Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity

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Colon carcinoma is one of the leading causes of death from cancer and is characterized by a heterogenic pool of cells with distinct differentiation patterns. Recently, it was reported that a population of undifferentiated cells from a primary tumor, so-called cancer stem cells (CSC), can reconstitute the original tumor on xenotransplantation. Here, we show that spheroid cultures of these colon CSCs contain expression of CD133, CD166, CD44, CD29, CD24, Lgr5, and nuclear β-catenin, which have all been suggested to mark the (cancer) stem cell population. More importantly, by using these spheroid cultures or freshly isolated tumor cells from multiple colon carcinomas, we now provide compelling evidence to indicate that the capacity to propagate a tumor with all differentiated progeny resides in a single CSC. Single-cell-cloned CSCs can form an adenocarcinoma on xenotransplantation but do not generate the stroma within these tumors. Moreover, they can self-renew and are capable of multilineage differentiation. Further analysis indicated that the lineage decision is dictated by phosphoinositide 3-kinase (PI3K) signaling in CSCs. These data support the hypothesis that tumor hierarchy can be traced back to a single CSC and provides clues to the regulation of differentiation in colon cancers in vivo.

colorectal cancer | tumor-initiating cell

Recent evidence suggests that various, if not all, tumors consist of heterogeneous populations of cells differing in marker expression and growth capacities (1, 2). The cancer stem cell (CSC) hypothesis suggests that this heterogeneity is because of ongoing differentiation within a tumor. In this model, only a small population of cells is clonogenic and contains tumor initiating potential whereas the majority of the tumor cells have undergone differentiation and lost this potential (1, 2). These clonogenic tumor cells are capable of inducing a new tumor in mice and are therefore defined as CSCs (3). In colorectal cancer (CRC), these cells have been reported to express CD133 and thus can be isolated by sorting the CD133+ fraction of tumor cells (4–6). Xenotransplantation of this CD133+ enriched fraction results in a tumor that closely resembles the original malignancy in both morphology and marker expression. Although this suggests that CD133+ cells signify CSC, it has also been reported that only one in 262 CD133+ cells would be a true CSC (4). In addition, none of these studies actually used 100% pure CD133+ cells, thus allowing for alternative explanations (2, 7).

Besides CD133, other cell surface proteins have been reported to mark colon CSCs. For instance, CD166 combined with CD44 (8) or CD24 combined with CD29 (R. Fodde, unpublished data) define the colorectal CSC population. It is currently uncertain whether these markers overlap and define a similar population or designate different populations of cells. These data show the need for a better definition of CSCs and the markers used, but above all raise the question as to whether the capacity to regenerate a heterogenic tumor resides in a monoclonal cell population or depends on multiple different CSCs. This is especially relevant when considering the multiple differentiation patterns observed in colon carcinomas, which could be a sign of multilineage differentiation by a CSC, but could also exemplify a polyclonal CSC population in which different CSCs drive separate differentiation programs. Neither can be excluded at this point because heterogenic colon carcinomas have so far been generated by xenotransplantation of a purified, but not necessarily monoclonal, population of CSCs.

Therefore, we set out to analyze the multilineage differentiation patterns of colon CSCs and further characterize these cells by using multiple markers. We show that all currently reported CSC markers are coexpressed on cells that contain a tumor-initiating capacity. More importantly, by using single-cell-cloned colon CSCs, we show that CSCs contain multilineage differentiation potential both in vitro and in vivo. Our in vitro experiments furthermore revealed that PI3K is a crucial determinant in this cell fate decision.

Results

Colon Sheroid Cultures Show Tumor-Initiating Capacity and Are Heterogeneous. Recent evidence indicates that spheroid cultures of primary cancer cells are superior to “regular” adherent grown cultures in medium containing serum (5, 9) because xenotransplanted tumors derived from such spheroid cultures more faithfully preserve the original gene expression profiles and tumor morphology (5, 9). In agreement, we have generated colon cancer spheroid cultures of primary colorectal cancers and liver metastases and these are consistently capable of inducing tumors upon xenotransplantation that resemble the original malignancies, both in morphology and marker expression (Fig. 1A) (6).

It is important to note that these cultures are not homogeneous, but consist of heterogeneous populations of cells with respect to markers associated with CSCs. Although the majority of cells are negative for the differentiation marker cytokeratin-20, we observe heterogeneity for CD133, which is associated with the stem cell compartment in a variety of tissues (data not shown) [Fig. 1B and supporting information (SI) Fig. S1]. Moreover, CD24, CD29, CD44, and CD166, which have all been described to enrich for CSCs in CRCs (R. Fodde, unpublished data) (8), were also expressed on a subpopulation in those spheroid cultures (Fig. 1B and Fig. S1). Importantly, we observed that the small percentage of CD133+ cells present in the primary tumor before culture also show expression of these markers (Fig. 1B) and that culture under stem cell conditions selects for cells bearing the above described CSC markers (Fig. 1B).

This suggests that the culture method allows for selective expansion of...
cells with an immature phenotype, without changing their marker expression profile. Importantly, staining for β-catenin revealed that only a minority of cells show clear nuclear localization of this protein (Fig. 1C), implying varying degrees of Wnt signaling activity. In normal colon tissue, active Wnt signaling is observed at the bottom of crypts and identifies colon stem cells. In apparent agreement, we also detected expression of the recently reported normal intestinal stem cell marker, Lgr5, in a defined subset of the spheroid cells in our cultures (Fig. 1C) (10). Combined, this illustrates that the spheroid cultures are heterogeneous with respect to marker expression and Wnt signaling activity. This heterogeneity could reflect the presence of multiple cell types within the cultures that may be responsible for the heterogeneity in the original malignancy and the xenotransplanted tumors derived from these spheroids. Alternatively, it could point to in vitro divergence from CSCs to more differentiated, less immature cells. Understanding this in further detail will provide important information on the origin of heterogeneity in solid tumors.

Clonogenicity of Colorectal CSCs. To elucidate whether a single colon cancer cell can undergo multilineage differentiation and has the capacity to generate a differentiated colon adenocarcinoma, we initiated a series of single-cell-cloning experiments. Using single-cell sorting of spheroid cells by flow cytometry we established that ∼1 in 20 cells has the capacity to induce a monoclonal culture as judged by the successful formation of spheroids (Fig. 2A). Moreover, we used a different CRC specimen and performed directly ex-patient single cell cloning by using single cell plating validated by microscopy (Fig. 2B).

Because CD133 is reportedly selective for CSCs in colon cancer, we tested whether clonogenicity was indeed present in the CD133+ subset of cells. We therefore FACS deposited a single GFP-transduced CD133+ spheroid cell into different amounts of GFP−CD133− cells from the same culture. This invariably resulted in GFP+ spheres (Fig. 2C). As transmission of the expression vector was excluded (Fig. S2), this indicated that the CD133+ and not the CD133− cells contain the clonogenic capacity. In agreement, limiting dilution experiments from a spheroid culture showed that ∼1 in 16 CD133+ cells has the capacity to generate spheroids, whereas a calculated 1 in 250 CD133− cells have this ability (Fig. 2D). To fully ascertain that this is also true for directly isolated tumor cells, we used a third primary CRC and initiated single cell cultures with purified CD133+ cells directly ex-patient. Also in this setting the CD133+ cell fraction generated single cell derived spheroids, whereas the CD133− cells were incapable of doing so (Fig. S3A and data not shown). This clearly confirmed that the clonogenic potential of our spheroid cultures resides in the CD133+ cells.

As stated above, the colon cancer spheroid cultures we obtained show heterogeneity for various markers related to the tumor initiating population in CRC even within the CD133+ population. We therefore determined whether any of the other CSC cell surface markers could improve the identification of the clonogenic population within the spheroid cultures. Coexpression of CD44, CD166, or CD29 with CD133 did not increase the selection of clonogenic cells. However, coexpression of CD133 and CD24 clearly identified the clonogenic cells with higher fidelity (∼1 in 5) (Fig. 2D), suggesting that the combination of these markers may provide a better selection of CSCs.

Single-Cell Propagation of Colorectal Cancer. The single-cell-derived cultures (SCDCs) we obtained by FACS deposition or single cell plating displayed similar expansion rates as the original culture, indicating that we did not select for rapid proliferating cells (Fig. S4). To determine whether these isolated and monoclonal cells could still grow out to form an adenocarcinoma, we injected these cells s.c. into mice. Irrespective of the method of subcloning, that is, either directly ex-patient or from spheroid culture, and either CD133-selected or unselected, all SCDCs formed tumors subcutaneously that had an adenocarcinoma appearance and resembled the primary human carcinoma from which they were derived (Fig. 2A–C and Fig. S3B). Crypt-like structures surrounded with epithelial cells were detected in all cases, which are derived from injected cells as evidenced by GFP expression in GFP+ SCDC-derived tumors (Fig. 2C). In contrast, the stromal cells were GFP− and therefore mouse derived. This indeed confirms our idea that the capacity to generate a morphologically differentiated colon adenocarcinoma resides in a single cell. To determine whether this single cell also retains self-renewal capacity after in vivo expansion, we...
examined whether tumors derived from these single cells express the CSC marker CD133, and found that a small proportion of cells had preserved this expression in vivo (Fig. 3 A and B, and Fig. S3A). Importantly, when spheroid cultures were rederived from GFP+ SCDC-induced xenotransplants, these were again GFP+ (Fig. 3C and Fig. S5A). This supports the hypothesis that the original GFP+ single cell had undergone massive expansion and differentiation in vivo, but also preserved clonogenic potential in a small subpopulation of its offspring. In agreement, rederived cultures expressed CD133, but not CK20 (Fig. 3C) and, on injection, induced growth of an adenocarcinoma in which a minority of the cells is again CD133+ (Fig. 3D). These observations were corroborated with cells derived from xenotransplants that were originally derived from directly ex-patient single cell cloned CSCs (data not shown), and thus prove that in vivo self-renewal is retained in these single cell cloned colon CSCs. To bolster our claim that the tumors we analyzed are truly single-cell derived we examined the GFP expression of the GFP+ SCDCs and the cultures that were derived from their xenografts. In contrast to the original line, which contained a broad expression range of GFP, we found that the GFP expression levels were restricted to a very limited intensity range in SCDCs. Importantly, this was unchanged even after mouse passage (Fig. S5A).

More importantly, Southern blot analysis was used to scrutinize GFP integration sites in the genome of the SCDCs and the cultures derived after xenotransplantation (SCDC.R1). The Southern blot profile of GFP integration sites shows complete similarity between the parental SCDCs and the cultures derived from the xenografts. In contrast to the original line, which contained a broad expression range of GFP, we found that the GFP expression levels were restricted to a very limited intensity range in SCDCs. Importantly, this was unchanged even after mouse passage (Fig. S5A).

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opportunity to confirm either of these models both in vitro and in vivo. We tested the in vitro differentiation of CSCs by plating them on tissue culture treated plastics and applying medium containing serum. This procedure results in loss of tumor initiating capacity of the cultures (6). Upon the onset of differentiation, CD133 and CD24 are rapidly down regulated followed by CD44, CD29 and CD166 show very limited change in cell surface expression levels. (B) Undifferentiated spheres were embedded in matrigel and were immediately snap frozen or allowed to differentiate for 10 days and then snap frozen. Sections were stained for CD133 (Upper) and CK20 (Lower). (Scale bars, 40 μm.) (A) Cells differentiated in matrigel show evidence for mucin production as shown here by Alcian Blue stain. (A and C) Data are representative for all clones analyzed (see also Fig. S6). (D) High-magnification microscopy reveals heterogeneous cell morphology in crypt-like structures of SCDCs. Both goblet cell-like (arrows) and enterocyte-like differentiation (arch) can be detected. (E) Staining for markers associated with different cell lineages in the colon epithelium of SCDC-derived xenografts (Upper) or in vitro-differentiated SCDCs (Lower). Villin indicates enterocyte differentiation. Alcian Blue and PAS staining reveal mucin production associated with goblet-like cell differentiation. Neuroendocrine differentiation is detected by Chromogranin A staining.

We therefore conclude that the distinct differentiation patterns detected within tumors are not because of the presence of multiple clones, but because of remnant differentiation patterns. As these SCDC-derived cells can also self-renew in vivo they fulfill the theoretical criteria of CSCs.

**SCDC Culture Differentiation can be Directed with PI3K Inhibition in Vitro.** The above data settle an important point in the origin of multiple distinct differentiation types within a tumor, but give no hint as to how this is orchestrated. In normal colon crypts and in adenomas, Notch signaling has been implicated in the decision to differentiate.

High-magnification microscopy of xenografts generated by SCDCs revealed heterogeneous cellular morphology (Fig. 4D). In analogy to normal colon crypts, goblet-like cells were detected next to enterocyte-like cells (Fig. 4D). In vivo differentiation in SCDC-derived tumors was also analyzed by staining for markers associated with the variety of differentiation lineages present in colon epithelium. Besides the readily detectable Alcian Blue staining, which points to goblet-like cell differentiation, membranous localized Villin- and Chromogranin A-immunoreactive cells, respectively, reveal that enterocyte-like and neuroendocrine-like cells are also present (Fig. 4E). Luminal surface staining for Villin was present in low amounts throughout the tumors, and Chromogranin A expression was present in only a small fraction of these tumor cells.

Intriguingly, when SCDCs are subjected to differentiation induced by adherent plates and serum containing medium, we also observed only a small number of Chromogranin A+ cells (Fig. 4E and Fig. 5D). Similarly, enterocyte-like differentiation as determined by membrane-localized Villin expression (Fig. 4E and Fig. 5 C and D) or intestinal fatty acid binding protein (I-FABP) was only detected in a fraction of cells (Fig. 5 C and D, and Fig. S7). Combined, our observations prove that CSCs from human colon cancer possess multilineage differentiation capacity. We therefore conclude that the distinct differentiation patterns detected within tumors are not because of the presence of multiple clones, but because of remnant differentiation patterns. As these SCDC-derived cells can also self-renew in vivo they fulfill the theoretical criteria of CSCs.
between goblet- and enterocyte-like programs (16). To elucidate how differentiation of colon CSCs is coordinated and to gain insight into the nature of differentiation modulating signals, we initiated a screen of inhibitors that were applied during *in vitro* differentiation. We determined that the highest dose of the inhibitors that did not result in apparent cell death or severe proliferation inhibition of the spheroid cultures (Fig. S8). Subsequently, we applied those concentrations of inhibitors to a colon spheroid cell culture that was subjected to adherent plate differentiation. For most inhibitors we observed no gross morphological changes in the differentiated cells (Fig. S7B). However, in the case of the PI3K inhibitor Ly294002, we observed a clear difference in morphology of the differentiated cells (Fig. S4). Cells were much more flattened and showed polarisation on the edges of cell aggregates. We confirmed that overall differentiation, as judged by loss of CD133 and gain of CK20 expression, was identical in the presence of PI3K inhibition (Fig. 5D and data not shown). Similarly, the low number of chromogranin A+ cells was unaltered in the presence of PI3K inhibition (Fig. 5D). However, cells treated with Ly294002 during differentiation showed increased intestinal alkaline phosphatase (1AP) activity (Fig. S8B and Fig. S8A and D), which is associated with the brush border membrane in enterocytic cells. Combined with the 4-fold increase in cells positive for membrane localized Villin, a border membrane in enterocytic cells. Combined with the 4-fold increase in CD133+ and gain of CK20 expression, was identical in the presence of PI3K inhibition (Fig. 5D). However, cells treated with Ly294002 during differentiation showed increased intestinal alkaline phosphatase (1AP) activity (Fig. S8B and Fig. S8A and D), which is associated with the brush border membrane in enterocytic cells. Combined with the 4-fold increase in cells positive for membrane localized Villin, a border membrane in enterocytic cells.

**Discussion**

In this study, we provide evidence to support an important aspect of the cancer stem cell hypothesis, which claims that a single CSC can self-renew and reconstitute a complete and differentiated carcinoma. This was not only observed when the starting material for the single cell sort was a spheroid culture, but was also apparent when CSCs were single cell cloned directly from a primary tumor. Importantly, we now formally confirm that the apparent when CSCs were single cell cloned directly from a primary tumor. Importantly, we now formally confirm that the apparent when CSCs were single cell cloned directly from a primary tumor. Importantly, we now formally confirm that the phenotype of the spheroid cultures from primary colon carcinoma displays only a limited number of cells with nuclear β-catenin despite APC mutations (18). Apparently, regulation of β-catenin in APC deficient cells still occurs in *vivo* and we now show that this is also true in these cultures in *vitro*. Whether this signifies the onset of differentiation is not clear but it could potentially help in defining CSCs within tumors as suggested by Fodde and Brabletz (18). Finally, the third argument for limited stemness is the fact that single-cell cloning is only ineffective for ~5% of the CD133+ cells and ~20% of the CD133+/CD24+ cells. This suggests that the remainder may have lost this capacity because of initial differentiation steps. A hypothesis supported by our data because we show a striking correlation between clonogenic potential of subpopulations in our cultures and the time at which the markers that define those populations are lost during differentiation. Both CD133 and CD24, for instance, are rapidly down regulated on differentiation, whereas their coexpression is the best designation of the clonogenic population (Fig. 2D).

Although this is an appealing model, it poses an interesting dilemma. It is currently completely unclear why these spheroid cells, under apparent identical conditions, would retain stemness in some cells and turn on differentiation programs in others. Not only did we observe this in *vitro*, but in *vivo* we detected that a single CSC could yield progeny displaying markers associated with goblet-like, enterocyte-like, and neuroendocrine-like cells. This characteristic is not shared with colon carcinoma cell lines which can be cloned, but have the tendency to form a tumor in which all typical morphology and differentiation is lost (19).

Previous observations have suggested that Notch and PI3K signaling are involved in lineage determination in normal crypts and homeostasis (16, 20). Our data support the idea that PI3K activity is crucial to lineage decision in CRCs and previous observations suggested a role for Notch in goblet cell fate in adenomas (16). However, neither observation provides a clue as to the actual signals that will differentially activate PI3K or Notch or, more importantly, the signals that will actually initiate differentiation of CSC offspring. *In vitro*, differentiation can be induced by changing growth factors and conditions for adherence (Fig. 4 and Fig. S6), but this appears to be an all-or-nothing signal and does not preserve self-renewal of CSCs. Intriguingly, PI3K activity, measured by pPKB, is observed in the spheroid cells and in differentiating cells and is prevented by Ly294002 (data not shown). This suggests that PI3K is active during differentiation but is unlikely to be solely responsible for the onset of differentiation even though PI3K inhibition dramatically changes the outcome (Fig. 5 and Fig. S8). *In vivo*, differentiation occurs alongside self-renewal, which is clear from the fact that CSCs are present in the xenotransplant (Fig. 3), suggesting that CSCs receive multiple signals that regulate their fate. When regarding normal stem cells as the paradigm, it seems logical to assume that this is regulated by a niche that provides coordinated signals (21, 22). *In vitro*, this niche can only be made up from neighboring tumor cells. Whether such a CSC niche really exists and which cells constitute this niche is an open question (23), but proving its existence or identification of the signals that regulate CSC proliferation and differentiation could be of vital importance for therapeutic strategies to prevent tumor regeneration (24, 25).

Combined, our data reveal that heterogeneity in colorectal carcinomas, with respect to both differentiation grade and differentiation phenotype, is a clonal trait. This is in contrast to the more classical genetic model in which ongoing accumulation of mutations is thought to result in the presence of multiple genetically distinct clones within a tumor (26). This process, which is referred to as tumor Darwinism, fuels the phenotypical heterogeneity present in a malignancy (19). However, our data now reveal that a large part of these assorted phenotypes can...
be explained by the CSC hypothesis that proposes a hierarch- 
chical organization of a malignant clone in addition to remnant 
responses to differentiation guiding signals from the micro- 
environment. This implicates a model in which carcinomas can 
be viewed as atypical organs, including a functional stem cell 
compartment in which crucial mechanisms for homeostatic 
control are lost but other characteristics are consistently 
present.

Materials Methods
Isolation of SCs. Colon CSC cultures were derived as described previously (6). 
SCCs were cultured in modified neurobasal A medium (27) containing N2 
supplement (Invitrogen), Lipid Mixture-1 (Sigma), and high levels of bFGF (20 
ng/ml) and EGF (50 ng/ml). A GFP− subculture was obtained by lentiviral 
transduction.

Generation of SCs. The FACSaria (BD Biosciences) was used for single-cell 
plating in 96-well, ultra-low-adhesion plates (Corning) containing stem 
cell medium. We stringently gated on single, PI-negative cells. For the GFP+/ 
CD133+ and GFP-CD133- mixing experiment, AC133-PE (Miltenyi Biotec; 1:100) 
staining was used to select positive cells. After visible spheres arose, 
they were transferred into ultra low-adhesion flasks (Corning) and expanded.

Direct Single-Cell Isolation. Tumor tissue was dissociated as described in ref. 6. 
CD45− cells were depleted by using double magnetic bead deple- 
tion (MACS). Cells were plated on ultra low-adhesion 96-well plates at a concentration of 
1 single cell per well, which was confirmed visually. Wells containing either 
one or more than one cell were excluded for further analysis. For enrichment of the 
CD133+ cells, we used microbeads conjugated with a CD133 antibody 
(AC133, cell isolation kit; Miltenyi Biotec).

Limiting Dilution Assay. Cells forming different subpopulations of a colon spher- 
ocids were deposited as 1, 2, 4, and 6 cells per well. Results were statistically 
evaluated by using the ‘limdil’ function of the R software package.

In Vitro Differentiation Assay. For in vitro differentiation, small spheres were 
plated in matrigel (GF reduced) and 2% FCS containing medium was layered 
in vitro

Flow Cytometry. Flow cytometry was performed on trypsin-dissociated CSC 
cultures with AC133 (Miltenyi Biotec), anti-Ck20 (Genetex), CD44 (BD Pha- mingen), CD166 (R&D Systems, clone 105901), CD24 (BD PharMingen), and CD29 
(BD PharMingen). For intracellular CK20 staining, 7-AAD (eBioscience) 
peptide
ubication was used to exclude dead cells.

In Vivo Tumor Propagation. For transplantation of cancer cells, we injected 30 
cells (−/100 cells/sphere) suspended in 100 µl of PBS/BSA subcutaneously 
short term
into nude mice. After 3–6 weeks, visible tumors arose. When tumor size 
reached 1 cm3, mice were killed, and tumors processed for either analysis or in 
vi tro
culture. For generation of tumors after direct single cell plating and short 
term in vitro expansion, 500 cells were injected all derived from the originally 
plated cell (Fig. 2B and Fig. 3B).

IAP Activity Assay. To measure IAP activity, we used Alkaline Phosphatase 
Substrate Kit 1 (SK-5100) from Vector Laboratories Inc. according to the 
manufacturer’s instructions. Quantification was performed with an inverted 
fluorescence microscope (Zeiss).

Inhibitors. Inhibitors were diluted in DMSO and used in adherent differen- 
tiation assays. Medium and inhibitors were refreshed every 48 h for 10 days.

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Corrections

COMMENTARY

The author notes that due to a printer’s error, in the legend for Fig. 1 on page 6893, the affiliation for Andrea Eberle should have appeared as “ETH Zürich, Switzerland.” In addition, in the fourth line of the legend, “inner membrane RND component AcrA” should instead read “inner membrane RND component AcrB.” The figure and corrected legend appear below. On page 6894, the final sentence of the commentary, “The AcrAB–TolC trinity would then represent machinery” should instead read “The AcrAB–TolC trinity would then represent a true peristaltic machinery.”

CHEMISTRY, BIOPHYSICS AND COMPUTATIONAL BIOLOGY

The authors note that due to a printer’s error, reference citations 22–50 appeared incorrectly in the text, starting on page 8102. The online version has been corrected.

ANTHROPOLOGY

The authors note that on page 19172, right column, fifth full paragraph, the last sentence is incorrect in part. “She then transfers this modified s to her own daughter—the grandmother of A,” should read “She then transfers this modified s to her own daughter—the granddaughter of A.” On page 19173, in the legend for Fig. 2, the last sentence is incorrect in part. “In this mode, direction and rate of change in r is determined by the effect of parental and societal intuitive preferences (ρ) on the transmission of cultural belief (σr), i.e., parameters C01, C10, V01 and V10” should instead read “In this mode, direction and rate of change in r is determined by the effect of parental and societal intuitive preferences (ρ) on the transmission of cultural belief (σr), i.e., parameters C01, C10, V01 and V10 in Table 2.” On page 19174, in Table 2, the last sentence of the legend, “Note that parameter subscripts refer to the direction of change in σr, not the state of parental σ,” should read “Note that parameter subscripts refer to the direction of change in σr, from the first subscript to the second, not the state of parental σ.” On page 19175, right column, first full paragraph, seventh line, “Its greatest effect ...” should read “Most likely, its greatest effect ...”. These errors do not affect the conclusions of the article.

CELL BIOLOGY

The authors wish to note the following: “We inadvertently overlooked an earlier report by S. C. Kirkland (28), relevant to our work.” The related reference citation appears below.


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Fig. 1. Schematic drawing of tripartite RND multidrug efflux system AcrAB–TolC of the Gram-negative bacterium Escherichia coli (courtesy of Andrea Eberle, ETH Zürich, Switzerland). Suggestions on the stoichiometry of the adaptor AcrA to inner membrane RND component AcrB (or to outer membrane channel TolC) vary between 1 and 4.

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Most likely, its greatest effect ...