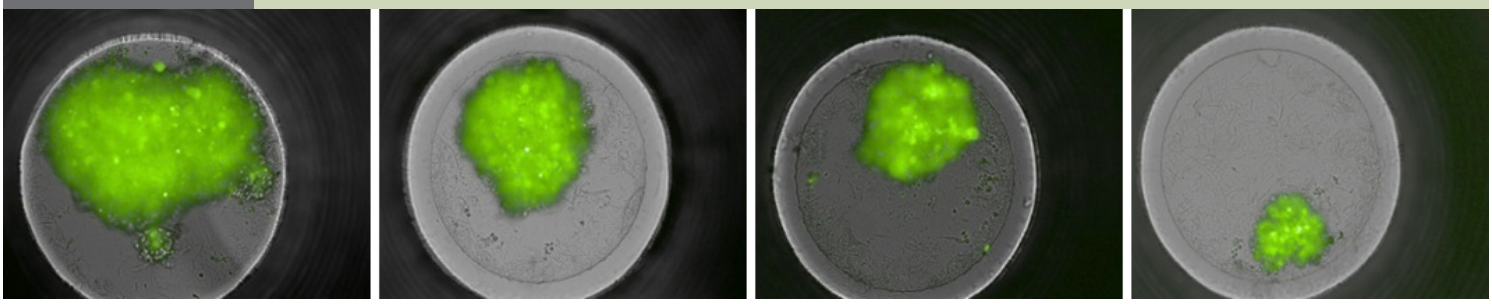
CASE STUDY

High Content Image Analysis



Inhibition of MicroRNA Sensitizes 3D Breast Cancer Microtissues to Radiation Therapy

Dr. Nataša Anastasov leads the Personalized Radiation Therapy Group within the Institute of Radiation Biology at the Helmholtz Center Munich. The group investigates tumor cell responses to ionizing radiation at the molecular, cellular and physiological level. Their research is contributing to the design of new strategies for the treatment of cancer.

Radiation therapy (radiotherapy) plays an important role in the treatment of breast cancer. It is mostly applied after surgery of the primary breast tumor to prevent tumor recurrence. However, two key problems of radiotherapy remain unresolved; the intrinsic radioresistance of some cancer cells and the occurrence of chronic health problems in collaterally irradiated tissue, although modern therapies do aim to reduce these side effects by limiting the area of the patient's body exposed to radiation. Strategies to improve the therapeutic impact of radiotherapy include increasing the efficacy of radiation, for example through the development of radiosensitizers, and reducing the side effects through the use of radioprotective agents.[1]

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MicroRNAs (miRNAs) are small non-coding RNA molecules involved in the transcriptional and post-transcriptional regulation of gene expression. They function by regulating the stability and translational efficiency of target messenger RNA (mRNA) molecules.[2] Altered expression of miRNAs has been demonstrated for several cancers, including breast cancer, and it has been suggested that miRNAs may be involved in the activation of signaling pathways and apoptosis.[3,4] Dr. Anastasov's group also recently showed that, in breast cancer cells, expression of an miRNA called miR-21 may contribute to radiation resistance by compromising cell cycle progression.[5]

This case study describes an investigation by researchers within the Personalized Radiation Therapy Group into the radiosensitizing potential of miR-21 down-regulation, using T47D breast cancer cells and T47D 3D breast cancer microtissues (InSphero AG). 3D tumor microtissues are widely accepted as being more physiologically relevant than conventional 2D monolayer cell cultures, and can therefore help generate more predictive data.[6]

Experimental Overview

Experiments were performed using radiation-resistant T47D breast cancer cells cultured as either a conventional 2D culture, or as a 3D microtissue. A lentivirus-based transduction system was used to modulate miR-21 expression, as previously described by Anastasov *et al.,* 2012.[5] Briefly, the pGreenPuro vector (pGP; System Biosciences, Inc.) encoding GFP was used as the backbone for specific miRNA oligonucleotide cloning. Vector inserts encoded either the miR-21 sequence, to over-express miR-21 (miR-21), or a short hairpin RNA (shRNA) targeting miR-21, to down-regulate expression of miR-21 (anti-miR-21). An empty pGP vector served as a negative control (EV = empty virus). For transduction, T47D cells were infected with 2 MOI (multiplicity of infection) of virus particles. Cell cultures were irradiated at room temperature using a ^{137}Cs irradiator (HWM-D 2000, Siemens, Germany).[5]

miR-21 Inhibition Induces Apoptosis in Irradiated 2D Cancer Cells

In the first experiment, to test the radiosensitizing potential of miR-21 over-expression or inhibition, 2D cultures of T47D cells were transduced with the different lentivirus types (EV, miR-21 or anti-miR-21) and irradiated with sham irradiation (0 Gy, control cells) or a dose of 5 Gy. Microscopic analysis of the cultures was performed 48 hr post-irradiation using phase contrast microscopy (Fig 1A). Cells in the control culture appear healthy and undisturbed, as do irradiated cells infected with EV, as a result of their radioresistance. Whilst over-expression of miR-21 has only a minor effect on the morphology of irradiated cells (+5 Gy, +miR-21), the expression of anti-miR-21 causes cells to become rounded following irradiation (+5 Gy, +anti-miR-21). Down-regulation of miR-21 therefore has a serious effect on cell health when combined with irradiation.

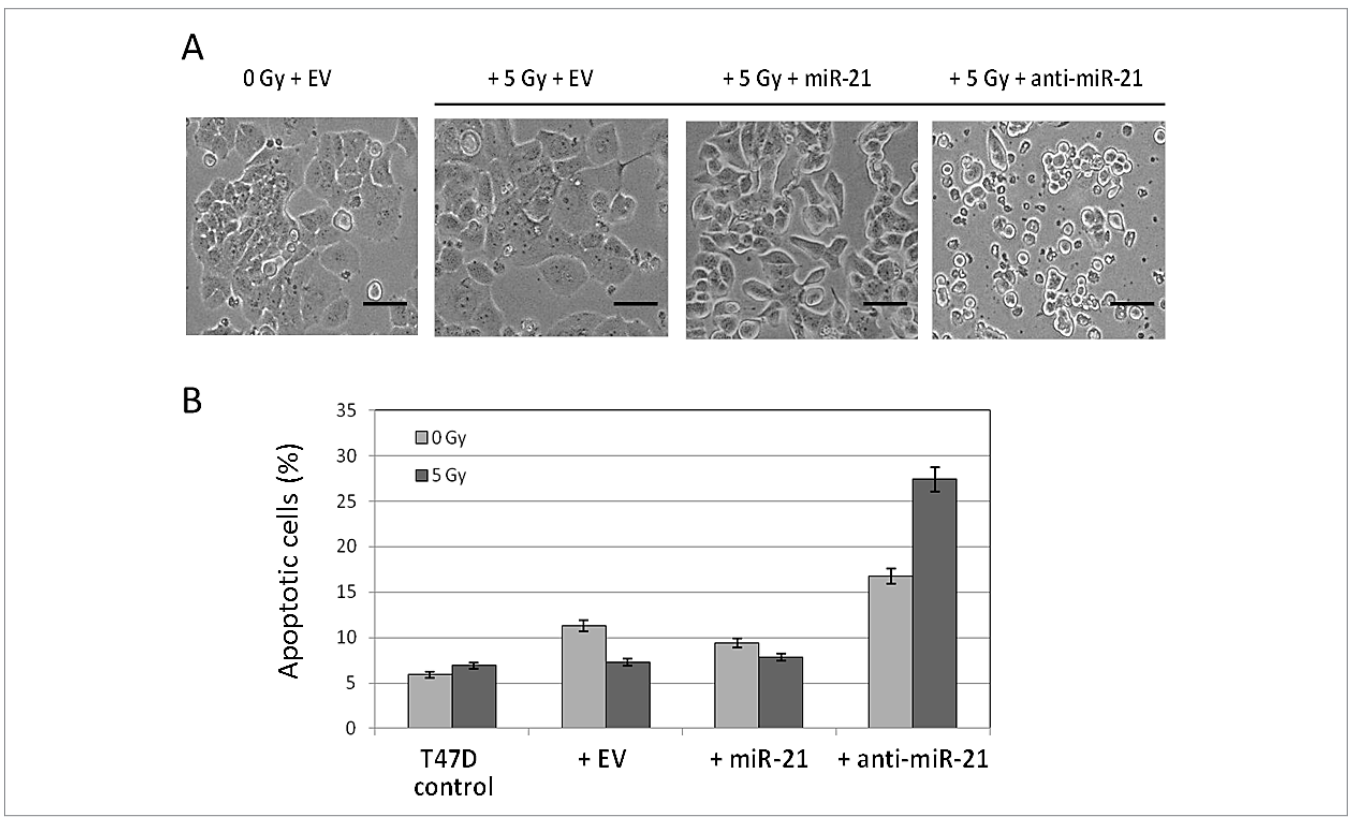

Figure 1. **Inhibition of miR-21 induces apoptosis in irradiated 2D cultured T47D cancer cells.** Radiation-resistant T47D breast cancer cells were transfected with different types of lentiviruses (EV = empty virus, miR-21 = over-expression of miR-21, anti-miR-21 = inhibition of miR-21) followed by sham irradiation (0 Gy, control) or irradiation with a dose of 5 Gy. (A) Phase contrast microscopic analysis of cells 48 hr post-irradiation. (B) DNA content analysis using flow cytometry 24 hr post irradiation. Cells with a DNA content less than G1 cells (<2n) were considered to be apoptotic.

To understand the effect of miR-21 on the cell cycle of 2D cultured T47D cells under the various treatment conditions, cells were stained with the DNA specific dye propidium iodide and a DNA content analysis was performed using flow cytometry. Under control conditions, irradiation induces a cell cycle arrest at the G2/M checkpoint in 2D T47D cells. Down-regulation of miR-21 (anti-miR-21), however, leads to a loss of radiation-induced G2/M arrest (data not shown).[5] The miR-21 knockdown also induces apoptosis in irradiated cells, as shown by quantitative analysis of the fraction of cells with a DNA content of less than G1 cells (<2n), which were considered to be apoptotic (Fig 1B). These results suggest a synergistic effect of miR-21 inhibition with radiotherapy.

miR-21 Inhibition Impairs Growth of Irradiated 3D T47D Microtissues

To investigate more physiologically relevant conditions, T47D cells were transfected with the three different lentivirus types and incubated for three days. Microtissues were then generated by seeding cells into the Insphero Gravity Plus™ plate (500 cells/well) and maturing them for two days in hanging drops, followed by transfer of the spheroids into Gravity TRAP™ assay plates. After one day of recovery, tissues were sham irradiated (0 Gy) or irradiated with a dose of 5 Gy. Imaging was performed at different timepoints post-irradiation, over a two week period, using the Operetta® High Content Imaging System.

Images from a single plane were acquired in the GFP and brightfield channels using the 10x NA objective in widefield mode (Fig 2A). Automated quantitative analysis of microtissue sizes at the different timepoints was then performed using Harmony® High Content Imaging and Analysis Software. The *Find Image Region* Building Block was applied to the GFP channel to detect the microtissues in the well and then the *Calculate Morphology* Building Block was added to calculate the tissue area (µm²) as the final readout (Fig 2B).

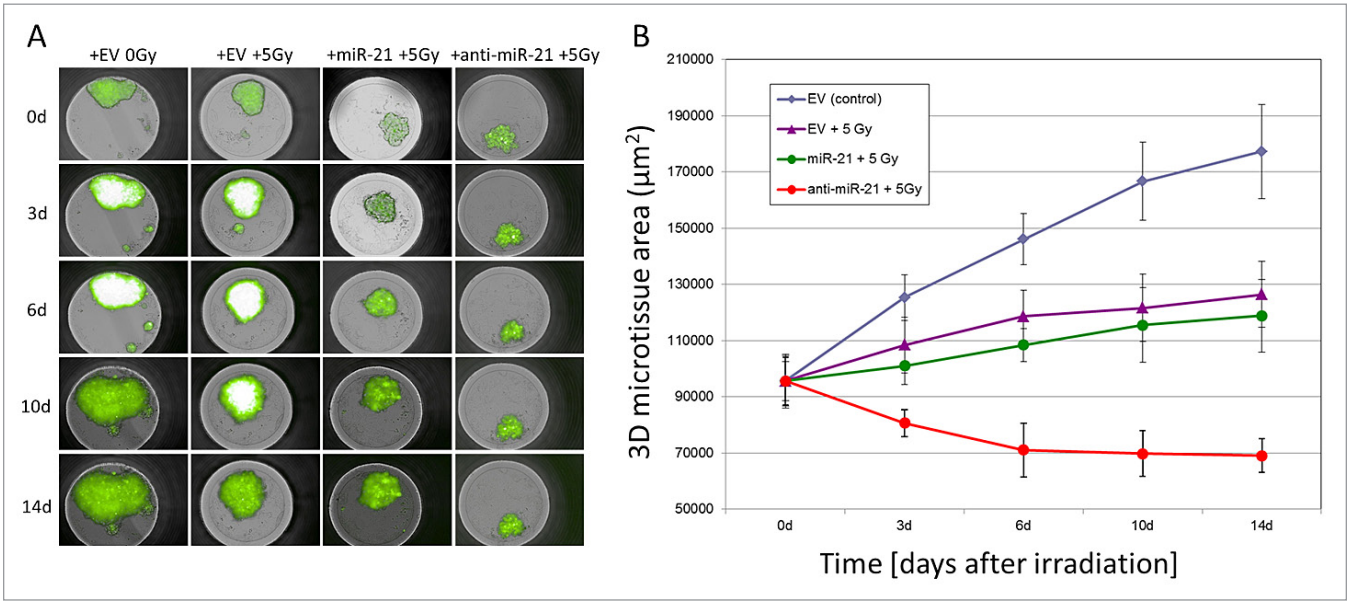

Figure 2. **Inhibition of miR-21 impairs growth of irradiated 3D T47D microtissues.** Radiation-resistant T47D breast cancer cells were transfected with different types of lentiviruses (EV = empty virus, miR-21 = over-expression of miR-21, anti-miR-21 = inhibition of miR-21) and seeded into Gravity Plus™ plates to form microtissues. Mature microtissues were transferred into Gravity TRAP™ assay plates and were either sham irradiated (0 Gy, control) or irradiated with a dose of 5 Gy. Microtissue growth was analyzed every three days over a two week period. (A) High Content Imaging of microtissues in the GFP and brightfield channel using the Operetta® High Content Imaging System (10x NA, single plane, widefield mode). (B) Quantitative analysis of microtissue size (area in µm²) using the Harmony® High Content Imaging and Analysis Software.

While the non-irradiated control tissues (+EV, 0 Gy) showed constant growth over the two week time period, the irradiated tissues (+EV, 5 Gy) showed only a moderate growth. This is most likely due to cell cycle arrest at the G2/M checkpoint, which controls DNA integrity, since radiation-induced G2/M arrest is a well-known effect of radiation therapy.[5] Irradiation is therefore clearly impairing the growth of 3D microtissues. Tissues over-expressing miR-21 show a comparable growth rate to that of irradiated tissues transfected with empty virus, suggesting that miR-21 over-expression has no significant impact on the cell cycle. On the contrary, irradiation shows a strong inhibitory effect on microtissue growth in cells expressing anti-miR-21. In fact, these tissues even show a size reduction over time, suggesting that the cells in the microtissue may be undergoing apoptosis or might die during the experiment (Fig 2B).

miR-21 Inhibition Induces Apoptosis of Irradiated 3D T47D Microtissues

To determine whether cells in the microtissue undergo apoptosis, an M30 Apoptosense® ELISA (PEVIVA®; TECOmedical AG) was performed using the microtissue supernatant. The M30 Apoptosense® is an ELISA based on a M30 monoclonal antibody which specifically binds caspase-cleaved keratin 18 (K18), a soluble human intermediate filament protein fragment. This assay therefore allows the measurement of apoptosis of K18-expressing cells. T47D cells were transfected with the different GFP-lentiviruses, cultured as 3D microtissues, and irradiated as described previously. Aliquots (30 µl) from the microtissue supernatants were collected immediately after irradiation (0 d), after six days, and after 12 days, from separate wells, and the ELISA performed. The colorimetric reaction was measured at 450 nm using a microplate reader. The resulting absorbance is directly proportional to the concentration of the K18 analyte. To determine apoptotic induction, a standard curve was plotted from known antigen concentrations versus measured absorbance. The induction of apoptosis was calculated in units per milliliters (U/ml).

When comparing the different apoptosis levels in the non-irradiated microtissues (0 Gy), it is clear that miR-21 over-expression has no significant effect on the level of apoptosis, while inhibition of miR-21 by anti-miR-21 induces apoptosis (Fig 3, upper panel). When the microtissues are irradiated (5 Gy), this effect becomes even more pronounced (Fig 3, lower panel). In combination with irradiation, anti-miR-21 expression strongly induces apoptosis, particularly 12 days post-irradiation. This strongly indicates a synergistic effect of miR-21 inhibition and radiation therapy.

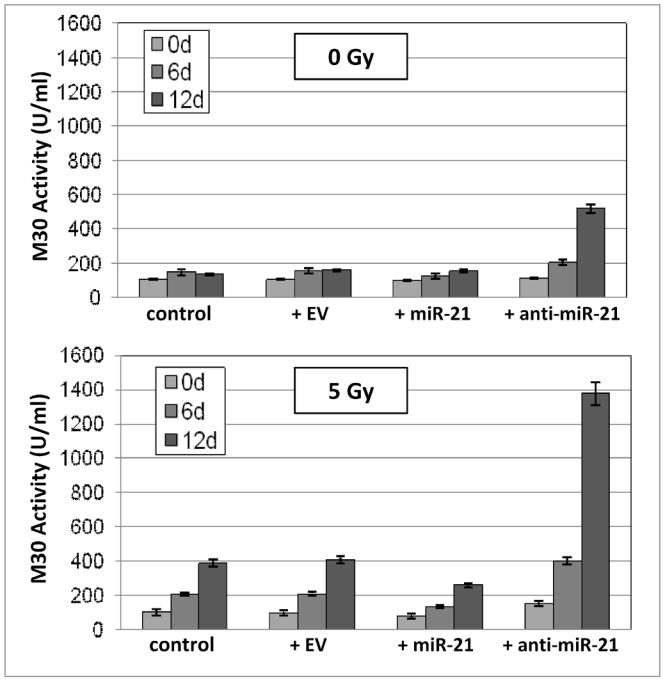

Figure 3. **Inhibition of miR-21 induces apoptosis of irradiated 3D T47D microtissues.** Radiation-resistant T47D breast cancer cells were infected with different types of lentiviruses (control = cells without lentiviral infection, + EV = infected with empty virus, + miR-21 = over-expression of miR-21, + anti-miR-21 = inhibition of miR-21) and seeded into Gravity Plus™ plates to form microtissues. Mature microtissues were transferred into Gravity TRAP™ assay plates and were either sham irradiated (0 Gy, upper panel) or irradiated with a dose of 5 Gy (lower panel). To test for apoptosis, aliquots of the microtissue supernatants were taken at different timepoints post-irradiation (0 d, 6 d and 12 d) and submitted to a M30 Apoptosense® ELISA.

The Operetta System and Harmony Software make it easy for radiation biologists such as myself to translate biology into unbiased quantitative data and to use alternative approaches to the classical colony formation assays, such as more sophisticated microtissue assays. To investigate radiation resistance, we need long term analysis of cultures following treatment, which is not easy with standard 2D monolayer cultures. These new cutting-edge technologies will contribute tremendously to the optimization of personalized treatment strategies for cancer patients.

- Dr. Nataša Anastasov

Conclusion

It has been previously suggested by Dr. Anastasov and colleagues that miR-21 knockdown and radiation therapy might have a synergistic effect in overcoming the radiation resistance of tumors.[5] In this case study, miR-21 was further confirmed as a potential target in breast cancer therapy by verification of the results obtained from a 2D culture, in a 3D environment, in 3D T47D breast cancer microtissues. Cells in microtissues have a more physiologically-relevant microenvironment and more closely resemble *in vivo* solid tumors.[6] As this case study demonstrates, microtissues are also a very good model system to study the radiation sensitivity of tumors and to test new strategies for either increasing radiation efficacy (radiosensitizers) or reducing the side effects of radiotherapy (radioprotectors), to further optimize patient care.

Using the Operetta® High Content Imaging System, the growth of microtissues was easily monitored by capturing the whole well of the Gravity TRAP™ plate using the 10x NA objective, followed by automatic analysis of tissue size over a two week time period with the Harmony® High Content Imaging and Analysis Software.

This case study exemplifies the combination of cutting-edge technologies such as shRNA, lentiviral transduction, 3D microtissues and high content imaging and analysis. Together, these technologies can contribute to the development of more personalized treatment strategies for cancer patients.

Acknowledgements

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