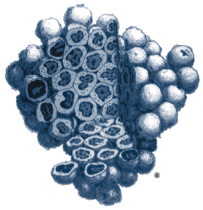

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# Bone Marrow Contributes to Epithelial Cancers in Mice and Humans as Developmental Mimicry

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**Key words.** Bone marrow cells, Malignancy, Differentiation, Plasticity, Hematopoietic stem cell, Bone marrow transplantation

## ABSTRACT

**Bone marrow cells have the capacity to contribute to distant organs. We show that marrow also contributes to epithelial neoplasias of the small bowel, colon, and lung, but not the skin. In particular, epithelial neoplasias found in patients after hematopoietic cell transplantations demonstrate that human marrow incorporates into neoplasias by adopting the phenotype of the surrounding neoplastic environment. To more rigorously evaluate marrow contribution to epithelial cancer, we employed mouse models of intestinal and lung neoplasias, which revealed specifically that the hematopoietic stem cell and its progeny incorporate within cancer. Furthermore, this marrow involvement in epithelial cancer does not appear to occur by induction of stable fusion. Whereas previous claims have been made that marrow can serve as a direct source of epithelial neoplasia, our results indicate a more cautionary note, that marrow contributes to cancer as a means of developmental mimicry.**

## INTRODUCTION

Transplantation studies in animals and humans have demonstrated that BMDCs contribute to epithelial cells of a variety of tissues [1-4]. The engraftment of epithelial cells of distant organs by bone marrow cells occurs at very low levels under normal physiologic conditions and has questionable functional significance [5]. However, contribution from marrow is enhanced in settings of injury or disease, which is likely related to the homing effects of inflammation [1, 6-9].

A recent murine study suggests that BMDCs contribute to cancer arising from the stomach lining [10]. Transplantation experiments performed in mice with chronic gastritis due to *Helicobacter* infection showed that resultant gastric carcinomas contained marrow-derived dysplastic and neoplastic glands. This study primarily emphasizes the importance of chronic inflammation in recruiting BMDCs. By unknown mechanisms, gastric glands nearby *Helicobacter* infection progress from dysplastic and then neoplastic morphology. Given the observation of marrow-derived cells within gastric neoplasia, the authors purport that marrow can be a primary source of epithelial cancer. Furthermore, the study was restricted to

**Correspondence:** Christopher R. Cogle, M.D., University of Florida, 1600 SW Archer Road, ARB R4-216B, P.O. Box 100277, Gainesville, FL 32610-0277, Telephone: 352-392-3058, FAX: 352-392-8530, Email: c@ufl.edu. #, * *Authors contributed equally.* *Received for publication March 8, 2007; accepted for publication April 20, 2007; first published online in Stem Cells Express May 3, 2007.* *©AlphaMed Press* *1066-5099/2007/$30.00/0 doi: 10.1634/stemcells.2007-0163*

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animal investigations, and leaves open questions regarding the clinical relevance.

These studies prompted us to more rigorously investigate marrow contribution to epithelial cancers in mice and humans. First, we address the question of clinical relevance by presenting human data which demonstrate minimal marrow contribution to secondary cancers after hematopoietic cell transplantations. However, given the low and sporadic levels of incorporation, the data is suggestive of developmental mimicry rather than marrow acting as a primary source of neoplasia. To validate our clinical findings, we employed animal models of cancer, which demonstrate that bone marrow can contribute, albeit at low levels, to neoplasias of the gut and lung; again, suggestive of developmental mimicry rather than marrow as a seed of cancer. We also directly address the question of which marrow cell participates in this contribution to cancer. Since the HSC has been observed to adopt the phenotype of several extramedullary tissues,[11, 12] we questioned whether this potential developmental mimicry in cancer may be due to the HSC and its progeny. Using single HSC-transplanted mice bearing lung cancer we show that the progeny of HSC can incorporate in cancer at low levels without evidence for stable fusion.

## RESULTS

### Human Bone Marrow Incorporates into Intestinal Adenomas in Transplant Patients

To address the clinical relevance of marrow contribution to cancer, we identified two women with neoplasias involving the colon after hematopoietic cell transplantation from male donors (Table 1). The colorectal adenomas were found shortly post-transplant (< 2 months) during colonoscopic evaluations for diarrhea. No infectious etiologies were found to explain the diarrhea; however, graft versus host disease (GVHD) was found in surrounding tissues of both patients. Coincident colonic adenomas were identified and resected. We questioned whether these adenomas were of host or donor origin. Because the adenomas were found shortly after transplant, and considering the long latency period of adenomas, it was likely that the adenomas were present in the colons before transplant and they were expected to be entirely of host composition.

We sectioned and stained the adenomas for the presence of donor-derived cells. As expected, the neoplastic tissues demonstrated donor-derived (Y chromosome positive) CD45 positive leukocytes, which were predominantly located in the lamina propria. To our surprise, however, the adenomas also contained donor-derived colonocytes in the adenoma epithelia (Figure 1). The donor epithelial cells displayed features consistent with neoplastic colonic adenoma cells including crowding and distortion, loss of polarity, and pronounced nuclear atypia. In addition, the donor adenoma epithelial cells did not express the hematopoietic surface protein, CD45 (leukocyte common antigen), and had surface protein expression typical of the surrounding adenoma epithelial cells (cytokeratin and mucin positive) (Figure 1 A, B). Moreover, the bone marrow derived columnar cells in the adenomas were located in the basal strata of adenoma epithelia, which potentially suggests recent immigration. In total, over 1000 adenoma epithelial cells were evaluated in both patients, demonstrating 1 – 4% of adenoma epithelial cells originating from donor BMDCs. In order to address whether these marrow-derived colonic adenoma cells were a result of stable fusion, we performed XY FISH on 40 marrow-derived adenoma cells. Confocal microscopy performed to enumerate X and Y chromosomes within each donor cell nucleus showed no evidence of a fusion sex chromosome karyotype (i.e., XXY or XXXY) (Supplemental Movie).

### Bone Marrow Incorporates Into Murine Intestinal Adenomas

Results from our human investigations suggested that marrow has the capacity to incorporate within tumors and adopt the phenotype of the

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surrounding neoplasia. However, we questioned if hematopoietic cell transplant prior to initiation of intestinal neoplasia would demonstrate more robust marrow contribution as reported by Houghton, *et al* in a previous report of gastric carcinoma [10]. Thus, we used adenomatous polyposis coli (APC) gene mutant mice as transplant recipients given their propensity to develop intestinal adenomas [13].

Female mice (n=4) harboring the *min* mutation of the APC gene ($APC^{min}$)were transplanted with whole bone marrow from male $APC^{min}$ mice. Three months post-transplant, the mice were sacrificed and small bowels and colons were resected. Adenomas were detected throughout the intestines of all animals. To address the question of whether the adenomas were of host or donor origin, we utilized a combination technique of immunohistochemistry and XY FISH to identify neoplastic cells of donor (male) origin. Analysis of the stained intestinal tissues demonstrated donor derived columnar-like epithelial cells in the adenomas of all small bowel and colon specimens (Figure 1C). These donor-derived adenoma epithelial cells were identified by neoplastic features of nuclear atypia, loss of polarity, and crowding. In addition these donor adenoma epithelial cells were cytokeratin positive (green) and harbored a Y chromosome (red dots) (Figure 1C). Due to the false positive concern of donor leukocytes overlapping cytokeratin positive adenoma cells, we performed triple surface protein staining (cytokeratin, CD45RB (lymphocytes), and F4/80 (granulocytes/macrophages)) in addition to Y FISH. Since CD45 immunostaining did not reliably highlight leukocytes in these lightly fixed tissues, which were paraffin embedded and then treated with a FISH preparative regimen, we found it necessary to use antibodies against CD45RB and F4/80 to adequately identify infiltrating leukocytes. Importantly, this triple surface protein staining, DAPI nuclear staining and FISH for Y chromosomes were performed on the same slide, ensuring no false positive interpretation due to overlapping leukocyte nuclei. The evaluation of over 600 adenoma sections using this more rigorous approach found evidence of donor-derived adenoma epithelial cells (Figure 1C). However, the frequency of marrow derived epithelial cells using this more rigorous staining method detected marrow contribution in intestinal neoplasias at a rate of only 10 for every 50,000 adenoma cells.

To address the question of whether the donor-derived adenoma cells represented fusion events between BMDCs and adenoma cells, we performed karyotype analyses using confocal microscopy. Analysis of 24 donor-derived adenoma cells did not show fusion karyotype (XXY, XXXY) (Figure 1D).

**Bone Marrow Does Not Contribute to Skin Cancer in Humans Post-Transplant**

The observation that bone marrow incorporates into intestinal neoplasias led us to consider whether BMDCs can contribute to other epithelial malignancies. Through a cooperative effort with the Center for International Blood & Marrow Transplant Research (CIBMTR, Milwaukee, WI) we identified four women who developed skin cancers after gender mismatched hematopoietic cell transplants (Table 1). The skin cancers developed 1 to 4 years after transplant, and the patients had histories of GVHD of the skin prior to cancer development. Paraffin embedded skin neoplasias of squamous and basal cell carcinoma histologies were stained for hematoxylin and eosin to confirm diagnoses (Figure 2A, 2D). Additional tissue sections were then stained for cytokeratin using immunohistochemistry and FISH for XY enumeration (Figure 2). Combined epithelial and karyotype analysis scrutinized over 5000 cells per specimen, which amounted to a total of 20,000 cells analyzed. None of the skin cancer cells from the four patients demonstrated male epithelial cells incorporating within the cancers (Figure 2C, 2F).

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### Human Bone Marrow Contributes to Lung Cancer

An additional woman who underwent gender mismatched hematopoietic cell transplant was found to have developed a lung cancer over four years post-transplant (Patient 6, Table 1). Prior to identification of her secondary squamous cell lung cancer, she was afflicted with pulmonary aspergillosis, and this was treated definitively with antifungal therapy. To determine whether the patient's lung cancer was of host or donor origin, specimens were analyzed by combined immunohistochemistry for cytokeratin and FISH for X and Y chromosomes. The lung cancer demonstrated donor origin as evidence by male cells co-expressing cytokeratin (Figure 3B). Approximately 20% of the lung cancer cells were of donor marrow origin. Confocal microscopy was used to perform karyotype analysis. XY FISH analysis of 12 donor marrow-derived lung cancer cells by confocal microscopy demonstrated no evidence of a fusion karyotype (i.e., XXY, XXXY).

### The Hematopoietic Stem Cell Contributes to Lung Cancer in Mice

Previous reports of marrow as a source of gastric cancer used *in vitro* testing to determine if the hematopoietic stem cell (HSC) or the mesenchymal stem cell (MSC) was the primary source of cancer. Given the limitations of *in vitro* systems, it was important for us to use an *in vivo* experimental model to more rigorously examine HSC contribution to cancer. Thus, we transplanted a single, male, GFP tagged HSC into (primary) female mice (n=120). After hematopoietic chimerism was achieved (n=3), we sacrificed these mice and transplanted their bone marrow into secondary female recipients (n=30). All secondary recipient mice achieved donor hematopoietic chimerism. Secondary recipient female mice (n=9) were then injected with female murine lung cancer intramuscularly in the hind limbs. As expected, analysis of the lung cancers after 14 days of growth demonstrated a prominent display of intratumoral donor HSC-derived cells. These cells were presumed to be inflammatory cells. However, staining of the lung cancer tissues for the pan-leukocyte protein CD45 only found small pockets of donor leukocytes, which appeared small and round. We then questioned whether the CD45 negative, HSC-derived cells were differentiating into cells expressing cell surface proteins normally associated with lung cancers. Thus, we evaluated the cells for evidence of cytokeratin surface protein expression and XY FISH. Since the host mice and the initial lung cancer cells had a female karyotype, this permitted us to use a combination of immunohistochemistry and FISH to track the male HSC and its progeny. The tumors demonstrated HSC-derived cells expressing cytokeratin and Y chromosome (Figure 3C). Even after a mere two weeks of tumor growth nearly 1% of over 10,000 cytokeratin positive cells contained a Y chromosome. To determine whether these HSC-derived tumor cells were the result of stable fusion, karyotype analysis was performed. XY FISH analysis of 15 of these donor-derived, cytokeratin positive cells found none with a fusion karyotype.

## DISCUSSION

We have previously shown that BMDCs can contribute to distant organs [1, 6, 14]. Whereas BMDC incorporation is minimal under normal physiologic conditions [5], it becomes more apparent in settings of injury and repair, likely reflecting the response to inflammation [1, 6-9, 15]. Indeed, recent reports have highlighted the role of chronic inflammation in promoting marrow incorporation into cancer [10]. Given these findings, we aimed to define the role of BMDC participation in epithelial cancers. The present study confirms previous reports, but offers an alternate explanation that marrow cells contribute to cancer as developmental mimicry rather than as a direct source.

Initially we questioned the clinical relevance of marrow contributing to epithelial neoplasias. Given our experience in finding marrow contribution to brains of women after hematopoietic cell transplantations [14], we used

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a similar study schema to address this question. Specifically, we identified women who received hematopoietic cell transplantations from male donors, and subsequently developed epithelial neoplasias. The risk of developing a new cancer after blood or marrow transplantation is estimated to be up to eight times higher than in aged-matched controls [16]. Predisposing risk factors such as radiation, chemotherapy and use of immunosuppressants have been recognized. However, another risk factor for secondary neoplasia in the post-transplant setting may be a history of antecedent epithelial inflammation due to GVHD or other inflammatory process. Our evaluation of post-transplant epithelial neoplasias demonstrated marrow engraftment, but at low levels of incorporation. Whereas previous murine studies have suggested that marrow can act as a direct source of cancer, our results offer an alternate explanation that marrow participates at the level of developmental mimicry, whereby hematopoietic cells have been called into a neoplastic environment where they can respond to developmental cues and adopt similar phenotypes to the surrounding neoplastic epithelial cells.

When chimeric tissues demonstrate low levels of graft incorporation, fusion is a consideration. We have previously demonstrated that the hematopoietic stem cell is capable of fusion [17]. Furthermore, Pawelek, *et al* recently put forward a theory of myeloid-cancer hybrid cells [18]. Using confocal microscopy and XY enumeration, we found no evidence of stable fusion between marrow-derived cells and neoplastic cells. Moreover, we developed a same-slide technique of staining for epithelial cells, leukocytes, nuclei, X chromosomes, and Y chromosomes to evaluate for overlap of myeloid and cancer cells. Using this technique, we found clear evidence of marrow-derived epithelial cells in adenomas and squamous cell lung cancer.

It has also been suggested that cells which arise as fusion products may undergo “reduction division”, dividing back into diploid cells. While that might still be an explanation for these findings, it should be noted that in models where fusion events have been described, most, if not all, of the fused cells persist in the tissues without complete resolution [19]. To address the question of reduction division, we scored a total of 40 Y positive human colonic adenoma cells and 12 Y positive human lung cancer cells with no evidence of hyperdiploidy. In the liver, where cell fusion has been demonstrated in severe disease stress states, it has been postulated that 28% of donor-derived hepatocytes are due to reduction division, resulting in diploid daughter cells [19]. Based on the probability of binomial distribution, the chance that we would find as many diploid donor-derived cancer cells amidst a background fusion resolution rate of 28% is over one in 1x10[22]. Thus, direct incorporation of human marrow cells, rather than fusion followed by absolutely complete and perfect resolution of every fusion event, is the most likely explanation of our current findings.

Fetal microchimerism is debatably an alternate explanation of marrow transdifferentiation. In this situation, male fetal stem/progenitor cells are transferred into the maternal circulation and persist for years.[20, 21] In this report, the transplanted female patients had varying obstetric histories. No definite correlation is present strongly linking fetal microchimerism as the cause of marrow involvement within neoplasias. Furthermore, multiparous control female human samples did not demonstrate Y chromosome cells within epithelia. Finally, our murine studies confirming marrow involvement in epithelial neoplasias were performed using nulliparous mice. Fetal microchimerism might be an explanation, but direct incorporation is more likely.

We also extend the clinical findings by employing murine models to more rigorously test the observed phenomenon of marrow contributing to epithelial cancers. Spontaneous adenomas in the small bowels and colons of bone marrow transplanted $APC^{min}$ mutant mice demonstrated epithelial cells within the adenomas that were of marrow origin. Several

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considerations should be made. First, incorporation of bone marrow cells into the intestinal adenomas occurred near the lamina propria, potentially suggesting recent immigration. Second, the low rate of marrow incorporation into murine adenomas was similar to the low rate detected in the human colonic adenomas, confirming our clinical observations. Another consideration is that BMDCs in the murine neoplasias may represent phagocytic events between the BMDC and resident adenoma cells. If this were true then resultant cells could display hematopoietic surface proteins as well as hyperdiploidy. However, using immunohistochemistry plus FISH, we show that these adenomas demonstrated differentiated donor cells that expressed cytokeratin and did not express hematopoietic surface proteins. In addition, XY FISH demonstrated no evidence for fusion karyotype.

Given the potential plasticity of the HSC we further questioned if this particular marrow cell participates in cancer development. Lung cancer grown in mice, which had been serially transplanted from single HSC donor mice, demonstrated cytokeratin positive cells of HSC progeny origin. Our immediate consideration was that these HSC-derived cells incorporating into lung cancer represented phagocytosis of cancer cells by macrophages. To our surprise XY FISH revealed no evidence of fusion. These results are the first to suggest that the HSC contributes to epithelial cells within a malignant tumor, answering a recent report which questioned which stem/progenitor cell population of the bone marrow contributes to carcinomas [10]. Mesenchymal cells may also contribute to marrow derived developmental mimicry, but the inability to perform long-term reconstitutions of the mesenchymal cell compartment within the marrow hamper the ability to definitively address this question experimentally. Another possibility is that pluripotent cells within the bone marrow may have contributed to tumor development. Recently, the Ratajczak laboratory identified a very small embryonic-like (VSEL) stem cell residing in the bone marrow of mice and humans.[22, 23] Marrow-inhabiting VSEL cells could be responsible for the developmental mimicry observed in both of our murine and human experiments.

There are two possibilities to explain HSC/marrow incorporation into cancer. As a recent study suggests, marrow derived cells may act as a direct source of cancer [10]. Serial tumor passaging is required to truly assess this possibility. However, our results offer an alternate explanation that marrow incorporates into epithelial neoplasias as developmental mimicry. Our results, demonstrating low rates of marrow incorporation into epithelial cancers of mice and humans, favor marrow participating as a developmental mimic rather than as a direct seed of cancer. In the setting of developmental mimicry, BMDCs are called into epithelial environments following inflammatory cues. Recently, the SDF-1/CXCR-4 axis has been recognized as crucial to hematopoietic progenitor recruitment in environments of ischemia and tumor initiation[15, 24]. Indeed, SDF-1 also plays a critical role in determining the metastatic destination of cancer cells.[25] Cross-talk could occur with marrow cells expressing CXCR-4, including HSC and VSEL cells.[26] After being called into neoplastic sites and through subjection to local growth factors and cell-cell contact the BMDCs undergo changes in cell fate, mimicking the surrounding neoplastic cells. Given the self-renewal and high proliferative potential of BMDCs, these marrow cells could go on to act as paracrine regulators, providing growth factors and immune evasion for the surrounding tumor. Increasing attention has been paid to the precursor role of hematopoietic progenitors in the tumor microenvironment. Within this niche, hematopoietic progenitor cells have been implicated in providing a permissive environment for tumor development and progression [27, 28]. Our findings that marrow cells incorporate within neoplasias of mice and humans presents another potential position from

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which marrow-derived cells could promote oncogenesis.

Marrow contributing to neoplasias also calls into question our current research practices. When establishing cancer cell lines, it is standard practice to use clinical biopsy specimens, which contain a heterogeneous mixture of cells. Microdissection and cancer cell isolation kits help in the enrichment process; however, these techniques still rely on cell differentiation proteins, which, according to our results, can be exhibited by BMDC mimics. Current cancer cell enrichment techniques could isolate multipotent BMDCs incorporated within neoplasias. Furthermore, the enrichment process requires several generations of passaging in culture conditions, which notoriously induce secondary cytogenetic abnormalities. If cultured in the right conditions, these adulterated cancer cell lines containing BMDC mimics could then be used for *in vitro* and *in vivo* experimentation, which determine the successes and failures of our anti-cancer efforts.

## MATERIALS AND METHODS

### Animals

Mice with genetic mutations in the APC gene (C57BL/6J-*Apc*$^{Min}$/J) and transgenic mice with ubiquitous GFP expression (C57BL/6-Tg(ACTbEGFP)1Osb/J) were obtained from Jackson laboratories (Bar Harbor, Maine). Wild-type C57BL/6 female mice were obtained from Charles River Laboratories (Wilmington, Massachusetts). The institutional animal care and use committees of Yale University and University of Florida approved all animal procedures.

### Human Subjects

Following IRB approval by the University of Florida Health Science Center paraffin embedded neoplastic tissues were obtained from female patients who received hematopoietic cell transplantation from male donors. Collaboration with the CIBMTR anonymously identified additional patients who underwent hematopoietic cell transplantation and developed secondary cancers. CIBMTR participating institutions were contacted and invited to participate in this trial. Local IRB approval was obtained at all sites participating.

### Murine Hematopoietic Cell Transplantation Studies

For the mouse adenoma experiments, bone marrow was harvested from a male APC$^{min}$ mutant mouse and $1x10^6$ cells were injected intravenously into recipient female APC$^{min}$ mutant mice (n=4). To prepare recipients, APC$^{min}$ female mutant mice received total body irradiation (1.1 Gy total from a $^{137}$cesium source) followed by marrow transplantation. All recipient mice were sacrificed 3 months post-transplant and intestines removed for fixation and staining.

For single HSC transplants Sca-1$^+$c-kit$^+$Lin$^-$ HSCs were enriched by FACS sorting before individual HSC selection with micromanipulators via fluorescent microscopy. Individual *Gfp*$^+$ HSCs were then mixed with $2x10^5$ non-*Gfp*$^+$ BM cells that had been depleted of Sca-1$^+$ cells by magnetic beads before transplant into irradiated (0.95 Gy total, $^{137}$cesium source) hosts. For the serial transplants, $1x10^3$ bone marrow cells were transplanted into irradiated (0.95 Gy total) secondary, female C57BL/6 recipients.

### Mouse Adenoma Immunohistochemistry

Isotype, serum, and no primary antibody controls were included for each sample in the immunostaining protocols. Negative and positive control tissues were processed in each staining run. For Y FISH, CD45 and cytokeratin, 3 µm sections were deparaffinized, hydrated, incubated in BD Biosciences Retrievagen A solution for 15 min at 100°C and then 20 min at room temperature, and incubated in 0.2 M HCL for 12 min and 1 M NaSCN at 80°C for 20 min. Y FISH was performed with digoxigenin-labeled Y chromosome probe and anti-digoxigenin-rhodamine antibody (Roche Molecular Biochemicals). After Y FISH, slides were



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incubated in 1:20 anti- CD45RB (Santa Cruz Biotechnology), 1:100 F4/80 (eBioscience, San Diego, CA) 1 h at room temperature, incubated with anti-rat alexa 647 (Molecular Probes), fixed in 2% PFA in 1x PBS for 8 min, digested with 0.5 trypsin for 1 min at 37°C, washed with 5% FCS to inactivate the trypsin, incubated with 1:200 anti-pankeratin (DAKO) overnight at 4°C, incubated in 1:500 anti-rabbit-FITC (Molecular Probes) for 1 h at 37°C, and coverslipped by using vectashield DAPI (Vector Laboratories).

**Immunohistochemistry on human specimens and mouse lung cancer**

Zinc formalin-fixed, paraffin-embedded adenoma sections were cut at 4 – 6 μm and air-dried overnight. After deparaffinization and rehydration, endogenous peroxidase activity was quenched by application of 3% hydrogen peroxide in methanol for 10 minutes at room temperature. Tissues to be stained for CD45 (leukocyte common antigen, LCA DakoCytomation, Carpinteria, CA) were antigen retrieved using Trilogy unmasking solution (Cell Marque, Hot Springs, AK). Sections stained for CK20 (cytokeratin 20, DakoCytomation, Carpinteria, CA) were sequentially retrieved with target retrieval solution (DakoCytomation,Carpinteria, CA) and trypsin (Digest-all 2, Zymed laboratories, San Francisco CA). Endogenous biotin was blocked with a kit (Dako, Carpinteria, CA), and primary antibody was then applied for one hour at room temperature (1:50 for CD45 and 1:25 for CK20). Primary antibody was detected using an LSAB2-HRP kit (DakoCytomation, Carpinteria, CA) and Diaminobenzidene (DAB). Isotype-matched negative controls were run with each of the antibodies, finding no non-specific binding. An appropriate positive control slide was also stained with each staining run.

**FISH Probing for X and Y Chromosomes**

For mouse tissues, slides were treated to two rounds of a five-minute incubation in Lugol's solution (Sigma, St. Louis, MO) followed by destaining in 2.5 M sodium thiocyanate. Tissue was further prepared by incubation in 0.2 N hydrochloric acid for 30 minutes at room temperature, (not done-tissue wasn't fixed in ZnF) Sections were retrieved by incubation in 1 M sodium thiocyanate for 30 minutes at 85 °C, followed by digestion in pepsin at 1 mg/mL (Sigma, St. Louis, MO) in 0.9% sodium chloride, pH 2.0 for 10 minutes at 37 °C. Slides were next rinsed with distilled water and equilibrated in 2x saline sodium citrate (SSC). After serial dehydration in ethanol, slides were placed on the heat plate of a Hybrite oven (Vysis Inc, Downers Grove, IL). Whole chromosome paint probes for murine X and Y chromosomes (OpenBiosystems, Huntsville AL) were pre-annealed following the manufacturer's directions and added to the sections. Coverslips were sealed over the slides with rubber cement. Tissue sections and probes were co-denatured at 60 °C for 10 minutes before being hybridized overnight at 37 °C. Slides were then washed in 50% formamide in 2x SSC at 46 °C thrice for 7 minutes each, followed by 2x SSC at 46 °C for 5 minutes, and the 4x SSC + 0.1% Igepal (Sigma, St. Louis, MO) at 46 °C for 5 minutes. Slides were air dried in the dark and then mounted with Vectashield containing 4,6-daminidino-2-phenylidole (DAPI) (Vector Laboratories, Burlingame, CA).

For human tissues, slides were treated to two rounds of a five-minute incubation in Lugol's solution (Sigma, St. Louis, MO) followed by destaining in 2.5 M sodium thiocyanate. Tissue was further prepared by incubation in 0.2 N hydrochloric acid for 30 minutes at room temperature, and incubation in 1 M sodium thiocyanate for 30 minutes at 85 °C. Pretreatment concluded with a digestion in pepsin at 4 mg/mL (Sigma, St. Louis, MO) in 0.9% sodium chloride, pH 2.0 for up to 60 minutes at 37 °C. Slides were next rinsed with distilled water and equilibrated in 2x saline sodium citrate (SSC). After serial dehydration in ethanol, slides were placed on the heat plate of a Hybrite oven (Vysis Inc, Downers Grove, IL). CEP probes for X and Y chromosomes (Vysis Inc, Downers Grove, IL) were added to the sections and coverslips were sealed over the

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slides with rubber cement. Tissue sections and probes were co-denatured at 75 °C for 6 minutes before being hybridized overnight at 37 °C. Slides were then washed in 50% formamide in 2x SSC at 46 °C thrice for 7 minutes each, followed by 2x SSC at 46 °C for 5 minutes, and the 4x SSC + 0.1% Igepal (Sigma, St. Louis, MO) at 46 °C for 5 minutes. Slides were air dried in the dark and then mounted with Vectashield containing 4,6-daminidino-2-phenylidole (DAPI) (Vector Laboratories, Burlingame, CA).

**Tissue Analysis**

Slides were analyzed using a Leica laser scanning spectral confocal microscope (Leica Microsystems, Bannockburn, IL). DAB staining for tissue specific antigens and characteristic cellular morphology were used to specifically classify cells. Paraffin-embedded adenoma blocks were sectioned and immunohistochemically stained with specific antibodies to identify epithelial neoplastic tissues (cytokeratin) and leukocytes (CD45). Basal cell skin cancer appeared below the epidermis as cubiodal cells and lacking a stratum spinosum Pallisading was evident at the periphery of the basal cell carcinomas. Neoplastic adenoma cells appeared elongated and large, with an epithelial orientation and positive staining with anti-cytokeratin antisera and periodic acid Schiff (PAS) staining. Squamous cell carcinoma cells were detected by their atypia, large size, angulated nuclei, evidence of keratinzation and invasion below the basement membrane. Leukocytes appeared small and round with positive anti-CD45 antisera staining. Y chromosome signal was punctate, green and regularly at the nucleus perimeter. X chromosome signal was similarly nuclear and punctate, but red.

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## REFERENCES

1. Krause DS, Theise ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell. 2001;105(3):369-377.
2. Okamoto R, Yajima T, Yamazaki M, et al. Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. Nat Med. Sep 2002;8(9):1011-1017.
3. Korbling M, Katz RL, Khanna A, et al. Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N Engl J Med. Mar 7 2002;346(10):738-746.
4. Brittan M, Hunt T, Jeffery R, et al. Bone marrow derivation of pericryptal myofibroblasts in the mouse and human small intestine and colon. Gut. Jun 2002;50(6):752-757.
5. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. Science. Sep 27 2002;297(5590):2256-2259.
6. Theise ND, Nimmakayalu M, Gardner R, et al. Liver from bone marrow in humans. Hepatology. Jul 2000;32(1):11-16.
7. Grant MB, May WS, Caballero S, et al. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. Nat Med. Jun 2002;8(6):607-612.
8. Lagasse E, Connors H, Al-Dhalimy M, et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med. 2000;6(11):1229-1234.
9. Theise ND, Henegariu O, Grove J, et al. Radiation pneumonitis in mice: a severe injury model for pneumocyte engraftment from bone marrow. Exp Hematol. Nov 2002;30(11):1333-1338.
10. Houghton J, Stoicov C, Nomura S, et al. Gastric cancer originating from bone marrow-derived cells. Science. Nov 26 2004;306(5701):1568-1571.
11. Goodell MA. Stem-cell "plasticity": befuddled by the muddle. Curr Opin Hematol. May 2003;10(3):208-213.



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12. Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cells. Blood. Nov 15 2003;102(10):3483-3493.
13. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science. Jan 19 1990;247(4940):322-324.
14. Cogle CR, Yachnis AT, Laywell ED, et al. Bone marrow transdifferentiation in brain after transplantation: a retrospective study. Lancet. May 1 2004;363(9419):1432-1437.
15. Butler JM, Guthrie SM, Koc M, et al. SDF-1 is both necessary and sufficient to promote proliferative retinopathy. J Clin Invest. Jan 2005;115(1):86-93.
16. Curtis RE, Rowlings PA, Deeg HJ, et al. Solid cancers after bone marrow transplantation. N Engl J Med. Mar 27 1997;336(13):897-904.
17. Terada N, Hamazaki T, Oka M, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature. 2002;416(6880):542-545.
18. Pawelek JM. Tumour-cell fusion as a source of myeloid traits in cancer. Lancet Oncol. Dec 2005;6(12):988-993.
19. Wang X, Willenbring H, Akkari Y, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature. Apr 24 2003;422(6934):897-901.
20. Liegeois A, Escourrou J, Ouvre E, Charreire J. Microchimerism: a stable state of low-ratio proliferation of allogeneic bone marrow. Transplant Proc. Mar 1977;9(1):273-276.
21. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci U S A. Jan 23 1996;93(2):705-708.
22. Kucia M, Reca R, Campbell FR, et al. A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia. May 2006;20(5):857-869.
23. Kucia M, Halasa M, Wysoczynski M, et al. Morphological and molecular characterization of novel population of CXCR4+ SSEA-4+ Oct-4+ very small embryonic-like cells purified from human cord blood: preliminary report. Leukemia. Feb 2007;21(2):297-303.
24. Jin DK, Shido K, Kopp HG, et al. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4(+) hemangiocytes. Nat Med. May 2006;12(5):557-567.
25. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature. Mar 1 2001;410(6824):50-56.
26. Ratajczak MZ, Zuba-Surma E, Kucia M, Reca R, Wojakowski W, Ratajczak J. The pleiotropic effects of the SDF-1-CXCR4 axis in organogenesis, regeneration and tumorigenesis. Leukemia. Nov 2006;20(11):1915-1924.
27. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell. Oct 27 2000;103(3):481-490.
28. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature. Dec 8 2005;438(7069):820-827.

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**Figure 1. Marrow contributes to intestinal neoplasias in humans and mice.** (A & B) Colonic adenomas from women who received hematopoietic cell transplantations from male donors. (A) Sections of the adenoma demonstrate a Y chromosome (green) within the nucleus (blue) of a gastrointestinal epithelial cell expressing cytokeratin (brown) and mucin (magenta). Magnification 60X. (B) Donor-derived adenoma cells (Y chromosome, green; nuclei, blue) were CD45 (brown) negative. Magnification 60X. (C & D) Intestinal adenomas spontaneously arising in female mice with $APC^{min}$ mutation after having received a bone marrow transplant from male $APC^{min}$ mice. (C) Fluorescent micrograph of an adenoma section stained for cytokeratin (green), leukocytes (pink), nuclei (blue) and FISH for Y chromosomes (red). Bracket indicates marrow-derived cells incorporated within adenoma epithelium. (D) Adenomas were stained for cytokeratin (white), nuclei (blue) and FISH for X (red) and Y (green) chromosomes. Using confocal microscopy, serial Z-steps through mouse intestinal adenomas at 0.5 micron intervals identified marrow-derived intestinal epithelial cells demonstrating no evidence for fusion (i.e., XXY, XXXY).

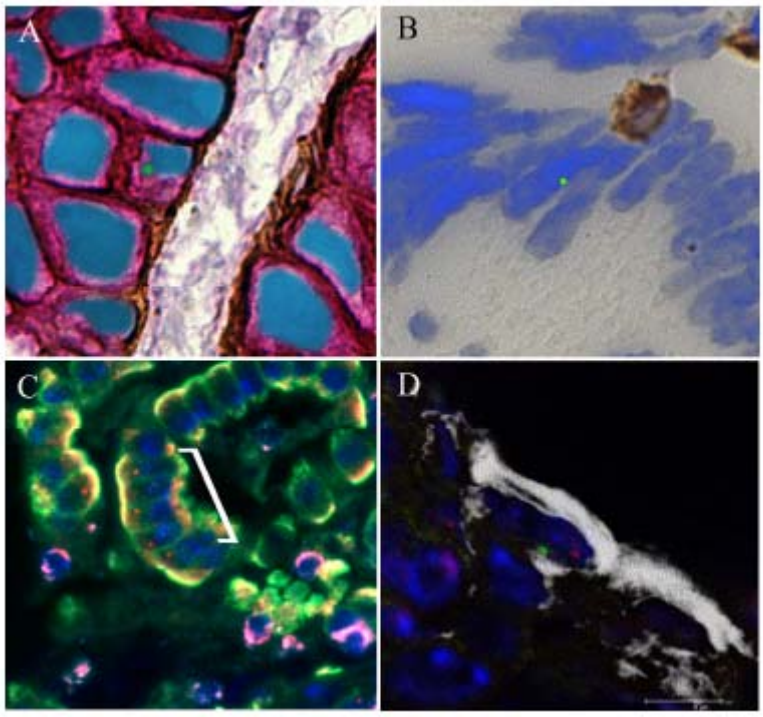


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**Figure 2. Human skin cancers without evidence of marrow incorporation.** Skin carcinomas found post-transplant in women who received hematopoietic cell transplantations from male donors. (A-C) Representative micrographs of a squamous cell carcinoma demonstrating hematoxylin & eosin staining (A) and cytokeratin staining (brown) (B). Magnifcation 60X. (C) Fluorescent micrograph of cytokeratin positive cells demonstrating nuclei (blue) with X chromosomes (red), but no Y chromosomes (green). Only female cytokeratin cells are found post-transplant in all squamous cell carcinomas. (D-F) Representative micrographs of a basal cell carcinoma demonstrating hematoxylin & eosin staining (D) and cytokeratin staining (brown) (E). Magnifcation 60X. (F) Fluorescent micrograph of cytokeratin positive cells demonstrating nuclei (blue) with X chromosomes (red), but no Y chromosomes (green). Only female cytokeratin cells are found post-transplant in all basal cell carcinomas.

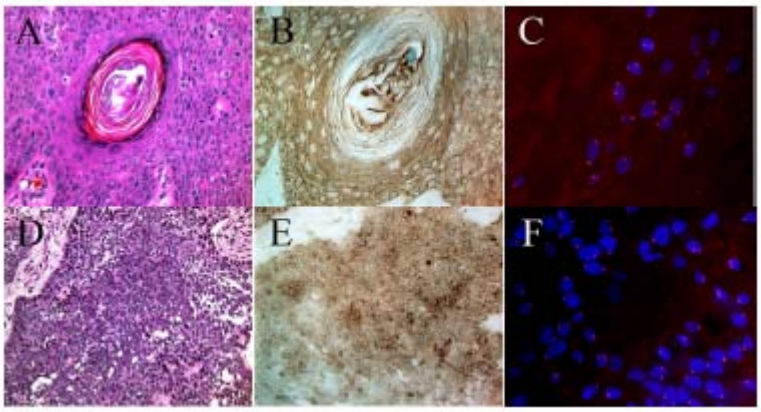


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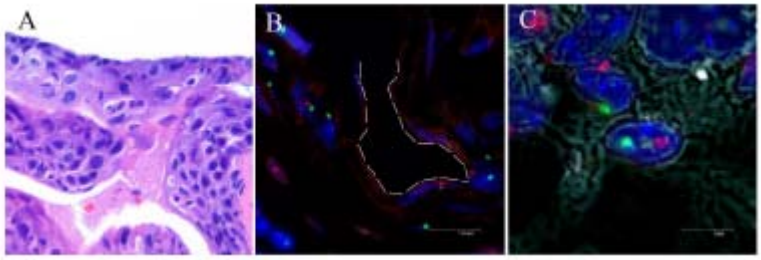
**Figure 3. The hematopoietic stem cell contributes to lung cancer.** (A & B) Secondary lung cancer found in a woman who received hematopoietic cell transplantation from her brother demonstrates lung cancer from donor marrow origin. (A) Hematoxylin and eosin staining of squamous cell carcinoma of the lung found after transplant. (B) Fluorescent micrograph showing immunostaining for cytokeratin (rust) and FISH for X (bright red) and Y (green) chromosomes. Magnification 63X. Dashed line indicates epithelial surface lining the squamous cell carcinoma. (C) Lung cancer from a female mouse which received secondary transplants from a single-HSC (male) transplanted donor. Lung cancer shows HSC-derived cells (Y chromosome, green; X chromosome, red) co-expressing cytokeratin (white).

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**Supplemental Movie.** Bone marrow contributes to murine adenomas without evidence for fusion. Intestinal adenomas spontaneously arising in female mice with $APC^{min}$ mutation after having received a bone marrow transplant from male $APC^{min}$ mice. Adenomas were stained for cytokeratin (white), nuclei (blue) and FISH for X (red) and Y (green) chromosomes. Using confocal microscopy, serial Z-steps through mouse intestinal adenomas at 0.5 micron intervals identified marrow-derived intestinal epithelial cells demonstrating no evidence for fusion (i.e., XXY, XXXY).

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**Table 1. Patient Characteristics**

| Patient | Age at transplant (years) | History of a male child | Primary Disease | Transplant | History of GVHD | 2nd Cancer | Days Post Transplant | Percent Donor in Neoplasia |
|---|---|---|---|---|---|---|---|---|
| 1 | 55 | Unknown | AML | BM | Yes | Colonic adenoma | 47 days | 1% |
| 2 | 28 | Yes | Hodgkin's Lymphoma | PBSC | Yes | Colonic adenoma | 30 days | 4% |
| 3 | 53 | No | NHL | PBSC | Yes | Squamous cell carcinoma of the skin | 1717 days | 0% |
| 4 | 43 | No | ALL | BM | Yes | Squamous cell carcinoma of the skin | 2656 days | 0% |
| 5 | 63 | No | AML | PBSC | No | Basal cell carcinoma | 327 days | 0% |
| 6 | 59 | Yes | AML | PBSC | Yes | Basal cell carcinoma | 1491 days | 0% |
| " | " | " | " | " | " | Squamous cell carcinoma of the lung | 1651 days | 20% |

GVHD, graft versus host disease; AML, acute myelogenous leukemia; PBSC, mobilized peripheral blood stem cell; BM, bone marrow

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**Bone Marrow Contributes to Epithelial Cancers in Mice and Humans as Developmental Mimicry**

Christopher R. Cogle, Neil D. Theise, DongTao Fu, Deniz Ucar, Sean Lee, Steven M. Guthrie, Jean Lonergan, Witold Rybka, Diane S. Krause and Edward W. Scott

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