Mesenchymal Stem Cells show high radioprotective activity in vivo

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Short title: radioprotective activity of mesenchymal stem cells

Abstract

Radiotherapy puts bones at risk of developing osteonecrosis. Irradiation has an impact on the viability as well as the differentiation capacity of mesenchymal stem cells (MSC), which play a pivotal role in bone regeneration.

To investigate the effect of irradiation on MSC, human bone-derived MSC were irradiated *in vitro*. With increasing doses the cells' self-renewal capabilities were greatly reduced. Notably however, mitotically stalled cells were still capable of differentiating into osteoblasts and preadipocytes. Next the pigs mandibles were subjected to fractionized radiation of 2x9 Gy within one week. This treatment mimicks that of a standardized clinical treatment regimen of a head and neck cancer patient (30x2 Gy). Fractures, which had been deliberately generated and subsequently irradiated showed retarded osseous healing. When isolating MSC from irradiated sites at different time points post irradiation, no significant changes in comparison to cells derived from un-irradiated specimens regarding proliferation capacity and osteogenic differentiation potential became apparent.

Therefore, pig mandibles were irradiated with 9 and 18 Gy *in vivo*, and MSC were isolated immediately afterwards. No significant differences between the untreated and 9 Gy -irradiated bone with respect to proliferation and osteogenic differentiation were unveiled. Yet, cells isolated from 18 Gy irradiated specimens exhibited a reduced osteogenic differentiation capacity, and during the first two weeks proliferation rates were greatly diminished. Thereafter, cells recovered and showed normal proliferation behaviour.

These findings imply that MSC can cope with irradiation up to high doses *in vivo*, and could be implemented in future therapeutic concepts to protect from osteonecrosis.

(250 words)

Keywords

Mesenchymal Stem Cells, osseous regeneration, fracture healing, radiation, osteoradionecrosis

Introduction

Radiotherapy bears the risk osteonecrosis, which is the most dreaded adverse side effect in treatment of head and neck cancer, one of the most common cancers worldwide [1]. During osteonecrosis, an impaired fibroblastic activity plays a decisive role, whereby the bony matrix is gradually converted into fibrous tissue [2]. In parallel, osteoblastogenesis becomes dysregulated leading to insufficient proliferation of osteoblasts. In consequence, this impediment often results in a high rate of myofibroblast proliferation within irradiated bone and surrounding tissues. It is generally assumed that irradiation causes a direct damage of tissue-borne multipotent progenitor cells. In fat, due to the massive generation of reactive oxygen species (ROS), it leads to the destruction of endothelia. This in turn initiates an acute immune response through cytokine release followed by an increased production of ROS via the recruitment of phagocytes. Vascular thrombosis and endothelial cell destruction eventually results in necrosis of microvascular structures, local ischemia, and consequently, tissue loss [2].

Investigations in the past demonstrated that irradiated bone can be supported by growth factors, although the response of cells residing in bone is decreased and their supply through a degenerating vascular system is compromised. Application of cytokines, such as bone morphogenetic proteins can greatly enhance bone regeneration of irradiated bone [3]. Besides other cells, also MSC respond to these type of cytokines [4]. Whether dormant MSC are capable of coping with radiation induced damage and are thus able to sustainingly contribute to wound healing and tissue regeneration after irradiation, is currently unknown.

Material & Methods

Isolation and cultivation of mesenchymal stromal cells

Human MSC were isolated from iliac bone biopsies, cultivated in long-term culture as described previously [5]. MSC from *Sus scrofa domestica* were harvested from cancellous and compact bone as well as from the periosteum of the mandible and the iliac crest. Samples were reduced in size to approximately 20 to 100 mm³ under sterile conditions. The specimens were stored in growth medium (minimum essential medium (MEM, GIBCO-BRL) containing 10 % fetal calf serum (FCS, Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin) and transported at room temperature. In a sterile work cabinet, the liquid was removed and the bone pieces were inserted into a pipette tip with its tappering end sitting in a 1.5 mL reaction tube, both within a 15 mL tube. This tube was centrifuged for 1 minute at 400 x g to collect the marrow. After centrifugation, the remaining pieces were treated with collagenase (2.5 mg/mL in MEM, Sigma) for 2 – 3 hours at 37 °C (Heraeus, Hera Cell 240) 20% O_2 and 5% CO_2 to render cells free from the tight extracellular meshwork covering the bony surface. The treated specimens were again centrifuged for 1 minute at 400 x g. The cell pellet was resuspended in growth medium as described above by gentle aspiration through syringe needles of different gauge sizes. In case of agglomeration of bone, bone marrow and cells, the fluid was separated from the rest by means of a 100 µm nylon mesh filter. Thereafter the resuspended cells were loaded on a Ficoll-Paque Plus® gradient (Amersham Biosiences) and centrifuged at 2500 × g for 30 minutes. The ratio between the resuspended cells containing liquid and those harvested after the Ficoll-Paque Plus® gradient was 1:1. The cells were harvested from the interphase (density < 1.075 g/mL). In order to remove the Ficoll-Paque Plus®, the cells were washed with growth medium and recovered by centrifugation at 1500×g for 15 minutes. The purified cells were further cultivated at 3% O_2 and 5% CO_2 (Thermo Electron Corporation 3110) at a cell density of 0.2 – 0.5x10E6 cell/cm^2. After 24 hours, the non-adherent cell fraction was removed by washing twice with PBS at 37 °C. The medium was changed every 3 to 4 days. After the primary culture had reached approximately 30–50% confluency, the culture medium was removed and the cells were washed twice with PBS for 3 minutes at the 3 % - O_2. Thereafter PBS

was removed and the cells were treated with 0.05% trypsin / 1mM EDTA (GIBCO) for 5 minutes at 37°C. Cells were harvested, washed once in media and further expanded at a density of 50 cells / cm^2. The number of population doublings during every passage was accounted.

Irradiation of cultivated mesenchymal stromal cells.

Cells were grown in 25 cm^2 flasks (2 cm in height) to a confluency of 50% and treated with 6 MeV photons, which are commonly used in clinical radiotherapy for treatment of cancer patients (ELEKTA Synergy Linear Accelerator; serial number: 131431, ELEKTA Oncology Systems installed at the Department of Therapeutic Radiology and Oncology / Medical University Innsbruck). For radiation of cell probes, an experimental setup was chosen which guaranteed broadly homogeneous dose delivery to the cell probes. The flasks were completely filled with medium. Four flasks were placed on a staple of Perspex as well as surrounded by Perspex and covered with a slab of 1 cm super flab material at a source-surface-distance of SSD=100 cm. Cell probes were irradiated with energy doses from 3 up to 18 Gy.

Flow cytometric analysis

Cell viability was examined with the aid of an argon laser-equipped flow cytometer (FACSCanto, Becton Dickinson) by monitoring 7-AAD fluorescence together with monitoring forward/ and side scattering in combination with the AnnexinV method as described previously [6]. Briefly, staining was performed as follows: cells were washed with PBS and stained with 20 µg/ml 7-AAD for 40 minutes at 37°C. Thereafter cells were washed with AnnexinV binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and stained with 5 µL AnnexinV-FITC (Becton Dickinson) in 100 µL AnnexinV binding buffer for 15 minutes at room temperature. Staining was stopped by adding 400 µL AnnexinV binding buffer. Data were analyzed with the aid of FACSDiva Software (Becton Dickinson). Cell cycle phases were assessed by analysing the amount of nuclear DNA by staining permalized cells with propidium iodide: cells were detached from the dishes as described and resuspended in de-ionized water containing 0.1% Triton X-100 and 50 µg/mL propidium iodide [7]. The proportions of each cell cycle stage were calculated with the aid of Cell Quest Pro (BD Biosciences).

Osteogenic and adipogenic differentiation *in vitro*

Human MSC were stimulated to differentiate *in vitro* as described previously [5]. Differentiation capacity was assessed in quadruplicates by initially growing cells for 10 days at 3% O_2 and 5% CO_2 (Thermo Electron Corporation 3110) and 37 °C in growth medium, and thereafter incubating the cells for 21 days with 10 mM β-glycerol phosphate disodium salt pentahydrat (Fluka, Vienna), 10 nM dexamethasone (Sigma Aldrich, Vienna), and 50 µg/ml 2-Phospho-L-ascorbic acid tri-sodium salt (Fluka) in growth medium at 37 °C, 20% O_2 and 5% CO_2 (Hera Cell 240, Heraeus). Eventually the cultures were fixed with 4% formaldehyde in PBS. After 5 minutes the specimens were washed twice with PBS, pH4. Then the cell layer was stained with Alizarin Red, pH4.1 for 20 minutes. Excessive stain was removed by several washing steps with PBS, pH4. For further analysis, the specimen was kept in PBS, pH4.0. The following classification was used to determine the differentiation grade: grade 3 – more than 60% cells engulfed by mineralized matrix; grade 2 - 40 – 60%; grade 1 - less than 40%; grade 0 – no differentiation in reference to negative control [5].

Mandible irradiation

All animal experiments were performed only after permission by the Austrian Government and National Ethics Committee (permission number: BMBWK-66.011/0143-BrGT/2006) and conducted in concordance to the EU directive 86/609/EEC.

To ensure broadly homogeneous dose delivery in the targeted volume of pig mandible *in vivo*, irradiation treatment was performed according to a standardized clinical workflow: first, the pig's head and in particular the jaws, separated with a bite block and placed in treatment position was examined by computed tomography (CT); next with the aid of the clinical treatment planning system, PrecisePLAN® (ELEKTA Oncology Systems, Crawley, UK) a standardized 3D model was compiled on the basis of the CT data set to correctly juxtapose two opposing wedged fields of 7 x 3 cm, thereby yielding a widely homogenous dose distribution during irradiation. Pig mandibles were irradiated with energy doses of 9 Gy and one week later another fraction of 9 Gy, which

altogether corresponds to a biologically effective dose of 60 Gy. Proper positioning of the radiation field was controlled by generating electronic portal images of each radiation field.

Under general anaesthesia a total of 20 pigs underwent an iatrogenic unilateral mandibular fracture. The fractures were stabilized with reconstruction plates and locking screws in general anaesthesia (Synthes 2.4, Synthes Austria, Salzburg, Austria). In the irradiation group (16 pigs) the fracture was set four weeks after irradiation, which followed the protocol described above. The irradiated pigs were sacrificed either right after irradiation, or 4, 5, 6, 8 or twelve weeks after irradiation. The non-irradiated pigs were sacrificed 1, 2, 4 and 8t weeks after the surgical treatment.

Histology

Biopsies were embedded in Technovit 9100 Neu (Heraeus Kulzer, Hanau, Germany) as described previously [4]. Performing the sawing and grinding technique, described by Donath et al. [8], histological sections of the fracture gaps were prepared with a thickness of 12 µm in average. Toluidin blue O staining was performed to assess the healing capacity of the irradiated and non irradiated fracture sites.

Results

Mesenchymal stromal cells (MSC) are rapidly proliferating when cultured in media containing high proportions of fetal bovine serum. When grown at low density, MSC readily form colonies, and they are capable of differentiating into multiple lineages when induced by appropriate means.

After treatment with increasing doses of ionizing radiation, cultured human mesenchymal stromal cells (hMSC), which had been derived from bone of systemically healthy individuals and which exhibited the above mentioned properties, exhibited increasing cell death rates (Figure 1). Colony formation was highly suppressed after treatment with doses of more than 9 Gy. When receiving 12 Gy or more, many cells survived and could actually be further differentiated into adipogenic and osteogenic precursors when incubated in appropriately stimulating media (data not shown). An effective dose of 18 Gy resulted in a greatly enhanced cell death rate.

These initial findings were corroborated with MSC derived from mandibular bone of pigs, which had been implemented as controls in experimental studies regarding irradiation treatment, osseous implant healing and induced osteoradionecrosis (Figure 2). In course of this *in vivo* experiment, in which the jaws of experimental animals were subjected to fractionated radiation of 2x9 Gy, which closely resembles the biologically effective dose of a standardized clinical treatment regimen for cancer therapy, we observed that an artificial fracture, which had been deliberately generated and subsequently treated with osteosynthesis plates and screws, showed retarded osseous healing (Figure 3).

This observation prompted us to investigate, whether *in vivo* irradiated MSC remain vital. In order to determine the impact of ionizing radiation on MSC within bone and bone marrow, mandible biopsies were taken from living animals directly after irradiation with 9 or 18 Gy. Notably, the long-term proliferation capacity of MSC isolates, which had actually been irradiated with 9 or 18 Gy was comparable to those of non-irradiated counterparts (Figure 4A). The number as well as the osteogenic potential of those MSC that had been isolated from mandibular bone irradiated with 18 Gy was greatly diminished (Figure 4 B, C).

When the MSC were isolated 4, 5 or 6 weeks post radiatio with 2 x 9 Gy, which accounts for a biological effective dose of 60 Gy, their respective number (Figure 5) as well as their long-term proliferation capacity (Figure 6) was indistinguishable from MSC isolated from non-treated control groups.

Discussion

MSC exhibit a high proliferation potential and a multipotent differentiation capacity [9]. In recent years many scientists were able to isolate MSCs from a large variety of specialized tissues. This naïve cell type could also be successfully differentiated *in vitro* into various tissue-specific precursors with phenotypes closely resembling that of osteocytes, chondrocytes, smooth muscle cells, skeletal muscle cells, cardiac muscle cells, neuronal cells, insulin producing cells, adipocytes, keratinocytes and endothelial cells. Inevitable damages during life-time, or other, yet intended harmful events during medicinal therapies may activate dormant stem cells in their niches, and it is also assumed that MSCs contribute to the regeneration of bone and bone marrow after injury *in vivo* through proliferation and controlled differentiation.

Harmful biological effects of irradiation are mediated via highly reactive radicals - such as the water ion H_2O^+ or the hydroxyl radical OH·, both of which are freely diffusible over cellular membranes and thus can damage any biomolecular entity, most important in this context DNA [10]. Cells are more radiosensitive during the M and G2 phase of the cell cycle, yet being most resistant in the late S phase [11]. The cell cycle of cancer cells is shorter than that of normal cells. Interestingly, cells residing in oxygenized tissues are 2 to 3 times more sensitive to radiation than cells at anoxic conditions [12], and in their regeneration phase after irradiation, normal somatic cells often proliferate faster.

In the treatment of head and neck cancer, osteoradionecrosis is a common consecutive complication of irradiation, which preferably occurs in the mandible [13]. It is conceivable to stimulate healing of irradiated bone through the application of cytokines such as bone morphogenetic protein (BMP), or vascular endothelial growth factor (VEGF) since it is well known that osseous bone healing *in vivo* is greatly enhanced by such bioactive factors. It is generally accepted that MSCs are responsive to BMPs [14-17]. As the influence of irradiation on the fate and proliferation of MSCs is only scarcely investigated, we first monitored the changing properties of cultured MSC, which were derived from porcine irradiated bone, by assessing their clonogenic growth potential, which after low density seeding serves as a reliable method to quantify the cell pool that

bears stem cell-like qualities. This is considered a good quantitative measure for the so-called stemness. Compared to non-irradiated controls, secondary colony-forming potentiality steadily decreased with increasing dosage, while the osteogenic and adipogenic differentiation of irradiated cells remained greatly unimpaired after the application of a high dose of 18 Gy, which corresponds to a biological effective dose of 60 Gy. These results are in good concordance with observations of Clavin et. al., who irradiated murine MSC with 0, 2, 6, and 12 Gy *in vitro* [18]. The cellular proliferation was clearly diminished after application of 12 Gy while adipogenic and osteogenic differentiation could still be achieved. Jing Li et. al. however reported that human MSC when irradiated with a single dose of 2, 4, 8 and 12 Gy in suspension and not as an adherent monolayer first ceased growth but restored their proliferation rate to normal levels after two weeks. Osteogenic and adipogenic potential was decreased with increasing doses of radiation [19]. Yet comparable *in vivo* data are missing up to now.

Considering the fact that bone marrow is a complex *in vivo* environment with many interactions of different cell types at various stages of differentiation, and secondly the marrow cavity being a complex three-dimensional structure, a scene that could be hardly re-enacted with cells in culture, we next studied the fate of MSC after irradiation *in vivo*. For that purpose, pig mandibles were irradiated with either 9 or 18 Gy, dosages which resulted in a greatly retarded osseous healing at the site of an artificial fracture. In order to examine the rate of damage in tissue-borne MSC, the animals were sacrificed immediately after irradiation and cells were isolated. The self renewal property and osteogenic potential of MSCs was clearly diminished after irradiation with a dose of 18 Gy. 9 Gy had only little impact on the MSC, which is in stark contrast to our observation regarding radiation sensitivity of *in vitro* cultured MSC. During common radiotherapy in the clinics, patients are subjected to a fractionized treatment regimen thereby receiving a biologically effective dose of 60 Gy. Working along these lines, we expected that an equivalent dose would lead to a sustainingly lasting effect on MSC in the animal model. In line with this assumption we actually accounted retarded bone healing during the recovery phase after treatment and also noticed 4 weeks post radiation, that the blood vessel density was greatly reduced in bone and muscle at irradiated sites (unpublished results).

Yet, viable MSC could be successfully isolated at several timepoints up to 8 weeks post radiation. The MSC number was comparable to non-irradiated control samples, their long-term proliferation potential was closely resembling that MSC from untreated bone and these cells also differentiated along the osteogenic lineage. Given these observations, radiation sensitivity appears to be greatly attenuated in MSC *in vivo*, which may be either due to intrinsic preventive measures such as enhanced repair mechanisms, or due to exogenous protective means of the stem cell niche. Cellular mechanism of radioresistance have been proposed for MSC by Chen et al., who demonstrated that MSCs exhibit high antioxidant ROS scavenging capacities together with an enhanced activity of the DNA double strand break repair system [20].

Fibrosis is considered key in the development of irradiation-related changes of bone [21]. In this context, hypoxia does not appear to be critical but is more a consequence of fibrosis in irradiated tissue [2]. Early after radiation, changes in endothelial cells go along with an acute inflammatory response, as endothelial cells become damaged directly through physical damage as well as through the action of ROS and free radicals. Injured endothelial cells produce chemotactic cytokines that increase the inflammatory response, which results in further ROS production. The destruction of endothelial cells together with micro-thrombosis results in local ischemia and the loss of the natural endothelial cell barrier. At that point myofibroblasts appear and persist [2], which in due course leads to fibroatrophic tissue layers, which are fragile and severely vulnerable. Yet our present study suggests that these tissues contain fully functional MSC, which may thus contribute to bone healing and regeneration and also take an active part in supporting and regulating hematopoeisis and thus sustaining organismic immune function.

Consistent with the notion that MSC survive radiation therapy, Friedenstein et. al. reported earlier that fibroblastic colony-forming units (CFU-F) reached normal values 25 days after whole body irradiation [22]. The notion of enhanced *in vivo* radioresistance of MSC was further substantiated by the observation that in patients, who underwent allogeneic bone marrow transplantation after irradiation, mesenchymal cells remained host-specific and virtually no transplanted stroma

cells were capable to home and engraft into the patients bone marrow; at the same time the entire hematopoietic system could be restored by donor-derived cells [23]. Besides these observations yet another conceptional view emerged, which refers to the possibility that undisturbed MSC residing in distant body parts are being mobilized and in a targeted fashion may engraft into lesioned tissues and empty niches. Mice that have been subjected to total body irradiation with 3.5 Gy and subsequently received hMSCs intravenously showed indeed enhanced engraftment into bone marrow, muscle, brain, heart, lungs and liver when compared to unirradiated litter mates [24]. Similar observations have been reported after low dose irradiation of tumors, where the recruitment of MSC into the tumor microenvironment was also increased [25].

A recently proposed concept [26, 27] which takes into account evidences that MSC-like cells reside in or close to the vessel wall, not only elegantly explains the broad tissue distribution of MSC. Yet in extrapolation of this privileged position, it is thus conceiveable that MSC contribute to vessel stability and, generally spoken, to tissue homeostasis [28]. In turn it is highly likely that during degeneration of blood vessels, MSC are being released into injured tissue, which is in line with our findings, demonstrating that MSC behave unaffected after irradiation [29]. It is further highly likely that in this case MSC become activated and proliferate whereby they also generate soluble bioactive factors. Besides increasing cellular mass, MSC secretion may in turn contribute to repair and/or regeneration of the injured tissue. By now, it became a well accepted paradigm that MSC are capable of modulating immune surveillance, thus controlling negative interferences of intruding T- and B-lymphocytes within the injury site [29]. By this token, MSCs may not only be a key in repair but even more in preservation of the affected tissue.

In conclusion, the here presented observations on cellular properties of MSCs after irradiation encompass analyses performed *in vitro*, *in vivo* and *ex vivo*, clearly demonstrating that MSC bear *in vivo* radioprotective activities higher than commonly believed. This evidence supports the notion that tissue-resident MSC can be effectively induced to promote bone healing after irradiation treatment, and thus the radioprotective property of MSC should be further considered in the

context of future therapeutic concepts. Further research is however required to determine whether the protective activity is based on intrinsic mechanisms or due to structural determinants of the niche or the surrounding tissues, or lastly, whether MSC from undisturbed sites are being activated to migrate and engraft to irradiated lesions.

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Figure legends

Figure 1: Irradiation of *in vitro* cultivated human mesenchymal stromal cells derived from cancellous bone of the iliac crest. (A) Irradiation of proliferating cells with the indicated dosage showed an impact on cell cycle progression of the surviving cell fraction. Cell survival (B) as well as colony formation (C) was decreased after irradiation treatment (n=3).

Figure 2: Culture and *in vitro* osteogenic differentiation of primary porcine mesenchymal stromal cells. (A) Fibroblastoid cells, which exhibited firm plastic adherence, clonogenic growth and multipotential differentiation capacity, were isolated from mandibular bone, bone marrow and periosteum as well as from cancellous bone of the iliac crest. (B, C) Osteogenic differentiation potential decreased after irradiation at the indicated dosage (for grading in panel C, see left panel, scale bar equals 1 cm).

Figure 3: Fracture healing in irradiated mandible of *Sus scrofa domestica*. Bone healing was investigated 8 weeks after a fracture gap was set. Samples A and C were irradiated, B, D are untreated controls. Representative examples of slow or poor healing (A, B) juxtaposed to a more rapid course (C, D) in both irradiated (A, C) and control mandibular bone; dashed line marks the former edge of the fracture gap (FG); connective tissue (CT) stains blue, local bone is labeled LB.

Figure 4: Properties of primary porcine mesenchymal stromal cells isolated from the mandible directly after irradiation with the indicated effective biological dosage. (A) The proliferation potential was monitored in long-term culture. (B) Colony formation was accounted in low density secondary culture. (C) After irradiation and subsequent cultivation in the presence of osteogenic induction medium, the differentiation potential was assessed (for grading see left panel), n=3.

Figure 5: Clonogenic growth of porcine mesenchymal stromal cells isolated from the mandible after fractionated irradiation with 2x9 Gy. (A) Colony formation of primary cultivated cells isolated 4, 5 and 6 weeks post irradiation and (B) integration of data accounted from all primary cultures isolated at the latter time points.

Figure 6: Proliferation potential of porcine mesenchymal stromal cells. (A) Growth kinetics of cells were isolated from the mandible 4 weeks after fractionated irradiation with 2x9 Gy, and grown in long-term culture (representative examples). (B) Proliferation index of cells isolated 4, 5 and 6 weeks post irradiation during their early stages of long-term cultures and integration of data accounted from all long-term cultivations. (C).

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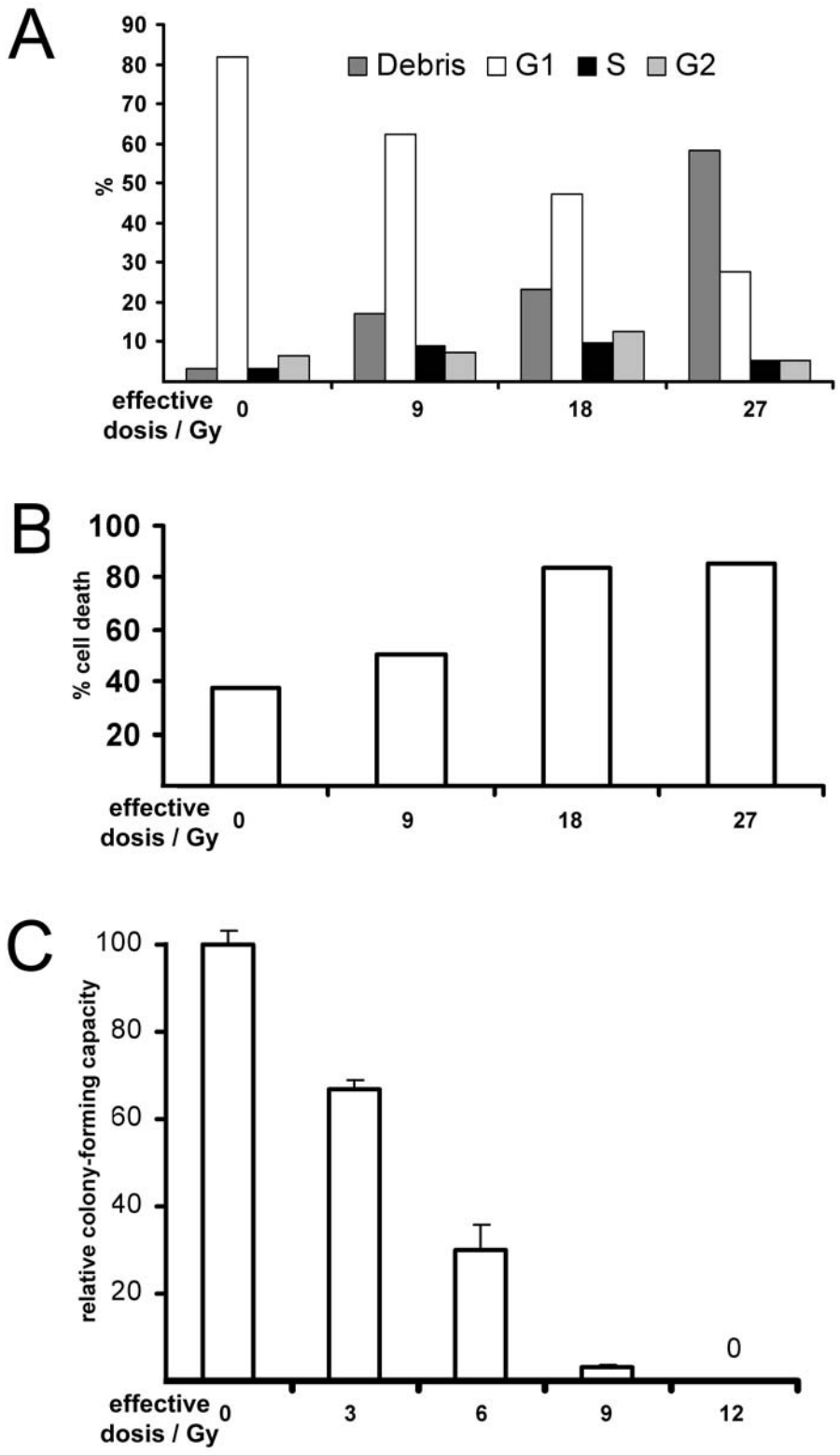
Figure 1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12

Figure 2

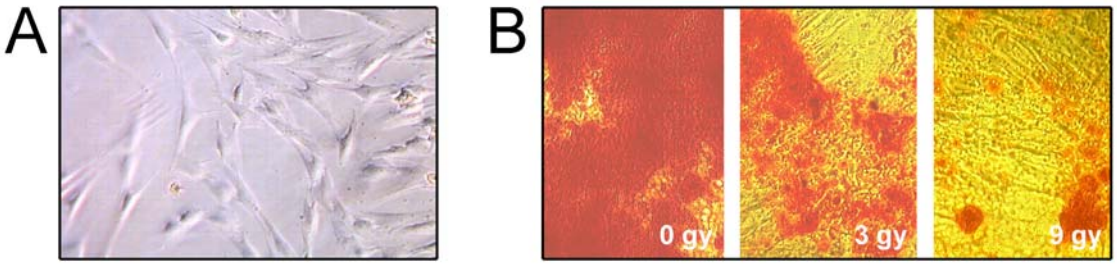


Figure 3

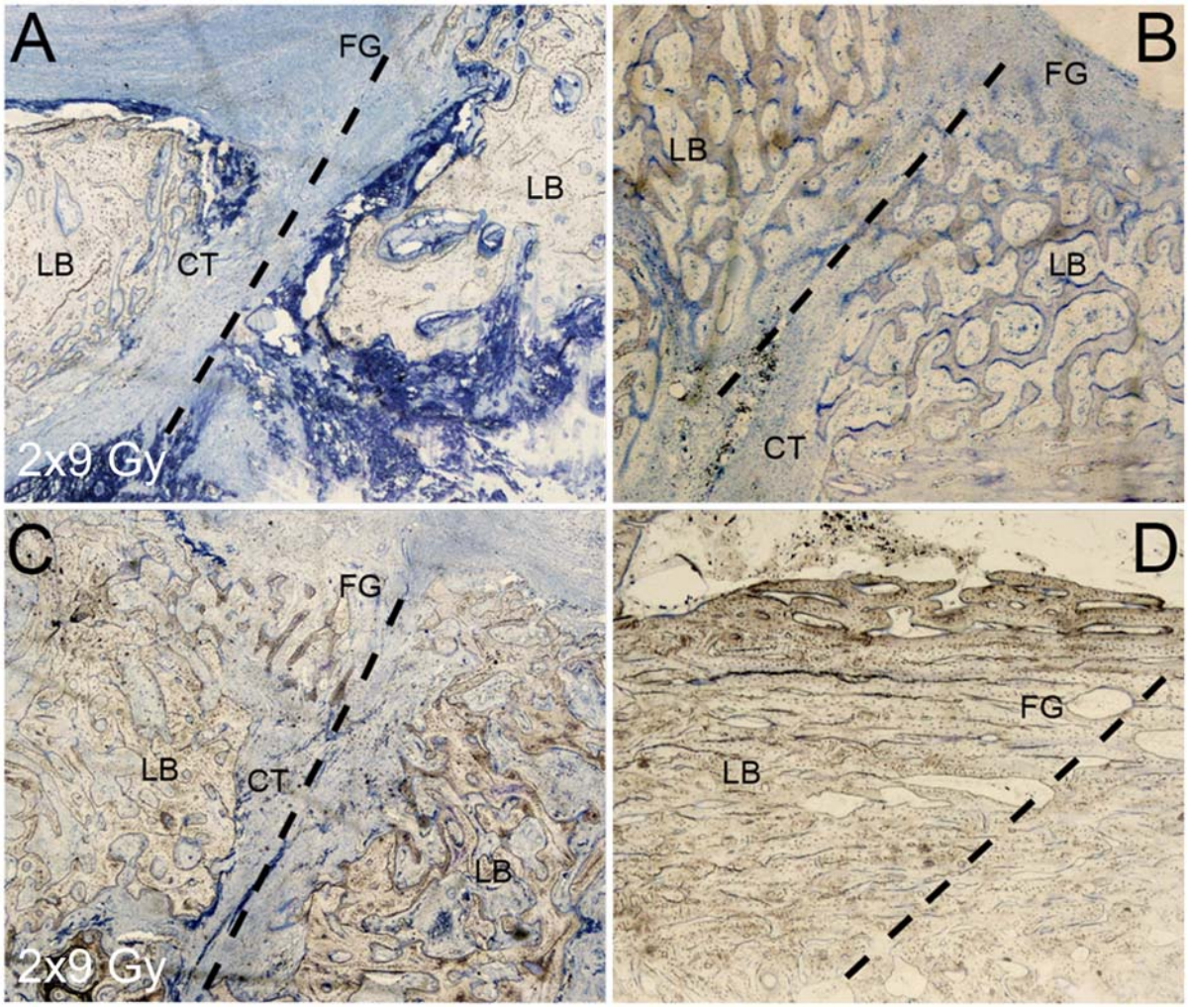


Figure 4

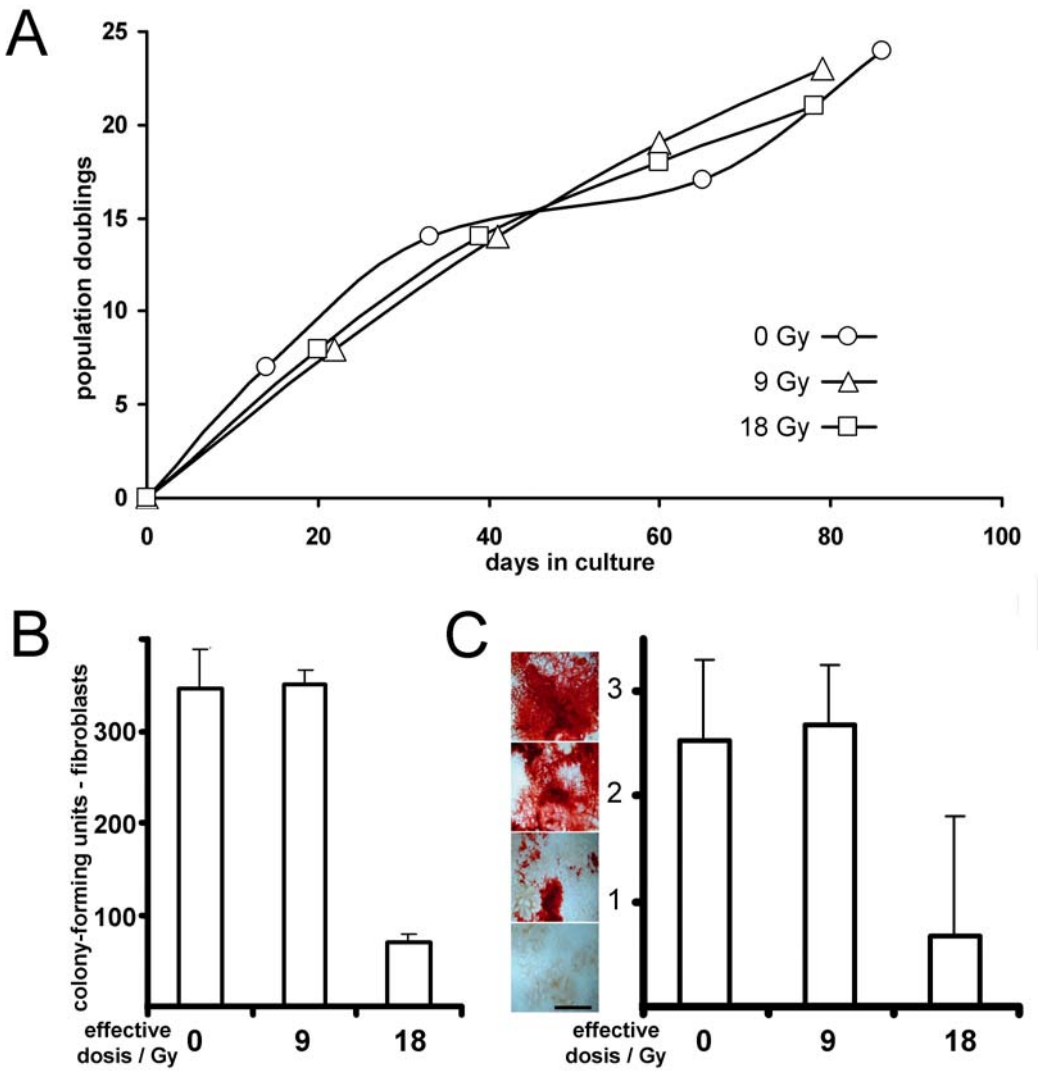

A
population doublings
days in culture
0 Gy
9 Gy
18 Gy
B
colony-forming units - fibroblasts
effective dosis / Gy
C
effective dosis / Gy

Figure 5

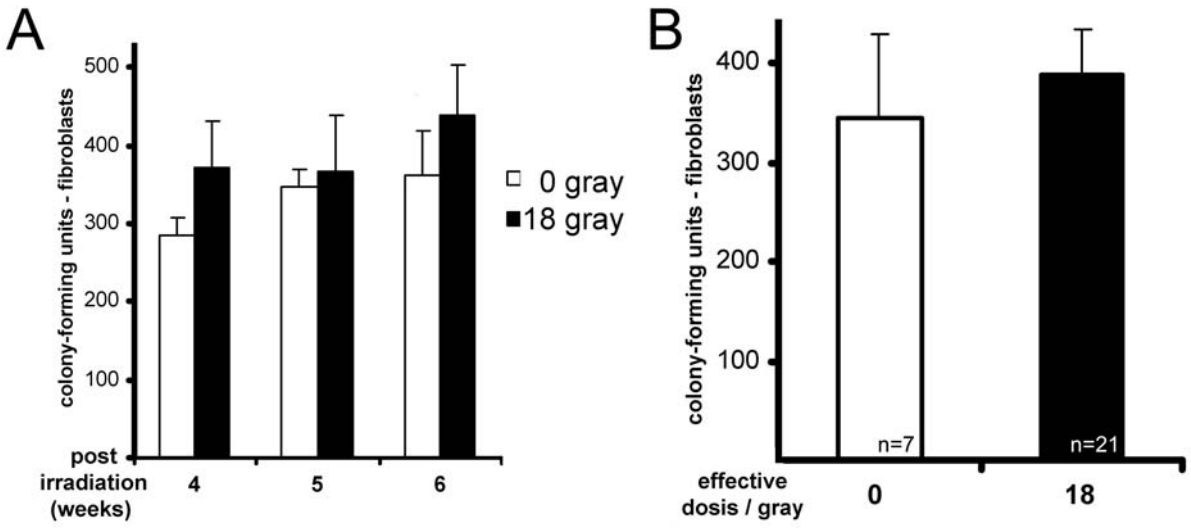
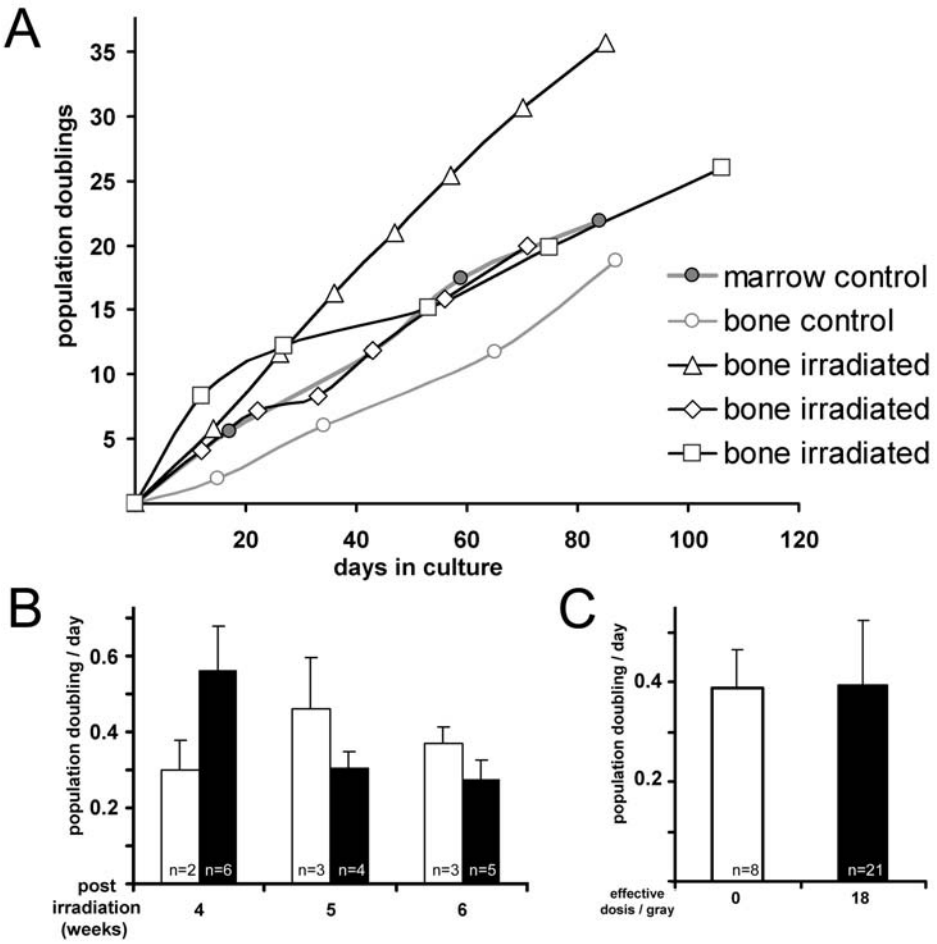

A
colony-forming units - fibroblasts
500
400
300
200
100
post irradiation (weeks)
4
5
6
0 gray
18 gray
B
colony-forming units - fibroblasts
400
300
200
100
n=7
n=21
effective dosis / gray
0
18

Figure 6


A
population doublings
35
30
25
20
15
10
5
20
40
60
80
100
120
days in culture
marrow control
bone control
bone irradiated
bone irradiated
bone irradiated
B
population doubling / day
0.6
0.4
0.2
n=2
n=6
n=3
n=4
n=3
n=5
post irradiation (weeks)
4
5
6
C
population doubling / day
0.4
0.2
n=8
n=21
effective dosis / gray
0
18