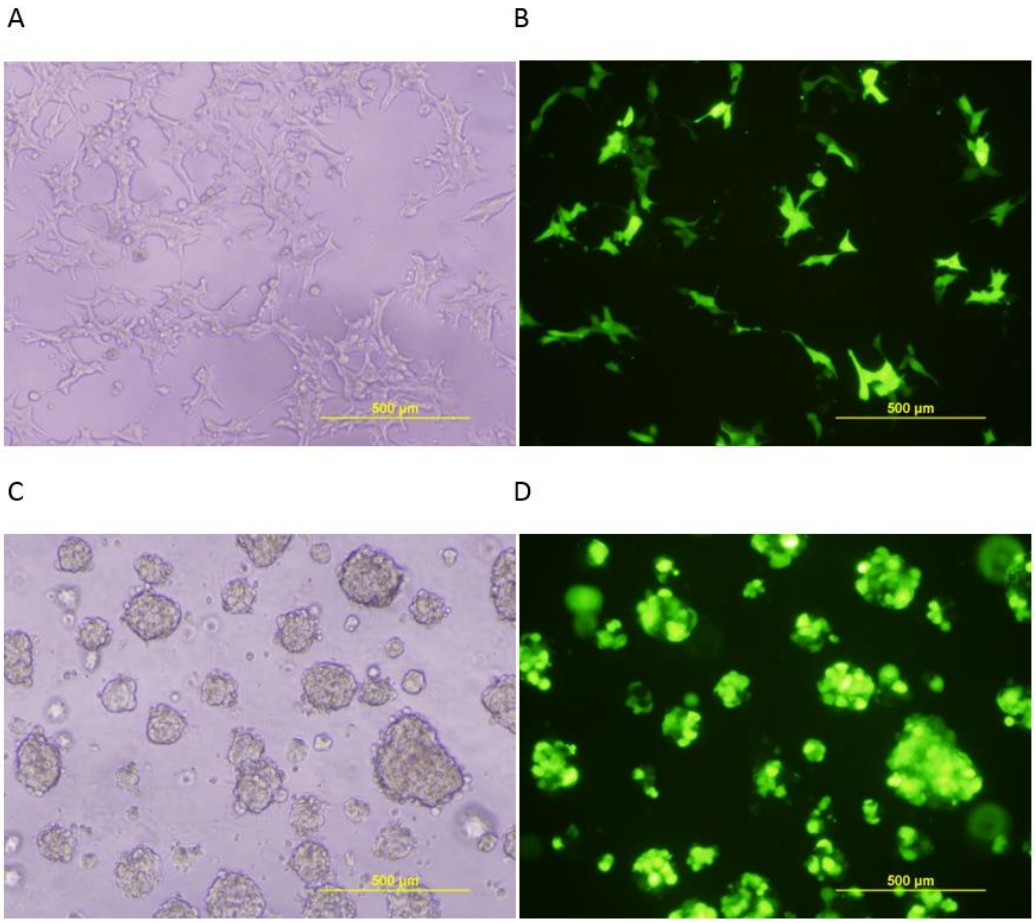


**Figure 11:** GFP-transfected 22Rv1 cells grown as adherent cells (A & B) and spheroids (C & D) 5 days after seeding. Images were taken with an Olympus CKX41 fluorescent microscope at 20× magnification using brightfield (A & C) or fluorescent (B & D) microscopy. Scale bar represents 500 µm.

***Cell viability following pPPE-CD/5-FC treatment***

Following characterisation, spheroids were used as a model to further test the activity of pPPE-CD. 22Rv1 cells were transfected with pPPE-CD or the constitutively active vector, pCMV-CD, or mock transfected as a negative control. The following day the cells were reseeded for growth as spheroids. Cells were also grown as an adherent monolayer and treated in the same manner as the spheroids throughout the experiment to compare the two models. The cells were subsequently treated with DHT and/or 2 Gy radiation under aerobic or hypoxic conditions and three days later with 5-FC. The cells were stained with Hoechst and high-content screening was used to assess the efficacy of pPPE-CD/5-FC treatment.

***Adherent 22Rv1 cells***

For the adherent 22Rv1 cells, the high-content images were analysed to measure cell number, which were compared between those treated with 5-FC and those without for the different transfectants under the various conditions. For those cells grown in air with and without DHT, there was no significant difference for untransfected cells or those transfected with pPPE-CD or pCMV-CD (Figure 9). However, when radiation was combined with DHT stimulation under aerobic conditions, the decrease in cell number for the pPPE-CD transfected cells treated with 5-FC, compared to untreated was significant ($p < 0.01$, Figure 10**).** This significant change in cell number in response to 5-FC treatment was not observed for the untransfected cells. Radiation treatment alone, without DHT, was not sufficient to achieve significant cell kill in pPPE-CD-22Rv1 (Figure 10**).**

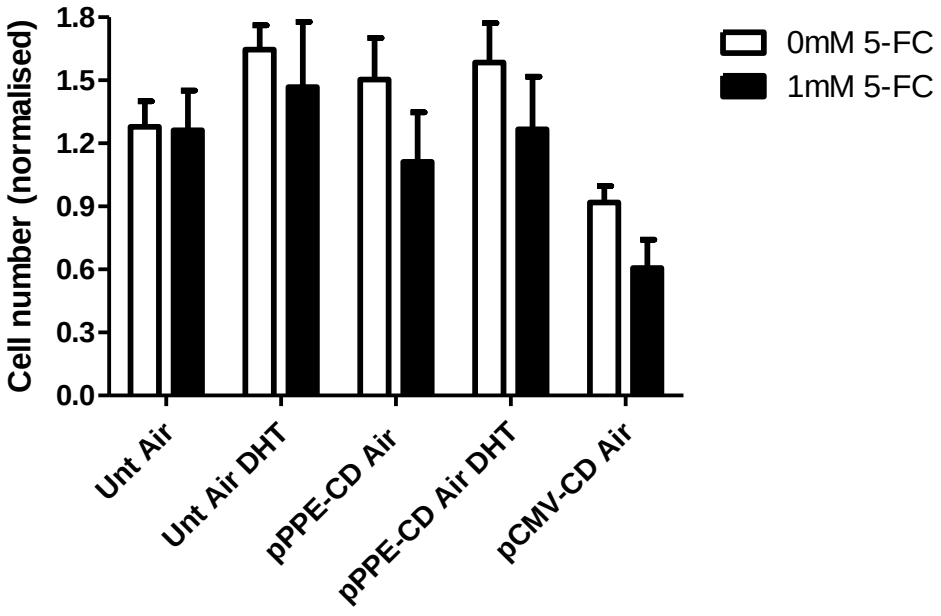


**Figure 9:** 22Rv1 cell count after treatment in air with and without DHT. Values represent the mean ± standard error of the mean. Unt, untransfected.

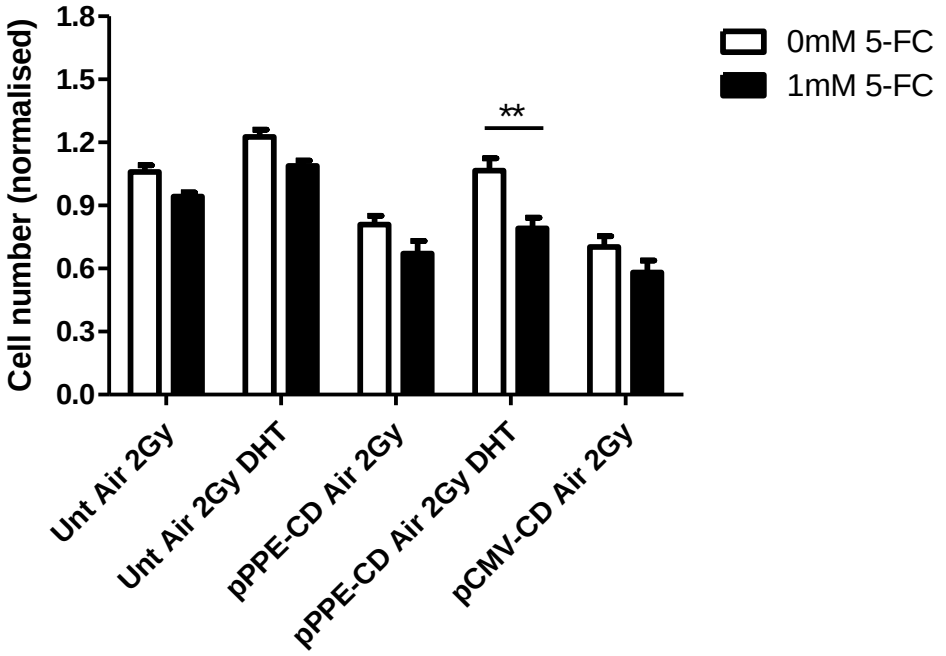


**Figure 10:** 22Rv1 cell count after treatment in air with radiation and with and without DHT. Values represent the mean ± standard error of the mean. Unt, untransfected; **, $p < 0.01$.

For cells grown under hypoxic conditions, both hypoxia alone and hypoxia in combination with DHT treatment resulted in a significant decrease in the number of pPPE-CD transfectants with 1 mM 5-FC treatment, which was not the case for untransfected cells ($p < 0.001$, Figure 11). The number of pCMV-CD transfected cells also decreased significantly with 5-FC treatment ($p < 0.01$). When hypoxia treatment was combined with radiation, 5-FC treatment also resulted in a significant decrease in cells expressing pPPE-CD but not untransfected cells or pCMV-CD transfectants. This was the case both with and without DHT stimulation ($p < 0.01$, Figure 12).

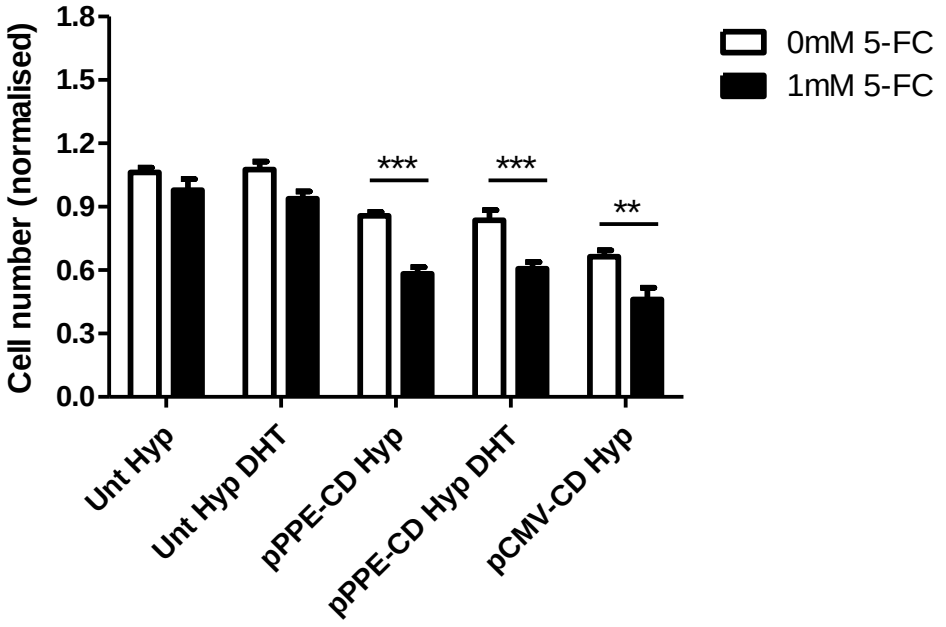


**Figure 11:** 22Rv1 cell count after treatment in hypoxia with and without DHT. Values represent the mean ± standard error of the mean. Unt, untransfected; ***, $p < 0.001$; **, $p < 0.01$.

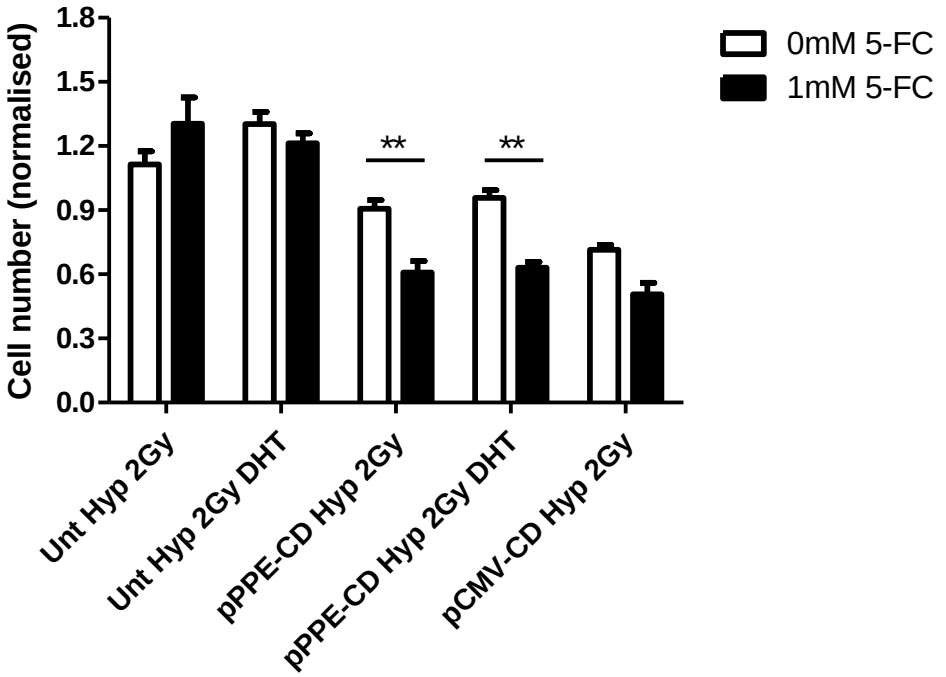


**Figure 12:** 22Rv1 cell count after treatment in hypoxia with radiation and with and without DHT. Values represent the mean ± standard error of the mean. Unt, untransfected; **, $p < 0.01$.

***22Rv1 spheroids***

A number of parameters were analysed to assess the efficacy of pPPE-CD/5-FC treatment in the spheroid model of 22Rv1. There was no significant difference in spheroid number with 5-FC treatment for any of the cell types (untransfected, pPPE-CD or pCMV-CD transfected) under the various growth conditions. Spheroid size was also analysed. A significant decrease in the average spheroid size was observed for pPPE-CD spheroids grown in air and treated with 1mM 5-FC compared to the equivalent samples without 5-FC ($p < 0.001$, Figure 13). 5-FC treatment under aerobic conditions resulted in a significant decrease in pCMV-CD spheroid size also ( $p < 0.05$) but this was not the case for untransfected cells. For spheroids grown under aerobic conditions with 2 Gy radiation treatment, there was a significant decrease in average size for radiation alone ($p < 0.05$) and the degree of significance increased for 2 Gy and DHT treatment combined ($p < 0.01$, Figure 14). Under hypoxic conditions, there was a significant decrease in the average spheroid size for pPPE-CD spheroids treated with 5-FC ($p < 0.5$, Figure 15). When hypoxia treatment was combined with radiation, the change in spheroid size in response to 5-FC no longer reached significance (Figure 16).

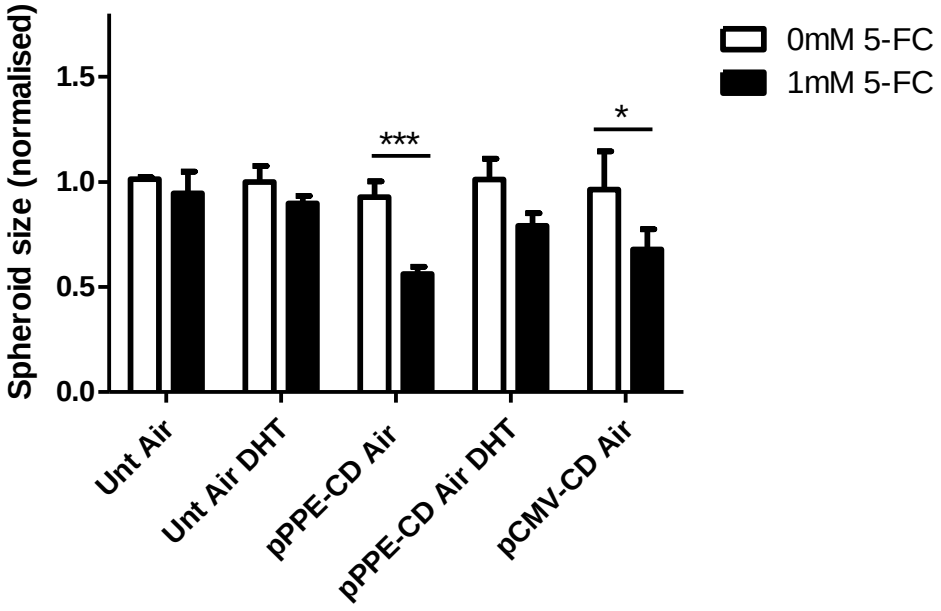


**Figure 13:** 22Rv1 spheroid size after treatment in air with and without DHT. Values represent the mean ± standard error of the mean. Unt, untransfected; ***, $p < 0.001$; *, $p < 0.5$.

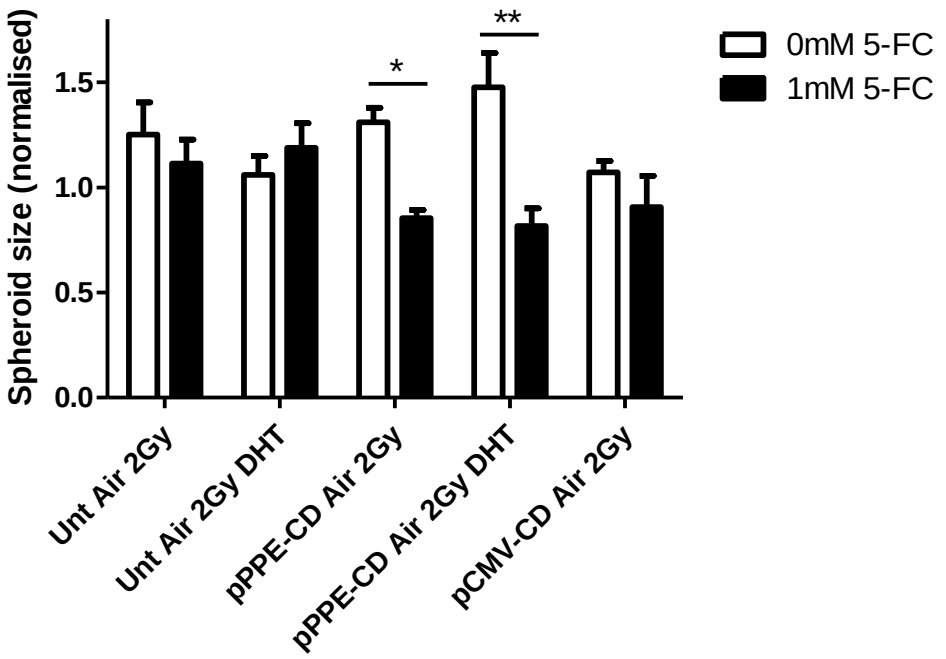


**Figure 14:** 22Rv1 spheroid size after treatment in air with radiation and with and without DHT. Values represent the mean ± standard error of the mean. Unt, untransfected; **, $p < 0.01$; *, $p < 0.5$.

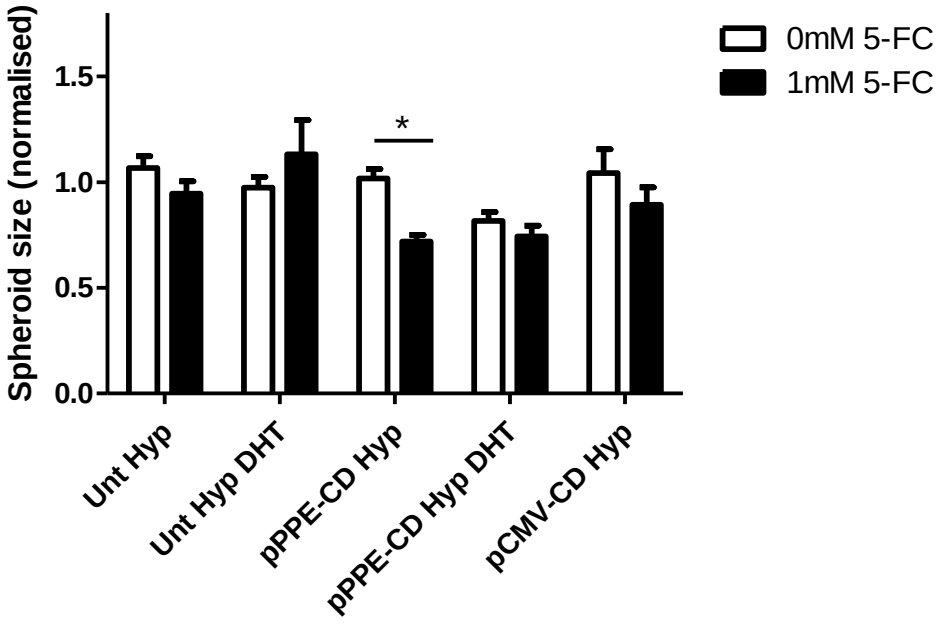


**Figure 15:** 22Rv1 spheroid size after treatment in hypoxia with and without DHT. Values represent the mean ± standard error of the mean. Unt, untransfected; *, $p < 0.5$.

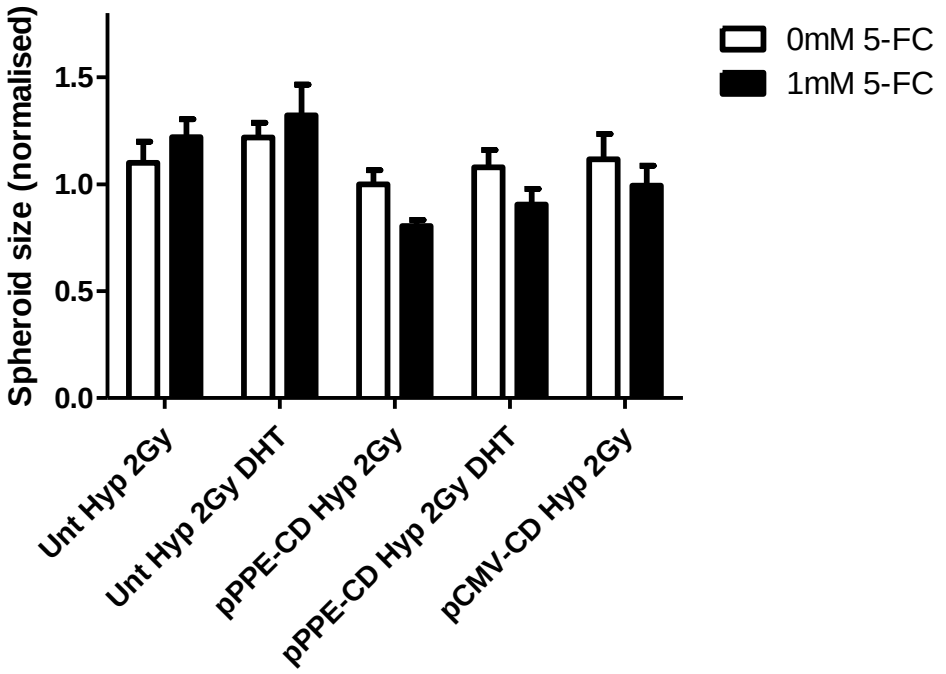


**Figure 16:** 22Rv1 spheroid size after treatment in hypoxia with radiation and with and without DHT. Values represent the mean ± standard error of the mean. Unt, untransfected.

**Discussion**

This research describes a novel technique for the formation of tumour spheroids. It has long been believed that growing tumour cell lines as spheroids is an improved model that is closer to the conditions *in vivo* compared to their growth as adherent monolayers. Despite this, their use has not become widespread and possible barriers may include the difficult and time-consuming nature of some of the current techniques and the need for specialist equipment. Also, if use of spheroids is to become standard practice for high-throughput screening, the method of formation needs to have ease of use and scalability. This technique for spheroid formation goes some way to addressing these issues. Once optimised, spheroid formation was as straightforward and simple as growing cells as a monolayer. Some techniques had to be adapted as the cells were in suspension in the media, meaning the media could not be removed and replaced as it would be with monolayer cell culture. Instead, drug-containing media was layered on top of the existing media to allow diffusion. This same method was used for the control 22Rv1 cells grown with the typical 2D cell culture.

It was important to establish that the cells had indeed formed spheroids and not aggregates (loosely packed bundles) of cells which lack cell-cell and cell-matrix interactions and will not have the pathophysiological gradients seen in genuine spheroids.[1] Unfortunately there are inconsistencies in the literature in the nomenclature used, with spheroids also being called spheres, organoids and tumoroids, but more importantly so-called spheroids actually being aggregates. Aggregates easily fall apart and cannot be manipulated. The 22Rv1 spheroids developed in this chapter remained intact when transferred from well to well and did not break apart easily.

As spheroids increase in size they develop a core of necrotic cells, something which models the situation *in vivo*, in which the peripheral cells of a tumour have access to nutrients and oxygen, whereas those further from a blood supply may die. In initial confocal microscopy, the stain propidium iodide, a membrane impermeant DNA dye, was restricted to the inner cells of the spheroid, suggesting they were necrotic. The nuclear dye Hoechst, showed the cells were healthy on the periphery of the spheroids and further confirmed the densely packed structure of the spheroids.

At sizes of 200 – 500 µm, chemical gradients (in oxygen or nutrients for example) will occur in the spheroids.[1] In the case of the 22Rv1 spheroids developed during the course of this research, their size suggests they would be large enough for chemical gradients to develop. Future work will involve using hypoxia markers, such as pimonidazole, to test this hypothesis. If these spheroids have gradients of oxygen concentration, it may not be necessary to use the hypoxia chamber and the model would be closer to *in vivo* conditions where levels of hypoxia vary in different parts of the tumour.[5] Khaitan *et al.* described spheroids as being "ideally suited for developing novel therapies that…exploit the endogenous oxidative stress in tumours." [5]

The cells were tracked from the point of seeding in HC media, to try to elucidate the mechanism of spheroid formation. Between time of seeding and 24 hours later the

number of individual cells had greatly decreased and the wells contained spheres of cells packed together suggesting that the cells travel through the media and aggregate in initial spheroid formation. Over the following days the spheroids became larger and more rounded suggesting cell division may also play a role. It would be informative to image the wells at shorter and more frequent intervals between seeding and Day 1 to gain more information on the movement of cells though the HC media. It may also be useful to assay secreted proteins in the media to help elucidate molecular cell signalling mechanisms behind spheroid formation and maintenance.

The experiments using high-throughput technology for automatic liquid handling were preliminary but were encouraging. HC media seems to be an improvement on normal DMEM media when it comes to the issue of cells sinking in the source cell suspension, resulting in decreased cell numbers over time during automatic liquid dispensing. Nanowell slides were used to test the capacity for spheroid formation on a nanolitre scale, which would be conducive to high throughput drug screening as it would allow large scale testing using very small amounts of the drug of interest. HC media and 22Rv1 spheroids showed potential in this regard and future work could involve testing established drugs in this setting to further develop this technique.

Previous attempts to increase PSA expression in 22Rv1 cells through the overexpression of the androgen receptor were not successful (Chapter 6). Before using the 22Rv1 spheroids as a model in which to test the pPPE-CD/5-FC system, levels of secreted PSA were measured to determine whether the growth of the cells in 3D cell culture had an effect on PSA expression. The cells were seeded and following spheroid formation, DHT was added to half of the wells, with ethanol added to the other half. Adherent cells were used as a control, with one set grown in HC media from the point of DHT addition onwards, to ensure any difference in PSA levels was due to growth as spheroids, not the media itself. Secreted PSA levels were measured after 3 days of DHT treatment and normalised to cell number to control for any disparity in cell proliferation rates between the 2D and 3D cultures. In the absence of DHT, the baseline level of PSA was significantly higher for the spheroids compared to the adherent cells ($p < 0.05$) and this increase was not seen for the adherent cells grown with HC media. With the addition of 1000 nM DHT, the PSA levels approximately doubled for both adherent cells and spheroids. Once again, PSA secretion was significantly higher for the spheroids ($p < 0.05$). The level of PSA for the spheroids with no DHT treatment was as high as the level of PSA for adherent cells with DHT treatment. This was an encouraging result, suggesting not only that 22Rv1 spheroids may be an improved model in which to test pPPE-CD but also that they may better reflect the PSA levels of primary tumour cell *in vivo* and be a good *in vitro* system in general.

The media used, both for spheroids and adherent cells, contained normal FBS, not charcoal-dextran stripped FBS as the HC media was a prototype and was still under development. Although this may have affected the levels of PSA produced by the cells, the adherent cells were grown in complete DMEM media as well as the spheroids, with the only difference being the additional additive in the HC media.

Therefore the significantly higher levels of PSA secreted by the spheroids should not be a result of any difference in hormone concentration between the normal and HC media. To further confirm this, future experiments will use HC media developed using CD-FBS.

The exact reasons for the higher PSA protein levels detected for spheroid samples are not clear. Differences in gene expression are quite likely, considering this has been demonstrated to be the case for a range of tumour types, including prostate, when spheroids were compared to the equivalent adhernent cells.[4, 6-8] It would be informative to examine genome-wide mRNA expression levels and their differences between 22Rv1 spheroids and adherent cells with and without DHT stimulation. This could be achieved through microarray transcription analysis which would involve a hybridisation-based pairwise assay of relative transcript expression levels between the two samples. Genome-wide expression arrays exist, which could give information about the expression levels of ~20,000 transcripts. Alternatively, genome-wide mRNA expression levels could be assayed through next-generation sequencing using RNAseq, which has the benefit over microarray-based methods that novel transcripts or those not included in the microarray design can be directly assayed.[13] These experiments would be informative as they would give detailed information on the genes up- and downregulated in spheroids compared to adherent 22Rv1, as well as in response to DHT treatment. Gene ontology (GO) and pathway (KEGG) analysis of overrepresented categories in the differentially expressed gene group would provide useful information and would help elucidate possible reasons for higher PSA expression levels in spheroids.

Transfection of 22Rv1 cells with a GFP-expressing vector, followed by growth in HC media, demonstrated that transfection of the cells did not affect their ability to form spheroids. The cells were tracked for up to 7 days after spheroid formation to ensure the GFP expression was still strong as this indicated that pPPE-CD expression would last sufficient time to allow growth of the spheroids after transfection followed by treatment of the cells with DHT/hypoxia/radiation, followed by 5-FC.

The activity of the pPPE-CD/5-FC system was tested in spheroids alongside adherent cells using Hoechst staining and high-content screening to assess the change in cell number with treatment. The results for the adherent cells showed the efficacy of this treatment strategy again, following on from the MTT and clonogenics results from Chapters 3 and 4. The number of cells were normalised within each replicate and the number of cells were compared between equivalent samples with and without 5-FC. For pPPE-CD cells grown in air, treated with DHT or 2 Gy radiation prior to 5-FC, the decrease in cell number did not reach significance compared to the samples without 5-FC. However, when DHT and radiation treatment were combined, the difference was significant ($p < 0.01$), serving as evidence for the benefit of a combined treatment strategy. pPPE-CD-22Rv1 grown in hypoxia alone, as well as in hypoxia with DHT, showed a significant decrease in cell number with 5-FC treatment ($p < 0.001$). Hypoxia activates pPPE-CD activity, resulting in 5-FC to 5-FU conversion and consequent cell toxicity. This decrease in cell number was also seen for cells grown in hypoxia with or without DHT that were irradiated ($p < 0.01$). These results are further confirmation of the increase efficacy of pPPE-CD under

hypoxic conditions and the potential benefits of a combined treatment strategy involving radiation and the pPPE-CD/5-FC system.

For the spheroid analysis, two parameters were measured, spheroid number and spheroid size, using the same Hoechst staining and high-content screening protocol as was used for the adherent cells. The pPPE-CD transfected spheroids showed no difference in number between 5-FC treated and untreated samples for any of the growth conditions. This was not surprising as the spheroids were allowed to form before their growth under various conditions (hypoxia, DHT) followed by 5-FC treatment. There were differences in spheroid size, however, with pPPE-CD/5-FC toxicity resulting in a significant decrease in size for spheroids grown in air and hypoxia. Interestingly, the difference did not reach significance if the spheroids were grown in air or hypoxia with DHT. When PSA levels were measured, the baseline expression for the cells grown in spheroids was as high as expression for the adherent cells treated with 1000 nM DHT. Therefore, it is not surprising that pPPE-CD was activated without DHT stimulation in this model. It is unclear why stimulation with DHT would not further increase the activity of pPPE-CD. Treatment of the aerobic spheroids with 2Gy radiation with and without DHT, also resulted in a significant decrease in spheroid size for the 5-FC treated samples. These results indicate that pPPE-CD/5-FC treatment can result in cell toxicity in a spheroid model and therefore shows potential in a system closer to *in vivo* conditions than cells grown in 2D cell culture.

There is debate over the most suitable and informative endpoint to measure after spheroid treatment and a range of different techniques have been used. An automated approach has definite advantages, especially for large sale drug screening. Changes in spheroid volume and growth delay after treatment are often measured and can be achieved using software capable of such image analysis. Automated screening was used during this project and measurement of spheroid size served as a straightforward indicator of degree of toxicity. Other groups have used the MTT assay, which was shown to be more sensitive than the lactate dehydrogenase (LDH) assay, cell growth or DNA assay.[14] However this has mainly been tested in the context of smaller spheroids (less than 200 µm). [2] A range of different assays commonly used in the study of monolayer cultures were compared for their suitability in spheroid analysis using the colon carcinoma lines HT29 and HCT-116. [11] It was found that the acid phosphatase assay is suitable as it does not require spheroid dissociation and it is linear and sensitive across different spheroid sizes. Future work could involve the use of this assay to further test the activity of pPPE-CD in prostate cancer spheroids.

5-FU, the toxic form of the prodrug used in this research, is a chemotherapeutic drug whose activity has been tested in spheroid models of cancer and compared to the equivalent 2D models. As discussed previously, the efficacy of 5-FU is reduced in 3D cell culture, the likely reason being the lower proliferation rates.[3] It was encouraging, therefore, to see a toxic effect with pPPE-CD/5-FC treatment in the spheroid model of prostate cancer. It would be informative to compare directly the effect of 5-FU between 22Rv1 cells in 2D and 3D culture using an assay such as the acid phosphatase assay. This would help in understanding the processes occurring

in 22Rv1-pPPE-CD spheroids treated with 5-FC – it is possible that the levels of activation of the PSA promoter are higher, leading to greater CD expression, but that the subsequent toxicity of 5-FU is lower.

This chapter discusses the development of a new technique for tumour spheroid formation and the results obtained indicate its potential as a model system. There are numerous experiments that could now be carried out to further develop and test these spheroids as well as the possibility of growing other prostate tumour cell lines as spheroids. It would be valuable to build up a panel of prostate tumour spheroids with which to test pPPE-CD/5-FC efficacy as well as having potential for wider use in the study prostate tumour growth and progression and for use in drug development.


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