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14. ABSTRACT Chemoresistance is a major hurdle against to successful breast cancer therapy. Although hypoxic tumor microenvironment has been associated with chemoresistance, molecular mechanisms driven by the tumor hypoxia remain not fully understood. The aim in this proposal is to elucidate the mechanism by which the hypoxic tumor microenvironment influences chemoresistance throughout <i>in vivo</i> monitoring and comprehensive profiling of cancer cells originated from the tumor hypoxia. During the first fiscal year, I have developed a method to trace tumor cells that were exposed to hypoxia by recruitment of hypoxia-responsible Cre (5xHRE-CREM) and multi-fluorescent gene (Brainbow) plasmids. In addition, I generated multiple fluorescent labeling technique and a transgenic mouse that would be useful to examine heterogeneous primary tumor cells in a single cell resolution. All of findings and developed methods would be useful to examine individual tumor cell behavior <i>in vivo</i> and for molecular profiling of cell population that originated from a specific pathologic condition, like as tumor hypoxia.					
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Introduction

Breast cancer is the most frequently diagnosed female cancer and one of leading causes of cancer death in the United States¹. According to National Cancer Institute's SEER (Surveillance, Epidemiology, and End Results) report, one in every eight women develops breast cancer in their lives². The mortality is decreasing because of the improved cancer diagnosis and advanced therapies, but paradoxically breast cancer was estimated as the most cost-consuming cancer by the national cancer cost projections (20.5 billion dollars in 2020), which represents a higher proportion of long term survivors². Furthermore, recent epidemiologic studies reported that 35~45% of survived patients may develop relapsed breast cancer 10 years after initial treatment³, which might be one of reasons to increase the medical cost used for breast cancer. To reduce medical cost to breast cancer care, it is therefore imperative to consider how to minimize chemoresistance and provide successful chemotherapies to breast cancer patient.

Most of our knowledge about chemoresistance comes from conventional approaches, such as cell-based assays or subcutaneous tumor mouse models. However, these assays do not reflect the complex tumor microenvironment of human tumors⁴. The progression of tumors involves a complicated interplay between cancer cells and their microenvironment, but the role of the tumor microenvironment in determining response to chemotherapy is not well understood. Different mechanisms can be responsible for the chemoresistance, including cancer cell intrinsic factors (ex. gene mutations) and extrinsic factors caused by the tumor microenvironment^{5, 6}. These two types of mechanisms likely co-exist and may cooperate with each other in tumors. For example, specific tumor microenvironment might allow the cancer cells to survive better after the initial drug dose, giving them time and opportunity to acquire genetic mutation(s) that can drive drug resistance.

Hypoxia has been associated with chemoresistance that is a major hurdle remaining to successful breast cancer therapy⁷. We hypothesize that hypoxic microenvironment influences the susceptibility of breast cancer cells to cytotoxic drugs, exemplifying cooperation between cancer cell intrinsic and extrinsic mechanisms of chemoresistance. The aim in this proposal is to establish the mechanism by which the hypoxic tumor microenvironment influences chemoresistance through profiling of cancer cells from hypoxic and normoxic areas of tumors. In this study, I want to develop a novel method to identify the cells exposed to hypoxia, and want to combine this method, as well as standard protocols for identification of hypoxia, with molecular profiling technologies to address how hypoxic tumor microenvironment confers chemoresistance to breast cancer cells. Furthermore, this study aims to identify potential gene mutations underlying the hypoxia-induced chemoresistance. The ultimate goal is translating my findings to unravel promising biomarkers and molecular targets to predict and prevent chemoresistance in breast cancer patients.

Body

Generation of hypoxia-inducible gene expression plasmid

As the first step, I intended to develop a robust method to track breast cancer cells originated from hypoxia, because conventional methods are inappropriate for our *in vivo* approaches. Hif1 α protein is a transcriptional factor involved in the hypoxia-specific transcriptional regulation and has been used as a critical endogenous marker for hypoxia⁸. To select suitable tumor cells to examine tumor hypoxia, I validated endogenous expression of Hif1 α in murine breast cancer cells that are available at our laboratory. Among 4 murine breast cancer cell-lines, 4T1 showed less expression of Hif1 α in normoxia and showed time-dependent upregulation of Hif1 α in hypoxic condition (**Figure 1A**). Using 4T1 cell-line, I next examined promoter activity of 5xHRE (hypoxia responsive element) that should induce gene transcription with response to Hif1 α stabilized in hypoxic condition⁹. After transient transfection of 5xHRE-Luciferase into 4T1 mouse breast cancer cells, the cells were incubated for additional 6 or 18 hr in normoxia and hypoxia (1.5% O₂) condition. The luciferase activity was evaluated with a CCD camera after treatment with luciferin. The results showed that the 5xHRE promoter was active under oxygen concentrations that represented hypoxia *in vivo* (**Figure 1B and C**). However, as shown in **Figure 1B**, incubation in normoxia condition for 6 hr also showed weak luciferase activity. This suggested a

possibility of leaky transcription of the Luciferase gene even in normoxic condition, thereby I decided to modify the original plasmid to enhance its specificity in hypoxic condition, as will be described in later section.

Examination of a reporter plasmid that switches expression of fluorescent proteins

I had planned to use the Cre-Lox technology to tag and trace cells that have been exposed to hypoxic tumor microenvironments. For example, carcinoma cells will be transduced with a construct that drives Cre expression in hypoxic condition (5xHRE-Cre) and a “GFP-to-RFP” construct that switches from green to red upon Cre expression by Hif1 α . To accomplish such a fluorescent labeling, it was essential for tumor cells to express stably both plasmids. Therefore, I tried to generate a 4T1 stable cell-line expressing a GFP-to-RFP cassette (pCMV-EGFP/RFP) that was previously reported and was available from a non-profit plasmid repository (Addgene, Cambridge, MA)¹⁰. However, this approach was not successful to establish a cell-line of stable expression probably due to low gene transfection efficiency and/or weak activity of pCMV promoter if introduced in 4T1 cells. For these reasons, I searched the other reporter cassette that can switch fluorescent proteins by the Cre-Lox mediated DNA recombination.

The Brainbow plasmid was developed for *in vivo* labeling of individual neuron cells (ref. 11). This plasmid contains multiple Lox sites and three coding genes driving expression of either red, yellow, or cyan fluorescent protein (RFP, YFP, or CFP). The expression of a specific fluorescent protein by any given cell clone was achieved by random Cre-LoxP DNA recombination (**Figure 2A**). To test this plasmid, I transfected the Brainbow 1.0L plasmid into 4T1 tumor cells and selected positive cells that express RFP as an initial fluorescent protein (**Figure 2B**). To test color switch by the Cre-Lox recombination, I exogenously introduced Cre expression by infection with a commercially available Adeno-Cre virus (Vector Biolabs, Philadelphia, PA). I was able to observe multiple fluorescent proteins in 4T1 tumor cells three days after infection, which indicated that the Brainbow plasmid was stably expressed and randomly recombined to express either of three fluorescent proteins (**Figure 2C**).

Modification of the hypoxia-inducible promoter to enhance transcription specificity in hypoxia

As mentioned earlier, the original 5xHRE promoter showed non-specific activity in normoxic condition (**Figure 1B**). To overcome this problem, I attempted to modify the promoter region by insertion or deletion of transcription regulatory DNA sequences, such as hCMVmp (human cytomegalovirus minimal promoter), a stop codon, and the Kozak sequence just before a start codon (**Figure 3A**). To test sensitivity and specificity of the modified promoters in hypoxic condition, I transiently transfected 4 types of plasmids (5xHRE-hCMV-stop/Kozak-CREM, 5xHRE-hCMV-CREM, 5xHRE-stop/Kozak-CREM, 5xHRE-CREM) into 4T1-Brainbow1.0L stable cells. As shown in **Figure 3B**, the most sensitive and specific induction of color change was detected in case of basic 5xHRE-CREM plasmid that was excluded with hCMVmp, a stop codon, and the Kozak sequence. Using this plasmid, I further examined a time-dependent induction of color changes in 4T1-Brainbow1.0L stable cells after transient transfection with the 5xHRE-CREM plasmid. As the results, I was able to observe a time-dependent increase of color changes from RFP to CFP or YFP that indicated tumor cells exposed to hypoxic condition (**Figure 4**). However, there were still challenges in using these plasmids because I also observed color switch in the cells of control condition, 30 hrs incubation in normoxia. In addition, transient transfection of 5xHRE-CREM has limited examination of confocal microscope by resulting in color change of only small population of cells. It was therefore necessary to improve the specificity of hypoxia-inducible plasmid and required to consider how to make stable cells expressing both plasmids, hypoxia-inducible Cre and the Brainbow plasmids.

Generation of primary tumor cells stably expressing the Brainbow plasmid

Although the 4T1-Brainbow1.0L stable cells have shown promising results by inducing color changes with respond to Cre activity, the other concern was that 4T1 is a kind of established cell-line that is consist of homogenous cell population and has lost original characteristics of tumor cells. In addition, it has a possibility of extopic expression of Hif1 α increased during repeated cell passages. With these reasons, I tried to use primary cells to reflect physiologic characteristics of tumor cells that are growing hetergenously. My first trial was to generate a lentiviral plasmid that could stably express the Brainbow plasmid. In collaboration with Werb laboratory (University of California, San Francisco) I got a lentiviral backbone plasmid (pSIN-Dest-51) that was previously used in mammary epithelial stem cell¹². However, by all means, the lentiviral plasmid was not

successfully generated because the backbone contains many restriction enzyme cutting sites and inserting ORF (open reading frame) of the Brainbow plasmid is very big.

Alternatively, I planned to generate a transgenic mouse that would simultaneously develop mammary tumors with expression of the Brainbow plasmid. In our laboratory, we have kept a mouse breast cancer model, mammary tumor virus promoter (MMTV)-polyoma middle T antigen (PyMT) model, which histologically and molecularly resembles human luminal type of breast cancer¹³. Fortunately, a transgenic mouse expressing Brainbow2.1R plasmid under promoter of CMV was developed¹⁴, and recently became available from the Jackson Laboratory (*R26R-Confetti*, #013731).

I purchased the *R26R-Confetti* mouse and did crossbreeding of a genetically engineered mouse breast cancer model, MMTV-PyMT with the *R26R-Confetti* mice that express the Brainbow 2.1R construct (**Figure 5A**). When tumor reached enough size to be surgically removed, I collected primary cancer cells and examined color changes by use of adeno-Cre virus. As shown in **Figure 5B**, primary cancer cells from the resulting mammary carcinomas expressed one of four fluorescent proteins (*RFP*, *YFP*, nuclear *GFP*, or membrane-targeted *CFP*) after infection with adeno-Cre virus. This approach was expected to be an effective way to distinguish individual primary tumor cells from heterogeneous population if combined with another plasmid that can induce Cre enzyme in a condition of interest.

Key Research Accomplishments

- (1) It was able to trace tumor cells that once exposed to hypoxic condition by recruitment of hypoxia-inducible Cre plasmid and fluorescent protein cassette that were recombined by the Lox-Cre system.
- (2) Although it had been tested in neuron cells or stem cells, the multiple-fluorescent labeling was firstly developed and assessed in murine mammary tumor cells.
- (3) A transgenic mouse was developed that could generate mammary tumors with endogenous expression of multiple fluorescent proteins. This mouse will be useful to examine individual tumor cell behavior and to do molecular profile in a single cell level.

Reportable Outcomes

**Poster Presentation at the Ninth AACR-JCA Joint Conference
Hyatt Regency Maui, February 21-25, 2013**

Tracing the influence of the tumor microenvironment on clonal selection using intravital microscopy

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Chemoresistance and metastasis are critical clinical issues responsible for most cancer deaths. The chemoresistant and metastatic cancer cells are thought to originate from aggressive subpopulations within the original tumor. Specific tumor microenvironments, such as exposure to hypoxia, likely contribute to the selective outgrowth of aggressive cell clones. However, it has been a challenge to trace how the microenvironment influences the clonal selection of cancer cells *in vivo*.

To address how the microenvironment influences clonal selection, we are developing methods based on the “brainbow” approach, originally developed for lineage tracing in neuronal development. Murine breast carcinoma cell lines were transduced with a plasmid that would drive expression of either red, yellow, or cyan fluorescent protein (*RFP*, *YFP*, or *CFP*). The expression of a specific fluorescent protein by any given cell clone was achieved by random Cre-LoxP DNA recombination. To temporally control the initiation of lineage tracing, we used tamoxifen-inducible Cre (Cre-ER^{T2}). To follow the clonal outgrowth *in vivo* over time, we used surgically implanted mammary imaging windows in immunocompetent mice and injected “brainbow” expressing, syngeneic 4T1 breast carcinoma cells under the windows. This allowed us to acquire multiple time-lapse imaging series by spinning disk confocal microscopy of the same tumor, done about 3 days apart and for up to one month after implantation. Using this technology, the outgrowth of cell clones expressing a specific fluorescent protein could be followed over time, as could the migration, proliferation, and cell death of individual cancer cells in the time-lapse image series. To trace clonal outgrowth in the hypoxic microenvironment, we are developing a construct for expression of tamoxifen-inducible Cre recombinase under 5x hypoxia response element (HRE).

Collectively, we have developed a method that will allow us to follow the outgrowth of cancer cells clones from specific tumor microenvironments - including areas of hypoxia - using a combination of lineage tracing involving fluorescent proteins and confocal imaging in live mice. Future plans include tracing clonal outgrowth during the development of chemoresistance and metastasis and genomic analysis of cancer cell clones isolated after imaging.

Conclusion

The chemoresistance and relapse of breast cancer is a critical issue for breast cancer treatment. Different mechanisms have been proposed for chemoresistance, but particularly the hypoxia-driven molecular mechanisms remain not fully elucidated. This study was proposed to result in the development of a new and robust method to retrospectively trace the cells that have been exposed to hypoxia. Moreover, this study was aimed to understand and target the hypoxia-driven chemoresistance with new strategies to design more effective breast cancer therapies.

In the limited term of conducting project, I tried *in vitro* and *in vivo* approaches to develop a robust method to follow the outgrowth of cancer cell clones from hypoxic tumor microenvironment. To do so, I have developed a combinatory way by recruitment of hypoxia-responsible Cre (5xHRE-CREM) and multi-fluorescent gene (Brainbow) plasmids. In addition, I generated a transgenic mouse that will be useful to examine heterogeneous primary tumor cells in a single cell resolution. Since recently advanced technologies have enabled high throughput profiling of individual tumor cells, the developed approaches from this study will contribute to lineage tracing of clonal outgrowth during the development of chemoresistance and elucidating genetic alterations in chemoresistant cancer cell clones that are originated from tumor hypoxia.

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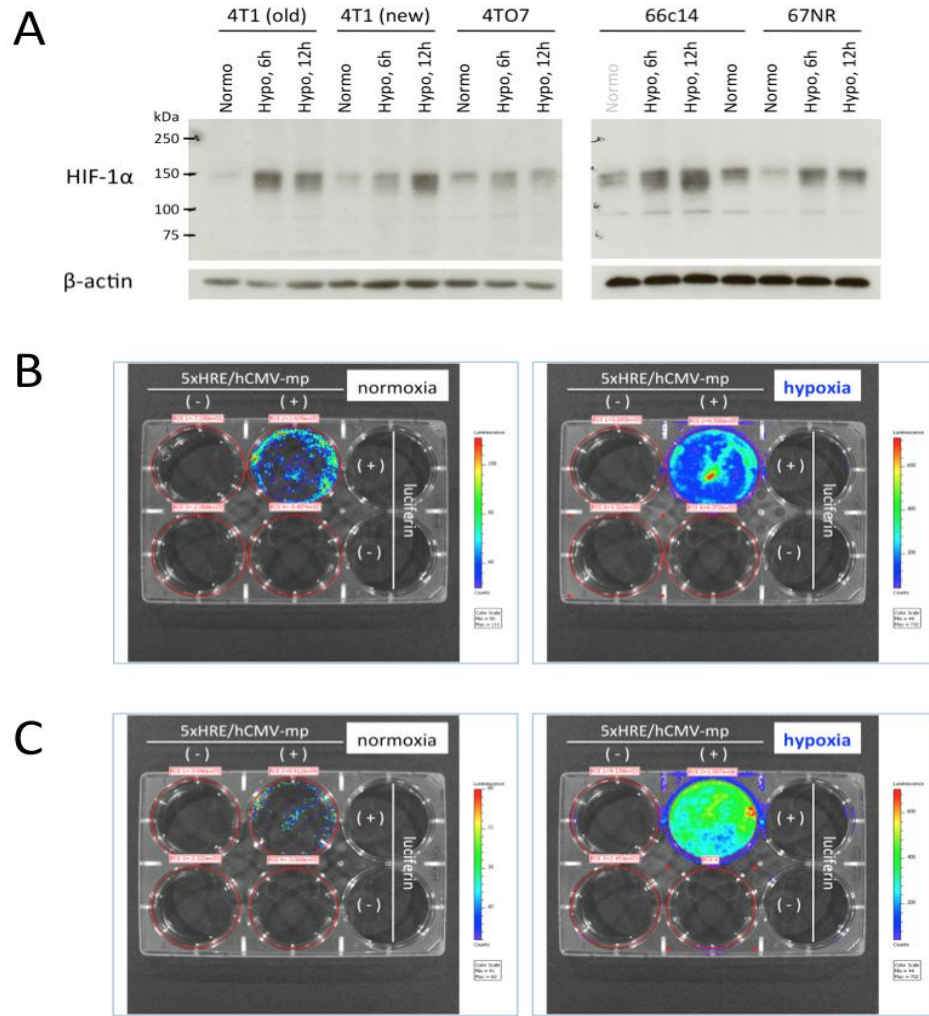


Figure 1. Hypoxia-inducible gene expression in 4T1 murine mammary tumor cell. (A) Endogenous expression of Hif1 α protein was examined by western blot. Murine mammary tumor cell-lines (4T1, 4TO7, 66c14, 67NR) were incubated for totally 18 hr with/without pre-incubation of 6 or 12 hr in a hypoxic incubator (1.5% O₂). The earlier (4T1 old) and later passage of 4T1 (4T1 new) cells were compared to exclude a possibility of extopic increse of Hif1 α as cell passage repeated. The expression of β -actin was assessed to serve as a control. (B and C) After transient transfection of 5xHRE-Luciferase into the 4T1 cells, the cells were incubated for additional 6 hr (B) and 18 hr (C) in normoxic or hypoxic condition. The luciferase activity was evaluated with a CCD camera 10 min after treatment with luciferin.

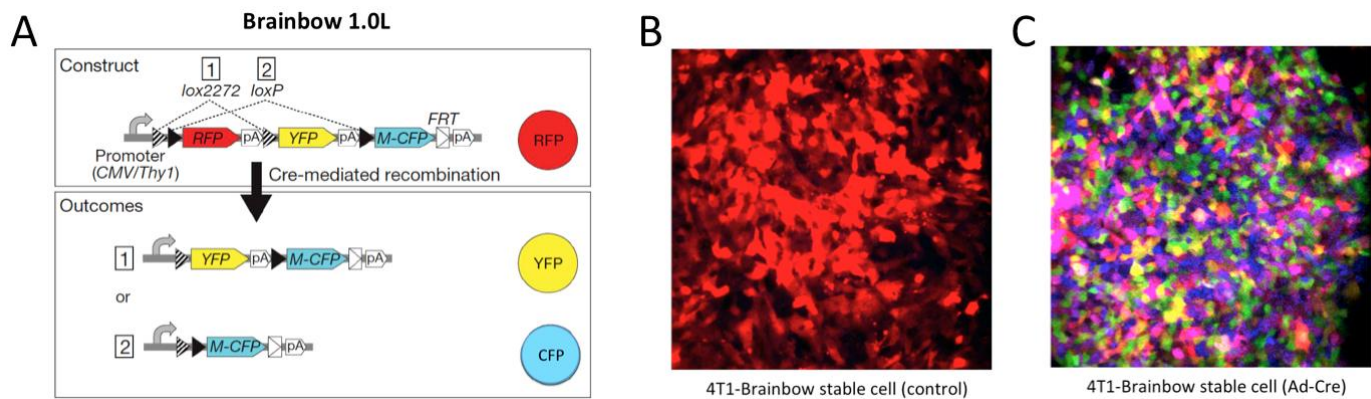


Figure 2. Induction of multiple fluorescent cell labeling by the Brainbow plasmid. (A) Schema of the Brainbow 1.0L plasmid (Addgene, #18721). The plasmid basically express RFP, but when the Cre recombinase is active, it generated switch of expression from RFP to YFP or CFP¹⁴. (B) Expression of RFP from established 4T1-Brainbow 1.0L stable cell was observed by a confocal microscope. (C) Exogenous introduction of Cre recombinase resulted in expression of multiple fluorescent proteins (RFP, YFP, CFP) in the 4T1-Brainbow 1.0L stable cell. The signal of YFP protein was converted to green color by ImageJ software. Before observation, the cells were cultured for three days after infection with adeno-Cre virus.

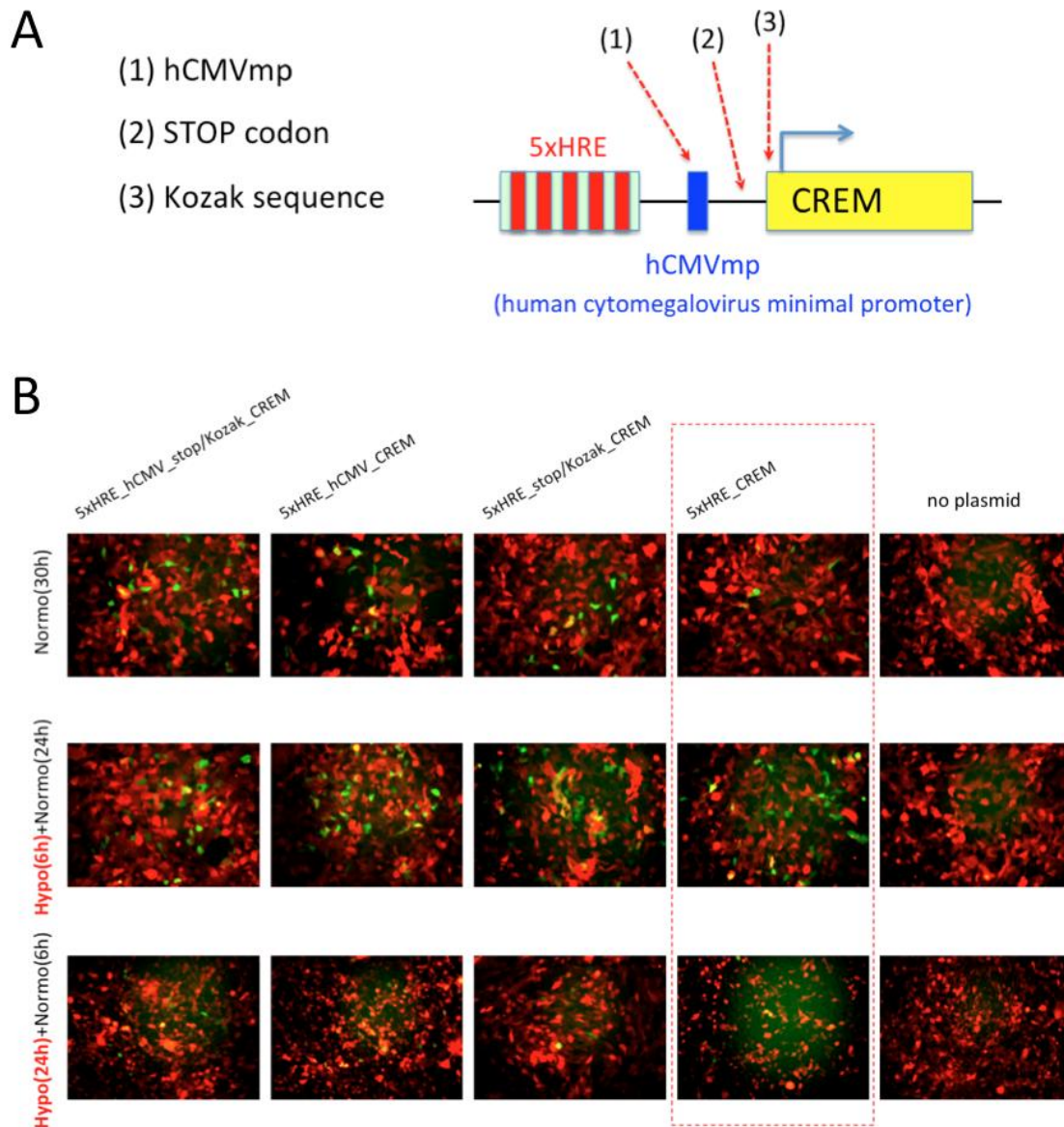


Figure 3. Modification of the hypoxia-inducible promoter to enhance transcription specificity in hypoxia. (A) Schema of promoter modification to enhance specificity of the original plasmid⁹. (B) The 4T1-Brainbow1.0L stable cells were transiently transfected with 4 types of plasmids, 5xHRE-hCMV-stop/Kozak-CREM, 5xHRE-hCMV-CREM, 5xHRE-stop/Kozak-CREM, and 5xHRE-CREM. The cells were further incubated for totally 30 hr with/without pre-incubation of 6 or 24 hr in a hypoxic incubator (1.5% O₂). Because of low transfection efficiency in 4T1 cells, less than 10% of cells could express 5xHRE plasmids.

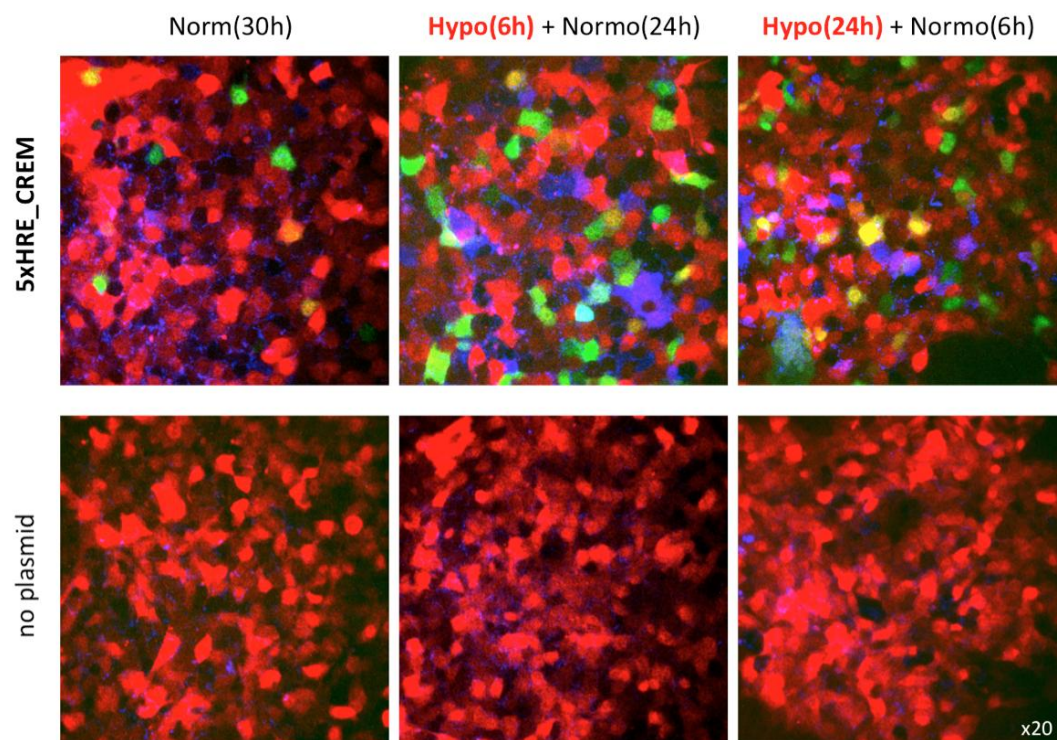


Figure 4. Multiple fluorescent labeling of tumor cells that were exposed to hypoxia. The 4T1-Brainbow1.0L stable cells were transiently transfected with 5xHRE-CREM plasmid, and then incubated for 30 hrs with/without pre-incubation in hypoxic condition as indicated on figure. Although expression of YFP and CFP was increased in a time-dependent manner, but those color switches were also observed in control, 30 hrs incubation in normoxia.

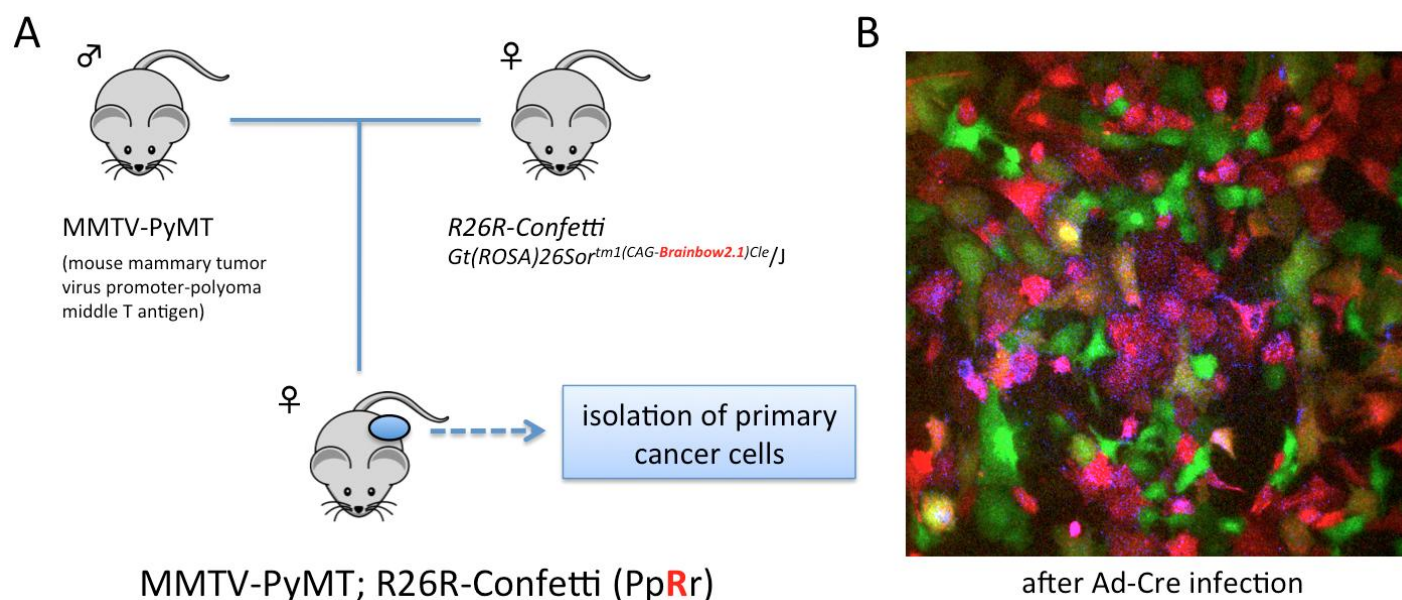


Figure 5. Generation of primary tumor cells stably expressing the Brainbow plasmid. (A) Breeding of MMTV-PyMT; *R26R-Confetti* mouse in background of the C57BL/6 mouse. When mammary tumor reached enough size to be surgically removed, primary cancer cells were isolated and kept frozen until next experiments. The *R26R-Confetti* mouse express Brainbow 2.1 plasmid that express either of four fluorescent proteins (RFP, YFP, nuclear GFP, or membrane-targeted CFP)¹⁴. **(B)** The isolated primary tumor cells were infected with adeno-Cre virus and further cultured for three days before observation with a confocal microscope.