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Discrimination of Colon Cancer Stem Cells Using Noncanonical Amino Acid

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Discrimination of colon cancer stem cells using noncanonical amino acid†

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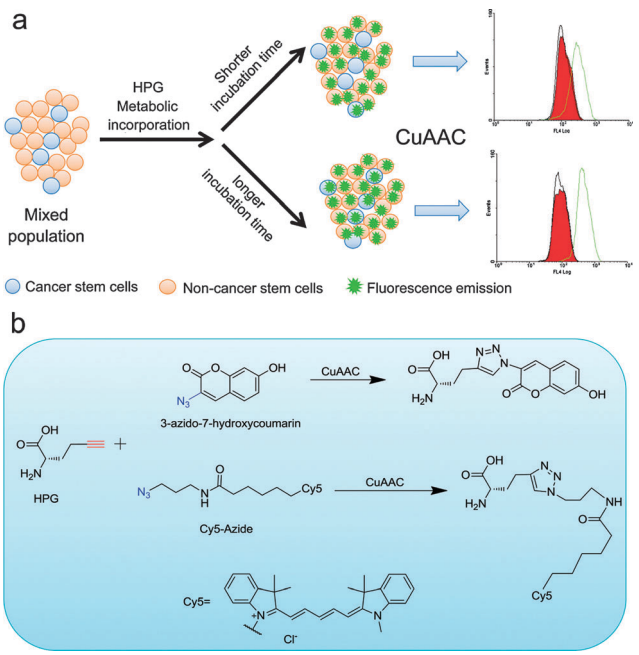
Cancer stem cells (CSCs) may be responsible for tumor recurrence. Metabolic labelling of newly synthesized proteins with non-canonical amino acids allows us to discriminate CSCs in mixed populations due to the quiescent nature of these cells.

Cancer stem cells (CSCs) exist in low population in primary tumor and cell lines. These cells can undergo self-renewal or differentiation into all the cell types seen in bulk tumors.[1] CSCs are resistant to chemo- and radiotherapies and thus might cause tumor recurrence. A better understanding of the proteome of these cells might offer a promising way to identify marker and therapeutic target. At present, most of proteomic studies of CSCs focus on glioblastoma stem cells because they have a well-established *in vitro* adherence culture model,[2–5] which is not available in other cancer types.[6] Otherwise, isolation of CSCs population *via* fluorescence activated cell sorting (FACS) from primary tumors or cell lines is necessary.[7,8] Therefore, developing a labelling method for discrimination of CSCs in a heterogeneous population while compatible with downstream proteomics analysis is highly desirable.

CSCs share the property of slow cycling with stem cells,[9] which generally have an extended G0 (quiescent) phase, and the rate of *de novo* synthesis of protein is lower than for other phases.[10] Because of the lower rate of cellular protein synthesis in CSCs, using pulse-labeling to label the newly synthesized proteins in mixed populations, non-CSCs will be labelled faster than CSCs. Conventional pulse-labeling method uses radioactive ^{35}S-methionine.[11] Recently, a large number of noncanonical amino acids (ncAAs) have been incorporated into cells site-specifically or residue-specifically,[12,13] and served as powerful tools for protein engineering and tagging.[14–23] As one of the most widely used methods, methionine analogues with side chains containing azide and alkyne groups have been employed as substrates for the natural translational machinery of cells.[24,25] Compared to radioactive ^{35}S-methionine, these analogues can undergo bioorthogonal ligation reaction, which allows further introduction of additional functionalities for downstream proteomics analysis of proteome.[22] For example, we previously have shown that coumarin based fluorogenic dye and reactive methionine analogue homopropargylglycine (HPG) could be used to investigate the spatial and temporal features of protein synthesis in *E coli* and mammalian cells by cell imaging.[21,23]

In this proof of concept study, as shown in Scheme 1, metabolic incorporation of HPG has been used to distinguish CSCs and non-CSCs in a heterogeneous population by controlling the incorporation time of HPG. Cell staining with fluorescent dye was used to monitor cell population changes *via* flow cytometry. By using 3-azido-7-hydroxycoumarin and azido cyanine5 to react with the alkyne group in HPG though copper(I) catalyzed alkyne–azide cycloaddition (CuAAC) reaction,[26,27] the successful incorporation of HPG in model cell line HCT-116 was confirmed by fluorescent microscopy and flow cytometry. Non-CSCs show distinctly higher fluorescence intensity than CSCs, which is due to a lower rate of protein synthesis in CSCs. Co-staining HPG with CSCs maker CD133 further confirmed the population with low HPG incorporation is the CSC population. For living cell applications, the potential cytotoxicity of copper is a major



Scheme 1 (a) Schematic representation of discrimination of colon cancer stem cells using noncanonical amino acid. (b) Cu(I) catalysed alkyne-azide cycloaddition (CuAAC) reaction between HPG with 3-azido-7-hydroxycoumarin and azido cyanine5 (Cy5-Azide).

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concern, however, the Cu(II)-bis-L-histidine complex used in our study shows low cytotoxicity.[28] In addition, we are seeking the possibility of new copper-free reaction conditions for living cells labelling.[29,30]

Initially, we tested HPG incorporation in colon cancer cell line HCT-116 by fluorescence microscopy. It has been reported that HCT-116 has CSCs subpopulations, which can be isolated by using well-studied CSCs maker CD133 and aldehyde dehydrogenase (ALDH) enzyme activity assay.[31,32] Incorporation of HPG in this model cell line was visualized by using 3-azido-7-hydroxycoumarin. The fluorescence of fluorophore in 3-azido-7-hydroxycoumarin is quenched by the azide group and will recover after the CuAAC reaction upon the formation of the triazole ring.[26,33] This fluorogenic nature ensures low background and high signal/noise ratio of the detection.[21,23,34–36] Compared to the Met control, as expected, HPG treated cells emit bright fluorescence (Fig. 1). These images indicate HPG can be successfully metabolically incorporated in our model cell line HCT-116.

To determine the difference of metabolic incorporation of HPG in the CSCs and non-CSCs population, these two populations were isolated by using a combination of CD133 and ALDEFLUOR assay, which can detect ALDH enzyme activity in living cells.[37] In general, the $ALDH^+CD133^+$ cells are considered as enriched in CSCs population. As shown in Fig. 2, the $ALDH^-CD133^-$ (*i.e.* non-CSC) population shows more fluorescent cells than the $ALDH^+CD133^+$ (*i.e.* CSCs) population. Based on the microcopy images, fluorescent cells have been counted and summed in both populations (Fig. 2g). Results indicate the rate of metabolic incorporation of HPG in CSCs is much slower than non-CSCs.

Based on the above results, the HPG positive population was determined using flow cytometry at different labeling time points. Cells growing at the log phase were used for the HPG labeling experiment in order to reduce the influence of the cell–cell contact and obtain an appropriate amount of cells for down-stream studies. To achieve this, cells were seeded as single cell suspensions 12 h before labeling at a seeding density of estimated 50% coverage of culture surface. Fig. 3a shows an overlap between HPG (green line) and L-Met (red histogram) and HPG with protein synthesis inhibitor anisomycin[16] (black line) treated cells, which indicate the existence of HPG negative

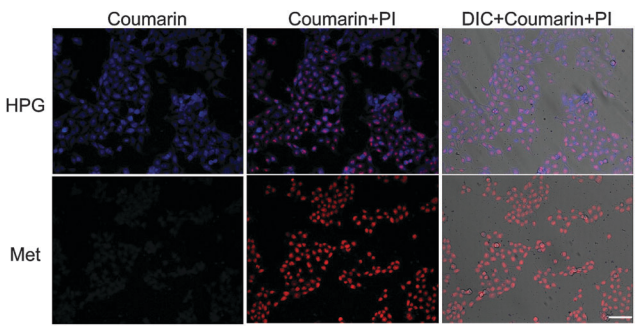

Fig. 1 Fluorogenic labelling of protein in colon cancer cell line HCT-116 with 3-azido-7-hydroxycoumarin. Imaging of HCT-116 cells pulse-labelled in media containing 100 μM HPG (top) or 100 μM Met (bottom). Blue represents coumarin and red represents propidium iodide (PI) which stains cell nuclei. Scale bar is 50 μm.

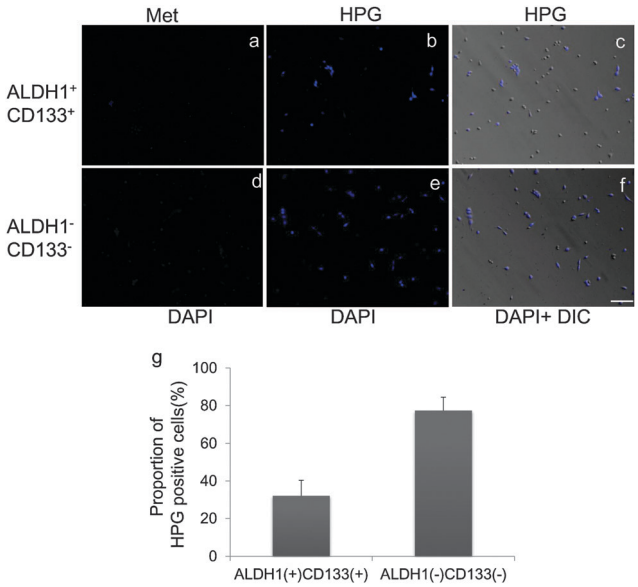

Fig. 2 Fluorogenic labelling of $ALDH^+CD133^+$ (CSCs) and $ALDH^-CD133^-$ (non-CSCs) subpopulations in HCT-116 with 3-azido-7-hydroxycoumarin. CSCs population is isolated based on high ALDH enzyme activity and surface marker CD133. Double positive $ALDH^+CD133^+$ and negative $ALDH^-CD133^-$ are considered as CSCs and non-CSCs, respectively. The top panels show fluorescence images of CSCs (a–c) and non-CSCs (d–f). Proportion of HPG positive cells in the two populations is shown at the bottom (g). In total more than 300 cells have been counted in both populations. Cells were treated with 100 μM HPG for 4 h and L-methionine treated cells were used as control. Scale bar is 100 μm.

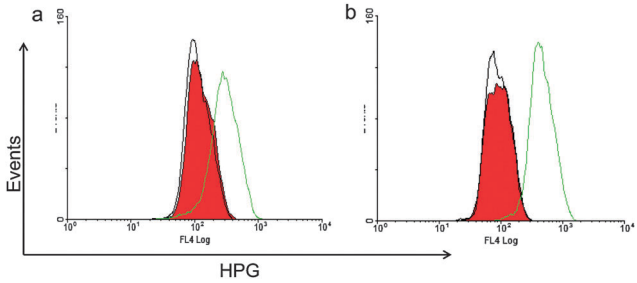

Fig. 3 Flow cytometry histogram of HPG labelling in HCT-116 cells. Cells were treated with 100 μM HPG (green line) for (a) 2 or (b) 4 h. L-Met (red histogram) and HPG with protein synthesis inhibitor (black line) treated cells were used as control. Azide-Cy5 was used to label HPG.

population (30%) in HCT-116. To confirm this HPG negative population only related to the protein synthesis rate of these cells, we doubled the HPG incubation time. As shown in Fig. 3b, the HPG negative population then decreased to less than 3%. According to our results (Fig. S1, ESI†) and literature reports,[31] the CSCs proportion in HCT-116 should be around 10–30%, as evaluated by CD133 antibody staining or ALDEFLUOR assay. Our results indicate that by simply changing the incubation time, we can label the whole population (4 h incubation) or only non-CSCs (2 h incubation). To further confirm the HPG negative population (after 2 h incubation) corresponds to the CSCs, we conducted the co-staining of HPG and the CSCs marker CD133. Fig. 4 shows CD133 positive

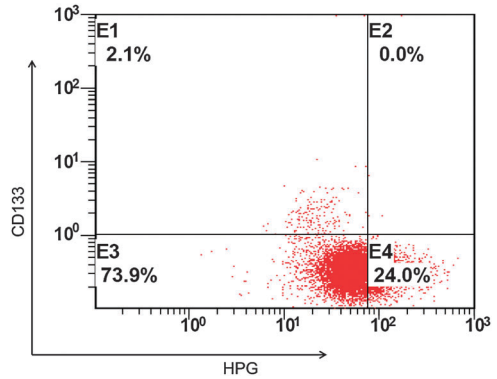

Fig. 4 Flow cytometry diagram of co-staining of HPG labelling (*x*-axis, FL4) and CD133 (*y*-axis, FL1) in HCT-116 Cells. Cells were treated with 100 μM HPG for 2 h. HPG positive population is gated by using L-Met and HPG with protein synthesis inhibitor as control. Azide-Cy5 was used to label HPG.

population only overlaps with HPG negative population, suggesting the CSCs population only co-exists in the HPG negative population.

In conclusion, bioorthogonal CuAAC reaction (a typical click chemistry) and homopropargylglycine incorporation has been used to distinguish CSCs and non-CSCs in a heterogonous population by controlling the incorporation time of HPG. Because the slower protein synthesis rate of CSCs, non-CSCs will be labeled at 2 h incubation while CSCs require more than 4 h. These results reveal the possibility of the rapid protein profiling of CSCs in heterogonous population. Since our method utilizes the universal property of CSCs with a lower rate of cellular protein synthesis, rather than surface proteins or other biomarkers to differentiate CSCs and non-CSCs, it has the potential to directly apply to any cancer cell model, including primary cancer cells.

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