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Original paper

Improved normal tissue protection by proton and X-ray microchannels compared to homogeneous field irradiation

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ABSTRACT

The risk of developing normal tissue injuries often limits the radiation dose that can be applied to the tumour in radiation therapy. Microbeam Radiation Therapy (MRT), a spatially fractionated photon radiotherapy is currently tested at the European Synchrotron Radiation Facility (ESRF) to improve normal tissue protection. MRT utilizes an array of microscopically thin and nearly parallel X-ray beams that are generated by a synchrotron. At the ion microprobe SNAKE in Munich focused proton microbeams ("proton microchannels") are studied to improve normal tissue protection. Here, we comparatively investigate microbeam/microchannel irradiations with sub-millimetre X-ray versus proton beams to minimize the risk of normal tissue damage in a human skin model, *in vitro*. Skin tissues were irradiated with a mean dose of 2 Gy over the irradiated area either with parallel synchrotron-generated X-ray beams at the ESRF or with 20 MeV protons at SNAKE using four different irradiation modes: homogeneous field, parallel lines and microchannel applications using two different channel sizes. Normal tissue viability as determined in an MTT test was significantly higher after proton or X-ray microchannel irradiation compared to a homogeneous field irradiation. In line with these findings genetic damage, as determined by the measurement of micronuclei in keratinocytes, was significantly reduced after proton or X-ray microchannel compared to a homogeneous field irradiation. Our data show that skin irradiation using either X-ray or proton microchannels maintain a higher cell viability and DNA integrity compared to a homogeneous irradiation, and thus might improve normal tissue protection after radiation therapy.

Introduction

In radiotherapy, the risk of developing normal tissue injuries often limits the radiation dose that can be applied in tumour patients. Radiation damage in the skin can reduce the patient's quality of life after tumour therapy. Microbeam Radiation Therapy (MRT), a spatially fractionated radiotherapy, uses an array of microscopically thin and nearly parallel synchrotron-generated X-ray beams [1–3]. In X-ray MRT the tumour is exposed to arrays of narrow 25–75 μm wide microplanar beams. These parallel orientated beams are separated by distances of typically 50–400 μm. The microarray geometry is maintained in the tumour, and comparisons between broad beam irradiations and MRT indicate a higher therapeutic index due to less toxicity in the normal tissue and a selective radiovulnerability of the tumour vasculature versus normal blood vessels by MRT [4–6].

Similar approaches use focused proton microbeams, here termed "microchannels", which remain separated in normal tissues but spread out until they reach the tumour to achieve a homogeneous dose distribution inside the target volume. The rationale for proton microchannels is therefore the reduction of normal tissue toxicity (as in X-ray MRT), while the response of the target volume is unaffected. The microchannel approach has been established at the ion microprobe SNAKE in Munich [7]. A microchannel

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irradiation at SNAKE with 20 MeV protons resulted in reduced inflammatory response in a human skin model compared to conventional homogeneous broad-beam irradiation [7]. They applied protons either focused in a matrix of 500 × 500 μm^2 (total field size: 4 × 4 mm^2) using 50 × 50 μm^2 wide channels with a channel dose of 200 Gy, or homogeneously with the same mean dose of 2 Gy. Inflammation was determined by measuring soluble inflammatory response parameters such as Interleukin-6, TGF-beta and Pro-MMP1 in the supernatant of the human skin tissue. The results showed a lower inflammatory response in the human skin model after microchannel compared to homogeneous irradiation. No significant cell death was observed in the skin after irradiation with 50 μm microchannels compared to non-irradiated controls, while homogeneously irradiated tissues showed a decrease in the cell viability of 48%.

In 2013, another group published Monte Carlo simulations for microchannel proton irradiations [8]. The authors evaluated peak-to-valley doses for several arrays of proton minibeams and concluded possible tissue sparing effects due to the spatial fractionation of the dose.

The major goal of the present study was to investigate if microchannel irradiation (using a similar concept as “pencilbeam” or “minibeam” irradiations, cf. [9] and [8]) in the micrometre size range of X-ray versus proton beams can also minimize the risk of normal tissue damage. Therefore, viability and genetic damage was determined in a three-dimensional human skin model after four different microbeam irradiation modes: a homogeneous field (HF), lines (LN), and small and large channels (SC and LC) with synchrotron-generated X-ray beams at the ESRF (Grenoble, France) and proton irradiation at the SNAKE microbeam (Munich, Germany).

Materials and methods

Tissue construct

The three-dimensional full-thickness skin model EpiDermFT™, which was used in previous studies [7], was obtained from MatTek Corporation, Ashland, MA, USA. For the irradiations at SNAKE the EFT-400 with a surface area of 1 cm^2 (thickness roughly half a millimetre) and for the irradiations at the ESRF the EFT-300 was used, which is the same as EFT-400 except the surface area was only 0.9 cm^2. This reconstructed human skin is a differentiated tissue consisting of cornified, granular, spinous and basal layer like the normal human epidermis, and a dermal layer. EpiDermFT™ is mitotically and metabolically active. The tissue, consisting of human-derived epidermal keratinocytes and dermal fibroblasts, is cultured on special cell culture inserts in 12-well plates, each containing 2.0 ml of fresh 37 °C New Maintenance Medium (NMM, MatTek Corporation, Ashland, MA, USA). The samples were incubated at 37 °C in a humidified atmosphere (5% CO_2), replacing the culture medium every 24 h.

Irradiation conditions at SNAKE (protons)

Proton irradiation was carried out at the Munich ion microprobe SNAKE (Superconducting Nanoprobe for Applied nuclear [Kern] physics Experiments) of the 14 MV Munich tandem accelerator, where defined cell nuclei can be irradiated with single or counted protons, thus allowing a precise dose application [10–12]. During irradiation, the skin construct was retained in a specially designed container (cf. [7,12]) and mounted directly behind the beam exit nozzle, with the dermis facing the beam. For an exact dose calculation, every proton was detected in a scintillator-photomultiplier detector after traversing the skin sample [7], with an LET value of 2.66 keV/μm at the position of the skin sample. The same irradiation setup was used to prepare the beam for two focused modes (channel modes) and for a homogeneous mode. In all three cases the average dose over the irradiated area was 2 Gy, with an uncertainty of approximately 4%, estimated mainly from the uncertainty of the field size (~2% in each dimension) and the accuracy of the LET value (~1–2%) [13,7]. For the microchannel irradiations, the channel size and distance was chosen such that 1% of the skin was irradiated. Small channels (SC, 50 × 50 μm^2, as in Ref. [7]) and large channels (LC, 180 × 180 μm^2) were irradiated with a local dose of 200 Gy and the distances between the channels were 500 μm (centre-to-centre) and 1800 μm, respectively. Three skin samples were used for each irradiation mode, and the whole experiment was performed in duplicate.

Irradiation conditions at ESRF (X-rays)

A very similar irradiation was performed with X-rays at the ID17 beamline of the European Synchrotron Radiation Facility (ESRF) in Grenoble/France, which is also used for the MRT activities at ESRF. It is equipped with a multi-slit tungsten collimator and a goniometer which can move the sample through the beam in order to generate microplanar beams as used in MRT [3]. The energy spectrum ranges from 50 to 350 keV with the maximum at around 100 keV. A specially designed tissue holder allowed the skin sample to be mounted on the goniometer, with the dermis facing the source. The skin sample was sandwiched between 2 mm of PMMA towards the source (to provide sufficient build-up) and 9 mm water (medium) followed by 6 mm of PMMA on the other side. A circular tungsten mask with 4.6 mm diameter was mounted immediately upstream of the sample.

For this setup, four irradiation modes were prepared: a homogeneous field (HF), lines (LN), and small and large channels (SC and LC). The vertical lines had a thickness of 51 μm and a distance (centre-to-centre) of 412 μm (defined by the multi-slit collimator), with the sample moving through the beam as for MRT. By using one horizontal line of 51 μm width with the same multi-slit collimator settings, a linear array of small channels of 51 × 51 μm^2 with a distance (centre-to-centre) of 412 μm could be generated. Vertical stepping of the sample with a step size (centre-to-centre) of 607 μm produced the desired SC pattern with one channel per 0.25 mm^2, very similar to the SC pattern realized at SNAKE. A large channel (LC) with 180 × 180 μm^2 was generated by primary slits (without the multi-slit collimator, and without the 4.6 mm collimator), and the sample was moved horizontally and vertically in steps of 1800 μm (centre-to-centre) in order to realize the same pattern as for large channels at SNAKE. Three skin samples were used for each irradiation mode.

In all four irradiation modes, the intended average dose over the irradiated area was 2 Gy. The required peak doses and output factors for irradiation (relative to a pin point ionization chamber measurement in an open field of 10 × 10 mm^2) were determined by detailed Monte Carlo simulations in Geant4 version 9.3p2 on a lateral calculation grid with a resolution of 5 μm. The Livermore low energy physics libraries were employed with 1 μm cut of lengths. These simulations were used to determine the mean dose over the nominal field size and to scale this dose to 2 Gy for all irradiation modes. Due to the shallower beam penumbra compared to protons, the resulting peak doses were lower than for protons (e.g. 186 Gy for SC and 15.1 Gy for LN compared to 200 Gy at SNAKE), with valley doses for X-rays of around 0.03 Gy.

MTT tissue viability test

The cell viability of the complete skin (i.e. keratinocytes in the epidermis and fibroblasts in the dermis) was quantified 40 h after

irradiation using the colorimetric MTT test (MTT-100, MatTek Corporation). The MTT substrate is only cleaved by active mitochondria and the amount of formazan generated in this enzymatic reaction correlates closely to the cell number [14]. It is assumed that the cleavage of the MTT substrate by active mitochondria is strongly correlated with the number of viable cells within 40 h after irradiation. In brief, the central part (diameter 4 mm) of the irradiated area was punched out using a centred mask and placed in 300 μl MTT solution for 3 h at 37 °C, followed by overnight extraction at room temperature in 2 ml extraction solution. After removal of the tissues, 200 μl of the sample was measured with a photospectrometer to obtain the optical density (OD) at 570 nm, subtracting background at 650 nm. The percentage viability was calculated for each tissue, dividing the corrected OD by the OD of untreated controls [7].

Micronuclei test

Genetic damage in epidermal keratinocytes was measured via the micronuclei assay as described earlier [12,7]. Immediately after irradiation, 3 μg/ml cytochalasin B was added into the culture medium and the cultures were incubated for a further 48 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. The 48 h incubation was considered to be the optimal time where up to 50% binucleated cells were reproducibly obtained. After cutting the central part (diameter 4 mm at ESRF, 5 mm at SNAKE) of the tissue by a biopsy punch, keratinocytes were isolated as described by Zlobinskaya et al. [7]. The number of micronuclei (MN) was only counted in binucleated cytokinesis-block (CB) cells containing detached MN in the cytoplasm in order to measure the frequency of MN only in the first division after irradiation. At least 500 binucleated CB cells with well-preserved cytoplasm were analysed for each sample of the proton and X-ray irradiations and from sham irradiated controls. Dermal fibroblasts were not scored because of their low density and long cell cycle duration.

Results

Dose distributions

Dose distributions for the proton microchannel irradiations at SNAKE were quantified using Gafchromic EBT2 films (International Speciality Products, USA), using a dedicated calibration curve [15,16] and by scaling the number of particles to fit the dynamic dose range of the films (i.e. increased total fluence to measure valley doses). The analysed irradiation pattern revealed that at least 93% of the area contained less than 1% of the applied mean dose in the two focused modes (SC and LC), indicating a valley dose of less than 0.02 Gy for 93% of the irradiated area (for details see Ref. [7]). The spatial dose distributions for X-rays at ESRF were also verified by EBT2 films, which were calibrated in the ESRF beam against an ion chamber. Again, exposure times were scaled in order to capture the valley doses in the dynamic range of the film. Figure 1 shows representative examples for homogeneous field (HF), lines (LN), small channel (SC) and large channel (LC) irradiation modes.

MTT tissue viability test

Cell viability of the EpiDermFT™ tissue was measured 40 h after irradiation following the MTT-100 protocol developed at MatTek Corporation by using the enzymatic, colorimetric MTT assay on the central part (diameter 4 mm) of the tissue. Pooled mean values of three independent experiments and the standard error are shown in Table 1 and Fig. 2. The larger uncertainty bars of the X-ray experiment are due to higher variation in the control samples, which are used for normalization of all other samples. To determine a statistical significance between the different application modes, an un-paired t-test was applied on the non-normalized data. Cell viability after homogeneous irradiation with protons was 39.8 ± 2.1% and 38.9 ± 6.1% for X-rays if compared to non-irradiated controls. After microchannel irradiation with small channels (50 × 50 μm^2, SC), the cell viability was significantly higher ($p < 0.05$; normalized values: 79.4 ± 0.5% for protons and 79.2 ± 13.6% for X-rays) if the same mean dose was used. Microchannel irradiation of large channels (180 × 180 μm^2, LC) and irradiation in 50 μm lines showed also significantly higher cell viability ($p < 0.05$) than after homogeneous irradiation for both protons and X-ray. However, the viability data of the two different microchannel irradiations (LC and SC) and the 50 μm lines (LN) did not show any significant differences. Furthermore, there was no significant difference between protons and X-rays for any of the irradiation modes.

Micronuclei assay

Genetic damage as determined by the measurement of micronuclei in keratinocytes was also significantly reduced after microchannel irradiation compared to homogeneous irradiation. Table 2 and Fig. 3 represent the results induced by protons and X-ray irradiation, which have been determined with the different irradiation modes ($n = 3$). To test for statistical significances, an unpaired t-test was carried out for the yields of micronuclei.

For the homogeneous irradiation mode (HF), the number of micronuclei per cytokinesis-blocked (CB) binucleated cell was 0.070 ± 0.005 for protons and 0.067 ± 0.009 for X-rays. Microchannel irradiation with small (SC) and large (LC) channels, as well as after irradiation in 50 μm lines (LN), significantly ($p < 0.05$) reduced the number of MN per CB cell at the same mean dose for protons as well as X-rays. The values for small channels (SC) were 0.030 ± 0.005 for protons and 0.031 ± 0.006 for X-rays, the values for large channels (LC) were 0.015 ± 0.003 and 0.027 ± 0.006, respectively. X-ray line irradiation (LN) induced 0.023 ± 0.005 MN per CB cell. No significant differences were found in the number of MN per CB cell between the two microchannel modes (SC, LC) and the 50 μm lines (LN). As for the MTT test, no significant difference between protons and X-rays was seen for any of the irradiation modes.

Discussion and conclusions

The risk of developing normal tissue injuries limits the radiation dose that can be applied to the target volume in radiotherapy. During the last decades the treatment techniques have evolved, and in modern radiotherapy damage to normal tissues can be limited by using highly conformal treatment planning techniques for photon and proton beams. However, even with most advanced radiotherapy techniques, many patients develop skin reactions at the irradiated area [17]. The severity of acute skin reactions is correlated to the delivered total dose, the dose per fraction and to the size of the treatment area. Acute radiation effects in the skin usually manifest initially as a slight erythema and can progress later from dry desquamation to confluent moist desquamation [17]. Therefore, patients often feel discomfort, which can range from a mild irritation to considerable pain. Since several decades it is known that acute radiation damage to skin involves both, the epidermis and dermis [18]. The basal layer of the epidermis proliferates very fast and is particularly sensitive to radiotherapy. Within 14 days after irradiation, the epidermis shows necrosis and inter-cellular oedema as a result of cell death of post-mitotic keratinocytes in the upper viable layers of the epidermis. Therefore, the

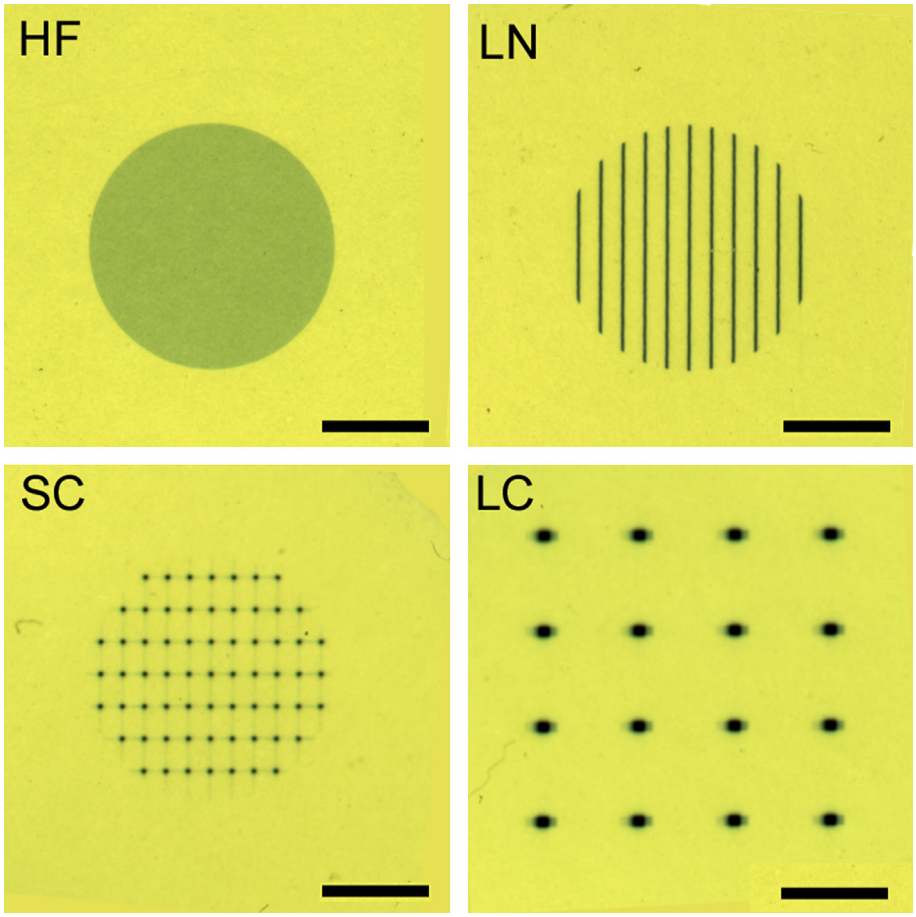

Figure 1. Dose distributions of four irradiation modes at ESRF: homogeneous field (HF), lines (LN), small channels (SC) and large channels (LC), measured with radiochromic film. Scale bars: 2 mm.

Table 1
MTT test results at SNAKE and ESRF.

	SNAKE	ESRF
CO	100.0 ± 0.5	100.0 ± 14.3
HF	39.8 ± 2.1	38.9 ± 6.1
LN	Not tested	77.9 ± 15.5
SC	79.4 ± 0.5	79.2 ± 13.6
LC	82.5 ± 1.2	81.4 ± 12.9

inflammation persists for the duration of radiation therapy and it takes another 2–4 weeks to heal after the end of radiotherapy.

The aim of our study was to investigate if microchannel irradiations with micrometre sized X-ray or proton beams can minimize the risk of normal tissue damage in the skin (independently of the biological response at the depth of the tumour, which might be quite different between X-ray MRT and proton microchannel irradiation due to the different scattering properties). It is well established that normal tissues can tolerate high doses of radiation over small volumes. Therefore, the "valley" dose in the gaps between adjacent microbeams has to be kept as low as possible to achieve an optimal normal tissue sparing effect. A previous study indicated that skin is highly resistant to X-ray microbeam irradiations [19]. The authors showed that even high doses up to 925 Gy (90 μm beams with 300 μm spacing, valley dose about 2.5%) were well tolerated in a rat skin model. No moist desquamation has been observed. The recovery of the damaged segments after microbeam irradiation of the skin was attributed to surviving cells, localized

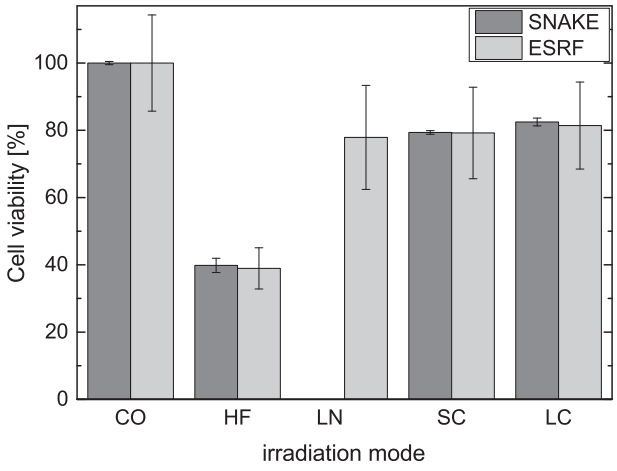

Figure 2. MTT test results at SNAKE and ESRF. Cell viability of homogeneous field (HF), line (LN), small channel (SC) and large channel (LC) irradiation was measured at the central part (diameter 4 mm) of the irradiated area after 40 h by the MTT assay. Uncertainty bars represent the SD of the mean value of three independently irradiated tissue samples, where negative control (CO) is sham treated.

between the irradiated areas with clonogenic potential that proliferate and lead to fast re-epithelialization [19]. Another study demonstrated that a peak entrance dose of 200 Gy with MRT produced similar histological responses in the skin to a broad beam irradiation with 11 Gy [20]. However, this study also showed that

Table 2
Micronuclei test results at SNAKE and ESRF.

	SNAKE	ESRF
CO	0.011 ± 0.004	0.012 ± 0.003
HF	0.070 ± 0.005	0.067 ± 0.009
LN	Not tested	0.023 ± 0.005
SC	0.030 ± 0.005	0.031 ± 0.006
LC	0.015 ± 0.003	0.027 ± 0.006

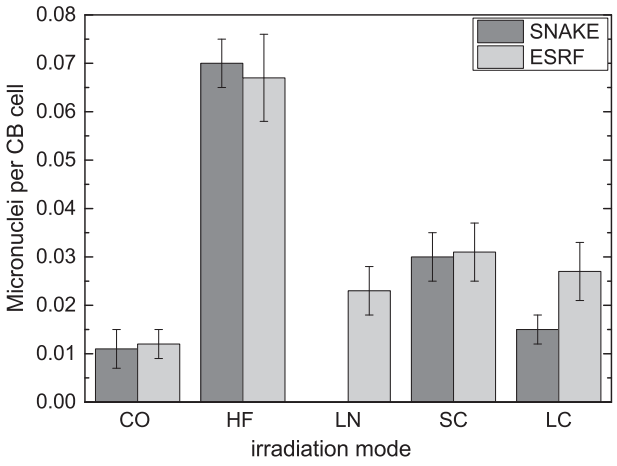

Figure 3. Micronuclei assay at SNAKE and ESRF. Mean number of micronuclei per divided, cytokinesis-block (CB) cell for control (CO), homogeneous field (HF), line (LN), small channel (SC) and large channel (LC) irradiations measured after 48 h. Uncertainty bars represent the SD of the mean value of three independently irradiated tissue samples.

apoptosis in epithelial cells was not confined to the path of the microbeams through the tissue. This might be attributed to high valley doses (2–16 Gy) which induces the migratory capacity of epidermal cells out of the irradiated area later.

Our investigations were performed in a human skin tissue model in order to account for the 3D geometry and the communication between different cell types such as keratinocytes and fibroblasts. This model has been proven to be valid for assessing the induction of micronuclei after proton and X-ray radiation [7,12,21,22].

Our comparative study proved the potential of proton as well as X-ray microchannel radiotherapy to spare normal tissue from acute and long-term side effects by spatial fractionation. Both, cell viability and genetic damage measured at SNAKE and ESRF showed the same superiority of microchannel irradiation compared to conventional homogeneous broad beam irradiation, for protons as well as for X-rays. This applies to all investigated micro-irradiation modes (lines, small and large channels). It is important to note that cell viability and genetic damage results were not significantly different after proton and X-ray irradiations in all irradiation modes. This indicates that rather the mode of radiation application than the radiation quality does impact cell viability.

It is also important to note that due to the higher survival rate of cancer patients in recent years, the risk to develop radiation induced second tumours has now become of greater importance than ever [23]. It was recently shown that the frequency of micronuclei can be predictive of the cancer risk, which suggests that higher amounts of genetic damage after irradiation is correlated with early events in carcinogenesis [24]. In our study, the lower number of micronuclei in skin keratinocytes after microchannel irradiation may imply a reduced risk to develop mutations in normal tissues, because there is a close relationship between chromosome aberrations and gene mutations and cancer predisposition after irradiation exposure [25]. Therefore the lower number of micronuclei in skin keratinocytes after microchannel irradiation may lead to a reduced risk to develop second tumours.

In conclusion, our study indicates that microchannel irradiations with either micrometre sized X-ray as well as proton beams can minimize the risk of skin damage in an *in vitro* human skin model. Future animal models are required to assess long-term effects of microchannel irradiation on normal tissues, *in vivo*.

Conflicts of interest

The authors declare no conflicts of interest.

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