SmartFlares fail to reflect their target transcripts levels

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SmartFlare probes have recently emerged as a promising tool for visualisation and quantification of specific RNAs in living cells. They are supposed to overcome the common drawbacks of current methods for RNA analysis: the need of cell fixation or lysis, or the requirements for genetic manipulations. In contrast to the traditional methods, SmartFlare probes are also presumed to provide information on RNA levels in single cells. Disappointingly, the results of our comprehensive study involving probes specific to five different transcripts, HMOX1, IL6, PTGS2, Nrg1, and ERBB4, deny the usefulness of SmartFlare probes for RNA analysis. We report a total lack of correlation between fluorescence intensities of SmartFlare probes and the levels of corresponding RNAs assessed by RT-qPCR. To ensure strong differences in the levels of analysed RNAs, their expression was modified via: (i) HMOX1-knockdown generated by CRISPR-Cas9 genome editing, (ii) hemin-mediated stimulation of HMOX1- and IL1β-mediated stimulation of IL6- and PTGS2 transcription, (iii) lentiviral vector-mediated Nrg1 overexpression. Additionally, ERBB4-specific SmartFlare probe failed to distinguish between ERBB4-expressing and non-expressing cell lines. Finally, we demonstrated that fluorescence intensity of HMOX1-specific SmartFlare probe corresponds to the efficacy of its uptake and/or accumulation.

The majority of quantitative analyses of expression of specific RNAs require cell fixation or lysis to isolate RNA; consequently the cells are lost for further experiments. Moreover, most of the procedures provide only information on average expression levels of individual genes in a given cell population. Therefore, a method that enables detection of a specific RNA in single living cells would be highly desirable. The most popular existing method for RNA visualization inside living cells requires modification of the chosen transcript to contain multiple copies of MS2 bacteriophage stem-loop motif and the introduction of MS2 coat protein (MCP) fused to a fluorescent protein into the cells. Because of the necessity of transcript tagging, this method is not applicable for the analysis of localization of endogenous transcripts and regulation of their expression. Lately, CRISPR-Cas9 system was successfully repurposed to allow endogenous RNA tracking. However, none of the aforementioned methods enable to sort the cells that exhibit the desired expression profile. Recently, a novel tool for RNA detection in living cells, namely SmartFlare, has been brought onto the market. It is based on previously developed Nanoflare technology. In brief, SmartFlare probes are ~13 nm gold nanoparticle-coupled single-stranded DNA (ssDNA) oligonucleotides designed to selectively bind a desired transcript. The oligonucleotides are hybridized to shorter ssDNA containing a fluorophore (Cy3 or Cy5). When a short oligonucleotide (called reporter strand) is bound to the longer one (called capture strand), fluorescence is quenched by the gold nanoparticle present in a close proximity to the fluorophore. However, when the target RNA is present, the short strand should be displaced and fluorescence is no longer quenched. The fluorescence intensity should correlate with the transcript level. Two additional probe types are available: scramble and uptake controls. The scramble probe is aimed at determining background fluorescence, as its capture strand is not complementary to any known transcript in human, mouse and rat. The uptake probe is a single oligonucleotide bound to a gold nanoparticle. This oligonucleotide is fluorescently labelled at a distal end, hence it is not quenched by the gold nanoparticle. The signal of the uptake probe, which is constantly fluorescent and is not sequence specific, should verify the ability of the cells to engulf the probes. According to the manufacturer, SmartFlare probes enable both live cell imaging as well as flow cytometry analysis and cell sorting.

Whenever a new technique emerges, rigorous and detailed tests are needed to avoid data misinterpretation. Here, we present a comprehensive evaluation of SmartFlare usefulness to analyse specific transcript levels in living cells. We took diverse approaches to alter certain mRNAs expression: (i) generation of HMOX1-knockdown cells by CRISPR-Cas9-mediated genome engineering, (ii) stimulation of gene expression with classical stimulators...
(hemin for HMOX1 or IL1β for IL6 and PTGS2), and (iii) transduction of cells with vectors coding for mouse neu-
regulin 1 (NRG1). Then, we compared SmartFlare HMOX1-Cy5, IL6-Cy5, PTGS2-Cy5, and Nrg1-Cy5 fluores-
cence intensities with the levels of corresponding mRNAs evaluated by RT-qPCR. We also measured ERBB4-Cy5 fluores-
cence signals in various cell lines and compared them with ERBB4 expression profile obtained by RT-PCR.

We showed that HMOX1-Cy5, IL6-Cy5, PTGS2-Cy5 and Nrg1-Cy5 SmartFlare fluorescence intensities do not correlate with mRNA levels measured by RT-qPCR. Using cell lines devoid of ERBB4 we prove that ERBB4-Cy5 SmartFlare probe is not able to selectively label ERBB4-expressing cells. Finally, we provide evidence that within the same cell type, SmartFlare fluorescence intensity is associated with the ability of the cells to internalize/accu-
mulate SmartFlare probes.

Results

SmartFlare probes do not discriminate between cells that strongly differ in specific transcript levels. We planned to use a SmartFlare probe to sort the population of 293T cells that became HMOX1-
deficient as a result of CRISPR-Cas9-mediated genome editing. 293T cells were transiently transfected with
HMOX1-targeting pX330-Pac-Cer vector coding for human codon-optimized Cas9 and human HMOX1-specific
sgRNA or with control empty pX330-Pac-Cer. Unlike control cells, the cells transfected with HMOX1-targeting
pX330-Pac-Cer showed highly efficient introduction of mutations in HMOX1 locus assessed by CELI mismatch
detection assay (Supplementary Figure S1). We expected that pX330-Pac-Cer would contain a sub-
population, in which indel mutations had caused a frameshift in all loci. It should result in strongly diminished
HMOX1 mRNA level due to degradation of transcripts containing premature stop codons. However, we were not
able to pinpoint this population using HMOX1-specific SmartFlare probe (data not shown).

Therefore, to generate cells with more discrete and uniform HMOX1 transcript levels rather than a hetero-
geneous population of wild-type, partial knockouts, and knockout cells, 293T cells were subjected to single cell
cloning. A fraction of single-cell-derived clones harbouring a mutation in HMOX1 locus showed HMOX1 expres-
sion reduced by 64–86% as measured by RT-qPCR (Fig. 1a). We also isolated a 293T cell clone with considerably
higher HMOX1 mRNA level compared with HMOX1 level in cells transfected with a control vector (Fig. 1a, clone
21–11). Surprisingly, despite significant differences in HMOX1 transcript levels between isolated cell clones and
control cells, there was no parallel difference in HMOX1-Cy5 SmartFlare fluorescence level evaluated by flow
cytometry (Fig. 1b,c). In clone 4–20 diminished HMOX1-specific SmartFlare signal was apparently accompa-
nied by decreased fluorescence of the uptake probe (further referred to as uptake-Cy5), therefore we decided to
normalize HMOX1-Cy5 fluorescence signal to that of uptake-Cy5 by dividing mean fluorescence intensity (MFI)
of HMOX1-Cy5 by MFI of uptake-Cy5 (Fig. 1c). The HMOX1/uptake ratios were almost identical across all
HMOX1-knockdown and control 293T cells, suggesting that all differences in HMOX1-Cy5 fluorescence between
cell populations reflected differences in the probe uptake rather than in HMOX1 transcript levels.

The disparity between RT-qPCR and HMOX1-Cy5 fluorescence could possibly be explained by low basal
HMOX1 transcript levels in 293T, possibly below SmartFlare resolution threshold. To verify this hypothesis,
we stimulated wild type 293T cells with hemin, a known activator of HMOX1 transcription, and examined
SmartFlare fluorescence using flow cytometry. Although HMOX1 levels increased about three- and seven times
in 293T cells treated with hemin for 3 or 6 h respectively, we did not detect any changes in SmartFlare fluorescence
intensity (Fig. 1d). Even more dramatic effect of hemin was observed in HeLa cells (Fig. 1e). However, even as
high as 77-fold increase in HMOX1 transcript level was not accompanied by any changes in HMOX1-specific
SmartFlare probe fluorescence (Fig. 1e). We concluded that HMOX1-specific SmartFlare probe does not discrim-
ininate between cells expressing high, normal, and negligible HMOX1 levels.

Another possible explanation for the lack of correlation between SmartFlare fluorescence and HMOX1 tran-
script levels could be that a degraded probe was used, in which fluorescence was already unquenched before its
application to the cells. However, a strong increase in the fluorescence of HMOX1-specific SmartFlare observed in
response to DTT, which is known to detach probes from gold nanoparticles6, ruled out this possibility. The effect
of DTT on HMOX1-Cy5 and three other probes used in this study (described below) is shown in Supplementary
Figure S2.

To verify whether or not HMOX1 represents a single case we investigated applicability of SmartFlares for analy-
sis of expression of a few other transcripts. First, we examined whether SmartFlare signals reflect changes in the
expression levels of inflammatory mediators, interleukin 6 (IL6) and prostaglandin-endoperoxidase synthase-2
(PTGS2, also known as cyclooxygenase-2), occurring in response to major proinflammatory cytokine, IL1β.
As expected, RT-qPCR analysis revealed strong (10 to 100-fold) increase in IL6 and PTGS2 mRNA levels in
IL1β-treated HeLa and U-373 MG cells in comparison with untreated counterparts (Fig. 2a,d; data concerning
PTGS2 in U-373 MG are not presented because, unlike in IL1β-stimulated cells, in control cells PTGS2 mRNA
was undetectable). However, these substantial differences in IL6 and PTGS2 levels were not accompanied by any
changes neither in IL6- nor in PTGS2-specific SmartFlare MFIs assessed by flow cytometry (Fig. 2b,c,e,f). To
address the question whether SmartFlare probes may affect IL1β-mediated changes in the expression of analysed
transcripts, we measured IL6 and PTGS2 mRNA levels in HeLa and U-373 MG cells preincubated overnight
(according to manufacturer’s instruction) with 100 pM PTGS2-specific probe and then stimulated with IL1β
for 3 h. The results showed that in HeLa cells the SmartFlare did not influence IL1β-induced upregulation of
IL6 and PTGS2 expression, however it slightly, by about 25%, diminished IL6 expression in IL1β-stimulated
U-373 MG cells (Supplementary Figure S3). We did not test whether this difference was due to the presence of
PTGS2-specific sequence or simply of gold nanoparticles; however, a potential influence of SmartFlare on ana-
lysed transcripts levels should be taken into consideration.

We also investigated the usefulness of SmartFlare probes for quantification of transcripts overexpressed due
to transduction of cells with lentiviral vectors. In our model, MC38CEA cells, which express endogenous NRG1,
dwere transduced with lentiviral vectors coding for mouse NRG1 type I or NRG1 type III, or with empty vector.
Type I and type III NRG1 are products of the same gene and Nrg1-specific SmartFlare probe used in this study should recognize both transcripts.

Transduction of the cells with vectors encoding NRG1 types I or III resulted, respectively, in 24-fold and 27-fold upregulation of Nrg1 expression as evaluated by RT-qPCR (Fig. 3a). Despite the evident differences in Nrg1 mRNA levels, MFI values of SmartFlare probe analysed by flow cytometry did not show any significant differences between cells with moderate and high Nrg1 mRNA levels (Fig. 3b,c). Moreover, MFI of Nrg1-specific probe was comparable to that of a scramble probe in all MC38CEA populations, although Nrg1 transcript was abundant (Fig. 3b and Supplementary Table S1). MC38CEA cells were able to internalize the probes since the uptake probe MFI reached values that were substantially higher than MFI in control, unflared cells (Fig. 3b). Thus, also in the case of Nrg1, SmartFlare technique failed to reflect substantial differences in the transcript levels (Fig. 3c).
ERBB4-specific SmartFlare probe fails to discriminate between ERBB4-expressing- and nonexpressing cell lines. SmartFlare probes are considered a useful tool for analysis of a gene of interest expression profile across different cell lines or cell subpopulations. We thus tested a number of human cell lines.
yet another transcript expression, namely ERBB4, using RT-PCR and compared the results with ERBB4-specific SmartFlare fluorescence signals. ERBB4 mRNA was detected in 293T, RH5 and RH30; weak band corresponding to ERBB4 transcript was also observed in PC3 and RH28 cells (Fig. 4a). ERBB4 mRNA was undetectable in HeLa, A549, BLM and DU145 cells. Discordantly, in all cell lines tested, ERBB4-specific probe signals exhibited higher MFI than that of the scramble probe (Fig. 4b), which is considered indicative of the transcript presence 5–7. When MFI of ERBB4-Cy5 in 293T (274) is compared with that of RH28 (675), one may infer that RH28 cells express higher level of ERBB4 than 293T, which is contradicted by the RT-PCR results. Unlike in our previous experiments performed on single cell lines, in this experiment, carried out across various cell lines, the fluorescence signals of both scramble and uptake probes differed significantly among the cell lines. Nevertheless, subtracting the scramble probe MFI values from ERBB4-specific MFI values did not change the picture (Fig. 4c). MFI values calculated in this way still failed to reflect differences in ERBB4 levels revealed by RT-PCR (Fig. 4c).

Cell-associated fluorescence of HMOX1-Cy5 SmartFlare reflects efficiency of its uptake rather than the transcript level. All our experiments indicated that even if cells strongly differ in particular transcript levels, they cannot be distinguished by SmartFlare probes. The results suggested that the differences in the intensities of intracellular SmartFlares fluorescence, rather than be related to the mRNA levels, may in fact reflect unequal abilities of the cells to accumulate or preserve the probes.

To verify this hypothesis we loaded 293T clone 4–8, which showed strongly diminished HMOX1 expression compared to control cells (Fig. 1a), with FITC-dextran and clone 21–11, which showed increased HMOX1 level to about 150% of that in control cells (Fig. 1a) with RITC-dextran. Then fluorescently labelled cells were mixed...
and incubated with HMOX1-specific SmartFlare probe. Two 293T subpopulations were gated based on Cy5 fluorescence intensity: FL4\textsubscript{low} with MFI = 309 and FL4\textsubscript{high} with MFI = 1142 (Fig. 5a). If SmartFlare fluorescence intensity correlated with HMOX1 transcript level, green fluorescence (channel FL1) should prevail in FL4\textsubscript{low} subpopulation due to low HMOX1 mRNA level in FITC-labelled 4–8 clone; conversely, red fluorescence (channel FL2) should be predominant in FL4\textsubscript{high} subpopulation as a consequence of enhanced HMOX1 expression in clone 21–11 (Fig. 5b). Instead, we observed equal distribution of cells with green and red fluorescence in both FL4\textsubscript{low} and FL4\textsubscript{high} subpopulations (Fig. 5c). Moreover, the mean intensities of both green and red fluorescence were higher in FL4\textsubscript{high} subpopulation than those in FL4\textsubscript{low} cells. The same effect was observed when clone 4–8 was loaded with RITC-dextran and clone 21–11 with FITC-dextran (data not shown). Therefore, we believe that SmartFlare fluorescence intensity does not correspond to target mRNA levels, but most likely is associated with the efficiency of SmartFlare probe uptake and accumulation.

Figure 5. Flow cytometry analysis of HMOX1-Cy5 SmartFlare fluorescence in 4–8 and 21–11 293T cell clones preloaded with fluorescently labelled dextrans. (a) The cell clones 4–8 (showing negligible HMOX1 expression) and 21–11 (showing high HMOX1 expression) were preloaded with FITC- or RITC-dextran, respectively, then mixed and incubated overnight with HMOX1-Cy5 SmartFlare probe. (b) The diagram presents the idealized hypothetical results of the experiment verifying the cause of differences in SmartFlare signal intensities. Green ovals represent 4–8 cells loaded with FITC-dextran, while red ovals represent 21–11 cells loaded with RITC-dextran. (c) Two subpopulations of the cells were gated based on Cy5 fluorescence intensity: FL4\textsubscript{low} and FL4\textsubscript{high} (left panel). Cell-associated FITC and RITC fluorescence in FL4\textsubscript{low} and FL4\textsubscript{high} subpopulations are shown as dot plots of FITC [FL1] vs RITC [FL2] (right panel). Representative result of 2 independent experiments is shown.
Discussion

Most of our findings contradict the conclusions of a number of studies that utilized SmartFlare and NanoFlare probes to evaluate differences in mRNA levels in living cells. However, to avoid strong background signal associated with cell autofluorescence disqualifies such probes for normalisation of target-specific signals.

In this type of experiments, it is essential to measure also MFI of a housekeeping transcript-specific SmartFlare, as MFI of a SmartFlare probe specific to a transcript of interest may differ in various cell types irrespective of the transcript content in these cells. The probes specific to a high-abundance transcripts (18S rRNA, β-actin, GAPDH), are currently available from SmartFlare manufacturer. The lack of proper uptake and/or housekeeping transcript controls makes us believe that sorting of mixed cell populations presented in Merck brochure (Cell sorting based on RNA detection in living cells using SmartFlare RNA Detection Reagents, Literature Code AN4665EN00), is actually based on differences in efficacy of a SmartFlare probe uptake and/or retention and not on differences in the levels of targeted mRNA. Moreover, our results indicate that cell-associated SmartFlare fluorescence strongly correlates with the applied probe concentration (Supplementary Figure S5) and in consequence, with the amount of the internalized probe.

According to the manufacturer's information and a general opinion presented in publications, the MFI of target-specific SmartFlare probe that is higher than MFI of a scramble probe is regarded as an evidence of the transcript presence. However, our observations contradict this notion, as the fluorescent signal of Nrg1-targeting probe is comparable to that of a scramble probe despite the abundance of Nrg1 transcript and strong ERBB4-Cy5 fluorescent signal, substantially higher than that of a scramble probe, is observed in cells that lack ERBB4 expression. This once again underlines that SmartFlare fluorescence does not correlate with targeted RNA levels.

One can argue that if our hypothesis is true, i.e. the fluorescence intensity of the SmartFlares reflects the efficiency of their uptake/accumulation and not RNA abundance, then the fluorescence signals of the scramble and specific probes should be similar. It would be true, provided that the probes were labelled to the same extent. As uneven labelling of the probes denies the principle of SmartFlare methodology, it did not cross our minds to routinely evaluate their labelling levels. To completely dispel any doubts concerning this issue, we performed additional analysis of a freshly purchased pair of SmartFlares: scramble-Cy5 and gene-specific probes for Cy3-attached probes (data not shown). This may explain the observed differences in fluorescence signals between scramble- and specific probes, otherwise puzzling in the light of our hypothesis.

A recent works support our belief that SmartFlare fluorescence does not depend on a specific transcript level. Using electron microscopy, groups of Lévy and Aurich independently demonstrated that SmartFlare probes are trapped inside intracellular vesicles and do not have a chance to interact with their target mRNAs. Thus, most probably the manufacturer of SmartFlare probes did not overcome the long-lasting problem concerning cytoplasmic delivery of RNA-targeting probes. The use of oligonucleotide molecular beacons that recognize specific mRNAs was proposed already in 1998 by Sokol et al. However, to avoid strong background signal associated with endosome-trapped probes, they microinjected the beacons directly to the cytoplasm. Curiously, the group of Mirkin developed NanoFlare probes confirmed prevalent vesicular localization of spherical nucleic acid (SNA). As they claim, the fluorescent signal detected in cells, may arise from lysosomal DNAse II digestion of SNA and release of the fluorophore. If it was the case, it would immediately disqualify SNA-based SmartFlares as an analytical tool. However, our preliminary results may suggest that a failure of SmartFlares does not depend on their lysosomal degradation, because cell treatment with moderate concentration of chloroquine that prevents endosome acidification, has minor effect of cellular fluorescence intensities of SmartFlares (data not shown).

The only exception is RAW264.7 cell line, in which chloroquine treatment leads to noticeable increase in SmartFlare cellular fluorescence (data not shown).
Is it possible to reconcile the previous results of SmartFlare-based experiments with our hypothesis? In our opinion, yes. We believe that in some cases, higher efficiency of internalization/accumulation of nanoparticles (including SmartFlares) may occur in parallel with other cell features, such as higher expression of anti-apoptotic and promigratory genes and, as a result, more aggressive phenotype of cancer cells. In this scenario, cells with high fluorescence of SmartFlare probe (due to high internalization rate) will also display higher expression of cancer-related genes than cells with low SmartFlare fluorescence. Thus, correlation between intensity of SmartFlare fluorescence and gene expression level would be entirely coincidental, as we believe is in the case of sorting of melanoma and prostate cancer cells based on fluorescence intensity of NODAL and AMACR SmartFlares, respectively. Similarly, higher internalization efficiency of NANOG-specific probe by so-called cancer stem cells (CSC) compared to cancer non-S with flow cytometry analysis of MC38CEA cells transduced with known pluri- potent stem cells. Interestingly, preliminary experiments performed on mouse V6.5 embryonic stem cells showed that fluorescence-activated cell sorting using Nanog- and Gdf3-specific SmartFlares resulted in the sub-populations that did not differ (Gdf3) or did not significantly differ (Nanog) in the transcript levels as determined by RT-qPCR. This observation, although not commented by the authors, is in agreement with our hypothesis.

Based on our results, we conclude that: (i) SmartFlare probes do not provide information on a particular transcript presence or absence; (ii) SmartFlare probes cannot distinguish the cells with high level from the cells with low level of a given mRNA; (iii) fluorescence intensity of a SmartFlare probe may be correlated with efficiency of the probe uptake; (iv) SmartFlare fluorescence intensity linearly correlate with the applied probe concentration.

We would also like to make use of this publication to appeal to the research community to provide, along with each new method and new research tool, a detailed protocol that explains the significance of all required controls as well as a guideline for the authors on minimal information that should be provided to allow reliable interpretation and critical evaluation of presented results.

Materials and Methods

Cell lines. 293T (human embryonic kidney cells, ATCC CRL-3216), U-373 MG (Uppsala) (human astrocy- toma cells, ECACC 08061901), PC3 (human prostate cancer cells, ATCC CRL-1435), DU145 (human prostate cancer cells, ATCC HTB-81) and MC38CEA (murine colon cancer cells expressing human carcinoembryonic antigen were cultured in DMEM (Lonza), HeLa (human cervical cancer cells, ATCC CCL-2) in EMEM (Lonza), A549 (human lung cancer cells, ATCC CCL-185) in F12 (Lonza), and BLM (human melanoma cells, HSF, RH28 and RH30 (human rhabdomyosarcoma cell lines, a gift from Dr. Peter Houghton, Greehey Children’s Cancer Research Institute) in RPMI 1640 (Lonza). All media were supplemented with 10% heat-inactivated fetal bovine serum (BioWest).

Plasmid construction. sgRNA targeting human HMOX1 gene was designed using CRISPR Design Tool (crispr.mit.edu). pX330-U6-Chimeric_BB-Cbh-hSpCas9 plasmid, a gift from Feng Zhang (Addgene plasmid #42230), was modified to express SpCas9, puromycin N-acetyltransferase (Pac) and Cerulean fluorescent protein (Cer) from a single promoter by inserting T2A and P2A coding sequences between SpCas9, Pac and Cer coding sequences (detailed cloning strategy is described in Supplementary Material and Methods and Supplementary Table S2). The resulted plasmid is hereinafter referred to as pX330-Pac-Cer. Phosphorylated and annealed oligonucleotides coding for HMOX1-targeting portion of the sgRNA were cloned into BbsI-digested and dephosphorylated pX330-Pac-Cer. Oligonucleotide sequences are listed in Supplementary Table S3.

cDNAs coding for mouse NRG1 type I and NRG1 type III were PCR-amplified from reverse-transcribed poly(A) fraction of RNA isolated from the brain of 3-week old C57BL/6 mouse (brain tissue was obtained from the animal house of the Faculty). RT and PCR were performed using ImProm II Reverse Transcription System (Promega) and KAPA HiFi polymerase (KAPA Biosystems) with following primers: NRG1(I)_XbaI restriction sites.

Lentiviral vectors production and cell transduction. Two days before transfection 1 × 10^6 293T cells were plated in a 10-cm dish. 293T cells were transfected with 10.5 μg of DNA: 6 μg of plLVX expression constructs, 3 μg of 2nd generation packaging plasmid psPAX2 and 1.5 μg of envelope plasmid pMD2.G (both were gifts from Didier Trono, Addgene plasmids #12260 and #12259, respectively) using Polyethylenimine HCl MAX, Linear, MW 40 000 (PEI; Polysciences) at a ratio of DNA to PEI 1:2. Media were renewed every 24 h for the next 3 days. Lentiviral vectors-containing media were pooled, filtered through a 0.45 μm filter and concentrated by centrifugation for 3 h at 23 000 g. Pseudoviral pellets were resuspended in 300 μl of serum-free DMEM; aliquots were stored at −80 °C. Pseudoviral stocks were titrated based on a concentration of viral-associated p24 protein using QuickTiter Lentivirus Titer Kit (Cell Biolabs). MC38CEA cells were transduced with 2000 lentiviral particles per cell (equivalent of MOI 10 as determined by flow cytometry analysis of MC38CEA cells transduced with known amount of lentiviral particles containing fluorescent marker ZsGreen). MC38CEA cells successfully transduced with plLVX-IRE5-puro vectors were selected using puromycin (Biozob, final concentration 5 μg/ml).
Transfection and generation of HMOX1-knockdown 293T cells. 293T cells (1 × 10^5) were plated in 24-well plates one day before transfection. The cells were transfected with 500 ng of HMOX1-targeting pX330-Pac-Cer plasmid using jetPRIME reagent (Polyplus Transfection) according to the manufacturer’s protocol. Puromycin (BioShop, final concentration 10 μg/ml) was added 24 h after transfection and selective pressure was maintained for the next 48 h. Thereafter, the cells were seeded into 96-well plates, on average one cell per well, in order to obtain single cell-derived clones. Genomic DNA from the cell clones was extracted using Blood/Cell DNA Mini Kit (Syngen) following the manufacturer’s protocol and subjected to a mismatch detection assay with CELI nuclease (detailed description is available in Supplementary Information). The cell clones with mutation within HMOX1 locus were subjected to RT-qPCR analysis of HMOX1 expression and those with the lowest level of HMOX1 transcript in comparison with the cells transfected with a control empty vector were selected for further experiments.

Cell stimulation. Hemin (Calbiochem) was dissolved in DMSO. Human recombinant IL1β (R&D Systems) was reconstituted in DMEM. Hemin (final concentration of 10 μM) or equal volume of DMSO was added to 293T and HeLa cell cultures 3, 6 or 24 h before RNA isolation or flow cytometry analysis. IL1β (final concentration of 10 ng/ml) was added to HeLa and U-373 MG cultures 3 h before RNA isolation or flow cytometry analysis.

RNA isolation, reverse transcription, RT-PCR and RT-qPCR. RNA was isolated from non-transduced cells by Chomczynski and Sacchi’s method and from the cells transfected with lentiviral vectors using DirectZol MiniPrep (ZymoResearch) with an in-column DNase I digestion step to avoid PCR amplification of genomically integrated Nrg1 cDNA.

RNA concentration was determined with ND-1000 spectrophotometer (NanoDrop Technologies). Equal amounts of RNA samples (1 μg) were reverse-transcribed using M-MLV reverse transcriptase (Promega) and oligo(dT) primer following manufacturer’s recommendations. RT-PCR was performed with Taq polymerase (KAPA Biosystems) for 40 cycles under the reaction conditions recommended by the manufacturer, using 1 μl of RT reaction per 20 μl of PCR reaction volume. RT-PCR products were resolved in 2% agarose gel containing SERVA DNA Stain G (Serva Electrophoresis GmbH) in TAE buffer. DNA bands were visualized using gel imaging system Quantum ST5 (Vilber Lourmat); the color of the images was inversed using Fusion Capt Advance Fx5 software (Vilber Lourmat). The acquired images were not processed in any other way. The original images are included in Supplementary Information (Supplementary Figure S7).

RT-qPCR was performed on Eco Real-Time PCR System (Illumina) using KAPA SYBR Fast qPCR Master Mix (KAPA Bioscience) in 10 μl reaction volume. Each sample was assayed in duplicate. Specific amplification was confirmed by analysis of the melting curves and agarose gel electrophoresis of PCR products. EF2 (human and mouse) and HPRT (mouse) were used as reference genes. The fold change in expression was calculated using REST2009 software; calculations included PCR efficiency correction. Primers used for RT-PCR and RT-qPCR are listed in Supplementary Table S4.

Flow cytometry. SmartFlare probes recognizing human HMOX1, PTGS2, IL6, ERBB4 and mouse Nrg1 transcripts as well as uptake- and scramble control probes, all labelled with Cy5 dye, were purchased from Merck (catalog numbers respectively: SF-1171, SF-1818, SF-456, SF-416, SF-1412, SF-137 and SF-102). The probes were resuspended in sterile, nuclease-free water (Sigma Aldrich). Various cell lines were plated in 24-well plates to reach 70–80% confluency on the next day. Then the SmartFlare probes were diluted in sterile PBS (Lonza) and added to the cell cultures to a final concentration of 100 pM. The cells were incubated for 16–24 h and analysed using FACSCalibur flow cytometer (BD Bioscience). For experiments with fluorescently labelled dextrans, SM-101 (catalog numbers respectively: SF-1171, SF-1818, SF-456, SF-416, SF-1412, SF-137 and SF-102) was added to the cell cultures to a final concentration of 1 mg/ml. Following overnight incubation, the cells were washed 5 times with PBS and trypsinised. Equal amounts of FITC- and RITC-labelled cells were mixed and plated onto 96-well plate (2 × 10^4 cells/well), then SmartFlare probes were added to a final concentration of 100 pM. After another overnight incubation the cells were harvested by trypsinisation and analysed using flow cytometer. All flow cytometry data were analysed with FlowJo v10.0.7 software (FlowJo LLC).

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M.C. conceived and performed all experiments and analysed all data; M.C. and J.B. discussed the results, wrote the manuscript, and accepted its final version.

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