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Identification of cells initiating human melanomas

Tobias Schatton¹, George F. Murphy², Natasha Y. Frank^{1,3}, Kazuhiro Yamaura¹, Ana Maria Waaga-Gasser⁴, Martin Gasser⁴, Qian Zhan², Stefan Jordan¹, Lyn M. Duncan⁵, Carsten Weishaupt⁶, Robert C. Fuhlbrigge⁶, Thomas S. Kupper⁶, Mohamed H. Sayegh¹, and Markus H. Frank¹

¹Transplantation Research Center, Children's Hospital Boston and Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

²Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

³Division of Genetics, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

⁴Department of Surgery, University of Würzburg Medical School, 97080 Würzburg, Germany

⁵Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114, USA

⁶Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA

Abstract

Tumour-initiating cells capable of self-renewal and differentiation, which are responsible for tumour growth, have been identified in human haematological malignancies^{1,2} and solid cancers^{3–6}. If such minority populations are associated with tumour progression in human patients, specific targeting of tumour-initiating cells could be a strategy to eradicate cancers currently resistant to systemic therapy. Here we identify a subpopulation enriched for human malignant-melanoma-initiating cells (MMIC) defined by expression of the chemoresistance mediator ABCB5 (refs 7, 8) and show that specific targeting of this tumorigenic minority population inhibits tumour growth. ABCB5⁺ tumour cells detected in human melanoma patients show a primitive molecular phenotype and correlate with clinical melanoma progression. In serial human-to-mouse xenotransplantation experiments, ABCB5⁺ melanoma cells possess greater tumorigenic capacity than ABCB5[−] bulk populations and re-establish clinical tumour heterogeneity. *In vivo* genetic lineage tracking demonstrates a specific capacity of ABCB5⁺ sub-populations for self-renewal and differentiation, because ABCB5⁺ cancer cells generate both ABCB5⁺ and ABCB5[−] progeny, whereas ABCB5[−] tumour populations give rise, at lower rates, exclusively to ABCB5[−] cells. In an initial proof-of-principle analysis, designed to test the hypothesis that MMIC are also

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Correspondence and requests for materials should be addressed to M.H.F. (mfrank@rics.bwh.harvard.edu).

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required for growth of established tumours, systemic administration of a monoclonal antibody directed at ABCB5, shown to be capable of inducing antibody-dependent cell-mediated cytotoxicity in ABCB5⁺ MMIC, exerted tumour-inhibitory effects. Identification of tumour-initiating cells with enhanced abundance in more advanced disease but susceptibility to specific targeting through a defining chemoresistance determinant has important implications for cancer therapy.

Human malignant melanoma is a highly aggressive and drug-resistant cancer⁹ that shows tumour heterogeneity^{10,11} and contains cancer cell subsets with enhanced tumorigenicity^{12,13}. We predicted that the melanoma chemoresistance mediator ABCB5 (refs 7, 8) could represent a molecular marker defining tumorigenic MMIC, because its expression also characterizes progenitor cell subsets in physiological skin¹⁴.

We first examined the relationship of ABCB5 to clinical malignant melanoma progression because of its close association with CD166 (ref. 7), a marker of more advanced disease¹⁵. This was assessed by ABCB5 immunohistochemical staining of an established melanoma progression tissue microarray¹⁶ representing four major diagnostic tumour types: benign melanocytic nevi, primary cutaneous melanoma, metastases to lymph nodes and metastases to viscera. We found that primary or metastatic melanomas expressed significantly more ABCB5 than benign melanocytic nevi, thick primary melanomas more than thin primary melanomas, and melanomas metastatic to lymph nodes more than primary lesions (Fig. 1a), identifying ABCB5 as a molecular marker of neoplastic progression. Apparent heterogeneity in ABCB5 expression was noted in metastases, with greater staining in the lymph node than in visceral metastases. When assayed in single-cell suspensions derived from clinical melanomas (Supplementary Table 1), ABCB5 was also found to be consistently expressed in 7/7 specimen, with ABCB5⁺ tumour cell frequency ranging from 1.6 to 20.4% ($10.1 \pm 2.9\%$, mean $\pm$ s.e.m.) (Fig. 1b, and Supplementary Table 1). Further characterization with respect to antigens indicative of a more primitive melanoma phenotype revealed expression of CD20 (also known as MS4A1)¹² in 4/7 specimens (cell frequency $0.4 \pm 0.2\%$, mean $\pm$ s.e.m.), nestin/NES^{17,18} in 7/7 ($28.7 \pm 7.3\%$), TIE1 (ref. 10) in 7/7 ($22.9 \pm 6.2\%$), CD144 (VE-cadherin; also known as CDH5)¹⁰ in 5/7 ($0.5 \pm 0.3\%$) and BMPR1A^{19,20} in 7/7 ($1.5 \pm 0.9\%$), and of the stromal marker CD31 (also known as PECAM1)¹⁰ in 6/7 specimens ($0.7 \pm 0.4\%$) (Fig. 1b). Preferential expression by ABCB5⁺ compared to ABCB5⁻ subpopulations, as previously identified for CD133 (ref. 7), was hereby demonstrated for nestin ($52.5 \pm 7.9\%$ versus $24.2 \pm 4.8\%$, respectively, mean $\pm$ s.e.m., $P = 0.026$), TIE1 ($64.5 \pm 7.6\%$ versus $22.5 \pm 6.5\%$, $P = 0.002$), VE-cadherin ($12.7 \pm 6.4\%$ versus $1.0 \pm 0.7\%$, $P = 0.016$), and BMPR1A (40.9 ± 6.9 versus $2.5 \pm 0.5\%$, $P = 0.001$), but not for CD20 ($0.0 \pm 0.0\%$ versus $0.8 \pm 0.8\%$, NS) or CD31 ($2.4 \pm 1.2\%$ vs. $0.3 \pm 0.2\%$, NS) (Fig. 1c). Expression of nestin, TIE1, VE-cadherin and BMPR1A by malignant ABCB5⁺ or ABCB5⁻ subpopulations within tumours was confirmed by analysis of genetically tracked fluorescent melanoma xenografts (Supplementary Fig. 1). Histologically, ABCB5⁺ cells correlated with non-melanized, undifferentiated regions, whereas melanized, more differentiated tumour areas were predominantly ABCB5⁻ (Supplementary Fig. 2a).

To determine whether the subset defined by ABCB5 was enriched for MMIC, we compared the abilities of ABCB5⁺ versus ABCB5⁻ melanoma cells to initiate tumour formation *in vivo*, using primary-patient-derived tumour cells in serial human-to-NOD/SCID mouse xenotransplantation experiments. ABCB5-dependent cell sorting was performed using immunomagnetic selection⁴⁻⁷, followed by confirmation of purity and viability of sorted populations as shown in Supplementary Fig. 3. Groups of mice were transplanted with replicate ($n = 6-11$) inocula of unsegregated, ABCB5⁺ or ABCB5⁻ melanoma cells representing four distinct patients over a log-fold range from cell doses unable to efficiently

initiate tumour growth (10^4 cells) to doses that consistently initiated tumour formation when ABCB5⁺ cells were used (10^6 cells) (Fig. 2a, b, and Supplementary Table 1). Of 23 aggregate mice injected with ABCB5⁻ melanoma cells, only one transplanted with the highest cell dose generated a tumour. In contrast, 7/23 mice injected with unsegregated populations, and 14/23 mice injected with ABCB5⁺ cells formed tumours ($P < 0.05$ and $P < 0.001$, respectively), including all mice injected with the highest cell dose of ABCB5⁺ cells (Fig. 2a, and Supplementary Table 1). ABCB5⁺ cells re-purified from ABCB5⁺-derived primary xenografts exclusively formed secondary tumours compared to their ABCB5⁻ counterparts, in 10/18 versus 0/18 recipients, respectively ($P < 0.001$) (Fig. 2b, and Supplementary Table 1). The MMIC frequency in unsegregated cell populations, calculated as described⁵, was 1/1,090,336 (95% confidence interval, 1/741,780 to 1/1,602,674). The frequencies in ABCB5⁺ inocula were 1/158,170 (95% confidence interval, 1/58,464 to 1/427,919) and 1/120,735 (95% confidence interval, 1/44,017 to 1/331,167) for primary and secondary tumour formation, respectively, demonstrating 71-fold and >359-fold enrichment compared to frequencies in ABCB5⁻ inocula (1/11,152,529 and $< 1/43,402,209$, respectively). Residual contamination with ABCB5⁺ cells (Supplementary Fig. 3a) may account for the single case of tumour formation by an ABCB5⁻ inoculum at the highest cell dose and indicates potential underestimation of MMIC enrichment among ABCB5⁺ populations. This is suggested by the presence of ABCB5⁺ cells in this tumour (Supplementary Fig. 2b) and the concurrent demonstration in genetic lineage tracking experiments that ABCB5⁻ melanoma cells do not generate ABCB5⁺ progeny (Fig. 2h). Comparison of the cellular diversity of clinical patient tumours with ABCB5⁺-derived primary and secondary xenografts revealed that ABCB5⁺ subpopulations re-established parent tumour heterogeneity as determined by flow cytometry (ABCB5 positivity $9.0 \pm 3.5\%$ (mean $\pm$ s.e.m.) in parent melanomas and $8.8 \pm 1.7\%$ and $13.1 \pm 3.2\%$ in corresponding primary and secondary ABCB5⁺-cell-derived xenografts, respectively) (Fig. 2c, and Supplementary Table 1) or ABCB5 immunohistochemistry (Fig. 2d). Regeneration of patient tumour heterogeneity for ABCB5 and the preferentially co-expressed markers of molecular plasticity and primitive melanoma phenotype CD144 and TIE1 (ref. 10) by primary and secondary ABCB5⁺-cell-derived xenografts was confirmed by immunofluorescent double staining of tumour sections (Supplementary Fig. 2c). In summary, these findings establish that MMIC frequency is markedly enriched in the melanoma minority population defined by ABCB5 and demonstrate *in vivo* self-renewal and differentiation capacity of this subset.

To examine the relative tumour growth contributions of co-xenografted ABCB5⁺ and ABCB5⁻ subpopulations directly, and to confirm ABCB5⁺ self-renewal and differentiation capacity, we isolated ABCB5⁺ or ABCB5⁻ cells from stably transfected G3361 melanoma cell line variants expressing either red fluorescent protein (DsRed) or enhanced yellow-green fluorescent protein (EYFP), respectively—a model system designed to allow *in vivo* genetic lineage tracking. We found that xenotransplantation of ABCB5⁺/DsRed and ABCB5⁻/EYFP fluorochrome-transfected co-cultures—reconstituted at $14.0 \pm 3.0\%$ and $86.0 \pm 3.0\%$ relative abundance (mean $\pm$ s.d., $n = 6$), respectively—to NOD/SCID mice resulted in time-dependent, serially increasing relative frequencies of DsRed⁺ tumour cells of ABCB5⁺ origin in experimental tumours compared to inoculates, up to a frequency of $51.3 \pm 1.4\%$ at the experimental endpoint of 6 weeks (linear regression slope 6.4 ± 1.0 , $P < 0.0001$) (Fig. 2e, f, g). These findings establish greater tumorigenicity of ABCB5⁺ subsets in a competitive tumour development model. They further indicate that tumour-initiating cells may also drive more differentiated and otherwise non-tumorigenic cancer bulk populations to contribute, albeit less efficiently, to a growing tumour mass. The capacity of non-tumour-initiating cancer cell populations to undergo a limited number of replications is consistent with previous findings in other solid tumours^{3–5,21}. Experimental tumours also contained DsRed/EYFP double-positive melanoma cells (Fig. 2e, g), indicating that ABCB5⁺-derived

tumour cells, like physiological ABCB5⁺ skin progenitors¹⁴, engage in cell fusion. When ABCB5⁺ cells were purified from experimental tumours, we found $92.9 \pm 6.4\%$ (mean $\pm$ s.d., $n = 3$) of fluorescent cells to be of DsRed⁺ phenotype (ABCB5⁺ origin) (Fig. 2h), confirming self-renewal capacity of this cell subset. EYFP⁺ DsRed⁻ cells were not found in repeat experiments ($n = 3$) at significant levels among purified ABCB5⁺ cells (Fig. 2h), and analysis of ABCB5 expression by triple-colour flow cytometry on DsRed⁺ (ABCB5⁺ origin) and EYFP⁺ (ABCB5⁻ origin) subpopulations derived from co-injected *in vivo* tumour xenografts confirmed that ABCB5⁺ cells were exclusively of DsRed⁺ phenotype, with no significant numbers of EYFP⁺ DsRed⁻ cells detected (median percentage 0%, results not illustrated). These results indicate that ABCB5⁺ tumour cells arose only from ABCB5⁺ inocula and that ABCB5⁻ cells give rise exclusively to ABCB5⁻ progeny. Moreover, fluorescent ABCB5⁻ isolates exhibited $52.5 \pm 0.8\%$ (mean $\pm$ s.d., $n = 3$) DsRed positivity (ABCB5⁺ origin) and $47.5 \pm 0.8\%$ EYFP positivity (ABCB5⁻ origin) (Fig. 2h), demonstrating that ABCB5⁺ melanoma cells possess the capacity to differentiate and give rise to ABCB5⁻ tumour populations. These findings confirm the existence of a tumour hierarchy in which ABCB5⁺ melanoma cells, enriched for MMIC, self-renew and give rise to more-differentiated ABCB5⁻ tumour progeny.

To dissect further and mechanistically whether the ABCB5-defined, MMIC-enriched minority population is also required for tumorigenicity when unsegregated cancer populations are xenografted, we examined whether selective killing of this cell subset can inhibit tumour growth and formation. We administered a monoclonal antibody directed at ABCB5 (refs 7, 14) in a human to the nude mouse melanoma xenograft model, because murine immunoglobulin G1 monoclonal antibodies trigger cellular immune effector functions²² and because nude as opposed to NOD/SCID mice are capable of tumour cell killing by antibody-dependent cell-mediated cytotoxicity (ADCC)²³. Anti-ABCB5 monoclonal antibody treatment resulted in significantly inhibited tumour growth compared to that determined in control-monoclonal-antibody-treated or untreated mice over the course of a 58-day observation period (tumour volume for anti-ABCB5-treated ($n = 11$ mice, no death during the observation period; $23 \pm 16 \text{ mm}^3$; mean $\pm$ s.e.m.) versus control-monoclonal-antibody-treated ($n = 10$ mice, excluding 1 death; $325 \pm 78 \text{ mm}^3$), $P < 0.01$; versus untreated ($n = 18$ mice, excluding 1 death; $295 \pm 94 \text{ mm}^3$), $P < 0.001$, see Methods for test used) (Fig. 3a). Control monoclonal antibody treatment showed no significant difference compared to no treatment (Fig. 3a). Anti-ABCB5 monoclonal antibody treatment also significantly inhibited tumour formation, with tumours detected in only 3/11 anti-ABCB5-treated mice versus 10/10 control-antibody-treated mice and 18/18 untreated control animals ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 3b). Human melanoma xenografts grown in untreated nude mice, like those in NOD/SCID recipients, showed tumour heterogeneity for ABCB5 (Supplementary Fig. 4a). Immunohistochemical examination of tumours that successfully grew in the presence of ABCB5 monoclonal antibody revealed that these tumours still contained ABCB5⁺ cells (Supplementary Fig. 4b), indicating that ABCB5⁺ MMIC had not been fully eradicated. On termination of monoclonal antibody administration, one tumour occurrence was noted among the eight ABCB5-treated mice that had not developed a tumour during an additional eight-month observation period, indicating prolonged inhibition of tumour-initiating cells.

To determine the mechanism of anti-ABCB5 monoclonal-antibody-mediated inhibition of tumour formation and growth, the immune effector responses ADCC and complement-dependent cytotoxicity (CDC) were assessed, as described²⁴. Anti-ABCB5 monoclonal antibody but not isotype control monoclonal antibody significantly induced ADCC-mediated melanoma target cell death ($2.1 \pm 0.4\%$ versus $0.2 \pm 0.2\%$, respectively, mean $\pm$ s.e.m., $P < 0.05$) in a melanoma subpopulation comparable in size to the ABCB5-expressing subset⁷ (Fig. 3c). Addition of serum to anti-ABCB5-treated cultures in the absence of effector cells,

or addition of monoclonal antibody alone did not induce significant cell death compared to controls (results not illustrated), precluding CDC or direct toxic monoclonal antibody effects as significant causes of tumour inhibition.

We next analysed the effects of ABCB5 targeting on established human-to-nude mouse melanoma xenografts ($n = 13$ derived from three distinct patients and $n = 10$ derived from established melanoma cultures) to test the hypothesis that negative selection for MMIC by ADCC-mediated ABCB5⁺ cell ablation inhibits tumour growth, as would be anticipated in a dynamic *in vivo* situation if the ABCB5⁺ melanoma subset is critical to robust tumorigenesis. *In vivo* anti-ABCB5 monoclonal antibody administration, started 14 days following tumour cell inoculation when xenografts were established (day 0), abrogated the significant tumour growth observed in isotype-control-monoclonal-antibody-treated or untreated groups over the course of a 21-day treatment period ($P < 0.001$ and $P < 0.001$, respectively) and significantly inhibited mean tumour volume compared to that determined in either control-treated or untreated mice (tumour volume for anti-ABCB5-treated ($n = 23$ mice; $32.7 \pm 9.4 \text{ mm}^3$; mean $\pm$ s.e.m.) versus control-treated ($n = 22$ mice; $226.6 \pm 53.8 \text{ mm}^3$), $P < 0.001$; versus untreated ($n = 22$ mice; $165.4 \pm 36.9 \text{ mm}^3$), $P < 0.01$, see Methods for test used) (Fig. 3d). The inhibitory effects of ABCB5 monoclonal antibody were also statistically significant when the subsets of freshly patient-derived melanoma xenograft tumours were analysed independently, with abrogation of the significant tumour growth observed in isotype-control-monoclonal-antibody-treated or untreated groups ($P < 0.05$ and $P < 0.001$, respectively) and significantly inhibited mean tumour volume compared to that determined in either control-treated or untreated mice (anti-ABCB5-treated ($n = 13$ mice; $29.6 \pm 9.2 \text{ mm}^3$) versus control-treated ($n = 12$ mice; $289.2 \pm 91.8 \text{ mm}^3$), $P < 0.05$; versus untreated ($n = 12$ mice; $222.9 \pm 57.5 \text{ mm}^3$), $P < 0.001$) (Fig. 3d). Control monoclonal antibody treatment showed no significant effects on tumour growth or tumour volume compared to no treatment in any of the groups analysed. The animals were euthanized following the treatment interval, as required by the applicable experimental animal protocol because of tumour burden and disease state in the patient-derived tumour control groups (measured maximal tumour volume, 971.5 mm^3). Immunohistochemical analysis of anti-ABCB5-treated patient-derived melanoma xenografts revealed only small foci of ABCB5 expression (overall $< 1\%$ of cells) (focal area of positivity shown in Fig. 3e), corresponding to *in vivo* bound anti-ABCB5 monoclonal antibody in an adjacent section. An additional adjacent section stained for CD11b disclosed macrophage infiltration, corresponding to regions of anti-ABCB5 monoclonal antibody localization, which frequently bordered zones of cellular degeneration and necrosis (Fig. 3e). In contrast, control-treated xenografts revealed 10–15% ABCB5-reactive cells, secondary anti-immunoglobulin monoclonal antibody failed to localize to the respective regions in an adjacent section but detected regions of intravascular murine immunoglobulin, and CD11b⁺ macrophages failed to infiltrate the tumour tissue (Fig. 3e). Similar effects were observed in cell-line-derived melanoma xenografts (Supplementary Fig. 4c, d), with enhanced tumour necrosis in anti-ABCB5-treated versus isotype-control-monoclonal-antibody-treated animals (30–40% versus $< 5\%$ necrotic cells, respectively) (Supplementary Fig. 4c). These findings further support the notion that the ABCB5-defined, MMIC-enriched minority population is required for tumorigenicity.

Because ABCB5 represents a possible chemoresistance mechanism^{7,8}, our findings provide evidence for a new, potentially critical link between tumour-initiating cells, cancer progression and chemo-resistance in a solid malignancy²⁵, raising the possibility that ABCB5⁺ MMIC may be responsible both for the progression and chemotherapeutic refractoriness of advanced malignant melanoma, and that MMIC-targeted approaches might therefore ultimately represent novel and translationally relevant therapeutic strategies to disseminated disease. Broader examination of a larger array of clinical specimen is

warranted to establish further ABCB5 as a universal MMIC marker and robust candidate therapeutic target. Whether related ABC members^{26,27} might also represent prospective markers of tumour-initiating cells, or whether ABCB5 might represent such a marker in additional malignancies, such as breast cancer, in which it is known to be clinically expressed and specifically downregulated with epigenetic differentiation therapy²⁸, requires further study.

Although MMIC are enriched in the melanoma subpopulations defined by ABCB5, clearly not every ABCB5⁺ cell represents a MMIC, because purified populations did not invariably form tumours. Our finding that ABCB5 serves as a molecular marker for MMIC is consistent with the demonstration that ABCB5 expression is closely co-regulated with melanotransferrin, a molecule also associated with melanoma growth²⁹. The tumour-initiating-cell frequency determined in malignant melanoma is approximately 19-fold lower than that, for example, determined in colon cancer⁵. Tumorigenicity in human-to-mouse xenotransplantation experiments, and as a result calculated stem cell frequency estimates, might vary with the applied experimental conditions, such as the tissue site of xenotransplantation, or the presence or absence of re-activated immune effector mechanisms in recipient immunodeficient mice³⁰. Alternatively, inherent differences between stem cell frequencies in distinct malignancies could account for the observed difference. The per cent positivity of tumour cells identified by the prospective marker ABCB5 in clinical melanomas parallels those obtained for the CD133 marker, which detects subpopulations enriched for tumour-initiating cells at similar relative frequencies in brain cancer⁴ and colon cancer^{5,6}, but likewise does not permit tumour-initiating-cell identification at the clonal level. Further studies are needed to reveal whether tumour-initiating cells can be molecularly defined at the single-cell level in a solid malignancy, or whether more than one cell is necessary for tumour initiation. Our results represent a significant step towards this goal in human malignant melanoma, and provide a basis to elucidate further, and eventually therapeutically target, the specific molecular pathways responsible for tumorigenicity, tumour progression and chemoresistance in tumour-initiating cells.

METHODS

Melanoma cells and culture methods

The ABCB5-expressing G3361 human malignant melanoma cell line^{7,14}, derived from a single tumour cell cloned in soft agar, was provided by E. Frei III and cultured as previously described⁷. The G3361/DsRed and G3361/EYFP cell lines were generated by stable transfection of G3361 melanoma cells with either *Discosoma* sp. red fluorescent protein (DsRed) or the enhanced yellow-green variant (EYFP) of the *Aequorea victoria* green fluorescent protein (GFP) in conjunction with the simian virus 40 large T-antigen nuclear retention signal, using pDsRed-Nuc or pEYFP-Nuc mammalian expression vectors also containing a neomycin resistance cassette (BD Biosciences) and the Lipofectamine 2000 reagent (Invitrogen), as previously described¹⁴. Clonal G3361/DsRed and G3361/EYFP cultures were generated from stably transfected cultures by limiting dilution. Clinical melanoma cells ($n = 7$ patients) were freshly derived from surgical specimens according to human subjects research protocols approved by the IRBs of the University of Würzburg Medical School or the Wistar Institute.

Antibodies

The specific IgG1 κ anti-ABCB5 monoclonal antibody (mAb) 3C2-1D12 (refs 7, 14) was used in the herein reported studies. Unconjugated or FITC-conjugated MOPC-31C mouse isotype control mAbs, FITC-conjugated goat anti-mouse IgG secondary Ab, phycoerythrin (PE)-conjugated anti-human CD20, anti-human and anti-mouse CD31, anti-human and anti-

mouse CD45, and isotype control mAbs were purchased from Pharmingen. Allophycocyanin (APC)-conjugated and PE-conjugated secondary mAbs were purchased from eBioscience. Unconjugated anti-human TIE1, anti-human BMPRI1A, PE-conjugated anti-human VE-cadherin and anti-human nestin mAbs were from R&D Systems. The following antibodies were used for immunohistochemistry or immunofluorescence staining: mouse anti-ABCB5 mAb^{7,14}, HRP-conjugated horse anti-mouse IgG secondary Ab, HRP-conjugated horse anti-goat IgG secondary Ab and HRP-conjugated goat anti-rabbit IgG secondary Ab (Vector Laboratories), FITC-conjugated rabbit anti-mouse IgG secondary Ab (ZYMED Laboratories), unconjugated rabbit anti-human VE-cadherin Ab (provided by Cell Signaling Technology), mouse control IgG Abs (DAKO), goat anti-human TIE1 Ab (Neuromics), rat anti-mouse CD11b Ab and rat anti-mouse CD31 Ab (BD Biosciences Pharmingen), rabbit anti-human CD31 Ab (Bethyl Laboratories), donkey anti-mouse IgG-AF488, donkey anti-rabbit IgG-AF594, donkey anti-rat IgG-AF594 and donkey anti-goat IgG-AF594 (Invitrogen), Texas Red-conjugated donkey anti-rabbit IgG secondary Ab, and rabbit control IgG Ab (all from Jackson ImmunoResearch).

Histopathology and immunohistochemistry

Five micron-thick melanoma cryosections were fixed in -20°C acetone for 5 min. Air-dried sections were incubated with $10\text{ }\mu\text{g ml}^{-1}$ ABCB5 mAb or $2.5\text{ }\mu\text{g ml}^{-1}$ CD11b mAb at 4°C overnight; 10 or $2.5\text{ }\mu\text{g ml}^{-1}$ mouse IgG were used as negative controls. Sections were washed with PBS 3 times for 5 min each and incubated with 1:200 peroxidase-conjugated secondary Abs for ABCB5 or CD11b staining. For ABCB5/VE-cadherin, ABCB5/TIE1, or ABCB5/CD31 fluorescence double labelling, $5\text{ }\mu\text{m}$ melanoma sections were fixed in -20°C acetone for 5 min. Air-dried sections were incubated with $10\text{ }\mu\text{g ml}^{-1}$ ABCB5 mAb and $2.5\text{ }\mu\text{g ml}^{-1}$ VE-cadherin, TIE1 or CD31 Abs at 4°C overnight; $10\text{ }\mu\text{g ml}^{-1}$ mouse IgG and $2.5\text{ }\mu\text{g ml}^{-1}$ rabbit IgG were used as negative controls. Sections were washed three times with PBS containing 0.05% Tween 20 for 5 min each and incubated with a 1:150 dilution of Texas Red-conjugated or AF594-conjugated secondary Abs and FITC-conjugated rabbit anti-mouse IgG Ab for 30 min at room temperature. After subsequent washings, the sections were mounted with VECTASHIELD mounting medium (Vector Laboratories) and covered with a cover slip. Immunofluorescence reactivity was viewed on an Olympus BX51/52 system microscope coupled to a Cytovision system (Applied Imaging).

Tissue microarray design and analysis

The Melanocytic Tumour Progression tissue microarray (TMA) is the product of a joint effort of three Skin SPORES (Harvard Medical School, MD Anderson Cancer Center, University of Pennsylvania). This array contains $480 \times 0.6\text{ mm}$ cores of tumour tissue representing four major diagnostic tumour types: benign nevi, primary cutaneous melanoma, lymph node metastasis and visceral metastasis. Cases were collected from the Pathology services of the three participating institutions. For quality control purposes, two duplicate cores are chosen at each distinct region. Nevi and primary melanomas had either one region or three regions of the tissue block sampled (2 or 6 cores), whereas metastatic tumours had one region sampled from each block. Therefore, the 480 cores represent 2 adjacent cores from 240 distinct histological regions. This array includes 130 cores from 35 nevi, 200 cores from 60 primary melanoma and 150 cores from 75 metastatic lesions. Operationally, thin nevi and thin melanomas involved only the superficial/papillary dermis, whereas thick nevi and thick melanomas had grown to involve both papillary and deep (reticular) dermis. This array was constructed in the laboratory of M. Rubin. Histological sections of the tissue array slide were baked at 58°C for 20 min and then treated with the following: xylene (twice for 1 h, then 10 min), 100% ethanol twice for 2 min, 95% ethanol for 2 min, and dH_2O three times for 2 min. Antigen retrieval was performed in 10 mmol l^{-1} citrate buffer, pH 6.0 with boiling in a pressure cooker for 10 min and then cooling to room temperature. After washing

with PBS twice for 5 min, tissue was blocked with 10% horse serum and 1% BSA in PBS at room temperature for 1 h then incubated with 5 $\mu\text{g ml}^{-1}$ ABCB5 mAb at 4 °C overnight. The tissue was then washed three times with PBS-0.05% Tween 20 for 5 min then treated with 3% H_2O_2 /PBS for 15 min. After rinsing in PBS, the sections were incubated with 1:200 biotinylated horse anti-mouse IgG Ab at room temperature for 30 min, rinsed in PBS-Tween three times for 5 min, and incubated with avidin–biotin–horseradish peroxidase complex (Vector Laboratories) for 30 min at room temperature. Immunoreactivity was detected using NovaRed substrate (Vector Laboratories). The Chromavision Automated Cellular Imaging System (ACIS) was used to quantify the immunostaining intensity of ABCB5 and mIgGIR on the HTMA 84 tissue microarray. The control slide intensity values (background plus intrinsic melanization) were subtracted from the experimental slide and the difference in the intensity values for each core was taken to be the true staining. The graph in Fig. 1a shows with 95% confidence interval the difference in intensity for each pathology diagnosis. *P* values between relevant groups were calculated using the independent/samples *t*-test. The number above each error bar shows the number of cases within each group.

Flow cytometric analysis

Analysis of ABCB5, CD20, CD31, CD45, VE-cadherin, BMPR1A, nestin, or TIE1 expression, or of co-expression of ABCB5 with the CD20, CD31, VE-cadherin or BMPR1A surface markers or the nestin or TIE1 intracellular markers in clinical patient-derived melanoma cell suspensions or in G3361 melanoma cells was performed by single- or dual-colour flow cytometry, as described previously⁷. Co-expression analyses of ABCB5 with the above-listed markers in single-cell suspensions derived from G3361/EYFP tumour xenografts and expression analyses of ABCB5 in G3361/DsRed-G3361/EYFP-derived tumours were performed by triple-colour flow cytometry, gating on EYFP-expressing melanoma cells or ABCB5-expressing cells, respectively. Clinical melanoma cells were incubated with anti-ABCB5 mAb or isotype control mAb or no Ab followed by counterstaining with APC-conjugated donkey anti-mouse IgG. Cells were then fixed in PBS containing 2% paraformaldehyde (30 min at 4 °C), and subsequently incubated with PE-conjugated anti-CD20, anti-CD31, anti-VE-cadherin, anti-nestin or PE-conjugated isotype control mAbs, or unconjugated anti-BMPR1A, anti-TIE1 or unconjugated isotype control mAbs followed by counterstaining with PE- or FITC-conjugated anti-immunoglobulin secondary antibodies. Washing steps with staining buffer or 1% saponin permeabilization buffer were performed between each step. Dual- or triple-colour flow cytometry was subsequently done with acquisition of fluorescence emission at the F11 (FITC, EYFP) and/or F12 (PE, DsRed) and F14 (APC) spectra on a Becton Dickinson FACScan (Becton Dickinson), as described⁷. Statistical differences between expression levels of the above-listed markers by ABCB5⁺ and ABCB5[−] patient-derived melanoma cells were determined using the nonparametric Mann–Whitney test. A two-sided *P* value of *P* < 0.05 was considered significant.

Cell isolation

Single-cell suspensions were generated from human melanoma xenografts on surgical dissection of tumours from euthanized mice. Each tumour was cut into small pieces (~ 1 mm³) and tumour fragments were subsequently incubated in 10 ml sterile PBS containing 0.1 g l^{−1} calcium chloride and 5 $\mu\text{g ml}^{-1}$ Collagenase Serva NB6 (SERVA Electrophoresis GmbH) for 3 h at 37 °C on a shaking platform at 200 r.p.m. to generate single-cell suspensions. Subsequently, tumour cells were washed with PBS for excess collagenase removal. ABCB5⁺-purified (ABCB5⁺) cells were isolated by positive selection and ABCB5⁺-depleted (ABCB5[−]) cell populations were generated by removing ABCB5⁺ cells using anti-ABCB5 mAb labelling and magnetic-bead cell sorting as described^{4,7,14}. Briefly, human G3361 melanoma cells or single-cell suspensions derived from human melanoma

xenografts or clinical melanoma samples were labelled with anti-ABCB5 mAb (20 $\mu\text{g ml}^{-1}$) for 30 min at 4 °C, washed for excess antibody removal, followed by incubation with secondary anti-mouse IgG mAb-coated magnetic microbeads (Miltenyi Biotec) and subsequent dual-passage cell separation in MiniMACS separation columns (Miltenyi Biotec), according to the manufacturers recommendations. Purity of ABCB5⁺ and ABCB5⁻ (ABCB5⁺ cell-depleted) clinical melanoma cell isolates or of ABCB5⁺ and ABCB5⁻ cell isolates derived from ABCB5⁺ patient cell-derived primary melanoma xenograft cells was assayed following magnetic-bead cell sorting by incubation with FITC-conjugated goat anti-mouse IgG secondary Ab and subsequent flow cytometric analysis of ABCB5 expression. ABCB5⁺ cell purification resulted in 10.4-fold enrichment of ABCB5⁺ melanoma cell frequency from $8.9 \pm 1.4\%$ in unsegregated samples to $92.4 \pm 2.8\%$ (mean $\pm$ s.e.m., $P < 0.001$, Supplementary Fig. 2a). Negative selection for ABCB5⁺ cells resulted in 6.7-fold depletion of ABCB5⁺ cell frequency to $1.3 \pm 0.6\%$ (mean $\pm$ s.e.m., $P < 0.05$, Supplementary Fig. 2a). CD31⁺ or CD45⁺ cell frequencies among unsegregated, ABCB5⁺ or ABCB5⁻ cell suspensions were determined by single colour flow cytometry, as above. Statistical differences in marker expression between unsegregated, ABCB5⁺, and ABCB5⁻ human melanoma cells were determined using parametric ANOVA or the nonparametric Kruskal–Wallis Test followed by Dun’s correction for comparisons of multiple groups, with two-tailed P values < 0.05 considered significant.

Animals

Balb/c nude mice and NOD/SCID mice were purchased from The Jackson Laboratory. Mice were maintained in accordance with the institutional guidelines of Children’s Hospital Boston and Harvard Medical School and experiments were performed according to approved experimental protocols.

Human melanoma xenotransplantation

Unsegregated, ABCB5⁺, or ABCB5⁻ clinical patient-derived melanoma cells (10^6 , 10^5 or 10^4 per inoculum), or ABCB5⁺ or ABCB5⁻ cells isolated from primary ABCB5⁺ patient-derived xenografts (10^6 , 10^5 , or 10^4 per inoculum) were injected subcutaneously uni- or bilaterally into the flanks of recipient NOD/SCID mice. Tumour formation/growth was assayed weekly as a time course, at least up to the endpoint of 8 weeks, unless excessive tumour size or disease state required protocol-stipulated euthanasia earlier, by determination of tumour volume (TV) according to the established formula $[\text{TV (mm}^3) = \pi/6 \times 0.5 \times \text{length} \times (\text{width})^2]$. With respect to tumour formation, mice were considered tumour-negative if no tumour tissue was identified on necropsy. Statistically significant differences in primary and secondary tumour formation were assessed using the Fisher’s Exact test. Differences in tumour volumes were determined using one-way ANOVA followed by the Bonferroni correction or the Kruskal–Wallis Test followed by Dun’s correction, with two-tailed P values < 0.05 considered significant. Tumour-initiating cell frequencies and respective confidence intervals were calculated as previously described⁵, using the L-Calcul version 1.1 statistical software program for limiting dilution analysis (Stemcell Technologies).

In vivo genetic lineage tracking

ABCB5⁺/DsRed and ABCB5⁻/EYFP human G3361 tumour cell populations, generated using magnetic-bead cell sorting as above, were reconstituted at the desired ratios on the basis of cell counting and the resultant relative abundance ratios in inocula were determined by dual-colour flow cytometry (F11 (EYFP) versus F12 (DsRed) plots) before xenotransplantation. G3361/DsRed and G3361/EYFP co-cultures were injected subcutaneously (10^7 cells per inoculum) into the right flank of recipient NOD/SCID mice. At 4 or 6 weeks post xenotransplantation, tumours were harvested and single-cell suspensions or frozen

tissue sections prepared as above, for determination of relative *in vivo* abundance of DsRed⁺ and EYFP⁺ melanoma cells by dual-colour flow cytometry or fluorescence microscopy of tumour-derived single-cell suspensions (on attachment in adherent tissue culture plates), and for analysis of 5 µm frozen tissue sections by fluorescence microscopy. Percentages were calculated as follows: %DsRed⁺ cells = (%DsRed⁺ / (%DsRed⁺ + %EYFP⁺) × 100) and %EYFP⁺ cells = (%EYFP⁺ / (%DsRed⁺ + %EYFP⁺) × 100). In additional experiments, the relative abundance of DsRed⁺ and EYFP⁺ melanoma cells was determined by dual-colour flow cytometry as above in ABCB5⁺ or ABCB5⁻ subsets purified from xenografts, or by triple-colour flow cytometry of unsorted, freshly dissociated xenografts gating on ABCB5-expressing cells (APC, F14 fluorescence), and the percentages of DsRed⁺ and EYFP⁺ tumour cells were statistically compared using the unpaired Student's *t*-test, with a two-sided *P* value of *P* < 0.05 considered statistically significant. FACS-sorting of tumour xenograft cells of ABCB5⁺/DsRed (F12 fluorescence) versus ABCB5⁻/EYFP (F11 fluorescence) origin for real-time RT-PCR analysis of BMPR1A, VE-cadherin, nestin and TIE1 expression was performed on a dual-laser FACSVantage flow cytometer (Becton Dickinson). Flow cytometric co-expression analysis of ABCB5 with the CD20, CD31, VE-cadherin, BMPR1A, nestin, or TIE1 markers was performed on single tumour cell suspensions prepared from xenograft tumours induced by inoculation of unsegregated G3361/EYFP tumour cells (10⁷ cells per inoculum) into recipient NOD/SCID mice.

Anti-ABCB5 mAb targeting

For targeting experiments directed at tumour formation, unsegregated human G3361 melanoma cells were xenografted subcutaneously into recipient Balb/c nude mice (10⁷ per inoculum). Animals were injected intraperitoneally with anti-ABCB5 mAb (clone 3C2-1D12)^{7,14} (500 µg per injection) or isotype control mAb (500 µg per injection) bi-weekly, or no Ab starting 24 h before melanoma xenotransplantation. Tumour growth was assayed bi-weekly as a time course by determination of tumour volume, as described above. For targeting experiments directed at established melanoma xenografts, unsegregated primary-patient-derived or human G3361 melanoma cells were xenografted subcutaneously into the right flank of recipient Balb/c nude mice (10⁷ per inoculum). Fourteen days post tumour cell inoculation (day 0), tumour volumes were determined, and mice were randomized into three treatment groups (anti-ABCB5 mAb treatment, isotype control mAb treatment or no treatment), with groups consisting of *n* = 21–22 animals, comprising *n* = 12–13 mice bearing primary-patient-derived tumours (*n* = 5 derived from patient P1, *n* = 3 from patient P3, *n* = 4–5 from patient P7, Supplementary Table 1) and *n* = 10 mice bearing human-cell-line-derived tumours. Tumour volumes at day 0 did not significantly differ among the groups (39.8 ± 9.3 versus 37.5 ± 6.7 versus 38.2 ± 5.9 mm³, respectively, mean ± s.e.m., NS), and furthermore did not significantly differ among the subgroups of primary patient-derived tumours (48.5 ± 15.7 versus 44.4 ± 11.1 versus 45.7 ± 9.4 mm³, respectively, mean ± s.e.m., NS) or cell-line-derived tumours (28.4 ± 5.8 versus 29.3 ± 6.1 versus 29.1 ± 6.0 mm³, respectively, mean ± s.e.m., NS). Subsequently, mice were injected intraperitoneally with anti-ABCB5 mAb (clone 3C2-1D12)^{7,14} (500 µg per injection) or isotype control mAb (500 µg per injection) or no Ab bi-weekly for the duration of the experiment. Tumour formation/growth was assayed weekly as a time course by determination of tumour volume as described above, until excessive tumour burden or disease state required protocol-stipulated euthanasia. Differences in tumour volumes were determined using parametric ANOVA or the non-parametric Kruskal–Wallis Test followed by Dun's correction for comparisons of multiple groups, with two-tailed *P* values < 0.05 considered significant. Differences in tumour volumes at different time points within experimental groups were determined using parametric ANOVA (repeated measures (paired) test) or the non-parametric Kruskal–Wallis Test (repeated measures (paired) test).

Assessment of ADCC and CDC

ADCC or CDC was determined by the established method of dual-colour flow cytometry. Briefly, human G3361 melanoma cell suspensions in serum-free Dulbecco's Modified Eagle's Medium (DMEM) (BioWhittaker) were labelled with 3,3'-diiodoacetylcarboxycyanine (DiO) (Invitrogen) according to the manufacturer's recommendations. DiO-labelled melanoma cells were then plated at a density of 3×10^5 cells per well in flat-bottomed 6-well culture plates in 3 ml and cultured in standard medium in a humidified incubator overnight. Thereafter, DiO-labelled melanoma target cells were pre-incubated in the presence or absence of anti-ABCB5 or isotype control mAbs ($20 \mu\text{g ml}^{-1}$, respectively) for 30 min at 37°C , 5% CO_2 , and subsequently co-cultured for additional 24 h at 37°C , 5% CO_2 with or without freshly isolated Balb/c nude mouse effector splenocytes (12×10^6 cells per well, 1:40 target to effector cell ratio) for assessment of ADCC, or in the presence or absence of 5% Balb/c nude mouse serum for determination of CDC. Subsequently, cells and their supernatants were harvested and analysed by dual-colour flow cytometry on a FACSCalibur machine (Becton Dickinson) immediately on addition of $10 \mu\text{g ml}^{-1}$ propidium iodide (PI) (Sigma), with lysed target cells recognized by a DiO^+PI^+ phenotype. ADCC levels for the three treatment groups were calculated as follows: $[\text{ADCC} (\%) = (\text{DIO}^+\text{PI}^+ \% \text{ sample positivity}) - (\text{mean Ab-untreated DIO}^+\text{PI}^+ \% \text{ sample positivity})]$. Differences in ADCC levels were determined using non-parametric one-way ANOVA (Kruskal–Wallis Test) followed by Dun's correction, with two-tailed P values < 0.05 considered significant.

Cell viability measurements

Cell viability was measured in tumour cell inocula before xenotransplantation using calcein-AM staining. Briefly, 1×10^6 unsegregated, ABCB5^+ , or ABCB5^- melanoma cells were incubated with calcein-AM (Molecular Probes) for 30 min at 37°C and 5% CO_2 to allow for substrate uptake and enzymatic activation to the fluorescent derivative. Subsequently the cells were washed and fluorescence measurements acquired by flow cytometry at the FL2 emission spectrum on a Becton Dickinson FACScan. Cells exhibiting generation of the fluorescent calcein-AM derivative compared to unexposed samples were considered viable. Cell viability was also determined using the trypan blue dye exclusion method.

RNA extraction and real-time quantitative reverse transcription PCR (RT–PCR)

Real-time RT–PCR for BMPR1A, VE-cadherin, nestin and TIE1 gene expression analysis were performed as follows: total RNA was extracted from melanoma cells using the RNeasy Micro kit (Qiagen). Total RNA ($5 \mu\text{g}$) in $20 \mu\text{l}$ RT reaction mix was transcribed into complementary DNA using the SuperScript III First-Strand Synthesis System for RT–PCR (Invitrogen). All reagents for real-time RT–PCR were from Applied Biosystems. The assay numbers for human β -actin, BMPR1A, VE-cadherin, nestin and TIE1 were 4310881E, Hs01034909_g1, Hs00174344_ml, Hs00707120_sl and Hs00178500_ml, respectively. Real-time quantitative RT–PCR was performed on a 7300 real-time PCR System (Applied Biosystems) in a $25 \mu\text{l}$ reaction mix containing $1 \mu\text{l}$ cDNA, $1 \times$ TaqMan Universal PCR Master Mix and $1 \times$ of each of the assays. Thermocycling was carried out at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All samples were run in triplicate. The relative amounts of BMPR1A, VE-cadherin, nestin and TIE1 transcripts were analysed using the $2^{-\Delta\Delta C_T}$ method, as described previously¹⁹. Statistical differences between messenger RNA expression levels of the above-listed markers by fluorescent xenograft cells of $\text{ABCB5}^+/\text{DsRed}$ origin and $\text{ABCB5}^-/\text{EYFP}$ origin were determined using the non-parametric Student's t -test. A two-sided P value of $P < 0.05$ was considered significant.

Quantification of DNA content by propidium iodide (PI) staining

Freshly sorted ABCB5⁺ or ABCB5⁺-depleted (ABCB5⁻) clinical melanoma cells or ABCB5⁺ patient cell-derived primary melanoma xenograft cells were fixed in ice-cold 65% (v/v) ethanol in PBS, washed in cold PBS, and incubated in a PI-staining mixture followed by determination of the cell fraction containing < 2n DNA by flow cytometry (Becton Dickinson FACScan), as described previously^{7,14}. The frequency of cellular fragments, non-viable cells and/or contaminating blood components containing < 2n DNA comprised 2.8 ± 0.3% versus 2.7 ± 1.9% (mean ± s.e.m.) in patient-derived ABCB5⁺ or ABCB5⁻ cell suspensions, respectively, and 1.4 ± 0.2% versus 0.6 ± 0.1% (mean ± s.e.m.) in ABCB5⁺ and ABCB5⁻ cell isolates derived from ABCB5⁺ patient cell-derived primary melanoma xenograft cells, respectively, with no significant differences detected among isolates, when subjected to the non-parametric Mann–Whitney test (Supplementary Fig. 2d).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ABCB5 expression analyses

a, Melanoma progression tissue microarray analysis for ABCB5, showing significant differences in ABCB5-staining intensities (mean $\pm$ 95% confidence interval (CI); thin or thick nevi versus thin or thick primary melanomas, or versus lymph node or visceral metastases, P values < 0.001 ; thin versus thick primary melanomas, $P = 0.004$; thin and thick primary melanomas versus lymph node metastases, $P = 0.001$; lymph node versus visceral metastases, $P = 0.025$; n , provided in figure). The picture colour map corresponds to sample types represented in the core array: green, thin nevi; orange, thick nevi; violet, thin primary melanoma; blue, thick primary melanoma; pink, lymph node metastases; yellow, visceral metastases. The scanning view of ABCB5 staining of the entire array corresponds to the colour key. **b**, Flow cytometry analysis of ABCB5, CD20, nestin, TIE1, CD144, CD31 or BMPR1a expression in $n = 7$ melanoma patients. **c**, Marker expression by ABCB5⁺ or ABCB5⁻ melanoma cells determined by flow cytometry (mean $\pm$ s.e.m., $n = 4-7$ patients).

Figure 2. Tumorigenicity, self-renewal and differentiation of ABCB5⁺ MMIC

a, Primary tumour formation of unsegregated (US), ABCB5⁻ or ABCB5⁺ cells. **b**, Secondary tumour formation of ABCB5⁻ or ABCB5⁺ cells. **c**, ABCB5 expression (mean $\pm$ s.e.m.) in parent tumours ($n = 3$) and respective ABCB5⁺-derived primary ($n = 11$) and secondary ($n = 7$) xenografts. **d**, ABCB5 immunohistochemistry (patient P3). **e–h**, *In vivo* genetic lineage tracking of human ABCB5⁺ melanoma cells. **e**, EYFP versus DsRed plots of a genetically labelled inoculum (left) and a corresponding 6-week-old tumour (right). Controls (small panels): non-transfected cells (top), DsRed⁺ cells (middle) and EYFP⁺ cells (bottom). **f**, Percentage of DsRed⁺ or EYFP⁺ cells (mean $\pm$ s.e.m.) in inocula ($n = 6$) and respective tumour xenografts ($n = 3$) as a function of time. **g**, Fluorescence microscopy of dissociated 6-week-old xenografts (top and middle rows) and a corresponding frozen tumour section (bottom row). Scale bars, 25 μ m. **h**, DsRed/EYFP positivity in ABCB5⁺ and ABCB5⁻ 6-week-old tumour subpopulations (left); quantified (right) as means $\pm$ s.d. ($n = 3$ replicate experiments).

Figure 3. ABCB5 targeting

a, Tumour volumes (mean $\pm$ s.e.m.) plotted against time. **b**, tumour formation rate in untreated ($n = 18$), control-monoclonal-antibody (mAb)-treated ($n = 10$), or anti-ABCB5 mAb-treated ($n = 11$) animals. **c**, Flow cytometric assessment of ADCC in anti-ABCB5 mAb-treated, control mAb-treated or untreated DiO-labelled melanoma target cultures counterstained with propidium iodide (PI). Left panels, representative flow cytometry results showing lysed, DIO⁺PI⁺ target cells in the right upper quadrants. Right panel, analysis of ADCC (mean $\pm$ s.e.m.) in $n = 6$ replicate experiments. **d**, Effect of anti-ABCB5 mAb on established melanoma xenografts. Tumour volumes (mean $\pm$ s.e.m.) for anti-ABCB5 mAb-treated ($n = 23$), control mAb-treated ($n = 22$), or untreated ($n = 22$) animals at days 0 and 21 of treatment. **e**, Immunohistochemistry of patient-derived melanoma xenografts treated with anti-ABCB5 mAb (top) or control mAb (bottom). Adjacent sections were stained with anti-ABCB5 mAb (left), secondary anti-Ig Ab (middle) or CD11b mAb (right), with zones of cellular degeneration in the top row shown below the dotted line.