Protein kinase C is required for Ras transformation and colon carcinogenesis in vivo

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Protein kinase C (PKC) has been implicated in Ras signaling, however, a role for PKC in oncogenic Ras-mediated transformation has not been established. Here, we show that PKC is a critical downstream effector of oncogenic Ras in the colonic epithelium. Transgenic mice expressing constitutively active PKC in the colon are highly susceptible to carcinogen-induced colon carcinogenesis, whereas mice expressing kinase-deficient PKC (kdPKC) are resistant to both carcinogen- and oncogenic Ras-mediated carcinogenesis. Expression of kdPKC in Ras-transformed rat intestinal epithelial cells blocks oncogenic Ras-mediated activation of Rac1, cellular invasion, and anchorage-independent growth. Constitutively active Rac1 (RacV12) restores invasiveness and anchorage-independent growth in Ras-transformed rat intestinal epithelial cells expressing kdPKC. Our data demonstrate that PKC is required for oncogenic Ras- and carcinogen-mediated colon carcinogenesis in vivo and define a procarcinogenic signaling axis consisting of Ras, PKC, and Rac1.

Introduction
PKC plays a requisite role in Bcr–Abl-mediated resistance to chemotherapy-induced apoptosis (Murray and Fields, 1997; Jamieson et al., 1999), and is critical for epithelial cell polarity (Suzuki et al., 2002) and cell survival (Murray and Fields, 1997; Jamieson et al., 1999). (PKC refers to the human gene. The corresponding gene in rodents, which is 95% homologous to PKC at the amino acid level, is termed PKC. For clarity, we will refer to both the human and rodent genes and gene products as PKC.) Although PKC has also been implicated in Ras-mediated signaling (Ubertall et al., 1999; Coghlan et al., 2000; Kampfer et al., 2001), nothing is known about its role in oncogenic Ras-mediated transformation. Activating Ras mutations occur in ~30% of all human cancers (Adjei, 2001), and in ~50% of human colon adenomas and carcinomas (Bos, 1989). Here, we investigate the role of PKC in Ras-mediated oncogenic transformation. Our data demonstrate that Ras-mediated transformation, invasion, and anchorage-independent growth of intestinal epithelial cells requires PKC activity. Furthermore, we demonstrate that PKC is critical for Ras- and carcinogen-mediated colon carcinogenesis in vivo.

Results and discussion
As a first step in examining the role of PKC in colon carcinogenesis, we assessed the expression of PKC in normal mouse colonic epithelium and azoxymethane (AOM)-induced colon tumors. Immunoblot analysis demonstrated that PKC is elevated in colon tumors compared with matched, uninvolved epithelium (Fig. 1 a). RT-PCR analysis demonstrated a corresponding increase in PKC mRNA in colon tumors (Fig. 1 b). PKC was also elevated in human colon carcinoma specimens when compared with matched uninvolved colonic epithelium (Fig. 1 c), demonstrating that elevated PKC is a common feature of both mouse and human colon tumors. Immunohistochemical staining confirmed the elevated expression of PKC in mouse colon tumors (Fig. 2 b) compared

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Abbreviations used in this paper: ACF, aberrant crypt foci; AOM, azoxymethane; caPKC, constitutively active PKC; kdPKC, kinase-deficient PKC; RacN17, dominant negative Rac1; RacV12, constitutively active Rac1; RIE, rat intestinal epithelial; RIE/Ras, Ras-transformed RIE.
PKC<sub>θ</sub> expression is elevated in AOM-induced colon tumors. Immunohistochemical analysis of sections from (a and c) normal, uninvolved epithelium and (b and d) an AOM-induced colon tumor from the same animal was performed using a specific PKC<sub>θ</sub> antibody in the (a and b) absence or (c and d) presence of competing PKC<sub>θ</sub> peptide as described in Materials and methods. Bars, 50 μm.

Figure 3. Transgenic caPKC<sub>θ</sub> mice are highly susceptible to AOM-induced colon carcinogenesis. (a and b) Total protein lysates from scraped colonic epithelium from nontransgenic (Ntg) and transgenic (a) caPKC<sub>θ</sub> (CA) or (b) kdPKC<sub>θ</sub> (KD) mice were analyzed for PKC<sub>θ</sub> protein (a and b, top) and PKC<sub>θ</sub> activity by immunoprecipitation histone kinase assay (a and b, bottom). (c) Colonos from AOM-treated mice were scored for ACF. CA/CA, homozygous caPKC<sub>θ</sub> mice; CA/+, heterozygous caPKC<sub>θ</sub> mice; Ntg, nontransgenic mice; KD/KD, homozygous kdPKC<sub>θ</sub>. Results represent the average ACF/animal ± SEM (n = 4–9; *P = 0.05 vs. Ntg; **P = 0.02 vs. Ntg). (d) H&E-stained section of a tubular adenoma from a nontransgenic mouse. (e) H&E-stained section of a carcinoma in situ from a caPKC<sub>θ</sub> mouse. (d and e) Bars, 100 μm.
ble changes in proliferation or differentiation markers in the colonic epithelium (unpublished data). Next, we treated transgenic caPKC₄, transgenic kdPKCᵦ, and nontransgenic mice with AOM to induce colon carcinogenesis (Murray et al., 1999; Gökmén-Polar et al., 2001) and analyzed the mice for preneoplastic lesions, aberrant crypt foci (ACF; Fig. 3 c). Heterozygous transgenic caPKCᵦ mice developed twice as many ACF, and homozygous caPKCᵦ mice developed three times as many ACF, as nontransgenic littermates (Fig. 3 c). In contrast, homozygous transgenic kdPKCᵦ mice developed significantly fewer ACF than nontransgenic mice. Thus, PKCᵦ activity in the colonic epithelium correlates directly with susceptibility to AOM-induced ACF formation.

Next, we assessed the effect of transgenic caPKCᵦ expression on colon tumor formation. Transgenic caPKCᵦ mice exhibited a threefold higher incidence of tumors than nontransgenic mice [63.6% (7/11) vs. 20% (2/10) tumor-bearing mice]. In addition, transgenic caPKCᵦ mice developed predominantly malignant intramucosal carcinomas (6/7 tumors; Fig. 3 e), whereas nontransgenic mice developed mainly benign tubular adenomas (2/3 tumors; Fig. 3 d). Therefore, elevated colonic PKCᵦ activity increases the number of preneoplastic lesions and subsequent colon tumors, and promotes tumor progression from benign adenoma to malignant intramucosal carcinoma. Due to the low tumor incidence in nontransgenic mice it was impractical to assess the effect of kdPKCᵦ on tumor formation.

Given the relationship between PKCᵦ and Ras signaling (Uberall et al., 1999; Coghlan et al., 2000; Kampl et al., 2001), we assessed whether PKCᵦ is important for Ras-mediated transformation of the intestinal epithelium. We and others (Sheng et al., 2000; Murray et al., 2002; Yu et al., 2003) have used rat intestinal epithelial (RIE) cells to study Ras-mediated transformation, and elucidate the molecular mechanisms by which PKCB₁H promotes carcinogenesis. Ras-transformed RIE (RIE/Ras) cells were transfected with FLAG-tagged, wild-type (wt) PKCᵦ or kdPKCᵦ. Both RIE/Ras/wtPKCᵦ and RIE/Ras/kdPKCᵦ cells expressed elevated levels of PKCᵦ when compared with RIE or RIE/Ras cells (Fig. 4 a, top). Immunoblot analysis using an antibody to oncogenic V12 Ras demonstrated that RIE/Ras, RIE/Ras/wtPKCᵦ, and RIE/Ras/kdPKCᵦ cells express comparable levels of oncogenic Ras (Fig. 4 a, second from top). Actin immunoblots confirmed that equal amounts of protein were loaded for each cell line (Fig. 4 a, third from top).

We next assayed RIE, RIE/Ras, RIE/Ras/wtPKCᵦ, and RIE/Ras/kdPKCᵦ cells for total PKCᵦ activity (Jamieson et al., 1999; Fig. 4 a, fourth and fifth from top). Although RIE and RIE/Ras cells expressed equivalent levels of endogenous PKCᵦ (Fig. 4 a, fourth from top), RIE/Ras cells exhibited elevated PKCᵦ activity (Fig. 4 a, fifth from top). Thus, expression of oncogenic Ras leads to activation of endogenous PKCᵦ while having no demonstrable effect on PKCᵦ expression. RIE/Ras/wtPKCᵦ cells expressed elevated PKCᵦ protein and activity when compared with RIE or RIE/Ras cells, whereas RIE/Ras/kdPKCᵦ cells exhibited elevated PKCᵦ protein, but no increase in PKCᵦ activity when compared with RIE/Ras cells (Fig. 4 a, fourth and fifth from top). Immunoprecipitation with an anti-FLAG antibody followed by immunoblot analysis confirmed the expression of FLAG-wtPKCᵦ and FLAG-kdPKCᵦ in RIE/Ras/wtPKCᵦ and RIE/Ras/kdPKCᵦ cells, respectively (Fig. 4 a, second from bottom). Assay of anti-FLAG immunoprecipitates for PKCᵦ activity confirmed that RIE/Ras/wtPKCᵦ cells express catalytically active FLAG-wtPKCᵦ, whereas RIE/Ras/kdPKCᵦ cells express catalytically inac-

![Figure 4](image)

**PKCᵦ is required for oncogenic Ras-induced Rac1 activation and invasion in vitro.**

(a) RIE cells were stably transfected with empty vector (RIE), Ras (RIE/Ras), Ras and wtPKCᵦ (RIE/Ras/wtPKCᵦ), or Ras and dnPKCᵦ (RIE/Ras/kdPKCᵦ). Total cell lysates were subjected to immunoblot analysis for PKCᵦ (top), oncogenic V12 Ras (second from top) and β-actin (third from top). Immunoprecipitates using a specific PKCᵦ antibody were analyzed for PKCᵦ expression (fourth from top) and PKCᵦ activity (fifth from top). Anti-FLAG immunoprecipitates were analyzed for PKCᵦ expression (second from bottom) and PKCᵦ activity (bottom). (b) Anchorage-dependent growth of RIE cells and RIE cell transfectants. Data represent the mean ± SD from three independent determinations. (c) Active (GTP bound) Rac1 was isolated from the indicated RIE cell transfectants: control empty vector; Ras; Ras and RacN17; Ras and kdPKCᵦ; and Ras and kdPKCᵦ and RacV12. Immunoblot analysis was performed for active Rac1 (top), total cellular Rac1 (middle), and actin (bottom). The asterisk indicates the migration of Myc-tagged, virally expressed Rac1 mutants. (d) The indicated RIE transfectants were assayed for invasion. Data represent the average number of cells invading into the bottom chamber ± SD from three independent experiments. *P = 0.02 versus RIE + control vector; **P < 0.02 versus RIE/Ras; ***P = 0.005 versus RIE/Ras/kdPKCᵦ.
tive FLAG-kdPKCζ (Fig. 4 a, bottom). These data demonstrate that oncogenic Ras activates both endogenous and transfected PKCζ, and confirm that our kdPKCζ construct is deficient in kinase activity.

RIE/Ras cells exhibited an increase in anchorage-dependent growth rate and saturation density compared with RIE cells (Fig. 4 b). Expression of wtPKCζ or kdPKCζ had little effect on the Ras-mediated increase in growth rate or saturation density (Fig. 4 b). RIE cells expressing wtPKCζ or kdPKCζ in the absence of oncogenic Ras exhibited no demonstrable change in growth rate compared with RIE cells, and no signs of cellular transformation (unpublished data).

Because Ras transformation is dependent on activation of the small molecular weight GTPase Rac1, RIE (Qiu et al., 1995), we measured Rac1 activity in RIE/Ras cells (Fig. 4 c). As expected, RIE/Ras cells exhibit elevated Rac1 activity when compared with RIE cells (Fig. 4 c). Expression of either a dominant negative Rac1 (RacN17) mutant (Qiu et al., 1995) or kdPKCζ blocked Ras-mediated Rac1 activation. In contrast, expression of a constitutively active Rac1 (RacV12) mutant (Qiu et al., 1995) had little effect on Ras-mediated activation of endogenous Rac1. Expression of wtPKCζ in the absence of oncogenic Ras was not sufficient to induce Rac1 activity (unpublished data). Thus, oncogenic Ras activates Rac1 in a PKCζ-dependent fashion.

Both Ras and Rac1 have been implicated in cellular motility and invasion (De Corte et al., 2002) and RIE/Ras cells exhibit an invasive phenotype (Fujimoto et al., 2001). Therefore, we assessed whether the invasive phenotype observed in RIE/Ras cells is dependent on Rac1 and PKCζ. As expected, RIE/Ras cells are highly invasive, whereas RIE cells are not (Fig. 4 d). Expression of RacN17 or kdPKCζ in RIE/Ras cells blocks Ras-mediated invasion (Fig. 4 d). However, expression of RacV12 in RIE/Ras/kdPKCζ cells partially restores invasiveness. Thus, oncogenic Rac-mediated cellular invasion is dependent on both Rac1 and PKCζ. Interestingly, expression of either wtPKCζ or caPKCζ in the absence of oncogenic Ras failed to induce invasion, indicating that PKCζ is necessary for Ras-mediated invasion, but is not sufficient to induce invasion in the absence of oncogenic Ras (unpublished data).

RIE/Ras cells exhibit anchorage-independent growth in soft agar, whereas RIE cells do not (Fig. 5 a and b). Expression of wtPKCζ significantly enhances, and expression of kdPKCζ blocks, soft agar growth of RIE/Ras cells (Fig. 5 a and b). Furthermore, expression of RacV12 in RIE/Ras/kdPKCζ cells restores soft agar growth (Fig. 5 c). Expression of RacV12 in RIE cells in the absence of oncogenic Ras does not induce soft agar growth, indicating that expression of active Rac1 alone is not sufficient to cause cellular transformation (Fig. 5 c), which is consistent with previous reports that RacV12 exhibits very weak transforming potential (Khorsravi-Far et al., 1995). These data demonstrate that PKCζ plays a critical role in Ras-mediated transformation of RIE cells because PKCζ is required for Ras-mediated activation of Rac1, cellular invasion, and anchorage-independent growth. Our data place PKCζ downstream of oncogenic Ras and upstream of Rac1 in a pathway that stimulates invasiveness and soft agar growth, two hallmarks of the transformed phenotype. Next, we assessed the importance of PKCζ in Ras-mediated invasiveness. Thus, oncogenic Ras-mediated invasion (Fig. 4 d). How-

Figure 5. **Expression of dnPKCζ blocks Ras-mediated transformation of the intestinal epithelium in vitro and in vivo.** (a) and (b) RIE cells were stably transfected with control empty vector (RIE), Ras (RIE/Ras), Ras and wtPKCζ (RIE/Ras/wtPKζ), or Ras and kdPKCζ (RIE/Ras/kdPKCζ), and evaluated for growth in soft agar. (a) Representative experimental results. Numbers in parentheses represents number of colonies/dish. (b) Values represent the average of three independent experiments ± SEM. *P < 0.001 versus RIE/Ras. (c) The indicated RIE cell transfectants were analyzed as described in panel a. Values represent the average of five determinations ± SEM. **P = 0.008 versus RIE/Ras; ***P = 0.0001 versus RIE/Ras/kdPKCζ. (d) 12-wk-old K-RasLA2 and K-RasLA2/kd PKCζ mice were analyzed for ACF in the proximal colon. Average number of ACF per mouse is plotted ± the SEM; n = 5; **P = 0.04.

Ras-mediated colon carcinogenesis in vivo using transgenic mice expressing a latent oncogenic K-ras allele (G12D) that is activated by spontaneous recombination (Johnson et al., 2001). Latent K-ras (K-RasLA2) mice develop Ras-dependent lung carcinomas and colonic ACF (Johnson et al., 2001). We bred our transgenic kdPKCζ mice with K-RasLA2 mice to generate bitransgenic K-RasLA2/kdPKCζ mice, and assessed them for spontaneous ACF development (Fig. 5 d). K-RasLA2/kdPKCζ mice developed significantly fewer ACF in the proximal colon than K-RasLA2 mice. These data are consistent with our results in RIE/Ras cells in vitro, and demon-
strate that PKC\(\alpha\) is critical for oncogenic K-ras–mediated colon carcinogenesis in vivo.

Our results provide direct evidence that PKC\(\alpha\) and Rac1 are necessary for the transformed phenotype induced by oncogenic Ras. Rac1 has been shown to be required for transformation by both H-Ras and K-Ras, the two most commonly mutated forms of Ras in human cancers. Our data demonstrate that PKC\(\alpha\) is also required for both H-Ras– and K-Ras–mediated transformation. Although H-Ras and K-Ras have both common and distinct effectors, both of these Ras isoforms activate Rac1, though K-Ras appears more effective than H-Ras (Walsh and Bar-Sagi, 2001). We have shown that H-Ras induces Rac1 activity through a PKC\(\alpha\)-dependent pathway and that PKC\(\alpha\) is required for K-Ras–mediated colon carcinogenesis. Given the increased propensity of K-Ras to activate Rac1, it is likely that the Ras→PKC\(\alpha\)→Rac1 pathway we have elucidated in RIE cells is also important for K-Ras–mediated colon carcinogenesis in vivo. Interestingly, PKC\(\alpha\) and Rac1 have also been implicated in epithelial cell polarity through formation of complexes containing PKC\(\alpha\), Par6, and Rac1 (Noda et al., 2001). Rac1 is thought to regulate PKC\(\alpha\) activity within these complexes to affect cell polarity (Noda et al., 2001). Our data now implicate signaling through PKC\(\alpha\)–Par6–Rac1 complexes in Ras-mediated transformation.

In this report, we present conclusive evidence that PKC\(\alpha\) is critical for colonic epithelial cell transformation both in vitro and in vivo. Interestingly, disruption of PKC\(\alpha\) signaling by kdPKC\(\alpha\) has little effect on normal intestinal epithelial cell homeostasis in vitro and in vivo, suggesting that PKC\(\alpha\) may be an attractive target for development of novel therapeutics against colon cancer.

Materials and methods
Analysis of PKC\(\alpha\) expression in mouse and human colon tumors
AOM-induced mouse colon tumors were produced in C57Bl/6 mice as described previously (Gökmen-Polar et al., 2001). Fresh frozen tissue from human colon carcinomas and uninvolved colonic epithelium was obtained from surgical specimens. Isolation of RNA and protein for RT-PCR and immunoblot analysis, respectively, was performed as described previously (Gökmen-Polar et al., 2001). Immunoblot analysis for PKC\(\alpha\) and actin was conducted using isozyme-specific antibody against PKC\(\alpha\) and actin (Santa Cruz Biotechnology, Inc.) as described previously (Murray and Fields, 1997; Gökmen-Polar et al., 2001). We determined previously that this PKC\(\alpha\) antibody recognizes PKC\(\alpha\) but not PKC\(\gamma\) (Murray and Fields, 1997). Primers for RT-PCR analysis were as follows: PKC\(\alpha\) forward primer, 5′-GCCTATGTGAGATGCGGCG-3′, and PKC\(\alpha\) reverse primer, 5′-GTCACAACCAAACGTGCGG-3′; and actin forward primer, 5′-GTCGCGCCGCCTGAGCAGCACA-3′, and actin reverse primer, 5′-CCTTCTGGATGTGCTGGTGGAG-3′. Colon tumors and uninvolved colonic epithelium from AOM-treated mice were fixed in 10% buffered formalin, sectioned, and subjected to antigen retrieval (Vector Laboratories). Immunohistochemical detection of PKC\(\alpha\) was performed using the specific PKC\(\alpha\) antibody (Santa Cruz Biotechnology, Inc.) and the DAKO LSAB2 (DAB) detection system (DakoCytomation). Specificity of immunostaining for PKC\(\alpha\) was demonstrated by inclusion of a fivefold molar excess of the peptide used to generate the PKC\(\alpha\) antibody (Santa Cruz Biotechnology, Inc.) in the antibody dilution. Digital images were acquired on a microscope (model DX51; Olympus) equipped with a DP70 digital camera using a 20× objective lens. Images were captured using the DP Controller software and processed in Adobe Photoshop.

Production of transgenic mice and carcinogenesis studies
Human cPKCs and kdPKC\(\alpha\) cDNAs were generated and characterized previously (Jamiesson et al., 1999; Lu et al., 2001). Transgenic cPKCs and kdPKC\(\alpha\) mice were generated on a C57Bl/6 background using the Fabpl4x at –132 promoter (Simon et al., 1997; provided by I. Gordon, Washington University, St. Louis, MO) to direct transgene expression to the colonic epithelium (Murray et al., 1999). Isolation of colonic epithelium, immunoblot analysis for PKC\(\alpha\) expression, and immunoprecipitation histone kinase assays were described previously (Jamiesson et al., 1999; Murray et al., 1999). Transgenic caPKCs, transgenic kdPKC\(\alpha\), and nontransgenic mice were injected with either 10 mg/kg AOM or saline as described previously (Gökmen-Polar et al., 2001). ACF analysis was performed 12 wk after the last AOM injection (Murray et al., 2002) using well-defined criteria (McLelland et al., 1991). Mice were analyzed at 40 wk for tumor number, size, location, and pathological grade as described previously (Gökmen-Polar et al., 2001). All tumors were classified as either tubular adenomas or intramusosal carcinomas in situ by histopathology. Digital images of the tumors were captured on a microscope (model Eclipse E600; Nikon) equipped with a ProgRes C14 camera (Jenoptik) using a 20× objective lens. Images were acquired using ProgRes C14 software with Microsoft Paint and processed with Microsoft Photoshop.

Transgenic K-rasLA2 mice (Johnson et al., 2001) provided by T. Jacks, Massachusetts Institute of Technology, Cambridge, MA were bred to transgenic kdPKC\(\alpha\) mice to obtain transgenic K-rasLA2 kdPKC\(\alpha\) mice. At 12 wk old, transgenic K-rasLA2 and bitransgenic K-rasLA2/dKDPC\(\alpha\) mice were assessed for spontaneous ACF formation (McLellan et al., 1991; Murray et al., 1999).

RIE cell transfections and cell analyses
RIE cells and derivatives were grown in DME containing 5% FBS as described previously (Ko et al., 1998). RIE/Ras cells were described elsewhere (Sheng et al., 2000; provided by H.M. Sheng, University of Texas Medical Branch [UTMB], Galveston, TX). Microarray analysis of RIE/Ras cells demonstrated that these cells do not express PKC\(\zeta\) (unpublished data). Human wtPKCs and kdPKC\(\alpha\) cDNAs were cloned into the pBabe/FLAGpuro retroviral expression vector and virus stocks were produced using Phoenix-E cells (provided by G. Nolan, Stanford University, Palo Alto, CA). Puromycin-resistant, stable transfectants were generated as described at http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html. Expression of FLAG-epitope-tagged PKC\(\zeta\) was confirmed by immunoblot analysis using anti-FLAG antibody (Sigma-Aldrich), and PKC\(\zeta\) kinase activity was determined by immunoprecipitation histone kinase assay as described previously (Jamieson et al., 1999).

Recombinant retroviruses containing Myc-tagged Rac1N7 or Myc-tagged Rac2V12 were generated by excising the Myc-tagged Rac1 construct from pSVK/Rac vectors (Qiu et al., 1995) with EcoRI and ligating them into the EcoRI site of the LZRS-GFP retrovirus. The entire coding sequence of each construct was confirmed by DNA sequence analysis. LZRS-GFP-Rac1 retroviruses were used to infect RIE cells and derivative cell lines using a protocol described previously (Ireton et al., 2002). Rac1 activity was assayed by affinity isolation of GTP-bound Rac1 using a protocol described previously (Sander et al., 1998). Active GTP-bound Rac1 assays were described previously (Jamieson et al., 1999).

Invasiveness of RIE cell transfectants was assessed in Transwell inserts populated with Matrigel (6.3-μm pore size; BD Biosciences). DME containing 10% FBS was added to the bottom chamber and 5 × 104 cells were suspended in 500 μl of serum-free DME and placed in the top chamber of the Transwell insert. Cells were incubated for 22 h at 37°C in 5% CO2, at which time noninvading cells were removed from the top chamber. Cells that had invaded through the Matrigel-coated filter were fixed in 100% methanol, stained with crystal violet, and counted on a microscope (Nikon) using a calibrated ocular grid. 15 representative areas of the bottom chamber were counted to determine the number of invasive cells in each well.

To assess anchorage-independent growth, RIE cell transfectants were suspended in DME supplemented with 10% FBS, 1.5% agarose, and a 1% insulin, transferrin, and selenium solution (Sigma-Aldrich), and plated (300 cells per Matrigel dish) on a layer of 1.5% agar containing the same supplements. Cell colonies were fixed with 20% methanol and stained with Giemsa after 7–14 d in culture and quantified under a dissecting microscope (Nikon).

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