Summary and Future Perspectives
Conventional radiotherapy, with photons/X-rays, is one of the most successful methods of cancer treatment, and is used to treat approximately half of all patients with cancer, either alone or together with surgery or chemotherapy (1). However, the success of radiotherapy is limited by tumor radioresistance and subsequent recurrences. Furthermore, conventional radiotherapy also results in the unavoidable co-irradiation of normal tissue surrounding the tumor (1, 2). Although radiotherapy increases a patient’s chance of survival, the co-irradiation of normal tissue may cause a dramatic decrease in the quality of life, due to irradiation-induced side effects (3). Means of reducing these side effects, such as high-precision particle radiotherapy, are continually being further improved (4). However, there is still much to be better understood to enhance efficacy of radiotherapy, minimise radiation-induced side effects and thus increase a patient’s quality of life post-treatment.

In this thesis, various aspects of the limitations of radiotherapy were studied and new models for studying radiation-induced side effects in vitro were described. Here, the main findings of the studies carried out are summarised and put into perspective.

**SUMMARY**

Radioresistance is associated with changes in many intrinsic signalling pathways, as well as alterations to the cellular microenvironment. One such pathway that has been associated with radioresistance is the TGF-β signalling pathway (5-7), while changes in p73 (a gene related to p53) signalling have also been associated with therapy resistance (8). In particular, the transactivation (TA) and N-terminus truncated (ΔN) isoforms of p73 are misregulated in many cancers, which in turn contributes to therapy resistance (8). In Chapter 2, the ability of p73 isoforms to modulate TGF-β signalling is shown. However, surprisingly, overexpression of the ΔNp73 isoform, which usually has dominant-negative inhibitory effects on p53/p73 (8), was found to have a more prominent effect in enhancing TGF-β downstream activation compared to mutated p53 or TAp73 overexpression. ΔNp73 was shown to stimulate TGF-β signalling via the formation of a complex with Smad Binding Elements. Furthermore, chromatin immunoprecipitation was used to show a direct interaction between ΔNp73 and Smad Binding Elements, which could potentially mediate the ΔNp73 effects on TGF-β signalling. These interactions between TGF-β and ΔNp73 may contribute to both tumor progression and therapy resistance.
Drugs are frequently used in order to modify tumor radioresistance (9), with many of these drugs targeting the DNA damage response (DDR) pathways (10). When a cell (or a tumor) has a mutation that causes a defect in a DDR pathway, other DDR pathways can compensate for the defect and cope with DNA damage. However, the inhibition of a second DDR pathway results in cell death, as cells are now unable to efficiently handle DNA damage. This is known as synthetic lethality (11). Radiotherapy can also take advantage of this concept, as radiotherapy induces large amounts of DNA damage, and thus inhibition of DDR pathways can enhance tumor radiosensitivity. The molecular chaperone Hsp70 is upregulated in many cancers, and therefore may offer an attractive drug target to enhance radiosensitivity. In Chapter 3, a previously unidentified role of Hsp70, and its co-chaperone DNAJB6, in the DDR was investigated.

Using clonogenic assays based on 3-dimensional spheroid culturing, in Chapter 3 it was found that inhibition of Hsp70 increased radiosensitivity of both cell lines and primary cells via an impairment of DNA double strand break (DSB) clearance. Furthermore, using reporter cell lines specific for the main pathways of DSB repair (non-homologous end-joining (NHEJ) and homologous recombination (HR)), a defect in NHEJ was identified upon Hsp70 inhibition. However, Hsp70 inhibition had no effect on HR, indicating a specificity of Hsp70 for NHEJ. Additionally, fluorescently-tagged DNAJB6 (a Hsp70 co-chaperone) was recruited directly to the sites of DNA damage following laser micro-irradiation, while knock-out of DNAJB6 resulted in increased radiosensitivity in clonogenic survival assays, indicating that this protein may also have a role in the DDR. While other members of the protein quality control system have been targeted in cancer treatment in the past (12, 13), the identification of a role for Hsp70 in NHEJ may offer a novel target in the DDR system to increase tumor radiosensitivity.

It has previously been suggested that cancer stem cells also contribute to a tumor’s radioresistance. Therefore, Chapter 4 focuses on the role of cancer stem cells (CSC) in the resistance of esophageal cancer tumors to radiotherapy and targeting of the mTOR pathway to enhance the effect of radiotherapy on this tumor subpopulation. Using flow cytometric analysis, a previously identified subpopulation of esophageal cell lines (CD44+/CD24-) was shown to be selected for under low oxygen and nutrient deprivation, two problems commonly found in cancerous tumors. Furthermore, inhibitors of the mTOR pathway (torin-1 and rapamycin) also selected for this population in both 2-dimensional and 3-dimensional cell culturing conditions, while stimulators of mTOR had the inverse effect. Finally, the CD44+/CD24- subpopulation was identified in patient-derived esophageal organoids. Stimulation of
the mTOR pathway was shown to diminish this subpopulation in patient-derived organoids, potentially offering a means to overcome the radiotherapy limiting factor of CSC radioresistance in esophageal cancer.

Increasing the radiosensitivity of a tumor is one means to broaden the therapeutic window of radiotherapy, while another option is to protect or enhance the regeneration of normal tissue surrounding the tumor. During treatment, normal tissue is unavoidably co-irradiated leading to normal tissue side effects (both early and late) (3). Normal tissue stem cells are essential for the homeostasis of tissue (14). Following damage, stem cells drive proliferation to restore tissue lost due to cell death. In the case of irradiation-induced damage, the DNA is damaged also resulting in an inability of cells to proliferate and also causes cell death. However, importantly, stem cells are also damaged and killed by irradiation, and thus leads to a reduction in cell replacement. In Chapters 5 & 6, models to study the effects of different forms of irradiation on normal tissue stem cells were established.

As mentioned above, the most important cells for tissue regeneration following radiotherapy are surviving stem cells. However, studying the effects of radiation on stem cells has previously been difficult under many different aspects. For many tissues, a stem cell population has not been clearly defined or characterised, it is not known where these cells reside, and some stem cells that have been identified have unidentified culture and growth conditions. In Chapter 5, a previously established model for mouse salivary gland stem cells was optimised for irradiation studies. Initially, the effects of irradiation with varying linear energy transfer (LET) on 2-dimensional and 3-dimensional cell cultures were compared. By performing clonogenic assays and modified gel-based clonogenic survival assays, 2-dimensional cultures were determined to be more radiosensitive than 3-dimensional cultures, while in both 2-dimensional and 3-dimensional cultures increasing LET reduced survival post-irradiation. Finally, mouse-derived salivary gland cells were irradiated with varying LETs, to give an indication of the effect of irradiation on normal tissue stem cells, again using a 3-dimensional clonogenic survival assay. This work showed the potential of a previously-established culture model for regenerative purposes to be used as an in vitro model for the study of normal tissue stem cell responses to various forms of irradiations.

The effects of low doses of irradiation on normal tissue-derived stem cells were studied in Chapter 6, using the in vitro organoid based model described in Chapter 5. Optimised photon radiotherapy
treatment plans aim to deliver the highest dose to a tumor, with the dose to tissue surrounding a tumor reducing with distance from the tumor. The goal of such treatment planning is to minimise the normal tissue complications following radiotherapy. However, previous studies on the impact of clinically relevant low radiation doses primarily focused on cancerous cell lines, which showed that low doses of irradiation have a disproportionally high impact compared to higher doses (15-17). Therefore, it is of importance to understand the effects of low irradiation doses on normal tissue stem cells.

In Chapter 6, it was first determined that a low irradiation dose to the high-density stem cell region of rat salivary glands results in a greater loss of in vivo function than higher doses. Next, it was shown, using mouse-derived salivary gland organoids, that this was due to a decreased clonogenic survival of stem cells following low doses of irradiation compared to higher doses, which in turn was due to a lack of activation of the DDR at lower doses. This was translated to a more clinical relevance, by demonstrating that a relatively low irradiation dose impacts greater on patient-derived normal tissue stem cells than a slightly higher dose.

In order to deliver an optimal dose of irradiation to a tumor, multiple fractions of radiation are administered. This results in multiple low dose fractions to normal tissue surrounding a tumor. Therefore, finally, in Chapter 6, mouse-derived salivary gland stem cells were irradiated with multiple relevant low dose fractions. Fractionated low doses were determined to be more detrimental to the survival of normal tissue stem cells than an equivalent higher dose. Thus, minimizing the dose of irradiation to normal tissue stem cells has a greater impact than a slightly higher irradiation dose and, if possible, it would be more advantageous to remove high-density stem cell regions from the radiation field completely to enhance the therapeutic index.

FUTURE PERSPECTIVES

Despite the success and common use of radiotherapy – approximately half of all patients who undergo cancer treatment will receive radiotherapy (1) – there are still many obstacles and limitations. The aim of this thesis was to establish and use new in vitro models to investigate the limitations of radiation treatment. Means of overcoming radioresistance, cancer stem cells and the effects of irradiation on normal tissue stem cells were all investigated.
The ability of a tumor to regrow is a major concern for cancer treatment planning. Many factors contribute to a tumor's capacity to repopulate following treatment, including pathways involved in tumor progression and radiosensitivity. Furthermore, cancer stem cells contribute to treatment resistance in many tumor types. Chapter 2 of this thesis looks at TGF-β signal modulation by p73 isoforms, which can contribute to tumor progression. Interestingly, ΔNp73, which has oncogenic properties and is associated with a poor patient prognosis (18), was found to have the largest effect on TGF-β signal enhancement and also directly interacted with the TGF-β signalling mediators, Smad binding elements. ΔNp73 is known to be upregulated in many tumor types, including thyroid carcinomas, gliomas, cervical cancers and head and neck squamous cell carcinomas (18). The work in this chapter was performed in the context of cell lines, and it remains to be seen if these effects would have an influence on tumor resistance to treatment or regrowth following treatment in a more translatable setting. Tumor-derived organoids, such as those described in Chapter 4, may offer a more appropriate in vitro model for such experiments.

Cancer cells frequently contain mutations in DDR pathways, which can be exploited to enhance tumor sensitivity by means of synthetic lethality by inhibiting further repair pathways, such as the case of PARP inhibition in BRCA-deficient tumors (11, 19). In Chapter 3, a novel role of the molecular chaperone Hsp70, and its co-chaperone DNAJB6, was identified in NHEJ (NHEJ). This newly identified role of Hsp70 in NHEJ may offer novel drug targets to enhance radiosensitivity. However, to make this finding most advantageous it would first be necessary to identify a more precise role of Hsp70/DNAJB6 in NHEJ. Hsp70 and DNAJB6 are most known for their roles in the protein quality control system (20), but are also involved in protein complex remodelling. In the case of DNA repair, Hsp70/DNAJB6 could potentially offer either of these roles, either folding/refolding repaired proteins or alternatively they could be involved in the recruitment/formation of the many protein complexes involved in NHEJ. Techniques such as mass spectrometry (21) or the recently developed proximity-dependent labelling biotinylated identification (BioID) method (22, 23) could be used to identify interacting protein partners for Hsp70 (or DNAJB6) after the induction of damage, using radiation for example, potentially providing mechanistic insight into the role of these proteins in NHEJ.

Although the existence and origin of cancer stem cells is often discussed (24-27), many groups have identified populations of cells within cancers with cancer stem cell (or at the very least cancer stem-like) characteristics (28-32). In terms of treatment, possibly the most important property of the cancer
stem-like cells is, in general, an increased resistance to therapies, including radiotherapy (26, 27). Importantly, in **Chapter 4**, methods of modulating a previously identified cancer stem-like population within esophageal cancer cell lines are described. While methods to increase the population were shown, importantly, methods to reduce this population were also identified (namely stimulation of the mTOR pathway). These are important findings, as diminishing the cancer stem cell-like population within a tumor would, in theory, decrease the resistance of cancers to many forms of treatment. This population was also shown in patient-derived organoid cultures. Analysis of this population of cancer stem-like cells in patient-derived organoids may offer the opportunity to determine a patient’s (radio-)sensitivity prior to treatment, allowing for a more personalised treatment. Furthermore, the use of patient-derived organoids may allow for identification of other compounds/medicine that can increase radiosensitivity on an individual basis.

As shown in **Chapter 4**, modulation of the autophagy pathway through hypoxia causes changes in the abundance of cancer stem cells. Furthermore, hypoxia also induces the unfolded protein response, which includes Hsp70 (33, 34). Increased Hsp70 in cancers is believed to be a method for oncogenic cells to ‘mask’ mutations that would be discarded under normal Hsp70 levels (35). While this could be true, the findings in **Chapters 3 & 4** taken together, could also indicate that increased levels of Hsp70 may play a role in cancer stemness. Indeed, increased levels of Hsp70 isoform levels have previously been associated with normal tissue stemness (36). Thus, it would be interesting to investigate the role of Hsp70 in cancer stemness, as targeting of Hsp70 (as proposed in **Chapter 3**) may not only target NHEJ, but cancer stemness as well, potentially increasing cancer stem cell radiosensitivity, an important parameter in long-term tumor control/eradication by radiotherapy. Furthermore, Hsp70 is upregulated in many different tumor types (35) and Hsp70 inhibition in healthy patient-derived salivary gland stem cells did not result in a significant increase in radiosensitivity. This potentially indicates that indeed the therapeutic ratio between tumor and normal tissue stem cells could be enhanced by Hsp70 inhibition. Indeed, another of the major limiting factors to radiotherapy is the normal healthy tissue surrounding the tumor, and thus understanding the response of the normal tissue stem cells is of particular importance following irradiation.

**Chapters 5 & 6** describe *in vitro* organoid models for investigating normal tissue stem cell response following irradiation. Under normal conditions, normal tissue stem cells are essential for the regeneration and functional restoration of tissue following damage. However, following irradiation
many of the normal tissue stem cells are damaged/sterilised (Chapter 5) to such an extent that they are functionally incapable of restoring homeostasis in damaged tissue. Intensity-modulated radiotherapy and particle therapy are high precision techniques that can deliver a more accurate dose to the target tumor, while better sparing the healthy tissue. However, IMRT still results in a large volume of healthy tissue being irradiated with low doses. Furthermore, while particles can be used to deliver a more accurate dose to the tumor, much less is known about the biological effects of particle therapy than photon irradiation. Therefore, understanding of the normal tissue stem cell response to different forms of irradiation (i.e. photons versus particles) will allow for better prediction of treatment limiting side effects in the future.

As shown in Chapter 6, it is not just the dose to surrounding normal tissue that is important, but rather the dose to critical (stem cell containing) regions that is important to the functionality of the co-irradiated tissue. If critical high-density stem cell containing regions of organs adjacent to the target tumor are within a low dose field, the functionality of the tissue in question can be greater impaired than if it were to be found within a higher dose field. Therefore, it may be necessary to avoid irradiation of such critical structures with low doses of irradiation. However, for this to be possible, there are two important requirements; (i) identification of the regions containing a higher density of stem cell within critical organs, and (ii) how these critical structures would differ in response to irradiation between different organs.

Using methods similar to van Luijk et al., who identified a high-density stem cell region of the parotid gland (37), high density stem cell regions within other critical structures should be identified, while organoid-based techniques such as those described in Chapters 5 & 6 could be used to investigate the response of these structures. When the function of an organ is identified to have a greater impairment at a relatively low dose compared to slightly higher doses, as with salivary glands (Chapter 6), it could be important to include this as a parameter for treatment planning. Although it does not completely eliminate co-irradiation of normal tissue, proton-based radiotherapy could be considered due to its better ability to spare healthy tissue. Furthermore, in Chapter 6, the greater impact on survival observed at lower doses (≤ 0.5 Gy) of photon irradiation than slightly higher doses (≥ 1 Gy) was diminished following irradiation with particles (carbon ions in this case).

The primary goal of radiobiology is to optimise the therapeutic index, i.e. increasing the effect of therapy
on a tumor while minimizing the normal tissue complications (2). The knowledge gained through the use of ‘traditional’ 2-dimensional cultures over the years and decades has been indispensable in the advancement of many fields of study in biology, including radiobiology and drug discovery. However, 2D culture models lack many crucial signalling factors, such as cell-cell and cell-matrix interactions, which contribute to essential cellular functions in proliferation, differentiation and survival (38, 39). Thus, the read-outs of conventional 2D models often misestimate the in vivo response to therapies. Organoid models, which possess many of the interactions lacking in 2D cultures, offer a potentially more realistic model to predict therapeutic responses (40, 41). Furthermore, comparing the response of tumor-derived organoids (Chapter 4) with normal tissue-derived biopsies (Chapters 5 & 6), or even co-cultures of the two, could potentially offer an individually personalised predictive therapeutic window.

Taken together, the work in this thesis contributes to the understanding of mechanisms which contribute to radioresistance and to radiation-induced side effects. Cancer stem cells and normal tissue side effects are crucial factors which limit the efficacy of radiotherapy. Means of modulating cancer stem cells may offer novel ways to tackle radioresistance, while the use of organoids offers an invaluable tool to further study these mechanisms in an in vitro setting. Radiotherapy is a powerful method of cancer treatment, and with the ever-increasing knowledge of the limitations of radiotherapy and advancements in means of overcoming these barriers, radiotherapy can only become more important in the future.
REFERENCES


