CD200-expressing human basal cell carcinoma cells initiate tumor growth

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Smoothened antagonists directly target the genetic basis of human basal cell carcinoma (BCC), the most common of all cancers. These drugs inhibit BCC growth, but they are not curative. Although BCC cells are monomorphic, immunofluorescence microscopy reveals a complex hierarchical pattern of growth with inward differentiation along hair follicle lineages. Most BCC cells express the transcription factor KLF4 and are committed to terminal differentiation. A small CD200−CD45− BCC subpopulation that represents 1.63 ± 1.11% of all BCC cells resides in small clusters at the tumor periphery. By using reproducible in vivo xenograft growth assays, we determined that tumor initiating cell frequencies approximate one per 1.5 million unsorted BCC cells. The CD200−CD45− BCC subpopulation recreated BCC tumor growth in vivo with typical histological architecture and expression of sonic hedgehog-regulated genes. Reproducible in vivo BCC growth was achieved with as few as 10,000 CD200−CD45+ cells, representing ~1,500-fold enrichment. CD200−CD45− BCC cells were unable to form tumors. These findings establish a platform to study the effects of Smoothened antagonists on BCC tumor initiating cell and also suggest that currently available anti-CD200 therapy be considered, either as monotherapy or an adjunct to Smoothened antagonists, in the treatment of inoperable BCC.

Results

Human BCC Express Hair Follicle Differentiation-Specific Keratins. BCCs typically arise on hair-bearing skin, and BCC cells resemble basal cells of the hair follicle outer root sheath (ORS), explaining the name and presumed origin of this tumor (Fig. 1A and B). Likewise, BCC tumors from transgenic mouse models also demonstrate hair follicle differentiation, even though lineage tracing experiments are divided as to the cell of origin between hair follicle bulge stem cells (22–26) and interfollicular epithelial cells (27). The process of hair growth is carefully choreographed and hair follicles consist of concentric cell layers characterized by distinct patterns of hair follicle specific keratin heterodimer.

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expression during each step toward terminal differentiation (28). We first sought to determine if human BCC expressed hair follicle-specific keratins by using RT-PCR and immunofluorescence to assess the extent of differentiation that would support the cancer stem cell model and the potential existence of TICs.

All human BCCs studied (N = 20) contained cells that expressed human hair follicle ORS keratins from the basal (K5, K14, and K19) and suprabasal (K16 and K17) layers (Fig. 1 C and D and SI Appendix, Fig. S1). K16, which is normally expressed in hair follicle suprabasal terminally differentiated cells, showed restricted expression within the tumor cell mass (Fig. 1D and SI Appendix, Fig. S1 C and E), suggesting that BCCs undergo inward differentiation. In normal hair follicles, SHH signaling from bulb matrix cells induced K17 expression in the ORS (29) (Fig. 1D). By contrast, K17 was ubiquitously expressed throughout BCC tumors (Fig. 1D and SI Appendix, Fig. S1 D and E), consistent with oncogenic SHH signaling in BCC (30). Whereas K16 and K17 expression is mutually exclusive in the hair follicle, coincident expression was observed within BCC (SI Appendix, Fig. S1E).

Human BCC samples also expressed hair follicle keratins typical of the companion layer, inner root sheath (IRS), cuticle, and medulla. The companion layer keratin K75 was expressed by six of 20 BCCs studied (Fig. 1 C and D and SI Appendix, Fig. S1D). Three of 20 BCCs also expressed IRS keratins, but only in a limited number of cells (Fig. 1 C and D). Hair shaft keratins were not observed in BCC. In summary, by conventional H&E staining, BCCs consisted of monomorphic cells disguising a complex pattern of hair follicle-specific keratin expression (SI Appendix, Fig. S1F) that implies hierarchical growth with differentiation and multiple tumor cell subpopulations.

**BCC Cells Express Human Hair Follicle Bulge Stem Cell Marker CD200.** BCC cells express a diverse pattern of hair follicle differentiation demonstrated by intracellular expression of hair follicle-specific keratins. One approach to enrich TIC subpopulations in human BCCs could involve characterization of human BCC heterogeneity by using cell surface differentiation markers that identify keratinocyte stem cells (KSCs). It has been assumed that BCC arises from KSCs because these cells are sufficiently long-lived to sustain the necessary mutations and they already possess the capacity for self-renewal. The hair follicle bulge region and other keratinocyte subpopulations have been defined by clusters of differentiation antigens: CD24, CD71, CD146, and CD200 (31). BCC tumor cell inward differentiation, as manifested by the potential hair follicle keratin expression, was also demonstrable by using cell surface markers of epidermal and hair follicle differentiation. CD24 localized to cells of the hair follicle IRS and also the interfollicular stratified epidermis, and was similarly restricted to the BCC inner tumor cell mass (SI Appendix, Fig. S2A). The transferrin receptor CD71 identified basal cells below the level of the bulge in hair follicles and was predominantly expressed in the outermost cell layers of BCC tumor nodules (SI Appendix, Fig. S2A). CD146 localized to the lower portions of the hair follicle basal layer and surrounding endothelial cells, whereas, in BCC samples, CD146 expression was limited to blood vessels and was absent from tumor cells (SI Appendix, Fig. S2A). Human hair follicle bulge KSCs reside within the ORS between the origin of the sebaceous gland and arrector pili muscle insertion, and express the cell surface protein CD200 (Fig. 2F). Small clusters of BCC tumors located in the basal and immediately adjacent suprabasal layers of occasional sections of tumor nodules also expressed CD200 (Fig. 2B and SI Appendix, Fig. S2A). Consistent with the inward pattern of differentiation, proliferation assessed by Ki67 labeling occurred in the outer cell layer of BCC tumor nodules (SI Appendix, Fig. S2B) and in cells that also expressed the antiapoptotic protein BCL2 (SI Appendix, Fig. S2C). We concluded that, if present, TICs might also be located in the outer cell layer of BCC.

BCC samples were efficiently dissociated into single cell suspensions with as many as 85% of cells viable (SI Appendix, Fig. S3A) similar to that observed after dissociation of normal skin and SCC (32). We found that not all BCC tumors expressed EpCAM as assessed by immunohistochemistry. When using BCC in which the majority of tumors expressed EpCAM, we determined that dissociated BCC tumor samples contained high numbers of EpCAM-positive tumor cells (49–62% of all cells isolated, n = 6; SI Appendix, Fig. S3B), confirming adequate dissociation and survival of BCC tumor cells, including a subpopulation of EpCAM−CD200+ cells (SI Appendix, Fig. S3C). All BCC samples contained a small CD200+ tumor cell population (1.11%; range, 3.96–0.05%; n = 21; Fig. 2C), irrespective of the histological type. BCC also contained CD45+ tumor-associated leukocytes that accounted for 13.81 ± 10.84% (n = 21) of all cells and included a subpopulation of CD200+ CD45+ cells (0.66 ± 0.7%; Fig. 2C). Thus, CD200+ BCC tumor cells could be distinguished by flow cytometry with the pan-leukocyte marker CD45 to exclude tumor infiltrating leukocytes. BCC CD200+ CD45+ and CD200−CD45− subpopulations were isolated by flow cytometry with greater than 86% and 98% purity, respectively (SI Appendix, Fig. S3D). To assess SHH signaling, flow-sorted BCC tumor cell cDNA was compared with cDNA from intact BCC tumor tissue and the GLI1-overexpressing sarcoma cell line SJS-1. Sustained SHH signaling leads to expression of hedgehog-regulated genes, including the transcription factor GLI1 that augments the pathway (33). Both CD200+ CD45+ and CD200− CD45− tumor cell populations expressed the human hedgehog-regulated genes K17, Pdgfrα, and GLI1 as expected (Fig. 2, B, D, and F and SI Appendix, Fig. S3E). Loss of GLI1 expression was apparent in the CD200− CD45− subpopulation. In contrast, the CD200+ CD45+ population maintained GLI2 expression similar to that observed in SJS-1 cells and hair follicles, highlighting a potential functional difference between these two populations. The CD200+ CD45+ subpopulation also exhibited almost twofold
more proliferating cells than the CD200– CD45– cells, 7.26% vs. 4.60%, respectively (SI Appendix, Fig. S3F). In summary, the CD200+ CD45− and CD200− CD45+ BCC tumor cell populations demonstrated activated hedgehog signaling consistent with the genetic basis for BCC.

The development of an in vitro colony forming efficiency assay, as was used to identify CD133+ primary human SCC TICs (32), could test BCC subpopulations before in vivo assessment. BCC cells formed cellular aggregates atop irradiated 3T3 feeder layers in tissue culture, similar to what was observed with primary human SCC and reminiscent of transformed cells, but BCC cells did not exhibit anchorage-independent growth (Fig. 3A). BCC spheroidal colonies could be quantified and were proportional to the number of cells plated, although the absolute number of colonies varied among the nine tumor samples and experiments (SI Appendix, Fig. S4A). In vitro, BCC colonies maintained active hedgehog signaling (Fig. 3B). Colonies from unsorted BCC cells could be passaged in vitro and when implanted into nude mice gave rise to tumors (Fig. 3C). When 10⁵ flow-sorted cells were plated from five different BCC samples, CD200+ CD45− sorted cells gave rise to threefold more colonies than the CD200− CD45+ subpopulation (P < 0.005), which also gave rise to fewer colonies than unsorted cells (P < 0.01; Fig. 3D). CD200+ CD45− sorted cells also gave rise to larger colonies in vitro (SI Appendix, Fig. S4B) that resulted in tumor growth in vivo, in contrast to the smaller colonies from the CD200− CD45+ cells that did not form tumors in vivo. Thus, human BCC contained a relatively small CD200+ CD45− tumor cell subpopulation that demonstrated increased colony forming efficiency relative to unfractionated tumor cells.

**Human BCC Growth in Vivo Is Dependent on a “Humanized” Stroma and Etoposide Pretreatment.** BCC growth in vivo has been difficult to achieve, even when tumor fragments containing stromal components were grafted into a variety of immunodeficient mice (SI Appendix, Table S1). We grafted 17 different human BCC tumor...
fragments into dorsal s.c. spaces of athymic primary human BCC cells were enriched for 5), and nonobese diabetic/SCID and BCC cells expressed K15 (14). After 12 wk, histological analyses of graft sites failed to demonstrate BCC in any recipient mice (SI Appendix, Fig. S5A). In vivo propagation of primary human SCC required the generation of a stromal bed before tumor grafting, achieved by implanting a glass disk or Gelfoam dressing into the s.c. space 2 wk before implantation of tumor tissue (34). This approach failed to allow propagation of BCC in athymic nude mice or SCID-beige mice (n = 4 each; SI Appendix, Fig. S5A). We hypothesized that residual inflammatory cells present in athymic nude mice and, to a lesser extent, SCID-beige mice might hinder tumor growth after initial creation of the stromal bed. Similar to the preparation of murine mammary fat pads with etoposide, which induced myelo-suppression before human breast cancer engraftment, we created stromal beds and administered i.p. etoposide 1 d before grafting BCC tissue. By using this approach, we achieved xenograft tumor growth after 12 wk from six of seven different primary human BCC samples (SI Appendix, Fig. S5A). Consistently, BCC growth occurred in only athymic nude but not SCID-beige mice, suggesting that a residual inflammatory milieu is essential for this in vivo BCC model. Tumor xenografts measured 3 to 8 mm in diameter, consistent with the slow growth of BCC in humans. Histology confirmed that the xenografts recreated the original BCC tumor architecture and maintained active SHH signaling (SI Appendix, Fig. S5B). Thus, BCC tumor growth in athymic nude mice was dependent on the creation of a stromal bed and etoposide pretreatment.

To begin to test the cancer stem cell hypothesis, it was necessary to successfully graft fractionated cell suspensions from primary human BCC. Analogous to our findings with grafting of primary human SCC cell suspensions (34), it was necessary to “humanize” xenograft stromal beds. One million (10^6) normal primary human fibroblasts were first suspended in Matrigel and implanted with glass discs or Gelfoam dressings. After 13 d, mice were treated with i.p. etoposide, and, on day 14, BCC xenograft cell suspensions were co-injected with an additional 10^6 primary human normal fibroblasts suspended in Matrigel into the prepared graft sites (Fig. 4A and B). This approach yielded successful xenograft tumor growth of 12 of 15 xenografts from 10 different primary human BCC when 3 million or more unsorted BCC cells were implanted (SI Appendix, Table S2). Tumor growth was not reproducible when 1 million unsorted primary human BCC cells or fewer were implanted, irrespective of the histological grade of the original tumor (Fig. 4C). The histological patterns of xenograft tumors matched the original primary human BCC histologies and tumors also maintained active SHH pathway signaling (Fig. 4D). The dose-dependence of engraftment supports the existence of a small number of TICs in human BCC. Based on a limiting dilution analysis, we calculated the TIC frequency in human BCC to be less than one per 1.5 million (SI Appendix, Table S3).

**CD200^+ CD45^− BCC Subpopulation is Enriched for TICs.** To determine if CD200^+ CD45^− primary human BCC cells were enriched for TICs, we grafted athymic nude mice with varying numbers of cells from 14 different BCC tumor samples (SI Appendix, Table S4) after isolation of CD200^+ CD45^− and CD200^+ CD45^− subpopulations. After 12 wk, xenograft sites were harvested and analyzed by histology. CD200^+ CD45^− cells did not give rise to tumors in xenografts (0 of 14) involving eight different BCC samples, even when 3 × 10^6 tumor cells were implanted. In contrast, CD200^− CD45^+ cells reproducibly formed tumors, initiated with as few as 10,000 cells in our in vivo assay (Fig. S4). CD200^+ CD45^− human BCC cells formed tumors resembling the original BCC and maintained active SHH signaling and differentiation (Fig. S6). Based on limiting dilution analysis, the TIC frequency in the CD200^+ CD45^− subpopulation approximated one in 822 (SI Appendix, Table S5). Thus, the CD200^+ CD45^− subpopulation was enriched for TICs more than 1,500-fold. Of equal importance, we determined that CD200^+ CD45^− BCC cells did not exhibit TIC activity. Because BCC xenografts were small and grew slowly, serial in vivo transplantation of the CD200^+ CD45^− population was not attempted. Taken together, these findings support the existence of CD200^+ TICs in human BCC.

The expression of CD200 on BCC TICs and human hair follicle bulge stem cells raised the possibility that BCC TICs arose from hair follicle bulge KSCs. Analogous to human hair follicle bulge stem cells, CD200^+ CD45^− BCC cells expressed K15 (SI Appendix, Fig. S6) (35). The ability of adult tissue stem cells and TICs to self-renew led us to study the expression of transcription factors involved in embryonic stem cell maintenance and self-renewal. Kruppel-like factor 4 (KLF4) has activator and repressor transcriptional activities and is a key regulator during embryogenesis, in which it prevents differentiation by regulating NANOG expression. However, in mature skin, KLF4 is normally expressed in the differentiated cell layers (36). Consistent with their differentiated state in BCC, KLF4 expression was restricted to the CD200^+ CD45^− subpopulation (SI Appendix, Fig. S6). The proto-oncogene C-MYC is associated with stem and transient amplifying cell proliferation, but continued expression leads to epidermal stem cell depletion and terminal differentiation (37). In BCC, C-MYC was expressed by CD200^− CD45^− and CD200^+ CD45^− subpopulations (SI Appendix, Fig. S6). In contrast, the POU domain transcription factor Oct3/4, homeobox transcription factor Nanog, and the telomerase reverse transcriptase Tert were expressed by tumor tissue but not the sorted BCC sorted populations (SI Appendix, Fig. S6). Hence, CD200^+ BCC cells clustered at the tumor periphery collectively do not express the...
regulator of keratinocyte differentiation KLF4 and are exclusively enriched with cells that can initiate tumor growth.

Discussion

BCC arise from keratinocytes with mutations leading to constitutively active SHH growth factor signaling. Unlike the multiple genetic lesions required during stepwise carcinogenesis in many other cancers, fewer “hits” are required for the development of BCC, perhaps explaining the absence of precursor lesions and why BCC is the most common malignancy in subjects of white race. SHH-expressing keratinocytes demonstrate continued proliferation and are resistant to p21CIP1/WAF1-induced replicative senescence (38). As BCCs grow, they continue to exhibit hair follicle differentiation with inward expression of differentiation-associated hair-specific keratins and the differentiation-associated protein CD24 in central regions, whereas cell proliferation mostly occurs at the tumor periphery. A small number of BCC cells identified by the cell surface marker CD200 reside as clusters at the tumor periphery, and are not transcriptionally programed toward terminal differentiation, as they do not express the transcription factor KLF4. These CD200+ BCC cells are also unique in that they lack expression of GLI2 in response to SHH signaling and instead rely on GLI1, contrary to the currently held view of SHH signaling in BCC. Collectively, these findings suggest that BCC cells are not uniform and undergone hierarchical differentiation as proposed by the cancer stem cell model, with TICs residing in a clustered and relatively undifferentiated CD200+ BCC cell precursor population.

CD200 is a highly conserved type-1 membrane glycoprotein that is expressed primarily by normal myeloid cells. However, CD200 expression is also observed in a number of malignancies, including renal carcinoma, ovarian carcinoma, colon carcinoma, melanoma, acute myeloid leukemia, multiple myeloma, and chronic lymphocytic leukemia (39, 40). Expression of the cognate receptor CD200R is restricted to myeloid cells and T lymphocytes (41). Ligand receptor interaction confers an immunosuppressive signal to immune cells. T lymphocytes down-regulate TH1 cytokines and instead express IL-10 and exhibit regulatory T-cell activity (42). CD200 KO mice exhibit expansion and activation of tissue specific macrophages, with rapid onset of experimental autoimmune diseases (41). The immune modulator protein CD200 is also expressed by human hair follicle bulge KSCs, presumably to protect these cells from immunological attack (31). Intriguingly, inter-spersed interfollicular keratinocytes that also express CD200 do not exhibit stem cell activity (43). As CD200+ CD45− BCC cells express K15, these cells may arise from mutated CD200+ human hair follicle bulge KSCs that also express the hair-specific keratin K15. Although expression of CD200 and K15 is not regulated by SHH, this does not exclude the possibility that BCC arises from transformed interfollicular or hair follicle differentiated keratinocytes. Putative TICs in multiple cancer cell lines have also been found to express CD200 (44). In human acute myeloid leukemia and multiple myelomas, CD200 expression is associated with poor prognosis (45–47). In summary, CD200 is expressed by BCC TICs and hair follicle bulge KSCs from which they may be derived, and may protect both cell populations from immunological attack.

To confirm the presence of BCC TICs, we developed a unique in vivo assay. Similar to many other cancers, BCC growth is dependent on the presence of stromal cells. We implanted glass beads or Gelfoam dressings together with 1 million primary human fibroblasts, a strategy we developed to propagate primary human SCC xenografts (33), to create a receptive stromal bed. i.p. administration of etoposide before tumor implantation was also required, in analogy to the method described for primary human breast cancer xenografts (48). With this approach, tumor growth was successful in athymic nude mice, which lack T lymphocytes, but not SCID-beige mice, which lack both T and B lymphocytes and have reduced natural killer cell numbers. We hypothesize that some inflammatory cells are important during the initial phase of stromal bed formation but then hinder tumor engraftment, as etoposide-induced myelosuppression was also found to be necessary. BCC grafts in this model grew slowly, consistent with the rate of growth of BCC observed in humans and mouse models. Thus, despite its complexity, the model we describe faithfully re-created human BCC growth from dissociated tumor cells and allowed characterization of TICs in BCC.

Recently, drugs that simultaneously inhibit multiple growth factor pathways (e.g., tyrosine kinase receptor inhibitors), single pathways (VEGF receptor, TGF-β receptor, EGF receptor, and SMO antagonists), mutated targets (B-Raf inhibitors), and downstream signaling targets (MEK inhibitors) have been developed. Although malignancies in patients often show initial responses to these drugs, cancer recurrence is frequently observed. This study demonstrates the existence of TICs that may drive BCC growth in patients as well as in mice, and these cells may be resistant to killing by SMO antagonists (SI Appendix, Fig. S7). Although not tested, our data would also suggest that currently available anti-CD200 neutralizing antibody alone or in combination with SMO antagonists might be beneficial in the treatment of inoperable and metastatic BCC.

Materials and Methods

Immunofluorescence and Immunohistochemistry. Immunofluorescence and immunohistochemistry were performed by using standard techniques, as previously described (25, 27). Immunofluorescence was performed on tissue sections (clone AE1/3; Dako), CD200 (clone MRC OX104; Serotec), GL1 (GTX27523; GenTex), GLI2 (GTX27195; GenTex), BCL2 (clone 124; Dako), CD24 (clone MLS; BD Pharmingen), CD71 (clone M-A712; BD Pharmingen), CD146 (clone P1H12; BD Pharmingen), CD200 (clone MRC OX104; Serotec), K67 (clone Mbl7; Dako), and human cytokeratins K14 (binding site, clone PHS03), K16 (gift from Rebecca Porter, Department of Dermatology and Wound Healing, School of Medicine, University of Wisconsin, Madison), and K75 (gift from Rebecca Porter). For cytospin analysis, cells were washed and suspended in PBS solution, 100-μL aliquots of cells were added to each slide in a Cytospin funnel (Shandon; Thermo Scientific) and spun at 100 × g for 5 min in a cyto centrifuge (Shandon Cytofunnel 2 cytocentrifuge; Thermo Scientific), fixed in acetone, and labeled with a pancytokeratin and anti-GLI1 antibody.

Flow Cytometry. Tumor samples were collected and enzymatic dissociation was previously described (27). Samples were analyzed and flow- sorted by using FACS-Calibur and a FACSAria flow cytometer (BD Biosciences) with mouse fluorochrome-conjugated IgG subtype isotype controls (BD Pharmingen). Live cell gates were created by using 7-aminocoumarin D (BD Pharmingen) to label dead cells. Cells were labeled according to manufacturer’s instructions with fluorochrome-conjugated antibodies: CD200-AF647 (Serotec), EpCAM-APC (BD Pharmingen), and CD45-FITC (BD Pharmingen). Cell cycle analysis was performed using propidium iodide/RNase staining buffer (BD Pharmingen), and data were analyzed by using FlowJo software (Tree Star).
In Vitro Assay and Tissue Culture. Unsorted or sorted cells were plated onto 50 g Irradiated 373 murine fibroblast feeder layers in 10-cm tissue culture Petri dishes (Corning). Human carcinoma xenograft cell lines (TNC-16B-1; TNC-16B-2; TNC-16B-3; TNC-16B-4; TNC-16B-5) were housed and used under conditions approved by the animal care and use committee at the National Cancer Institute or carried out under the terms of a UK Government Home Office project license (ethics approval is included in the UK Home Office project license). Mice were anesthetized, and Gefolmo dressings (Johnson and Johnson) or sterilized glass discs were implanted into the dorsal s.c. space, together with 10⁶ primary human fibroblasts suspended in 100 μL of Matrigel (BD Biosciences), for single cell suspension experiments, and wounds were closed with surgical staples (Mikron). After 13 d, etoposide (30 μg/kg) was administered (diluted in serum-free HBSS for a final injection volume of 200 μL). On day 14, mice were anesthetized, glass discs were removed (as appropriate), and BCC cells together with 10⁶ primary human fibroblasts that had been suspended in 100 μL of Matrigel were injected into s.c. spaces or, alternatively, into residual Gefolmo dressings. After 12 wk, mice were euthanized using CO₂ inhalation, and tumors were removed for analysis.

Transplantation of Disseminated Human BCC Cells. Athymic nude homozygous female SCID (Jackson Labs), SCID-beige, and nonobese diabetic SCID (Taconic) mice were housed and used under conditions approved by the animal care and use committee at the National Cancer Institute or carried out under the terms of a UK Government Home Office project license (ethics approval is included in the UK Home Office project license). Mice were anesthetized, and Gefolmo dressings (Johnson and Johnson) or sterilized glass discs were implanted into the dorsal s.c. space, together with 10⁶ primary human fibroblasts suspended in 100 μL of Matrigel (BD Biosciences), for single cell suspension experiments, and wounds were closed with surgical staples (Mikron). After 13 d, etoposide (30 μg/kg) was administered (diluted in serum-free HBSS for a final injection volume of 200 μL). On day 14, mice were anesthetized, glass discs were removed (as appropriate), and BCC cells together with 10⁶ primary human fibroblasts that had been suspended in 100 μL of Matrigel were injected into s.c. spaces or, alternatively, into residual Gefolmo dressings. After 12 wk, mice were euthanized using CO₂ inhalation, and tumors were removed for analysis.

RT-PCR. Tissue specimens were microdissected to remove the overlying epidermis. Tissue or cultured cells were homogenized in TRIzol (Invitrogen) followed by RNA isolation using an RNeasy kit (Qiagen) per the manufacturer’s instructions. Superscript III (Invitrogen) or iScript cDNA synthesis (Bio-Rad) were used to create cDNA. All PCR reactions were carried out using Platinum Taq (Invitrogen) and specific primers (SI Appendix, Tables S6 and S7). Total human- and mouse-specific GAPDH were used as the housekeeping genes for the amplifications.

Statistical Analysis. Paired t tests were used to compare the colony forming efficiencies of unsorted vs. CD200⁺ CD45⁻ vs. CD200⁺ CD45⁻ subpopulations. For in vivo limiting dilution assays, the frequencies of cancer-initiating cells were calculated by using L-Calc software (Stem Cell Technologies), with χ² analysis to determine internal consistency.

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