in vivo *20*: 573-582 (2006)

Reduced Irradiation Pulmonary Fibrosis and Stromal Cell Migration in Smad3–/– Marrow Chimeric Mice

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Abstract. *Pulmonary irradiation fibrosis involves migration to the lungs of bone marrow origin myofibroblast progenitor cells (marrow stromal cells (MSCs)). Smad3–/– mice display decreased ionizing irradiation-induced skin fibrosis, defective osteochondrogenesis and other abnormalities thought to be associated with a defective stromal cell response(s) to transforming growth factor-beta (TGFβ). Clonal bone marrow stromal cell lines were derived from the adherent layer of continuous bone marrow cultures of homozygous deletion recombinant negative Smad3–/– mice and Smad3+/+ littermates. Quantitation in an Automated Cell Tracking System of the in vitro single cell migratory capacity over five days demonstrated a significant decrease in locomotion in microns per 24 h of Smad3–/– compared to Smad3+/+ clonal MSC lines. Reexpression by retroviral vector transfection of the Smad3 but not control ds-red transgene restored in vitro migratory capacity. Intravenously injected GFP transgene product labeled Smad3–/– (MSCs) seeded 10-fold less effectively than ds-red transgene product labeled Smad3+/+ cells to the 80 days post 20 Gy irradiated lungs of C57BL/6J mice and proliferated less significantly for 60 days after cell injection. Female mice chimeric for male Smad3–/– compared to Smad3+/+ marrow showed decreased irradiation pulmonary fibrosis, Y+ stromal cell migration to the lungs, and improved survival. The data show that the reduced in vitro and in vivo migratory capacity of Smad3–/– bone marrow stromal cells correlates with decreased radiation pulmonary fibrosis observed in mice chimeric for Smad3–/– marrow.*

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Key Words: Bone marrow stromal cells, hematopoietic microenvironment, Smad3, TGFβ signal transduction, gene transfer.

The late effects of total body irradiation (TBI) for preparation of experimental animal or human recipients for bone marrow transplantation include pulmonary and bone marrow fibrosis, which is detected months after irradiation (1-4). The mechanism of irradiation fibrosis is not known, but is thought to involve signal transduction through the TGFβ receptor pathway (1, 5). Fibroblastic cells of bone marrow origin are induced to migrate to the irradiated or bleomycin-treated lung by signaling which includes the TGFβ receptor pathway through the Smad3/Smad4 complex (6-8). Homozygous deletion recombinant negative Smad3–/– mice have been demonstrated to display decreased ionizing irradiation-induced skin fibrosis (9, 10, 12), and have multiple defects in mesenchymal cell function including decreased osteochondrogenesis (11), and abnormalities of the immune system (12).

The absence of a negative regulatory TGFβ signaling pathway in Smad3–/– mice is further revealed in continuous bone marrow cultures which display bone marrow stromal cell mediated improved support for hematopoietic progenitors leading to increased longevity of hematopoiesis *in vitro* (13). Furthermore, clonal bone marrow stromal cell lines derived from Smad3–/– long term bone marrow cultures show constitutive adipocytogenesis and resistance to TGFβ mediated induction of osteoblast differentiation (14), as well as intrinsic radioresistance attributed to a relative block in cell cycle progression through the G2/M phase (15).

In previous studies uncloned lines of Smad3–/– bone marrow stromal cells were shown to migrate less effectively *in vitro* compared to Smad3+/+ cells (15). In the present studies using clonal cell lines labeled with fluorescent proteins, we sought to determine whether the decreased ionizing irradiation-induced skin fibrosis in Smad3–/– mice correlated with a reduced irradiation pulmonary fibrotic response. Clonal Smad3–/– and Smad3+/+ cell lines were labeled with GFP+ or ds-red fluorochrome markers and tested for ability to home to and proliferate in irradiated

lungs at 100 days after irradiation, the time of development of pulmonary fibrosis (6). Since Smad3–/– mice demonstrate life shortening and die at 6-7 months (11, 12), *in vivo* study of the role of Smad3–/– MSCs in pulmonary fibrosis required the preparation of marrow stromal cell chimeric mice, and pulmonary stromal cell line seeding experiments using the established 100-day late irradiation fibrosis model in C57BL/6J mice (6, 16). We correlated *in vitro* marrow myofibroblast cell migration to *in vivo* pulmonary seeding and proliferation studies using clonal cell lines in C57BL/6J mice. The results demonstrate both significantly reduced migratory capacity of clonal lines of MSCs from Smad3–/– mice *in vitro* and decreased *in vivo* migration of fluorochrome labeled cells to and proliferation in irradiated lungs. Furthermore, recipient mice chimeric for Smad3–/– marrow (compared to Smad3+/+ marrow chimeric mice) showed decreased irradiation pulmonary fibrosis.

Materials and Methods

Mice, animal irradiation and cell line injection. Smad3$^{ex8/ex8}$ mice were generated by targeted disruption of the Smad3 gene by homologous recombination. Targeted embryonic stem-cell clones were microinjected into C57BL/6 blastocysts to obtain germline transmission. Mice heterozygous for the targeted disruption were intercrossed to produce homozygous offspring (12). These mice were genotyped and referred to as Smad3-/- as published (9).

C57BL/6J mice were obtained from Jackson Laboratories, Bar Harbor, ME. The mice were housed five per cage, fed standard laboratory chow, and managed according to the Institutional Animal Care and Use Committee. C57BL/6J female mice were irradiated using a Varian 6 MeV linear accelerator (Varian Medical Systems, Palo Alto, CA, USA). The mice were irradiated to 14 Gy or 20 Gy at a dose rate of 1.8 Gy per min. with the head and abdomen shielded so that only the pulmonary cavity was irradiated. Mice irradiated to 20 Gy 100 days previously were intravenously (*i.v.*) injected with $1x10^5$ to $5x10^6$ cells of clonal bone marrow stromal cell lines (6). Dose-response curves documenting the optimization of lung irradiation doses have been published previously (6). Chimeric C57BL/6J female mice, reconstituted with male Smad3+/+ or Smad3-/- marrow were prepared according to published methods (6). Mice were tested 30 days after 10 Gy total body irradiation and marrow transplantation for the percent of peripheral blood cells positive for donor marrow by Y probe analysis as published (6).

Retroviral vector transfer of the Smad3, GFP, or ds-red transgenes into the Smad3-/- cell line and selection of clonal sublines. The Smad3, GFP, or ds-red transgene was cloned into a pRevTRE retrovirus (Clontech Laboratories, Inc., Mountain View, CA, USA) containing the hygromycin resistance gene. Clonal cell lines of Smad3+/+ and Smad3–/– bone marrow stromal cells (14) were vector-transfected with retrovirus by plating $1x10^5$ cells in each well of a 6-well plate 24 h before transfection. To each well was added 1 ml of DMEM medium plus 2 ml of retroviral medium from the packaging cells. Polybrene was added to each well for a final concentration of 4-8 μg/ml. Twenty-four hours later the medium was replaced with DMEM medium containing 10% FCS, L-glutamine, penn/strep and hygromycin at a concentration of 200 μg/ml for the first 7 days. Beginning on day 8, the cells were grown in the presence of 100 μg/ml hygromycin and cells resistant to hygromycin were selected. Clones were isolated by limiting dilution in 96 well plates. Subclonal lines were established using the limiting dilution method (13). Expression of the Smad3 transgene was demonstrated by RT-PCR using primers specific for the human Smad3 transgene (9, 10). Those clones of Smad3–/– cells expressing the Smad3 transgene were designated Smad3–/–(3), those expressing ds-red or GFP were termed Smad3–/– (ds-red), or Smad3–/– (GFP).

Cell tracking experiments. Clonal sublines of Smad3+/+, Smad3–/–, and Smad3–/–(3) cells, as well as uncloned lines from each were plated in duplicate, at a density of 100 cells/well in 60 μl of DMEM + 10% FBS in a 384 well plate. The plate was placed on the Cytoworks automated cell culture imaging system (17). This system consists of an environmentally controlled biochamber on an electronically controlled x-y motorized stage (Ludl Electronics, Ltd.) mounted upon an inverted microscope (Nikon TE 300). The stage moves precisely to each well in the multi-well plate or to any number of locations within each well with a positioning repeatability of + 1.5 μm over the longest distance traveled by the stage. The biochamber temperature, humidity, and CO_2 content are controlled by a custom instrument control program, which integrates control of the microscope stage, filters, shutters, camera, image storing functions, and thermal zones, through a specialized serial interface (17).

Video time-lapse imaging and analysis. Images were acquired over 5 days, at 18 min intervals, from two different locations in each of 42 wells (total of 84 imaging locations) using a 10x objective and a Photometrics SenSys high resolution CCD camera (Roper Scientific). Image sequences were processed using a custom software program that identifies and records the location and morphological characteristics of cell-like objects. Both magnitude and directional velocity information are output from the linked positions of objects in sequential images. This average velocity represents the actual distance traveled, as determined by the movement of the centroid of the cell, divided by the elapsed time (17).

Statistical analysis of cell motility data. In each well, values for stationary objects were removed, assuming that they have high (x, y) density. Values for large objects also were removed based on the distribution of cell area at early time points. The cumulative distribution functions of cell velocity were plotted and compared across the 18 cell clones. On each day, cell velocity of each cell line was summarized with sample mean and standard deviation. Cell velocity was compared across the three cell lines with one way ANOVA and Tukey's multiple comparison procedures. Linear mixed models were fitted for the velocity data using PROC MIXED in SAS, where cell line and time were assumed to be fixed effects and well was assumed to be a random effect (17).

Irradiation survival curves. Cells from Smad3+/+, Smad3–/–, Smad3–/–(3), or Smad3–/– ds-red lines were irradiated to doses ranging from 0 to 8 Gy and plated in 4-well tissue culture dishes at concentrations of 500, 1,000, or 5,000 cells/well. Five days later the cells were stained with crystal violet and colonies of greater than 50 cells were counted. The data was analyzed by linear quadratic and multi-target, single-hit models (18-20).

Histopathology. The lungs were removed, frozen in optimum cutting temperature (OCT) medium and sectioned. The slides were then

Table I. *Cell migratory velocity of Smad3+/+, Smad3–/– and Smad3–/–(3) cells and their subclones in vitro.*

Day	Uncloned cell lines				
	Smad3+/+	Smad3–/–(3)	Smad3–/–	*p*-value comparing +/+ with –/–	*p*-value comparing –/– (3) with –/–
1	0.93±0.48	0.44±0.32	0.43±0.31	<0.0001	1
2	0.93±0.48	0.49±0.33	0.46±0.34	<0.0001	<0.0001
3	0.85±0.48	0.58±0.39	0.49±0.35	<0.0001	<0.0001
4	0.79±0.46	0.70±0.51	0.52±0.35	<0.0001	<0.0001
5	0.74±0.46	1.06±0.75	0.50±0.37	<0.0001	<0.0001
6	0.76±0.46	1.15±0.76	0.53±0.41	<0.0001	<0.0001
Pooled data (day 1-6)	0.85±0.48	0.57±0.43	0.48±0.35	<0.0001	<0.0001
Day	Pooled data of 5 clones				
	Smad3+/+	Smad3–/–(3)	Smad3–/–	*p*-value comparing +/+ with –/–	*p*-value comparing –/– (3) with –/–
1	0.71±0.39	0.64±0.41	0.46±0.34	<0.0001	<0.0001
2	0.77±0.44	0.64±0.42	0.47±0.33	<0.0001	<0.0001
3	0.73±0.46	0.68±0.43	0.49±0.33	<0.0001	<0.0001
4	0.68±0.47	0.70±0.44	0.51±0.35	<0.0001	<0.0001
5	0.74±0.51	0.71±0.47	0.52±0.37	<0.0001	<0.0001
6	0.82±0.55	0.68±0.46	0.55±0.39	<0.0001	<0.0001
Pooled data (day 1-6)	0.74±0.44	0.66±0.43	0.50±0.35	<0.0001	<0.0001

The values are mean±standard deviation, and the unit of these values is Microns/min. For the data corresponding to different rows of this table, statistical analysis was performed separately but with the same method. These analyses included one-way ANOVA of log (velocity) and pairwise comparison between the three uncloned or 5 representative clonal cell lines derived from each line. The *p*-values show that Smad3+/+ and Smad3–/–(3) cells always moved faster than did Smad3–/– cells except on day 1 where the velocities of the uncloned Smad3–/–(3) and uncloned Smad3–/– cells were not significantly different.

washed in PBS, mounted with an antifade histomount, cover-slipped, and examined under a fluorescent microscope. Quantitation of ds-red or GFP fluorescent positive cells was performed as previously published (6).

Statistical analysis of mouse experimental data. Data were analyzed using a Student's *t*-test or Log-Rank test according to published methods (6).

Animal welfare. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh in strict accordance with the Institutional Animal Care and Use Committee of the University of Pittsburgh guidelines.

Results

Restoration of signal transduction through the TGFβ receptor by reinsertion of a functioning Smad3 gene into Smad3–/– bone marrow stromal cells. Clonal cell lines of Smad3–/– bone marrow stromal cells were co-transfected with a retrovirus containing the Smad3 transgene and a hygromycin resistant gene according to published methods (19). Cells were grown in the presence of hygromycin and subclones selected in 100 μg/ml hygromycin and then tested for expression of the Smad3 transgene. Five subclonal lines of Smad3–/– cells which were positive for the transgene RNA for the Smad3 gene, so selected, were designated Smad3–/–(3). Five subclones of Smad3+/+ cells were also selected for expression of the ds-red transgene inserted by plasmid transfection. Five subclonal lines were designated Smad3–/–(ds-red). Subclonal lines of Smad3–/– cells expressing its GFP transgene Smad3–/– (GFP) were similarly selected.

Decreased migratory capacity in vitro of multiple subclonal lines of Smad3–/– cells is restored by reinsertion of a functioning Smad3 gene. Five subclonal lines of each of three cell lines: Smad3–/–, Smad3+/+, and Smad3–/–(3), respectively, were plated at 100 cells per well in each of 42 wells of a 384-well plate and placed on the Cytoworks Automated Cell Tracking System according to published methods. Cell motility was tracked over five days. The results are shown in Table I and Figure 1. There was heterogeneity in migration capacity between clonal lines within each group; however, Smad3–/– clonal lines demonstrated a significantly reduced motility compared to the Smad3+/+ clonal lines. Reinsertion of the Smad3 gene into Smad3–/–(3) cells shifted migratory capacity to a level closer to that of Smad3+/+ cells (Table I and Figure 1). The present results confirm and extend those of a

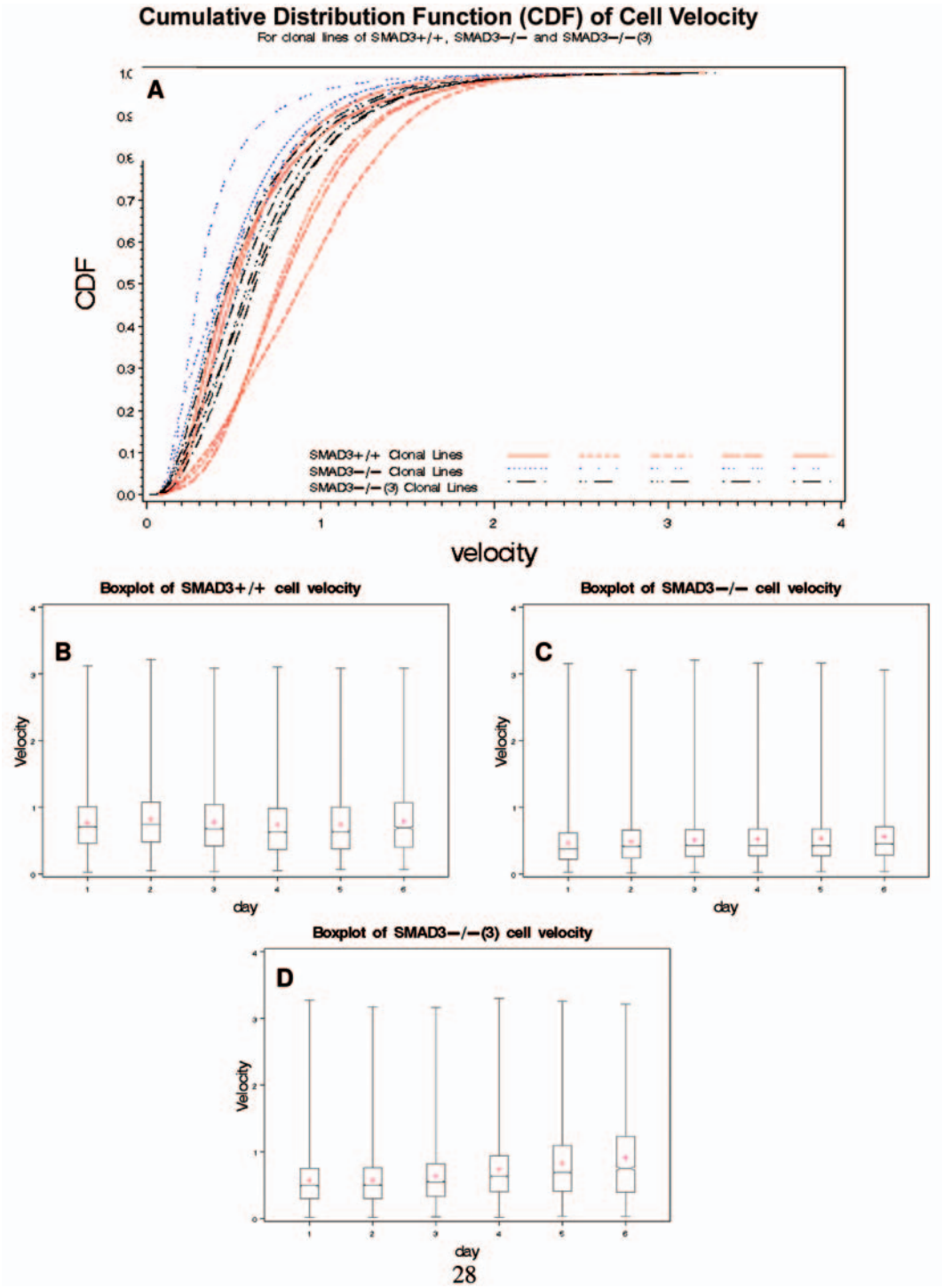

Figure 1. *A functioning Smad3 gene product improves marrow stromal cell migration in vitro. Five clonal cell lines from Smad3–/–, Smad3+/+ or the transgene "corrected" Smad3–/–(3) stromal lines were evaluated for migration over 5 days in the Cytoworks automated cell tracking system. A) Five clonal lines of Smad3+/+ cells (red lines) moved the fastest. Smad3–/– clonal lines (blue lines) moved slowest. The transgene corrected Smad3–/–(3) cell line velocity increased relatively faster with time compared to Smad3–/– clonal lines (black). The plots show how the cell velocity changed with time. Smad3+/+ clonal cell lines velocity was first rapid, then decreasing; Smad3–/– cell line velocity increased slowly with time. Using one-way ANOVA, for Smad3+/+ over 5 days the average cell velocity was respectively 0.77, 0.82, 0.78, 0.74, 0.74 (microns/24 h). The p-values comparing average cell velocity between these days were respectively <0.0001, <0.0001, <0.0001 and 1. For Smad3–/–, the average cell velocity was 0.47, 0.49, 0.51, 0.52, 0.53 (microns/24 h), and the p-values were all <0.0001. For the transgene "corrected" Smad3–/–(3) (D), the average cell velocity of Smad3–/– was 0.57, 0.58, 0.63, 0.74, 0.83 (microns/24 h) and the p-values were 0.22, <0.0001, <0.0001 and <0.0001.*

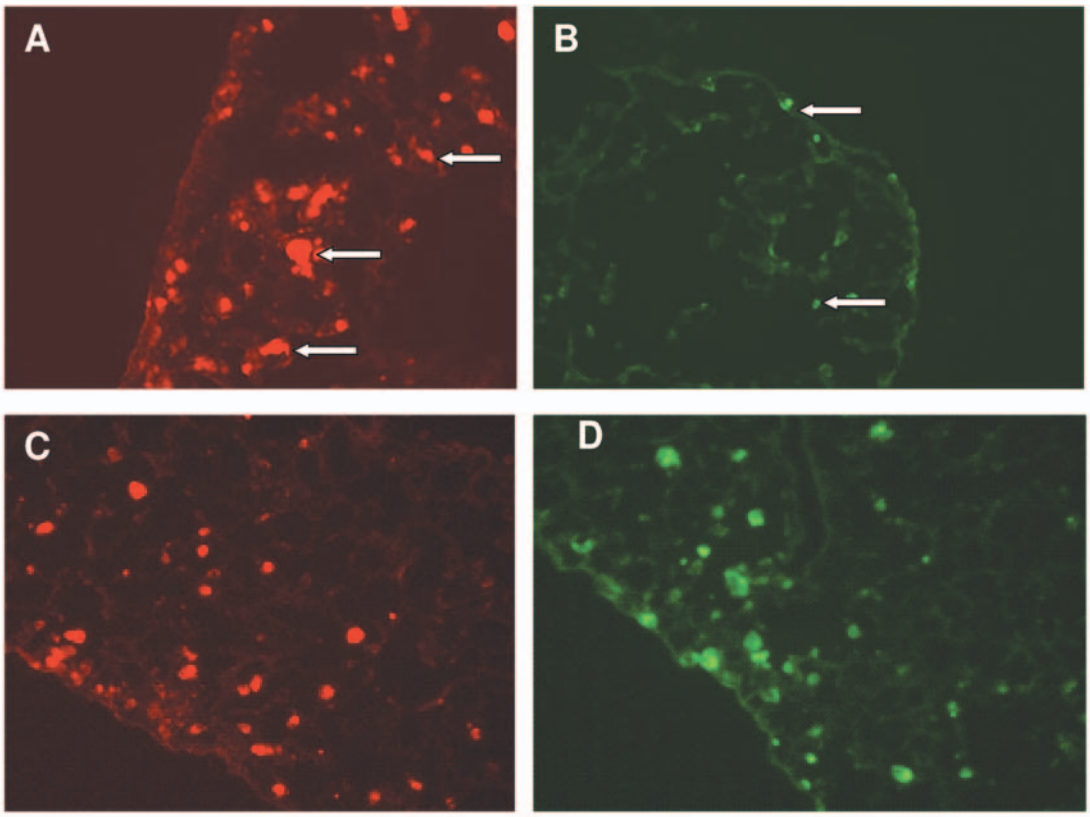

Figure 2. *Reduced pulmonary seeding to the irradiated lung of Smad3–/– bone marrow stromal cells. Seeding of the lung with bone marrow stromal cells labeled with GFP or ds-red and injected at 100 days after 20 Gy, then sacrificed 24 hours later. A) Results equal number of 1x10^5 Smad3+/+ ds-red compared to B) Smad3–/– (GFP+) cells. C) Results using correction factor from calculations in Figure 3, C) 5x10^5 Smad3+/+ (ds-red) compared to D) 4.07x10^6 Smad3–/– (GFP) cells in explanted lung sections at 24 hours after i.v. injection show equivalent number of seeded cells. (x500).*

previous publication using uncloned lines (15) and establish that cell motility *in vitro* which is reduced in clonal cell sublcones of Smad3–/– cells is restored at a clonal level by the reinsertion and functioning of the Smad3 transgene.

Smad3–/– marrow stromal cells (MSCs) demonstrate decreased seeding capacity to the irradiated mouse lung in vivo. One of the histopathologic markers of late ionizing irradiation effects is migration to the irradiated lung of bone marrow origin stromal myofibroblast progenitors (6). C57BL/6J mice were irradiated to 20 Gy to both lungs as described in the methods and as published (16). At 100 days after irradiation, when macrophage infiltration and bone marrow stromal cell migration is known to occur to initiate the late effect of fibrosis (6), mice were injected *i.v.* with an equivalent number of 1x10^5 clonal Smad3–/– (GFP) or Smad3+/+ (ds-red) cells. At 24 hours after injection mice were sacrificed and lung sections quantitated for the number of ds-red positive cells per high power field. Smad3–/– cells migrated less effectively to the lungs than did Smad3+/+ cells. Seeding of the lung was 10-fold more effective with 1x10^5 ds-red Smad3+/+ (Figure 2A) compared to Smad3–/– GFP cells (Figure 2B). In this experiment, mice were *i.v.* injected with cell lines at 100 days after 20 Gy to both lungs. In nonirradiated control mouse lungs of similarly injected mice, few cells were seen with either line (not shown).

Seeding of the irradiated lung by Smad3–/– clonal cell line cells is 100-fold relatively decreased compared to Smad3+/+ clonal cell line cells. Previous studies demonstrated that the plating efficiency, doubling time and plating density of Smad3–/– cells were not significantly decreased compared to Smad3+/+ cells (15). Thus, the decreased seeding efficiency to the lungs of Smad3–/– cells may have been attributed to their known decreased motility and/or decreased TGFβ responsiveness. If this were the case then injection of a greater number of Smad3–/– cells might equalize seeding. To test this hypothesis, we next injected varying numbers of Smad3–/– (ds-red) or Smad3–/–(3) ds-red cells compared to Smad3+/+ ds-red cells using clonal lines which were each >98% red by visualization *in vitro* to attempt to derive a number to equalize seeding to the lungs with each line.

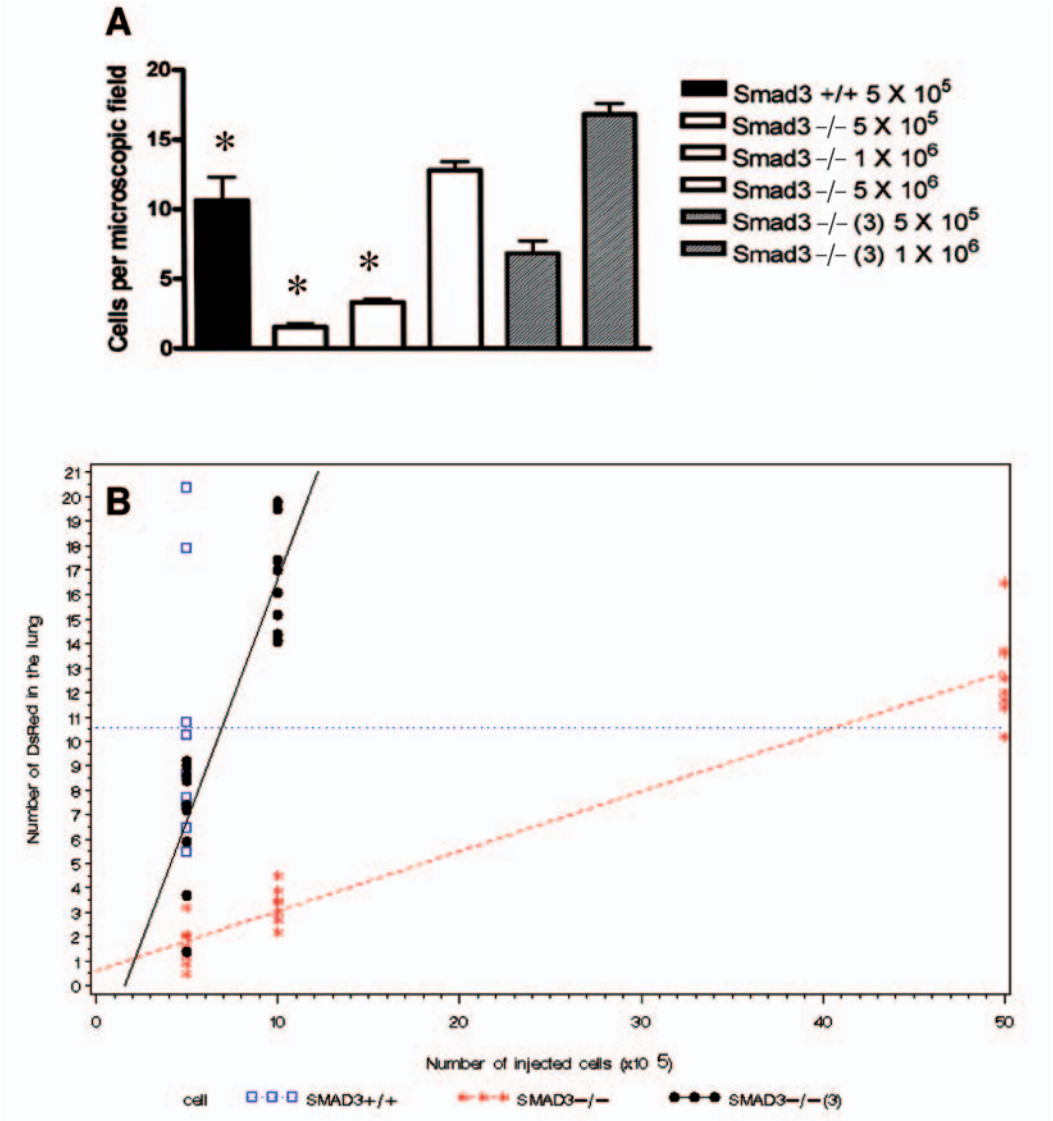

Figure 3. *Decreased relative pulmonary migration of intravenously injected Smad3–/– ds-red compared to Smad3+/+ ds-red bone marrow stromal cell lines. Mice (20/group) were irradiated to 20 Gy to the pulmonary cavity and injected on Day 120 after irradiation with 5x10^5 ds-red-Smad3+/+ cells as the standard. Other groups received ds-red-Smad3–/– cells at doses from 5x10^5 to 1x10^6 or ds-red-Smad3–/–(3) bone marrow stromal cells at each dose level. Twenty-four hrs later the mice were sacrificed, lungs expanded in OCT, excised, frozen in OCT and sectioned. The sections were examined and 90 microscopic fields were examined and the number of ds-red cells counted. Mice injected with 5x10^5 or 1x10^6 Smad3–/– cells had significantly lower number of ds-red cells compared to the mice injected with 5x10^5 Smad3+/+ cells ($p<0.0001$ or 0.0007, respectively). The lungs from mice injected with 1x10^6 Smad3–/– (3) cells had significantly increased number of ds-red cells compared to the equivalent number of Smad3–/– cells ($p=0.0064$). (*) Subgroup with 250 μg TGFβ injected with cells showed no difference from others. B) Plot of the fitted lines for the relationship between the number of ds-red cells in each high powered field of the lung vs. number of seeded cells for Smad3–/– (red dashed line) and Smad3–/–(3) (black solid line) compared to 5x10^5 SMAD3+/+ cells (blue data points).*

As shown in Figure 3, seeding could be equalized by injecting more Smad3–/– ds-red cells. When the number of *i.v.* injected ds-red Smad3+/+ cells was 5x10^5 the mean number of ds-red cells seen per lung field at 24 h was 10.578. For ds-red Smad3–/– cells, the fitted equation was the relationship between the number of ds-red cells (denoted as nDsRed) and the number of seeded cells (denoted as nSeeded) and was nDsRed=0.596+0.00000245*nSeeded. For the transgene restored Smad3–/–(3) cells, using the linear regression model, the relationship between the number of ds-red cells (denoted as nDsRed) and the number of seeded cells (denoted as nSeeded) was nDsRed = –3.111 + 0.0000197*nSeeded. Therefore, to obtain equivalent numbers of 10.578 ds-red Smad3–/– cells per high powered field

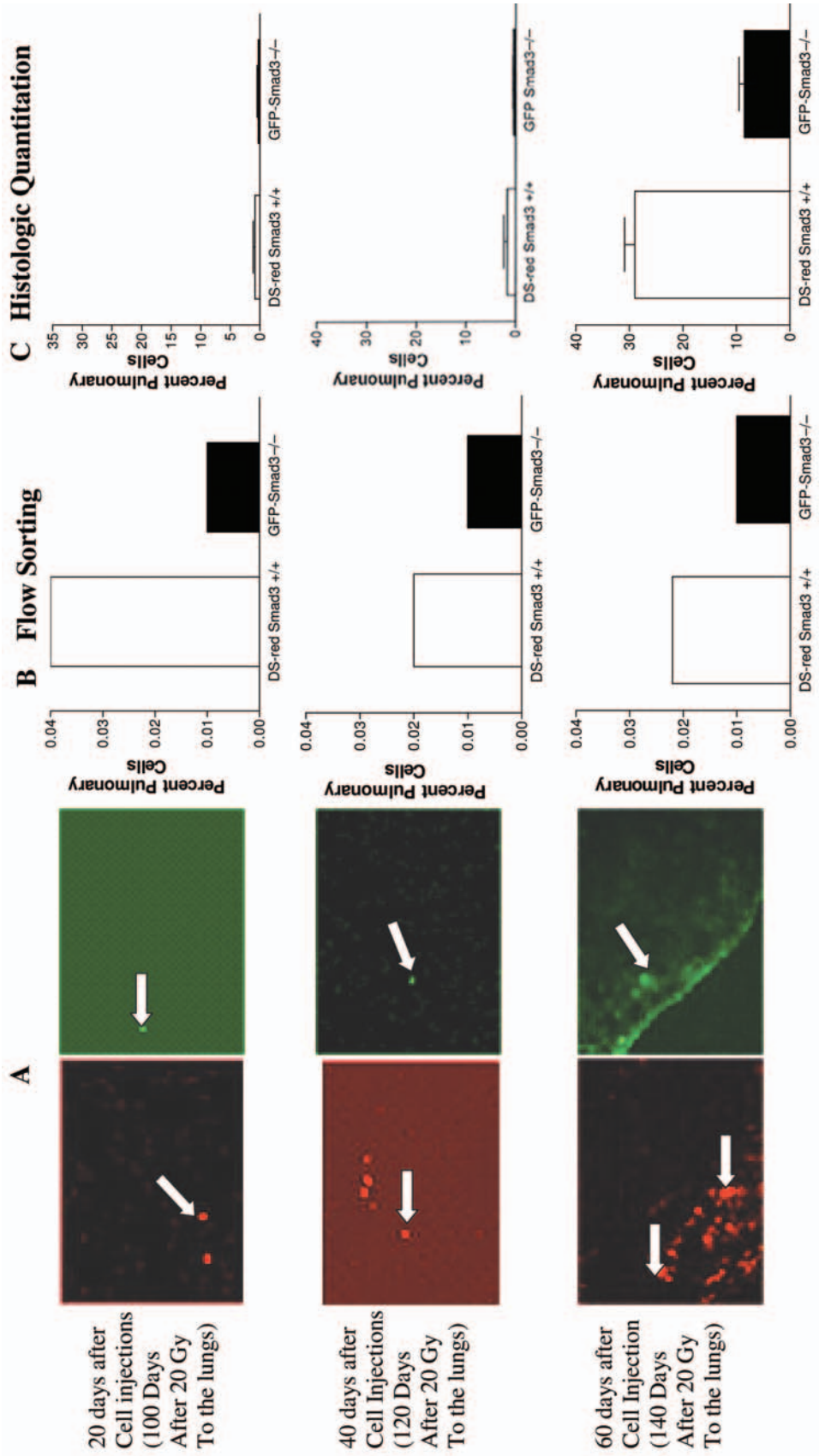

Figure 4. *Reduced proliferation of Smad3–/– myofibroblasts in the irradiated lungs (after equivalent seeding) compared to Smad3+/+ cells. C57BL/6J mice were irradiated to 20 Gy to pulmonary cavity and injected 80 days later with $5x10^5$ ds-red Smad3+/+ or $5x10^6$ GFP+ Smad3–/– cells. Subgroups of five mice were sacrificed 20, 40 or 60 days later at day 100, 120 or 140 after irradiation. The lungs were removed and two of the lobes prepared as single cell suspension by digesting with dispase and collagenase, then analyzed for the percent ds-red-Smad3+/+ or GFP-Smad–/– cells. The other three lobes were expanded in OCT, frozen in OCT and sectioned. The sections were examined under a fluorescent microscope and the percent ds-red Smad3+/+ cells or GFP-Smad 3–/– cells in areas of organizing alveolitis/fibrosis determined. Column A shows photographic appearance of representative sections of lung showing ds-red Smad3+/+ or GFP-Smad3–/– cells. (x500) Column B shows the percent of ds-red or GFP positive cells in the total lung by sorting $2x10^6$ cells by flow cytometry. Column C shows the percent of ds-red or GFP cells in areas of developing organizing alveolitis/fibrosis by histopathologic scoring of 100 high powered fields per lung for each of 5 mice. Results for Column B and C showed Smad3–/– cell numbers significantly lower than those for Smad3+/+ cells at each time point and in each assay ($p<0.05$).*

(equivalent to injection of $5x10^5$ SMAD3+/+ cells in the reference experiment) the calculated number was $4.07x10^6$ Smad3–/– cells (ds-red or GFP labeled) relative to $6.95x10^5$ Smad3–/–(3) cells. When these formula derived numbers were utilized, equivalent seeding was observed at 24 h (Figure 2C-D). Thus, around 10-fold more ds-red Smad3–/– cells were required by *i.v.* injection to give an equivalent number of cells seeding the lungs when scored 24 h later.

Proliferation of pulmonary seeded SMAD3–/– GFP+ cells is decreased compared to equivalent numbers of pulmonary seeded SMAD3+/+-ds-red cells. The above data demonstrated that seeding of the lung with fluorochrome labeled SMAD3–/– cells 24 h after injection was normalized by a ten-fold increase in cell number, to the levels seen with SMAD3+/+-ds-red labeled cells. We next sought to determine whether proliferation of seeded cells (once adjusted for equivalent seeding efficiency), was decreased with SMAD3–/– fluorochrome labeled clonal marrow stromal cell lines. The data in Figure 4 show the representative photographic appearance, cell sorting and histopathologic quantitation of proliferation of seeded cells into the lungs at 20, 40 and 60 days after *i.v.* injection. C57BL/6J female mice with the inoculum size adjusted to have same number of cells seeded into the lungs at 24 hours after injection (in this experiment at 80 days after 20 Gy irradiation to both lungs), were then followed for 20 or 40 days, or 60 additional days, then lungs removed, separated to each of the five lobes, and analyzed for areas of developing organizing alveolitis/fibrosis and for the percent and numbers of ds-red Smad3+/+ or GFP Smad3–/– cells in each area of organizing alveolitis/fibrosis.

The results demonstrate that initially seeded cells of both types, ds-red Smad3+/+ and GFP-Smad3–/–, were partially cleared from the lungs at day 20 after injection then both proliferated (or reseeded from extra pulmonary sites) at days 40 and 60. However, the Smad3–/– cells proliferated (or reseeded) less effectively compared to Smad3+/+ cells at each time point in the irradiated lung microenvironment after an initial equivalent cell number adjusted seeding.

C57BL/6J female mice chimeric for Smad3–/– male marrow show decreased irradiation pulmonary fibrosis and improved survival. The above data established that Smad3–/– marrow stromal cell lines migrated to the irradiated lungs less efficiently than did Smad3+/+ cells. We next tested whether this data correlated to a decrease in irradiation fibrosis mediated by Smad3–/– bone marrow stromal cells. C57BL/6J female mice were subjected to 10 Gy total body irradiation and were then reconstituted by injection *i.v.* of $5x10^5$ whole marrow cells from male Smad3–/– or male Smad3+/+ marrow which was harvested from equal age, one month old donor mice. Recipients were tested at 30 days post transplant for percent Y probe positive (donor) nucleated cells in the peripheral blood according to published methods (6) and were found by this measure to be >85% donor marrow reconstituted.

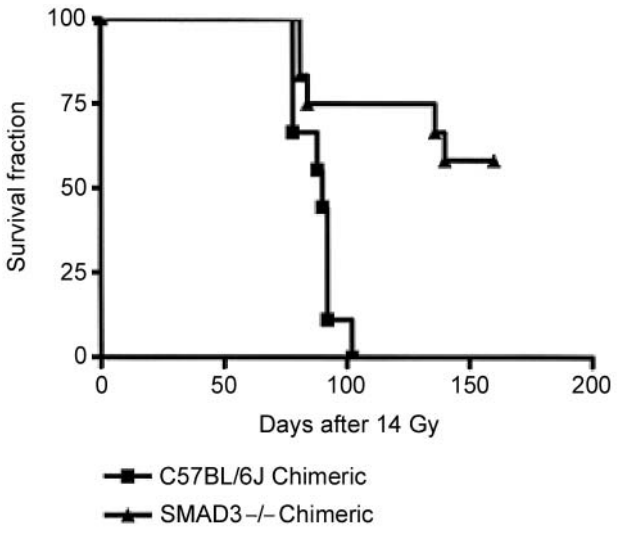

Figure 5. *Increased survival following 14 Gy pulmonary irradiation of C57BL/6J female mice chimeric for male Smad3–/– compared to Smad3+/+ marrow. C57BL/6J female recipients received 10 Gy TBI at day -30, then i.v. injection of $5x10^5$ Smad3+/+ or Smad3–/– whole male mouse marrow at day -29. Chimerism was demonstrated by >85% Y probe positive cells in peripheral blood at day -1. At day 0 mice received 14 Gy irradiation to both lungs. All mice were followed for survival.*

Chimeric mice then received 14 Gy irradiation to the lungs as published (lower dose chosen to take into account the TBI 10 Gy prior dose) (6) and were followed for survival, then upon premorbid condition or death as defined by our IACUC, were evaluated by histomorphometric analysis of lung sections from all 5 lobes of at least 10 mice per point for percent of the lung replaced with fibrotic areas characterized as organizing alveolitis/fibrosis (6). The results are shown in Figure 5. Chimeric mice with Smad3+/+ transplanted marrow were all dead by day 100. (This earlier time of death reflects the prior 10 Gy TBI dose). The organizing alveolitis/fibrosis response (and time of death) from 20 Gy pulmonary irradiation is usually around 120-140 days in C57BL/6J female mice (16). All Smad3+/+ marrow chimeric dying mice in the present study had >20% pulmonary organizing alveolitis/ fibrosis and contained Y+ cells within fibrotic areas at a level of around 8.9% recipient origin Y probe negative cells (Table II). These results confirm and extend those in a previous publication (6). In contrast, over 50% of Smad3–/– marrow chimeric mice were still alive at 170 days. Furthermore, those Smad3–/– marrow chimeric dying mice had reduced areas of pulmonary fibrosis that contained relatively fewer (around 4.5%) marrow origin Y+ vimentin positive fibroblasts. The results in Table II show that decreased Smad3–/– cell migration to the lungs correlated with improved survival. The data provide further evidence that the Smad3 gene mediated step in TGFβ signal transduction is

Table II. *Donor marrow origin fibroblastic cells in areas of organizing alveolitis/fibrosis in female C57BL6/J mice chimeric for male Smad3+/+ or male Smad3–/– marrow.*

Time	% Fibrosis		% Y+ cells in areas of organizing alveolitis/fibrosis	
	Smad3+/+ Chimeric	Smad3–/– Chimeric	Smad3+/+ Chimeric	Smad3–/– Chimeric
Day 80	7.7±2.0	6.0±2.9	2.5±0.7	1.1±0.3
Prior to death or Day 150	26.7±2.8	13.4±3.6 (p=0.0007)	8.9±0.9	4.5±0.7 (p<0.0008)

Mice from the experiment shown in Figure 5 were analyzed at day 80 after 14 Gy irradiation to the lungs, or prior to the time of death (or at day 150) for the contribution of male donor fibroblasts to the fibrotic areas in the lungs. Results are for 5 mice per group and show mean % of the total lung displaying fibrosis, and relative percent of fibrotic areas containing male Y+ probe positive cells in the areas of organizing alveolitis/fibrosis. Y+ male cells were fibroblastic in morphology and were vimentin positive by dual staining (6). Mice chimeric for Smad3–/– marrow had less marrow origin and overall pulmonary fibrosis. *P*-value compares Smad3–/– with Smad3+/+ chimeric mice at death (or day 150 for Smad3–/– chimeric survivors).

critically required not only for efficient seeding of marrow stromal cells to the lungs but also for their proliferation in the process of pulmonary irradiation fibrosis.

Discussion

The present studies establish that bone marrow stromal cells from Smad3–/– homozygous deletion recombinant negative mice display decreased migratory capacity *in vitro* and *in vivo* to irradiated recipient lungs, and that these deficiencies are restored by re-expression of a functioning Smad3 transgene. Smad3–/– bone marrow stromal cells were previously demonstrated to have pleotrophic effects of deficiency of the Smad3 gene product (9-12, 14-15, 21-23). Phenotypic changes described with Smad3–/– MSCs include constitutive adipocyte differentiation (14), decreased responsiveness to osteogenic stimulation (14), and enhanced ability to support hematopoietic cells in co-cultivation experiments in which both the hematopoietic and stromal cells are defective in Smad3 mediated TGFβ signal transduction (13). Smad3 re-expression in Smad3 deficient bone marrow stromal cells was previously shown to restore the biological properties of radiation sensitivity, intact migratory response, release from G2M block in a cell cycle (15).

Previous studies have demonstrated that bone marrow derived myofibroblasts contribute to 10 to 20% of the fibrotic lesions in the periphery of the lungs of irradiated C57BL/6J mice at 100 days after 19 Gy irradiation (6). This model of murine organizing alveolitis/fibrosis has been shown to be highly analogous to clinical radiation fibrosis in irradiated patients, post-total body irradiation, or high dose irradiation to the lungs for the treatment of lung or esophagus cancer (24). By injecting *i.v.* variable numbers of Smad3–/– or Smad3+/+ cells using a ds-red label on each, into mice 100 days after 19 Gy irradiation to both lungs, then 24 hours later removing lungs and imaging for the number of ds-red cells per high power field in multiple lobes of the lung and in multiple mice it was possible to derive a numerical factor for normalizing the reduced seeding efficiency of Smad3–/– cells compared to that of the Smad3+/+ cells. These experiments showed that injection of a ten fold greater number of Smad3–/– cells resulted in equivalent seeding at 24 h to the lungs. With seeding efficiency normalized, we addressed the question of whether GFP-Smad3–/– cells proliferated less effectively compared to Smad3+/+ ds-red cells in the TGFβ rich environment of the irradiated lung after seeding. At 20 days after equivalent seeding at 80 days, Smad3–/– cells cleared nearly completely from the lungs while some Smad3+/+ cells were still detectable. We do not know whether this decrease represented apoptosis, other forms of cell death, or reflected clearance of cells to an extra pulmonary site(s). Flow sorting of bone marrow at this 20 day post injection time point did not reveal detectable ds-red or GFP positive cells. By 40 days after pulmonary seeding, increased numbers of cells were detected in the lungs of both Smad3–/– and Smad3+/+ cell line injected mice, with further increases detected at day 60. These increases were always greater in Smad3+/+ cell injected mice and may reflect proliferation of initially seeded cells, reseeding from another site or both mechanisms. Under each condition and at each time point, Smad3+/+ cells were present in relatively increased numbers compared to Smad3–/– cells.

The present data demonstrate that mice chimeric for Smad3–/– marrow had less irradiation pulmonary fibrosis and less MSCs seeding to the lungs than that observed in Smad3+/+ chimeric mice, resulting in improved survival. The relevance of the present data to *in vivo* radiation pathology of Smad3–/– mice may be significant (9, 10). Cutaneous irradiation is known to induce a fibrotic response which reproducibly occurs between three and six months after single fraction of irradiation in the mouse model (9). Recent studies on the biology of radiation fibrosis have demonstrated a significant component of marrow stromal cell migration into the irradiated area tissue, involving fibroblast progenitor cells traveling from distant sites, principally from bone marrow, to the periphery of the irradiated pulmonary field (6). TGFβ signaling from irradiation damaged tissues has been thought

to be a component of the signal eliciting marrow myofibroblast or fibroblast progenitor migration into irradiated target volumes (6, 8). Other studies have demonstrated that Smad3–/– mice and cells derived from these animals display an intact irradiation induced TGFβ secretion response (9, 10). The present studies establish that clonal sublines of Smad3–/– cells are deficient in migratory response *in vitro* and *in vivo* and that these properties are restored by reintroduction of a Smad3 gene product.

Further studies are required to determine the mechanism by which an intact TGFβ signaling transduction pathway restores cell migratory capacity *in vitro* and *in vivo* of bone marrow stromal cells which are involved in ionizing irradiation pulmonary fibrosis.

Acknowledgements

This research was supported by NIH Grant #R01- CA119927-08.

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Received July 3, 2006
Accepted July 12, 2006