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Iron and thiol redox signaling in cancer: an exquisite balance to escape ferroptosis

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Abbreviations:

aCGH, array-based comparative genome hybridization

AID/APOBEC, activation-induced cytidine deaminase

ACSL4, acyl-CoA synthetase long chain family 4

CBS, cystathionine β-synthase

CGL, cystathionine γ-lyase

CTH, cystathionine

ETC, electron transport chain

Fe-NTA, ferric nitrilotriacetate

GPX, glutathione peroxidase

GSH, reduced glutathione

ICGC, International Cancer Genome Consortium

8-OHdG, 8-hydroxy-2′-deoxyguanosine (8-oxodG)

MM, malignant mesothelioma

5hmC, 5-hydroxymethylcytosine

5mC, 5-methylcytosine

mtDNA, mitochondrial DNA

·NO, nitric oxide

NRF2, nuclear factor erythroid 2-related factor 2

NTA, nitrilotriacetate

NTP, non-thermal plasma (low-temperature plasma)

8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG)

PGRMC1, progesterone receptor membrane component 1

PHGPX, phospholipid glutathione peroxidase (GPX4)

RNS, reactive nitrogen species

ROS, reactive oxygen species

TET; ten-eleven translocation (dioxygenases)

Abstract

Epidemiological data indicate a constant worldwide increase in cancer mortality, although the age of onset is increasing. Recent accumulation of genomic data on human cancer via next-generation sequencing confirmed that cancer is a disease of genome alteration. In many cancers, the *Nrf2* transcription system is activated via mutations either in *Nrf2* or *Keap1* ubiquitin ligase, leading to persistent activation of the genes with antioxidative functions. Furthermore, deep sequencing of passenger mutations is clarifying responsible cancer causative agent(s) in each case, including aging, APOBEC activation, smoking and UV. Therefore, it is most likely that oxidative stress is the principal initiating factor in carcinogenesis, with the involvement of two essential molecules for life, iron and oxygen. There is evidence based on epidemiological and animal studies that excess iron is a major risk for carcinogenesis, suggesting the importance of ferroptosis-resistance. Microscopic visualization of catalytic Fe(II) has recently become available. Although catalytic Fe(II) is largely present in lysosomes, proliferating cells harbor catalytic Fe(II) also in the cytosol and mitochondria. Oxidative stress catalyzed by Fe(II) is counteracted by thiol systems at different functional levels. Nitric oxide, carbon monoxide and hydrogen (per)sulfide modulate these reactions. Mitochondria generate not only energy but also heme/iron sulfur cluster cofactors and remain mostly dysfunctional in cancer cells, leading to Warburg effects. Cancer cells are under persistent oxidative stress with a delicate balance between catalytic iron and thiols, thereby escaping ferroptosis. Thus, high-dose *L*-ascorbate and non-thermal plasma as well as glucose/glutamine deprivation may provide additional benefits as cancer therapies over preexisting therapeutics. (251 words)

Keywords: iron, cancer, carcinogenesis, redox signaling, ferroptosis

Next-generation sequencing of cancer

Ever since their origin, humans have been fighting against infectious agents, such as microbes, fungi and viruses, whether the events are major or minor [1, 2]. Tubercle bacilli has been the most formidable enemy thus far, as it can live serenely in the phagosomes of macrophages [3]. Historically, tubercle bacilli has killed millions of humans all over the world. However, effective antibiotics (streptomycin, isoniazid, rifampicin, ethambutol, *etc.*) were discovered during the 1940s to 1960s, and this major infection has finally been mostly stopped in developed countries [4, 5].

Currently, cancer is one of the leading causes of human mortality in many countries [6]. Since 1981, cancer is the leading cause of death in Japan, and the number of deaths is still increasing, presumably due to the elongation of the average lifetime. Fortunately, the 5-year survival rate for cancer overall has gradually increased and reached 62.1% in Japan for patients diagnosed between 2006-2008 (http://ganjoho.jp/en/index.html), reflecting the impact of early detection with biomarkers/medical check-ups, cancer prevention and novel therapeutics (protein kinase inhibitors [7] and humanized antibody therapies [8]).

Recent accumulation of genomic data on human cancer via next-generation sequencing strongly confirmed that cancer is indeed a disease of genome alteration (http://cancer.sanger.ac.uk/cosmic) [9]. With this technique, accurate analysis of the whole genome in cancer has become feasible for the first time, in contrast with previous studies that sequenced just a small fraction (several genes) of the genome. Furthermore, we can accurately analyze the frequency of certain mutations, reflecting tumor heterogeneity. This is especially evident for a cancer with a definite causal gene, such as renal clear cell carcinoma with the *VHL* tumor suppressor gene [10]. Detailed analyses of a clinical case revealed that each daughter cell or metastasized tumor nodule harbored additional mutations for each progression, similar to the illustration of the origin of the species proposed by Sir Charles Darwin **(Fig. 1)**. In

addition, the importance of genome information is ever increasing, because it dramatically changes therapeutics for advanced cancer, such as the *ALK*-fusion gene in lung cancer [11, 12] and the *BRAF* mutation in metastatic malignant melanoma [13, 14].

The International Cancer Genome Consortium (ICGC) was established to catalog and share a database of cancer genome mutations (http://icgc.org/). The success of this activity is very much based on the following two factors: 1) unexpected huge success in molecule-targeted cancer therapies, starting with imatinib in chronic myeloid leukemia [15] and gastrointestinal stromal tumor [16], and gefitinib/erlotinib in lung adenocarcinoma with the *EGFR* mutation [17]; 2) dramatic innovation of sequencers, which has enabled the efficient sequencing of target genes in paraffin-embedded clinical samples with an exon capture technique [18]. The ICGC, established in 2008, is the third international genome project after the Human Genome Project in 1990 [19] and the International HapMap Project in 2000 [20]. The purpose of the ICGC is to comprehensively record 50 different kinds of human cancer genome alterations, with at least 500 cases for each type of alteration. Currently, the US, China, the UK, Canada, France, Germany, Australia, Japan, Spain, Italy, Korea, India, Saudi Arabia and Singapore are working together [21].

Simultaneously, with the decreased cost of sequencing, ~7,000 cases with cancer genome data have been collected, and meta-analysis of passenger mutations was performed for the first time in 2013. This study classified the mutational patterns into 22 signatures (1A/1B to 21) and others, depending on the mutation itself and the bilateral adjacent bases, linking them with possible causative factors, including aging, activation of APOBEC family of cytidine deaminases, smoking and UV [22]. AID/APOBEC, known to be induced with inflammation via NF-κB transcription factor [23, 24], is associated with the handling of the oxidized form (5-hydroxymethylcytosine; 5hmC) of 5-methylcytosine to generate

5-hydroxymethyluracil [25], eventually causing C:G to T:A transition mutation [24]. According to these classifications, it is most likely that oxidative stress is a major cause of carcinogenesis, with the involvement of essential molecules for life, such as iron and oxygen [26, 27].

Thiol redox signaling in tumor biology

Of note, evolution of life on earth went through the era of Fe(II) and H_2S prior to the age of O_2 **(Fig. 2)** [28]. Approximately 15% of the genome consists of genes associated with oxidative metabolism, so its disruption via genetic/epigenetic alterations by any cause most likely leads to an increased oxidative stress. Superoxide is known to facilitate iron mobilization from ferritin to increase labile iron pool of catalytic Fe(II) [29]. Hypothetically, these events could drive the mutator phenotype [30] in a broad sense, contributing to carcinogenesis and its progression. An initiating event both in cancer and aging could be a disruption of important molecular structure for oxidative metabolism, leading to inefficiencies of electron flows and fluxes. In this review, we will follow this context, focusing on the role of iron and thiols in cancer.

Cancer cells are often under persistent oxidative stress [31]. This concept was established in the mid-1990s by the measurement and immunostaining of a most frequent oxidatively modified DNA 2'-deoxyribonucleoside, 8-oxo-7,8-dihydro-2’-deoxuguanosine (8-oxodGuo [32]; also as 8-hydroxy-2’-deoxyguanosine [8-OHdG] [33]) in combination with the analysis of the activity of the repair enzymes for 8-OHdG, OGG1 [34, 35] and the MutT homologue [36]. An increase in both 8-OHdG and repair enzymatic activity indicated that tumor cells are exposed to oxidative stress with increased ROS generation **(Fig. 3)**. This may be attributed to the increased expression of various oxidases, including NADPH oxidase [37], lysyl oxidase [38], cytochrome c oxidase [39] and monoamine

oxidase [40] under diverse tumor microenvironments. Recently, it was recognized that cancer cells and stromal cells, especially M2 macrophages [41-43] and myofibroblasts [44, 45], are interacting with each other in cases with poor prognosis, including relapse and epithelial-mesenchymal transition. In contrast, a recent paper reports that ablation of these myofibroblasts causes reduced survival in pancreatic carcinoma via immunosuppression [46], indicating that the contributions of stromal cells to cancer are not that simple. It is of note that both of the cells have an association with iron metabolism. Namely, the hemoglobin receptor (CD163) is associated with the M2 macrophage and myoglobin is associated with the myofibroblast.

In many cancers, the Nrf2 (nuclear factor erythroid 2-related factor 2) transcription system is persistently activated to induce numerous antioxidant enzymes via somatic mutations either in *Nrf2* or *Keap1*, resulting in inactivation of Keap1 ubiquitin ligase or the inability of Keap1 to approach Nrf2 [47-50]. This finding is one of the achievements of next-generation sequencing. Nrf2 was discovered during the study of maturation in erythroid lineage (putative transcriptional activator for beta-globin) [51, 52] and is a master transcription factor for coping with oxidative stress [53, 54]. Because most of the carcinogenic processes go through oxidative stress for initiation and promotion with selection processes, hijacking the Nrf2-Keap1 system is ideal for cancer cells to outgrow the other surrounding cells **(Fig. 4A)**. It is of note that the Nrf2 discovery is similar to that of nuclear factor erythroid 2, which is obviously associated with iron in the genesis of hemoglobin [55-57].

CD44, a cell adhesion molecule, is involved in various physiological processes, such as hematopoiesis, lymphocyte activation/homing, tissue remodeling and cell migration [58]. Alternative splicing of *CD44* mRNA gives rise to many different isoforms. The shortest isoform is known as the standard form or hematopoietic

isoform (*CD44s*) and is encoded by the standard exons of the *CD44* gene (exons 1-10). The insertion of variable exons may give rise to the various *CD44* variant isoforms (*CD44v*). *CD44s* is expressed ubiquitously by most vertebrate cells, whereas *CD44v* is expressed in some epithelial cells and in lymphocytes during maturation/activation, as well as in various cancer cells. Recent findings indicated an association between *CD44v* expression and tumor progression [59]. This is based on the stabilization of cystine/glutamate antiporter (system x_c^- [60, 61]) via CD44v, leading to an increase in reduced glutathione levels in cancer cells **(Fig. 4B)** [62] .

CD44v expression has been intimately associated with tumor progression and metastasis in many types of human cancer, such as pancreatic cancer, bladder cancer, colon cancer, and head and neck squamous cell carcinoma [63-66]. Moreover, *CD44v* expression is associated with cancer stem-like cell properties and thus is responsible for the chemoresistance of this subpopulation of cancer cells [67-69].

However, recent reports suggest that the functions of CD44 are more complex and cancer cell-specific. Most of the previous studies on the role of CD44 in malignant mesothelioma (MM) have centered on CD44s (standard form), not on the splicing isoforms. For example, Chew *et al.* are the first to show the coexpression of *CD44v* and *CD44s* isoforms in the majority of human MM cell lines, which differ from the non-tumorigenic MeT-5A human mesothelial cells that express only the *CD44s* isoform at a considerably lower level. Among the 14 MM cell lines examined, Y-MESO-12 was the only MM cell line that lacked *CD44s* expression and expressed only the variant isoforms. Nonetheless, *CD44s* appeared to be the predominant isoform expressed in the other MM cell lines, despite the expression of *CD44v*, indicating the rheostatic nature of *CD44* isoform expression [70]. CD44 is a known target of the Wnt signaling pathway [71]. As our previous study showed that β-catenin, an important downstream effector of the Wnt signaling pathway, is activated in rat MM cells through CTGF (CCN2) [72], the overall upregulation of

CD44 expression in MM cells might be correlated with the activation of β-catenin. Activation of the Wnt/β-catenin pathway in MM cells was previously reported [73, 74].

Glutathione (GSH) and thioredoxin are two major antioxidant systems in the cell; therefore, it is natural to think that they are protective against carcinogenesis. However, there are recent reports that GSH and thioredoxin synergistically promote carcinogenesis [75, 76]. This could be another example of cancer hijacking the antioxidant system other than the Nrf2-Keap1 system, but we suspect that there are some problems that need be considered in the animal models. In these works, genetically engineered mice were used in which certain carcinogenic steps, especially mutations, are omitted. It is possible that the GSH and thioredoxin systems work every day to prevent mutations by decreasing/stopping ROS reactions and promoting genome repair processes. In human carcinogenesis, a mutation is a stochastic process [77], which has to be fully considered. On the other hand, it is generally true that the GSH [78] and thioredoxin [79] systems are activated in cancer cells. Thus, if specific inhibition of these two systems is possible only in cancer cells, it may work as a cancer therapy [80, 81].

Evidence of iron involvement in human carcinogenesis

Many pathological conditions result in oxidative stress in tissues and cells. These conditions, summarized in **Table 1**, are all associated with an increased risk for carcinogenesis except for reperfusion injury, where acute clinical effects are usually so overwhelming that the incubation period for carcinogenesis would not be sufficient. **Figure 5** represents the current classification of carcinogenic agents. We believe that each agent is similar to the architectural structures or mountains on the earth and that the huge bottom portion of the earth represents endogenous oxidative stress derived from the use of iron and oxygen.

Iron is the most abundant heavy metal in mammals. Adult human males contain ~4 g of iron, and as much as ~60% of the iron exists as a heme cofactor in hemoglobin in erythrocytes. Life on earth is impossible without iron as far as we know. Because iron has been so inaccessible during evolution, we do not have any active metabolic pathway to release iron from our body. Iron is absorbed from the diet via DMT1 (SLC11A2) [82, 83], by duodenal villi or via parenteral injection as a medical therapy. Daily excretion of iron is possible only through unnoticed hemorrhage or cellular loss from the skin or mucosa, which accounts for ~1 mg per day, whereas ~1 mg of iron is absorbed from the diet through duodenal epithelial cells every day [84]. Thus, iron metabolism in an individual is a semi-closed system. Monthly menstruation in females of reproductive age and pregnancy, which provides iron to the fetus, are two typical causes of iron loss in women. Of note, inner circulation of iron is incredibly fast, considering that the lifetime of red blood cells in humans is ~120 days; therefore, approximately 0.8% is degraded every day in the spleen or other cells belonging to reticuloendothelial system. Iron is taken out of heme in red blood cells by heme oxygenase in splenic macrophages and is sent to the bone marrow via serum transferrin, which adds up to ~19.2 mg per day. Various pathological conditions produce excess iron in or nearby parenchymal cells, including genetic alterations in the iron sensing system (hereditary hemochromatosis), repetitive hemorrhage in an isolated small organ (ovarian endometriosis), long-standing inflammation (viral hepatitis B/C) and atmospheric exposure to a nanosized foreign body (asbestos) [84, 85]. Each condition is associated with carcinogenesis of specific target epithelial/mesothelial cells (**Table 2**).

The most efficient way to remove iron from our body is phlebotomy or blood donation. It was reported in 2008 that phlebotomy of 500 ml of blood twice a year for 5 years significantly decreased cancer incidence (hazard ratio, 0.65) and cancer-specific mortality (hazard ratio, 0.39) in healthy volunteers except for the

presence of peripheral arterial disease [86]. More specifically, in chronic hepatitis C patients, elevated hepatic 8-OHdG levels were normalized with phlebotomy and low iron diet [87], which have been one of the registered therapies in Japan. Indeed, phlebotomy lowered the incidence of hepatocellular carcinoma in these patients in a prospective study [88]. In this sense, excess iron can be the most common but underscored cause of oxidative stress and carcinogenesis, though we need more confirmation with epidemiological studies.

Ferric nitrilotriacetate (Fe-NTA)-induced renal carcinogenesis as a versatile model

Now that gene-editing techniques for mice and rats have become well developed [89-91], they are often used to demonstrate a high incidence of cancer by skipping the processes of mutations. However, we ourselves are essentially wild-type animals except for rare cases of familial cancer syndromes, such as germ line mutation of *BRCA1* or *BRCA2* [92]. In the 1990s, our research group confirmed the high reproducibility of renal carcinogenesis in wild-type rats by repeated intraperitoneal administration of Fe-NTA for 12 weeks, where approximately 90% of male Brown-Norway, Fischer-344 and Wistar strains develop renal cell carcinoma after 1 year, with half of the cases metastasizing to the lungs [93-96]. This model can be applied to mice [97]. Such a reproducible carcinogenesis model of a highly malignant solid tumor in wild-type animals has never been reported to our knowledge. Furthermore, an acute-phase experiment is highly reproducible such as Fenton reaction-induced oxidative renal tubular damage [98-113], which eventually leads to renal tubular carcinogenesis. Regarding mice, we obtained a relatively lower incidence of carcinogenesis: 60% in *A/J* mice [97] and 10% or less for *C57BL/6* mice (unpublished data). There is a marked strain difference, which requires further investigation to identify the mechanisms involved.

Hydroxyl radicals (·OH), the most reactive species in the biological system, are the major chemical species responsible for Fe-NTA-induced renal carcinogenesis via Fenton-like reactions *in vivo* [101, 114, 115]. Accordingly, they react with any biomolecule nearby, leading to aldehyde formation [101, 102, 116, 117] and oxidatively generated DNA modifications [103, 118, 119] as mentioned earlier, together with DNA/protein strand breaks [120, 121] and the resulting cross-links between them [122]. There have been discussions for decades over the actual amounts of oxidized products, where those measured by gas chromatography/mass spectrometry [103, 122] might show higher amounts, thus necessitating a care when comparing data among different methodologies [123]. Theoretically, transition metal has to be located in the close vicinity of DNA in order to trigger genomic damage. These are indeed complex *in vivo* reactions involving lipids, proteins and nucleic acids, and a full understanding of these reactions is difficult with the current methodologies, leaving some black-box reactions. Notwithstanding, this is some of the strongest evidence that repeated iron-catalyzed Fenton reactions [124] have the ability to induce cancer *in vivo*, which contributed tremendously to the establishment of currently used oxidative stress markers [27, 125].

The next question was whether there were any target oncogenes or tumor suppressor genes in Fenton reaction-induced carcinogenesis. Because there was no whole genome data for rats available in the 1990s, we used a microsatellite analysis strategy to determine the common target genes. By analyzing tumors produced in F_1 hybrid rats, we reached the conclusion that the *p15/p16* tumor suppressor gene is a major target gene [96]. Its homozygous deletion or hemizygous deletion with methylation of the promoter region was observed in one third of tumors, respectively. We observed the hemizygous deletion at the *p16* locus in the renal tubular cells as early as a few weeks after the start of Fe-NTA injections [126]. The *p15/p16* tumor suppressor gene is the second most frequently silenced tumor suppressor gene in

human cancers after *p53* [127].

Coming into this century, we could use array-based comparative genome hybridization (aCGH). We reanalyzed the genomic alteration of the Fe-NTA-induced renal cell carcinoma with the aCGH method [128]. In addition to confirming our previous results, we found amplification of common target oncogenes, such as *c-Met* (hepatocyte growth factor receptor) and *Ptprz1* (tyrosine phosphatase) [129]. Notably, recurrent fusion of these two oncogenes, adjacently located on a single chromosome, were reported in human secondary glioblastomas [130]; second, alterations in terms of genomic deletions and amplifications were prominent at the chromosomal level, which was in marked contrast with other animal cancer models except for massively genetically engineered mice [131]; and lastly, the genomic alterations were most similar to human renal cell carcinoma, when compared with the Oxford grid information [132], with the second most similar being human malignant mesothelioma [128]. These results suggest that iron excess and the ensuing oxidative stress may also be responsible for human cancers at least partially, especially for the deletion of the *p15/p16* tumor suppressor genes. These results continue to apply to the pathogenesis of asbestos-induced malignant mesothelioma [133-136] and its similarity to that of multiwall carbon nanotube-induced malignant mesothelioma in rats [137, 138], in which exactly the same tumor suppressor genes, *p15/p16*, are the targets, concomitant with local iron excess near the target cells. We believe that there are target genes, such as *p15/p16*, in excess iron (oxidative stress)-induced carcinogenesis [139-141].

Then, the third question was whether there are any portions in the genome susceptible to iron-catalyzed oxidative stress [140, 142]. There are various types of DNA damage, including single and double strand breaks, base modifications, abasic sites and cross-links [143]. More likely, DNA double strand breaks, followed by erroneous repair processes, are directly associated with the amplification and

deletion of genes. Based on our previous data on the proportional association of strand breaks and 8-OHdG in plasmid DNA [144], we concentrated on the 8-OHdG localization in the genome. As a strategy, we employed immunoprecipitation, using a monoclonal antibody against 8-OHdG (N45.1) [118, 145]. Fortunately, the affinity of N45.1 monoclonal antibody to 8-OHdG in DNA fragments was high enough to allow for specific immunoprecipitation of the DNA fragments containing 8-OHdG [146].

First, we used the mouse kidney after intraperitoneal Fe-NTA administration for immunoprecipitation, followed by sequencing each fragment after cloning. We also used subsequent PCR reactions to amplify the target genomic loci. We found that the extra-gene area of the genome accumulates more 8-OHdG, and that exposure to oxidative stress or knockout of *Ogg1* (repair enzyme of 8-OHdG) accumulates additional 8-OHdG. We also observed that the *p15*/*p16* loci are indeed more susceptible [146]. We believe that this ground-breaking observation opened up a novel research category called *OXYGENOMICS*, the study of the susceptibility of fragile genomic loci to oxidative stress [142] **(Fig. 6A)**. This phenomenon is associated with the concept of chromosome territory [147, 148], the transcriptional status of the loci and the chemical species generated (oxidative stress), which are expected to be different among the various cell types, cell cycle stages and pathological conditions.

We have recently applied this technique in combination with the aCGH method to the genome of the rat kidney, which revealed another rule. 8-OHdG is concentrated on the lamina-associated domain, which sustains the nuclear membrane structure [149]. ROS are abundant in the cytoplasm; therefore, the results support the bodyguard hypothesis where the genomic region closest to the nuclear membrane protects the rest of the more important genome being transcribed deep inside the nucleus **(Fig. 6B)**. Susceptibility of each genomic locus to oxidative

stress and the following dysfunctional repair and selective processes for proliferation appear to be the critical pathogenic events in oxidative stress-induced cancer. Other models include malignant mesothelioma, which is described in detail elsewhere [133, 135, 136, 150].

Ferroptosis

For the past few decades, two types of cell death processes, necrosis and apoptosis, have been intensively discussed; generally, necrosis is an unintended, passive and diffuse form of cell death, whereas apoptosis is a programmed, strictly regulated, restricted to single cells and energy-consuming form of cell death. However, this paradigm in the research area of cellular death has been drastically altered recently as a novel form of iron-dependent, non-necrotic, and non-apoptotic cell death has come to light. Ferroptosis, an emerging concept proposed in 2012, is defined as non-apoptotic programmed cell death that can be inhibited by Fenton-reaction inhibitory iron chelators such as desferal [151] **(Figure 7)**. Two opposing types of iron chelators exist. The aforementioned nitrilotriacetate (NTA) used in the Fe-NTA-induced renal carcinogenesis model is an iron chelator that promotes the Fenton reaction [120].

Ferroptosis was first reported by a set of small molecules in genetically engineered human fibroblasts overexpressing oncogenic *H-Ras*. Erastin was used to induce this special type of programmed cell death, and lipid peroxidation-derived signals promoting cell death were recognized as a hallmark of ferroptosis [151]. Glutathione peroxidase 4 (GPX4; formerly as phospholipid glutathione peroxidase, PHGPx), a selenoprotein, is distinct from the other GPXs in that GPX4 alone can directly regulate membrane-associated lipid peroxidation [152]. GPX4 was reported first to inhibit apoptosis [153, 154] and thereafter as a key regulator of ferroptotic cellular death [155]. The phenotype of GPX4 knockout mice is embryonic lethal

[156] but spermatocyte-specific depletion causes infertility due to the low sperm motility [157], which explained some fraction of human male infertility [158, 159]. Of note, the inactivation of GPX4 triggers acute renal tubular lumen cell death [160].

These data suggest a crucial role for membrane-associated iron in initiating the Fenton reaction in ferroptosis. Accordingly, the study of the pathogenesis of excess iron-induced carcinogenesis led to one important concept, resistance to ferroptosis, in addition to iron addiction [27]. In other words, cancer cells can resist iron-induced oxidative stress through genetic alteration but still require more iron for proliferation and experience persistent oxidative stress, albeit at a low level [31, 36, 161, 162]. The metabolic balance of ROS in cancer cells is indeed maintained within a narrow range, indicating a delicate balance between proliferation and dilution effects through mitosis **(Figure 8)**. Furthermore, reports revealed that glucose deprivation [163, 164] and recently glutamine deprivation [165, 166] selectively cause metabolic oxidative stress in cancer cells *versus* normal cells because glucose and glutamine provide precursors for nucleic acids, proteins and lipids, and reducing equivalents to detoxify increased steady-state levels of oxidants in cancer cells in comparison to normal cells. In this regard, involvements of macropinocytosis [167] and autophagy [168] are under hot discussion.

It is of note that the idea of ferroptosis strongly depends on the battle between iron (oxidants) and thiols (antoxidants). Thus, its origin goes back to the discovery of cystine transporter system (cystine/glutamate antiporter; system x_c^- [60, 61]). **Table 3** summarizes the historical findings associated with ferroptosis and Fe-NTA-induced renal cancer model, which we now understand fit well each other in many aspects. Recently, two independent groups suggested, based on CRISPER-based genome-wide screening [169] and/or mass spectrometric lipidomics approaches [170], that doubly and triply oxygenated species of arachidonic- and adrenic acid-containing phosphatidylethanolamines generated through acyl-CoA

synthetase long chain family 4 (ACSL4) are responsible for directing cells into ferroptosis.

Catalytic iron inside and outside cells

Catalytic (labile; free) iron is defined as iron that can catalyze the Fenton reaction and may be chelated by small molecules. Catalytic ferrous iron is recognized as an important initiator of the Fenton reaction. Historically, detection via bleomycin-dependent lipid peroxidation has been used for decades [174]. However, a biochemical method of this kind does not allow for localization analysis in cells and tissues. Turn-on type visualization of catalytic Fe(II) for morphological analyses became available for the first time in 2013. Hirayama *et al.* have succeeded in synthesizing a novel fluorescent probe, RhoNox-1, starting from the rhodamine backbone structure [175, 176]. This probe is extremely useful for analyzing both live cultured cells and frozen sections **(Figure 9)**. We have shown the dose- and time-dependency of catalytic Fe(II) in the kidney in the Fe-NTA model, using frozen sections [177]. Of note, Fe(II)-NTA did not react with RhoNox-1 *in vitro*; therefore, we realized that chelation was lost in the renal tubular lumen after an intraperitoneal injection, followed by an interaction between catalytic Fe(II) and the luminal membrane *in situ* under reducing conditions [114].

Recently, we studied all kinds of organs/cells in rats to evaluate the distribution of catalytic Fe(II) and found that eosinophils show the highest abundance. Among the organelles, the lysosomes have the highest abundance, sharing ~40-80% of RhoNox-1 fluorescence. This mainly depends on the acidity inside the lysosomes, where higher amounts of iron can be solubilized. Of interest, proliferating cells in general, especially cancer cells, harbor more catalytic Fe(II) in the cytosol and mitochondria [178]. We then used an ovalbumin-induced allergic peritonitis model to study the dynamics of catalytic Fe(II) *in vivo*. Peritoneal lavage, including cells

and fluid, revealed that an increase in the number of inflammatory cells (macrophages, neutrophils, eosinophils and lymphocytes) resulted in increased "total" iron content within the peritoneal inflammatory cells, whereas the "total" iron content per cell was significantly decreased. Notably, macrophages, eosinophils and neutrophils exhibited significantly increased "catalytic" Fe(II) with increased *DMT1* expression and decreased *ferritin* expression, whereas "catalytic" Fe(II) was significantly decreased in the peritoneal lavage fluid. Thus, catalytic Fe(II) *in situ* is a more straightforward reflection of cellular activity, indicating that inflammation basically works to maintain intracellular iron with a higher fraction of the catalytic form functionally available so as not to feed iron to microorganisms [178].

Here we will discuss the role of ferritin in iron storage and mobilization from the context of catalytic ferrous iron. In mammalians, ferritin, 24 subunits assemble to form a hollow symmetrical protein with a molecular weight of ~480 kDa; the multimer is a shell with an outer diameter of 12 nm with a 8-nm-diameter cavity accommodating up to 4,500 Fe(III)OOH [179]. Three different types of monomer are in mammalian cells: the H (FtH, heart/heavy, ~21kDa) and the L (FtL, liver/light, ~19 kDa) monomers are constituents of the abundant cytosolic ferritins, whereas mitochondrial subunit (FtMt, ~21kDa) is found exclusively in the mitochondrial ferritin. H monomer works as ferroxidase to store iron in the shell [179]. Recently, it was shown that ferritinophagy via NCOA4, a selective cargo receptor, plays a role in recruiting Fe(II) for use in erythropoiesis by combining autophagy and proteasome-dependent proteolysis of ferritin via HERC2 [180, 181]. Thus far, various factors have been reported to increase catalytic Fe(II) (iron mobilization from ferritin), including *L*-ascorbate [182], superoxide [29, 183], proteasome [184] and chelating agents [185]. Hypoxia induces ferritin expression [186] presumably via higher iron solubility at lowered pH and ferritin-mediated iron sequestration then stabilizes hypoxia-inducible factor 1-α [187]. In these circumstances, turn-on

fluorescent probes described above would be useful for future studies.

Modulators in thiol redox signaling

Major modulators of thiol redox signaling include three distinct gaseous molecules: nitric oxide, carbon monoxide and hydrogen (per)sulfide, which can reversibly compete or strengthen sulfhydryl functions **(Figure 10)**.

It is accepted that cancers in general express features of inflammation driven both by microenvironmental and chemical factors and the intrinsic production of various cytokines by tumor cells. Inflammation causes oxidative stress via various ROS and reactive nitrogen species (RNS). Peroxynitrite, generated through the chemical reaction of $O_2^{\cdot -}$ and nitric oxide ($^{\cdot}NO$), works in a similar fashion to hydroxyl radicals, generated through the iron-catalyzed Fenton reaction, and causes oxidatively generated damage or may react with available tyrosine or thiol-containing proteins to form 3-nitrotyrosine or reversible nitrosylation of thiols (S-NO) [188-190]. More precisely, peroxinitrite may react first with carbon dioxide/bicarbonate into unstable nitrosoperoxicarbonate, the precursor of carbonate anion radical and $^{\cdot}NO_2$ radical [191, 192]. Thereafter, highly oxidizing guanyl and tyrosyl radicals, the products of one-electron oxidation mediated by carbonate anion radical, are able to recombine with $^{\cdot}NO_2$ radical.

However, despite the extensive work on the role of NO in carcinogenesis and tumor biology, NO has been shown to promote or inhibit carcinogenesis/malignancies, depending on the types of cells and situations [193]. Recently, NO has been suggested to support the surrounding environments for cancer proliferation, and the concentration of NO is important for macrophage differentiation, either to the tumoricidal M1 phenotype (~nM order) or to the protumor M2 phenotype (~μM order) [194]. Furthermore, according with technical advancements, many proteins were identified as undergoing S-NO modification.

For example, many GTPases within the Ras superfamily contain redox-sensitive Cys residues that are susceptible to S-nitrosylation, which is associated with the initiation of tumorigenesis and the maintenance of established tumors. We should not forget that all three NO synthases are heme enzymes. Recently, it was found that glutathione *S*-transferase P1-1 and multidrug resistance-associated protein 1 (MRP1) form an integrated NO storage and transport system that protects cells against NO toxicity via dinitrosyl-diglutathionyl iron complexes [195].

Among the diverse electrophilic molecular species that are endogenously generated, 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP) is a unique second messenger whose formation, signaling and metabolism in cells was recently clarified. Importantly, these studies revealed that reactive cysteine persulfates that are formed abundantly in cells are critically involved in the metabolism of 8-nitro-cGMP [196, 197].

Carbon monoxide (CO) has emerged as an important regulator of inflammation recently [198]. CO appears to stop cancer proliferation and chemoresistance through a putative membrane receptor. Progesterone receptor membrane component 1 (PGRMC1) is a multifunctional protein with a heme-binding moiety related to that of cytochrome b_5, which is a putative progesterone receptor. Dimerization is required for association with the epidermal growth factor receptor and the cytochrome P450 enzymes, which mediate chemoresistance to doxorubicin and may be responsible for PGRMC1's anti-apoptotic activity. Kabe *et al.* recently found that CO inhibits this dimerization [199]. PGRMC1 exhibits involvement in diverse functions, including regulation of cytochrome P450, steroidogenesis, vesicle trafficking, and regulation of the mitotic spindle and the cell cycle. Its wide range of biological functions is associated with its emerging relationship with cancer and progesterone-responsive female reproductive tissues. PGRMC1 exhibits all the hallmarks of a higher-order nexus signal integration hub protein. It appears

capable of acting as a detector that integrates information from kinase/phosphatase pathways with heme, CO levels and probably redox status [200].

Lastly, UV radiation that mostly acts on DNA by inducing pyrimidine dimers is a poor direct inducer of oxidative reactive species at the exception of UVA component, which is able to generate singlet oxygen through photosensitization II mechanism. However, UV radiation as most genotoxic agents is able to trigger a delayed generation of superoxide anion radical and NO as part of inflammation response [201].

Metabolomics and cystathionine

Recently, Sen *et al.* have identified cystathionine, a sulfur-containing metabolite, to be selectively enriched in human breast cancer tissues (~50-100 pmoles/mg protein) compared with undetectable levels in normal breast tissues [202]. The accumulation of cystathionine, specifically in human breast cancer, was attributed to the overexpression of cystathionine β-synthase (CBS), its synthesizing enzyme, and the undetectable levels of its downstream metabolizing enzyme, cystathionine γ-lyase (CGL). Of interest, both CBS and CGL were not detected in normal breast tissues. They further observed that cystathionine protected human breast cancer cells against excess ROS and chemotherapeutic drug-induced apoptosis. Moreover, cystathionine promoted both mitochondrial and endoplasmic reticulum homeostasis in human breast cancer cells. As both the mitochondria and the endoplasmic reticulum are the key organelles involved in regulating apoptosis, they reasoned that endogenous cystathionine could be contributing to the increased apoptotic threshold in human breast cancer cells. It may not be a coincidence that CBS/CGL are, at least partially, responsible for the generation of cysteine persulfide [197].

Mitochondrial iron in cancer

It is believed that the relationship between mitochondria and the host cells comes from the α-proteobacterial endosymbiont [203]. Mitochondria are the synthetic sites of heme [204] and iron-sulfur (Fe-S) clusters [205] as cofactors and generate ATP by oxidizing lipids, amino acids and glucose and by transferring the electrons derived from these reactions to the electron transport chain (ETC), which ultimately delivers them to molecular O_2. A normal membrane potential of -180 millivolts across the inner membrane of 7-nm-thickness produces strong electrical field strength within mitochondria. Under normal conditions, mitochondria trigger redox signaling in the cell through the release of ROS from ETC. However, they can become dysfunctional by a variety of different mechanisms and decrease in number in cancer cells, which is closely associated with continuous proliferation presumably through excess ROS generation [206, 207].

Mitochondria are equipped with special iron transporting and storing molecules. Mitoferrin 1 and mitoferrin 2, homologous members of the mitochondrial solute carrier family, contribute to mitochondrial iron delivery in a variety of cells [208]. Reduction in mitoferrin 1 and/or mitoferrin 2 by knockdown results in decreased mitochondrial iron accumulation, heme synthesis and iron-sulfur cluster synthesis. It appears that mitoferrin 1 is more important in erythroid cells. Although cytosolic ferritins are ubiquitous in mammals, mitochondrial ferritin expression is restricted mainly to the testis, neuronal cells and islets of Langerhans, presumably protecting important cells (involved in reproduction, intelligence and sugar metabolism, respectively) from iron-catalyzed oxidative damage [209]. Recently, it was reported that mitoferrin-2-dependent mitochondrial iron uptake sensitizes human head and neck squamous carcinoma cells to photodynamic therapy [210].

Fe-S clusters are classical ubiquitous cofactors composed of iron and inorganic sulfur. The combination of the chemical reactivity of Fe and S, together with the many variations of cluster composition, valence status and proteins, enables the Fe-S

cluster to participate in numerous biological processes, including mitochondrial respiration, regulation of iron metabolism, the oxidative stress response and maintenance of the nuclear genome. Of note, diseases caused by defects in Fe-S cluster biogenesis are all severe metabolic diseases in either muscle, heart, brain or erythrocytes [211], and a cancer-prone syndrome is not included.

Heme homeostasis is a highly coordinated process, consisting of biosynthesis, transport and degradation. The biogenesis and degradation processes are well characterized, but the pathways for heme trafficking and incorporation into hemoproteins remain poorly understood [212]. Free heme is a cytotoxic molecule that generates ROS and disrupts lipid bilayers and organelles. Accordingly, cytosolic carriers or chaperones that function to sequester or transport heme are important. Currently, many intra- and extra-cellular heme transporters/binding proteins have been reported, including glutathione *S*-transferase, heme-binding proteins, fatty acid-binding proteins (intracellular trafficking) as well as HRG-1, HCP1, FLVCR1/2 and ABCG2 (importer/exporter) [212]. Among those, it was recently reported that hemopexin-dependent heme uptake via endocytosis regulates the Bach1 transcription repressor and heme oxygenase gene activation [213]. A recent study using human non-small-cell lung cancer cells showed substantially increased levels in an array of proteins promoting heme synthesis, uptake and function, knockdown of which significantly reduced oxygen consumption, proliferation, migration and colony formation [214].

Mitochondria have their own genome (mitochondrial DNA; mtDNA), which is derived from oocyte mitochondria and shows extensive sequence variability within the population. A recent systematic study using conplastic mice revealed that the mtDNA haplotype profoundly influences mitochondrial proteostasis, ROS generation, insulin signaling and aging parameters [215]. Some human cancers, such as hepatocellular carcinoma [216] and breast cancer [217], reveal a high

incidence of somatic mutation in mitochondrial DNA. The accumulated mtDNA mutations reflected the degree of malignancy (poor differentiation) in hepatocellular carcinoma [216]. Somatic mutations in mtDNA are found in 73.7% of breast cancer, with a dramatic decrease in mt DNA content per cell and a positive correlation between mtDNA mutational burden and patient survival [217].

Comparison to pregnancy regarding iron uptake

Although carcinogenesis and fetal development are completely different biological phenomena, there are numerous common aspects, especially in the speed of cellular proliferation to produce a mass, including a fetus and the placenta in pregnancy. In a clinical setting, it is extremely rare to observe a 3-kg tumor mass that has grown in 9 months despite the monoclonality of the cells. Trophoblast cells in the placenta, much like cancer cells, metabolize glucose without the use of molecular oxygen by a phenomenon called the Warburg effect. This constitutes a unique acidic environment that favors the secretion of cytokines and growth factors that initiate angiogenesis and tissue repair [218], in which a2 V-ATPase appears to play a role [219, 220].

The precise molecular mechanisms underlying iron transfer from the maternal liver, the major iron storage organ, to the fetal liver via the placenta are largely unknown. Transferrin and its receptor system [221] are used in trophoblasts in the placenta as well as in other cells [222], but it was shown that DMT1 (Slc11a2) is not essential for iron transport across the placenta [223]. It is interesting to note that another iron transporter works during organogenesis in the fetus. A lipocalin superfamily member (lipocalin 2, 24p3, Ngal) delivers iron to the cytoplasm where it activates or represses iron-responsive genes and is differentially expressed from transferrin during the conversion of the mesenchyme into epithelia [224]. Lipocalin 2 is now known as a siderophore-binding protein, and its major task is to collect iron

from infected bacteria [225]. A possible mammalian siderophore is 2,5-dehydroxybenzoic acid [226]. Furthermore, lipocalin 2 is overexpressed in a variety of cancers and plays a role in supporting proliferation [109, 227-230].

Hypothetical essence of iron and thiols in cancer

We humans cannot live without oxygen, and the internal milieu of our cells has a mildly reducing condition with pH 7.4. This indicates that our cells are under a relatively electron-rich condition, and oxygen is used as a mediator for persistent electron flow [27], in which the use of iron for oxygen transport (hemoglobin) is the most integral part. As described above, we have obtained fine, complicated but organized metabolic networks during the evolutionary processes. After the recent major victory over the infectious agents, now "wear and tear with friction" or the side effects of ROS are becoming the major problem in the course of ever-increasing average lifetime of humans.

Iron as a transition metal (Fe(II)/Fe(III)) has a characteristic of relatively high solubility as Fe(II) but has much higher reactivity with H_2O_2 to generate $^{\cdot}OH$. Iron works in the form of Fe(II) as a cofactor or heme, whereas iron is stored (ferritin) or transported (transferrin) in the form of Fe(III). Therefore, catalytic Fe(II) should be minimally low in the cytosol of normal state non-tumorous cells. However, in tumor cells or highly proliferating cells, recent data suggests higher catalytic Fe(II) in the cytosol [178]. This has to be counteracted by reducing agents (thiols) or by decomposing ROS, which is consistent with the overall findings regarding cancer metabolism.

Simultaneously, we would like to note the dilution effects (**Figure 8**). This word was originally used to predict the prevalence of certain microbial diseases (such as Lyme disease induced by spirochete via tick bite, where mice, deer and birds propagate infected ticks). The original dilution effect means that an increase in the

number of non-host species would decrease the prevalence of the disease [231]. Here, we mean that rapid proliferation allows for the presence of a higher concentration of toxic substances, such as catalytic Fe(II), in accordance with the higher metabolism and more volume/media per time units. Accordingly, we believe that cancer cells maintain a delicate balance between oxidative toxicity and dilution effects through proliferation, leading to a low level of persistent oxidative stress as a result [31], still escaping ferroptosis.

By understanding the characteristic features of cancer cells described, high-dose *L*-ascorbate and low-temperature (non-thermal) plasma may produce additional benefits over preexisting cancer therapeutics. Recent two articles support this view of ours in that catalytic iron may be a unique characteristic of lung, brain and breast cancer cells that can be targeted with pharmacological doses of *L*-ascorbate [232] or *D*-penicillamine [233] for cancer therapy.

Role of *L*-ascorbate in epigenetics and cancer therapy

Emerging evidence suggests that *L*-ascorbate [234], the dominant form of vitamin C, exerts influence on genome activity via demethylating 5-methylcytosine (5mC). *L*-Ascorbate serves as a cofactor for ten-eleven translocation (TET) dioxygenases that catalyze the oxidation of 5mC into 5-hydroxymethylcytosine (5hmC), which is further modified to 5-formylcytosine and 5-carboxylcytosine, which are ultimately replaced by unmodified cytosine. An abundance of 5mC in the promoter region of tumor suppressor genes is closely associated with the absence of their transcription and thus are one of the critical processes for carcinogenesis and tumor biology [127]. The JmjC domain-containing histone demethylases also require *L*-ascorbate as a cofactor for histone demethylation [235, 236]. TET dioxygenases are also dependent on Fe(II) and α-ketoglutarate (2-oxoglutarate), which do not affect the enzymatic activity at a variety of concentrations [237]. In contrast to the relatively high level of

5hmC in embryos, cancer cells have very low or undetectable levels of 5hmC [238-241]. Recent meta-analysis shows that higher intake of *L*-ascorbate might have a protective effect against lung cancer [242].

Due to the benefits of ascorbate re-expressing the shutdown tumor suppressor genes described above, high-dose ascorbate is suggested as a cancer therapy. Furthermore, there is another action, depending on the prooxidant activity of *L*-ascorbate in combination with Fe(II) [120, 144, 234], which is observed in higher amounts in cancer cells in comparison to non-tumorous cells as described [178]. This was suggested already in the 1970s, but oral administration of 10 g *L*-ascorbate per day failed in a clinical trial [243]. Presently, this is performed with parenteral administration and is exhibiting good tolerance and outcomes with standard therapies in many clinical trials [244-246]. Recently, a novel mechanism was suggested; *L*-ascorbate may target the GAPDH enzyme with oxidation, thus killing only *KRAS*- and *BRAF*-mutant colorectal cancer cells [247]. A recent report is promising for pancreatic cancer [248].

Non-thermal (low-temperature) plasma

During the last ten years, an exciting cooperative movement between engineering and biology/medicine has occurred to apply non-thermal plasma (NTP) to medical instruments and therapeutics [249-252]. Plasma is the fourth condition of physical states, beyond the normal solid/liquid/gas phase and is a mixture of gas/radicals/electrons/cations/anions/UV [253, 254]. The sun, lightning and the aurora represent plasma in nature, which emits an extraordinary amount of energy. Non-thermal atmospheric plasma has been developed recently **(Figure 11)**.

This is indeed an innovative research area. Now it is becoming clear that we can load cells with adjustable near-natural oxidative stress with a handy machine at a much milder degree than radiation [255]. Many chemical species/agents have

already been detected with NTP exposure, including ·OH, H_2O_2, $O_2^{\cdot-}$, ·NO, electrons and UV. We believe that the major species are hydroxyl radicals and UV [256]. One of the critical issues in this area is that standardization of the apparatus has not been organized worldwide thus far. Despite these situations, there is sufficient evidence to demonstrate that NTP works to promote would healing [257, 258] and possibly kills cancer cells [259, 260]. The theoretical basis for NTP is that cancer cells are oxidatively stressed [31]. Therefore, additional oxidative stress is expected to kill cancer cells *in situ* but not non-cancer cells. We recently noticed the dual effects of *L*-ascorbate when exposing cancer cells to NTP; specifically, the addition of *L*-ascorbate to media immediately prior to NTP exposure enhances cancer cell ablation, whereas incubation with *L*-ascorbate for more than a few hours decreases the cytotoxicity, suggesting a key role of iron in the mechanisms involved [261]. Furthermore, media or infusion solution exposed to NTP appears useful for many applications, including age-related macular degeneration in the retina [262] and cancer therapy [252, 263]. Not only hydrogen peroxide and nitrite/nitrate but novel organic species appear to work in these situations, and intensive studies are now in progress.

Conclusion

Chronic iron overload is associated with carcinogenesis. Cancer cells are under persistent oxidative stress with a delicate balance between catalytic iron and thiols, in other words, iron addiction with ferroptosis-resistance. Thus, high-dose *L*-ascorbate and low-temperature plasma may provide additional benefits as cancer therapies over preexisting therapeutics. For cancer prevention, we believe that appropriate iron reduction with phlebotomy/blood donation may lower cancer risk in the presently healthy population, especially in middle-aged males. Further clinical trials are warranted to confirm the merits of these interventions. Recently,

poly(rC)-binding protein 2 is suggested as an intracellular iron chaperone molecule [264, 265]. Considering the emerging concept of ferroptosis and novel techniques to visualize catalytic Fe(II), this area is of much interest.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Carcinogenesis as multiple and additional somatic mutations through similar mechanism to Darwinian evolution. A-H are independent somatic mutations that occurred either on oncogenes (+, activation) or tumor suppressor genes (-, loss of function). Note that this is a simplified scheme and cancer in adulthood in general has obtained 5-6 effective somatic mutations prior to its establishment.

Figure 2. Time line of evolution relative to ferrous iron, hydrogen sulfide and oxygen on earth. Modified based on [28]. GOE, great oxidation event when Fe(II) in ocean was oxidized to Fe(III) sedimentation (iron ore). Note that this figure is conceptual.

Figure 3. Persistent oxidative stress in cancer. Simplified scheme of the relation between the generation of reactive oxygen species and the repair of the oxidative damage induced thereby.

Figure 4. Two cases of hijacking preexisting antioxidant systems by cancer cells. A: Nrf2-Keap1 system. Keap1 is an E3 ubiquitin ligase for Nrf2, which is a master transcription factor for antioxidative enzymes/molecules. Somatic mutation either in *Nrf2* or *Keap1* to block the interaction of these two proteins causes persistent activation of Nrf2 transcription factor, which is a big advantage for proliferation of cancer cells in comparison to the other non-tumorous cells. Ub, ubiquitination. **B: CD44v.** Alternative splicing of CD44 generates CD44v, which eventually increase reduced glutathione in the cell by stabilizing cystine/glutamate antiporter. GSH, reduced glutathione. Refer to text for details.

Figure 5. Classification of carcinogenic agents. We may be looking at the superficial portions of carcinogens whereas the real major fraction resides in the endogenous oxygen and iron metabolism. PM2.5, particulate matter in the air with the diameter less than 2.5 μm. The items and the carcinogenic agents are representative.

Figure 6. Oxygenomics. A: Concept of oxygenomics. Genome loci susceptible to oxidative insults depends at least on three factors, namely, reactive species generated, chromatin structure and chromosome territory in the cell. **B: Bodyguard theory of genomic loci associated with nuclear membrane.** Certain parts of genome are anchored at the nuclear membrane through lamina-associated domain (LAD), where oxidative DNA damage, such as 8-oxo-7,8-dihydroguanine (8-oxodGuo or 8-OHdG), is left unrepaired and abundant. Refer to text for details.

Figure 7. Ferroptosis. Differences from other types of cell death are conceptually described. GPX4, glutathione peroxidase 4. Refer to text for details.

Figure 8. Cancer cells maintain exquisite balance to escape ferroptosis despite Warburg effect and active iron uptake. Refer to text for details.

Figure 9. Visualization of catalytic Fe(II) with RhoNox-1 in human fibroblast. A human embryonic lung fibroblast cell line, IMR-90 SV, was stained with RhoNox-1 (a; 10 μM, 60-min incubation; magenta) concomitant with (b) Lyso Tracher Green (200 nM; 60-min incubation, green). (c) Merge picture of (a) and (b), indicating that white color areas are catalytic Fe(II) in the lysosomes. (d) Magnification of merge picture (c), showing the square portions of (a)-(c). (e) Quantitative image profile

(magenta, catalytic Fe(II); green, lysosome; arrow heads, overlapping areas based on the analysis of rectangular portion in d). Scale bar, 20 μm. Note that this is an example of application to cultured live cells. Refer to text for details.

Figure 10. Interaction between iron-catalyzed oxidative stress with thiols, nitric oxide and carbon monoxide. There is a threshold between redox signaling and oxidative molecular damage. In general, thiols are counteracting oxidative stress. Nitric oxide (NO) and carbon monoxide (CO) modulate oxidative stress through reaction with heme for NO/CO and also with nucleotides for NO. H_2S and persulfide are now recognized important for redox signaling. Refer to text for details.

Figure 11. Non-thermal (low-temperature) plasma for treating cells in 96-well plate. Arrow, flame of non-thermal plasma. Refer to text for details.

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Table 1. Association of Oxidative Stress and Cancer

Pathology	Organ	Cancer	Social/scientific Significance
Radiation exposure			
(mainly, hydroxyl radical)	Bone marrow	Leukemia	Atomic bomb, atomic power plant accident, space mission, cancer therapy
	Thyroid gland	Thyroid cancer	
	Breast	Adenocarcinoma	
	Soft tissue	Sarcoma	
Ultraviolt exposure			
(mainly, singlet oxygen)	Skin	Squamous cell carcinoma Basal cell carcinoma Malignant melanoma	Whites more susceptible, due to paucity in melanin pigments
Chronic inflammation			
(various reactive species)			
Infection			
Helicobactor pylori	Stomach	Adenocarcinoma	Antibiotics effective for prevention
		Malignant lymphoma (mainly, mucosa-associated lymphoid tissue lymphoma)	Antibiotic therapy may cure the disease
Ebstein Barr virus	Pharynx	Nasopharyngeal carcinoma	Endemic in China
	Craniocervical region, mainly	Malignant lymphoma (Burkitt lymphoma)	Endemic in Central Africa; may occur in immunodeficient condition, such as after transplantation
	Stomach	Adenocarcinoma	
Hepatitis B/C virus	Liver	Hepatocellular carcinoma	Prevalent in Asian countries; vaccine or antiviral drug available
Papilloma virus	Uterine cervix	Squamous cell carcinoma	Vaccine available
River fluke	Bile duct	Cholangiocarcinoma	
Tubercle bacillus	Lung/pleura	Malignant lymphoma	
Autoimmune			
Hashimoto disease	Thyroid gland	Malignant lymphoma	Most popular autoimmune disease
Sjogren's disease	Salivary/lacrimal gland	Malignant lymphoma	
Crohn's disease	Colon	Adenocarcinoma	Included in inflammatory bowel disease
Ulcerative colitis	Colon	Adenocarcinoma	Included in inflammatory bowel disease
Foreign material			
Silica	Lung	Lung cancer	Miner, pottery maker
P M2.5	Lung	Lung cancer	Air pollution
Asbestos	Lung/pleura	Lung cancer/malignant mesothelioma	Miner, asbestos factory worker, construction
Mechanical (unfitted false teeth)	Gingiva/tongue	Squamous cell carcinoma	
Deep burn	Skin	Squamous cell carcinoma	Longstanding inflammation of decades
Excess transition metal			
(mainly hydroxyl radical)			
Iron	Summarized in **Table 2**		
Copper	Intestine, kidney	Intestinal tumor in Min mice; renal cell carcioma by cupric nitrilotriacetate	Animal model data only; no significant increase of hepatocellular carcinoma in patients of Wilson's diease
Cromium	Lung	Lung cancer	Miner, chromate production plant
Cadmium	Lung	Lung cancer	Miner
Chemicals			
(various reactive species)			
Tobacco smoke	Larynx, lung, oral mucosa, esophagus, stomach, bladder, etc.	Various carcinoma (squamous cell carcinoma, adenocarcinoma, urothelial carcinoma, *etc.*)	Various chemicals included, such as nicotine, addictive, passive smoking
Benzene	Bone marrow	Leukemia	Chemical factory worker
Genetic deficiency in repair enzymes for oxidative DNA damage			
p53 (Li-Fraumeni syndrome)	Lymphoid tissue, soft tissue	Malignant lymphoma, sarcoma	Loss of apototic pathway (autosomal dominant, inactivation of the remaining allele necessary)
BRCA1/2	Breast, ovary	Adenocarcinoma	Loss of repair of DNA strand breaks (autosomal dominant, inactivation of the remaining allele necessary)
Mutyh	Colon	Adenocarcinoma	Loss of excsion repair for 8-oxoguanine (autosomal dominant, inactivation of the remaining allele necessary)
XPA (xeroderma pigmentsum)	Skin	Squamous cell carcinoma	Loss of repair of pyrimidine dimers (autosomal recessive)

This table presents examples of each category and are not comprehensive.

Table 2. Association of Excess Iron and Cancer

Disease/Intervention	Pathogenesis of excess iron	Organ	Cancer	Social/Scientific Significance
Hereditary hemochromatosis	Genetic, 6 types with different clinical manifestations, germline mutation of genes associated with iron metabolism (*HFE, HJV, HAMP, TFRC2, SLC40A, FTH1*) to cause dysregulated excess iron uptake from duodenum	Liver	Hepatocelular carcinoma	Genetic analysis useful; if identified at an early age, phlebotomy is the best therapy and the patients can live with no symptoms/complications
Viral hepatitis B/C	Hepatocyte loss leads to decrease in hepcidin, followed by unregulated iron uptake from deodenal vollous epithelia	Liver	Hepatocellular carcinoma	Vaccination for viral hepatitis B and antiviral drugs for viral hepatitis C are available now, prevalent in Asian countries
Ovarian endometriosis	Local repeated hemorrhage for several decades, leading to massive iron deposition in	Ovary	Adenocarcinoma (clear cell carcinoma, endometrioid adenocarcinoma, serous papillary adenocarcinoma)	Endometriosis as a whole is popular among reproductive-age women (~10%)
Malignant mesothelioma	All kinds of asbestos fibers adsorb histones, actin and hemoglobin on the surface (known as asbestos body by pathologsits) and phagocytosed by parietal mesothelial cells when the fibers reach pleural cavity decades after respiratory exposure	Mesothelial cells, lung	Malignant mesothelioma, lung cancer	Exposure to asbestos is intimately associated (IARC, group 1), multiwalled carbon nanotube of 50-nm diamater causes malignant mesothelioma in animals with similar mechanisms to asbestos (IARC, group 2B)
Leukemic transformation from myelodysplastic syndrome	Iron deposition in bone marrow due to dysfunction in iron usage for erythrocytes	Bone marrow	Leukemia	Still controversial
Clinical intervention by phlebotomy	Phlebotmy decreases iron storage	Many organs	Cancer decreased in general, 35% decrease in incidence and 60% decrease in mortality	Phlebotomy of 500 ml twice a year for 5 years, triple merits of blood donation, especially to middle-aged men, refer to text for details

IARC, International Agency for Research on Cancer; only representative reports are summarized; refer to text for details.

Table 3. Association Between the Research Field of Ferroptosis and Ferric Nitrilotriacetate-Induced Renal Carcinogenesis

Year	**Ferroptosis**		**Fe-NTA-induced renal carcinogenesis**	
Concept	Iron-dependent, non-necrotic , non-apoptotic cell death		Iron-dependent persistent oxidative stress causes aggressive cancer similar to those of humans	
1980	System Xc- discovered	Bannai *et al.* [60]		
1982	Discovery of PHGPX(GPX4) enzymatic activity	Ursini *et al.* [152]	Fe-NTA-induced renal carcinogenesis model discovered	Okada and Midorikawa [171]
1990			Lipid peroxidation in the target cells localized	Toyokuni *et al.* [101]
1993			Involvement of GSH cycle in carcinogenesis found	Okada *et al.* [114]
1994	Human *PHGPX* cloned	Esworthy *et al.* [172]	HNE and HNE-modified proteins identified in the target cells	Toyokuni *et al.* [102]
1999			One of the target genes identified as *p16* tumor suppressor gene	Tanaka *et al.* [96]
2006			Oxygenomics proposed	Akatsuka *et al.* [146]
2008	Non-apoptotic cell death observed with *GPX4* depletion	Seiler *et al.* [173]		
2012	Ferroptosis proposed	Dixon *et al.* [151]	Similarity of genetic alterations in this carcinogenesis of wild-type animals to those of human cancers in general suggested	Akatsuka *et al.* [128]
2014	GPX4 as key regulator of ferroptosis	Yang *et al.* [155]		
2016			Cancer (general; prior to therapy) as iron addiction with ferroptosis-resistance suggested	Toyokuni [27]
2017	Importance of doubly and triply oxygenated species of arachidonic- and adrenic acid-containing phosphatidylethanolamines in ferroptosis through ACSL4 suggested	Doll *et al.* [169]; Kagan *et al.* [170]		

Fe-NTA, ferric nitrilotriacetate; GSH, glutathione; HNE, 4-hydroxy-2-nonenal; PHGPX, phophplipid glutathione peroxidase

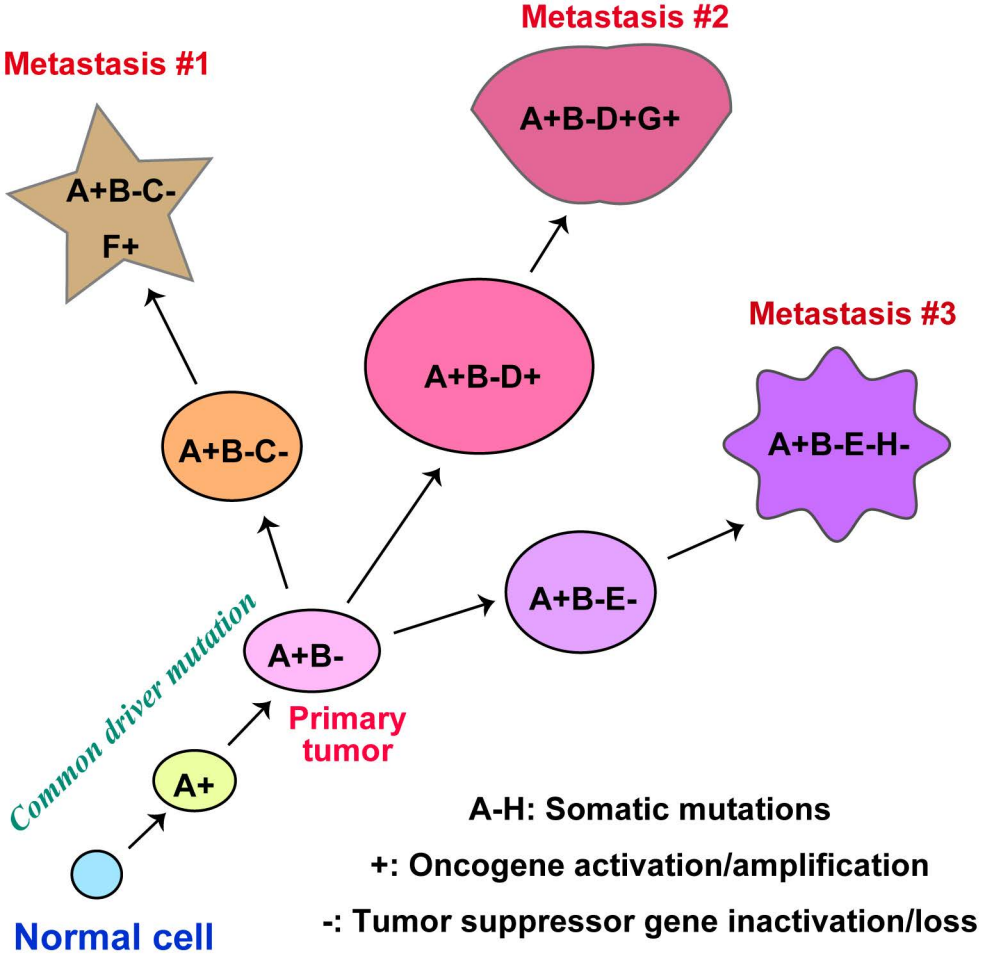
Metastasis #1
Metastasis #2
A+B-D+G+
A+B-C-
F+
Metastasis #3
A+B-D+
A+B-C-
A+B-E-H-
A+B-E-
A+B-
Common driver mutation
Primary
tumor
A+
A-H: Somatic mutations
+: Oncogene activation/amplification
-: Tumor suppressor gene inactivation/loss
Normal cell

Figure 1

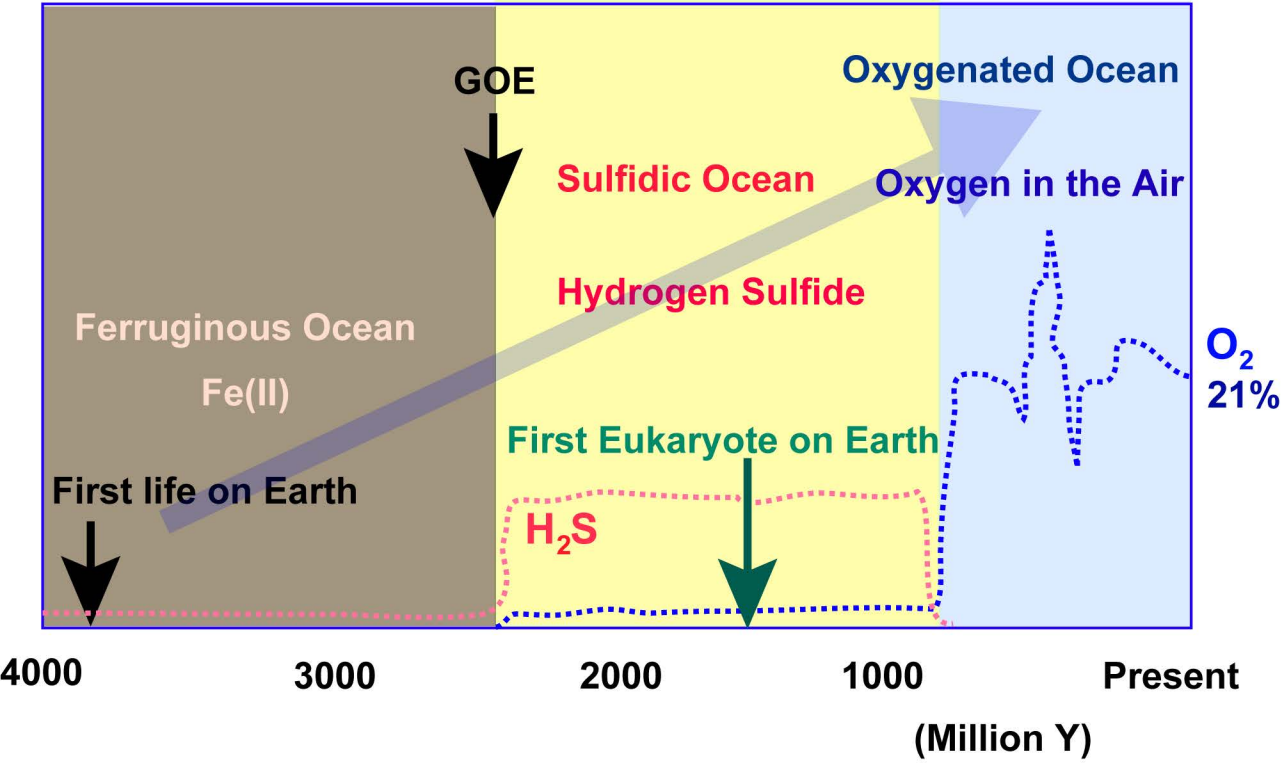
GOE
Oxygenated Ocean
Sulfidic Ocean
Oxygen in the Air
Hydrogen Sulfide
Ferruginous Ocean
Fe(II)
O_2
21%
First Eukaryote on Earth
First life on Earth
H_2S
4000
3000
2000
1000
Present
(Million Y)

Figure 2

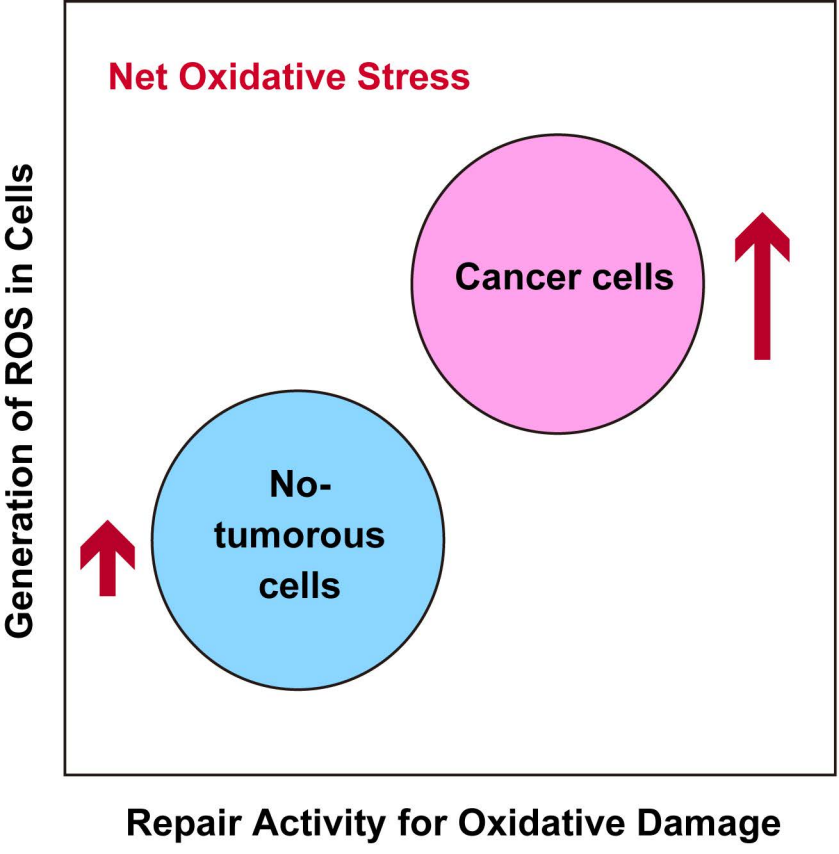
Net Oxidative Stress
Cancer cells
No-
tumorous
cells
Generation of ROS in Cells
Repair Activity for Oxidative Damage

Figure 3

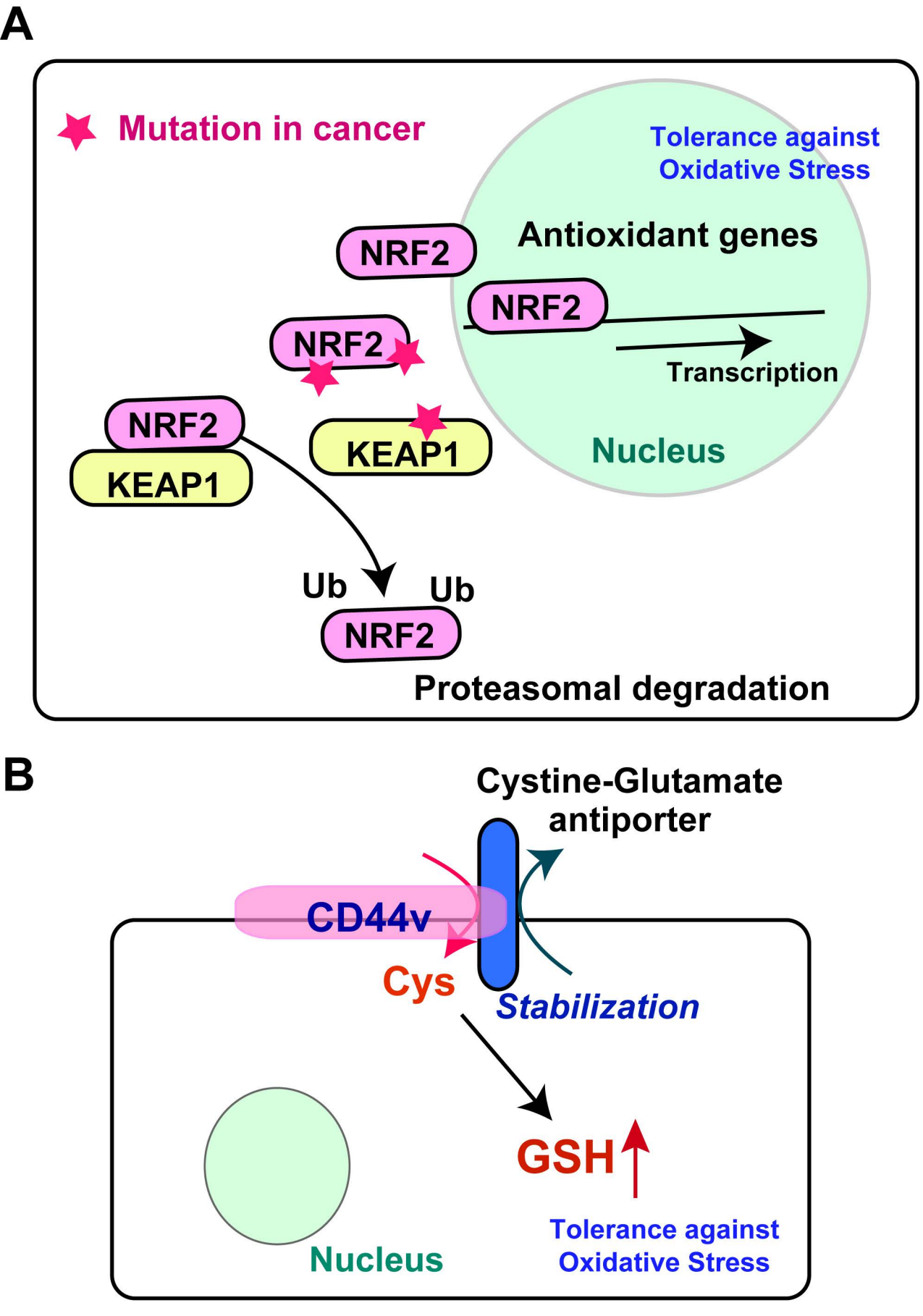
A
Mutation in cancer
Tolerance against
Oxidative Stress
Antioxidant genes
NRF2
NRF2
NRF2
Transcription
NRF2
KEAP1
KEAP1
Nucleus
Ub
Ub
NRF2
Proteasomal degradation
B
Cystine-Glutamate
antiporter
CD44v
Cys
Stabilization
GSH
Nucleus
Tolerance against
Oxidative Stress

Figure 4

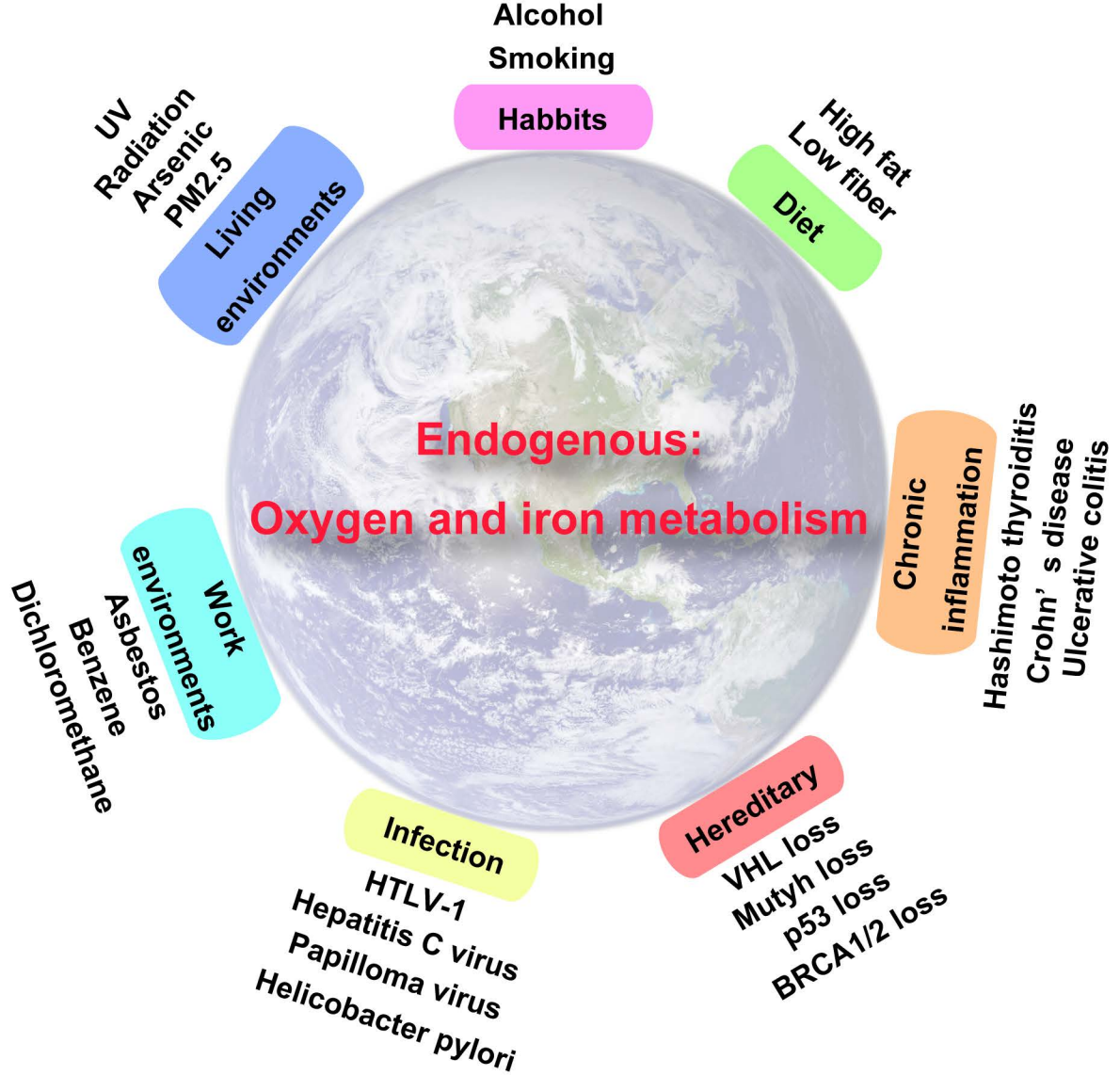
Alcohol
Smoking
Habbits
UV
Radiation
Arsenic
PM2.5
Living environments
High fat
Low fiber
Diet
Endogenous:
Oxygen and iron metabolism
Chronic inflammation
Hashimoto thyroiditis
Crohn' s disease
Ulcerative colitis
Work environments
Asbestos
Benzene
Dichloromethane
Infection
HTLV-1
Hepatitis C virus
Papilloma virus
Helicobacter pylori
Hereditary
VHL loss
Mutyh loss
p53 loss
BRCA1/2 loss

Figure 5

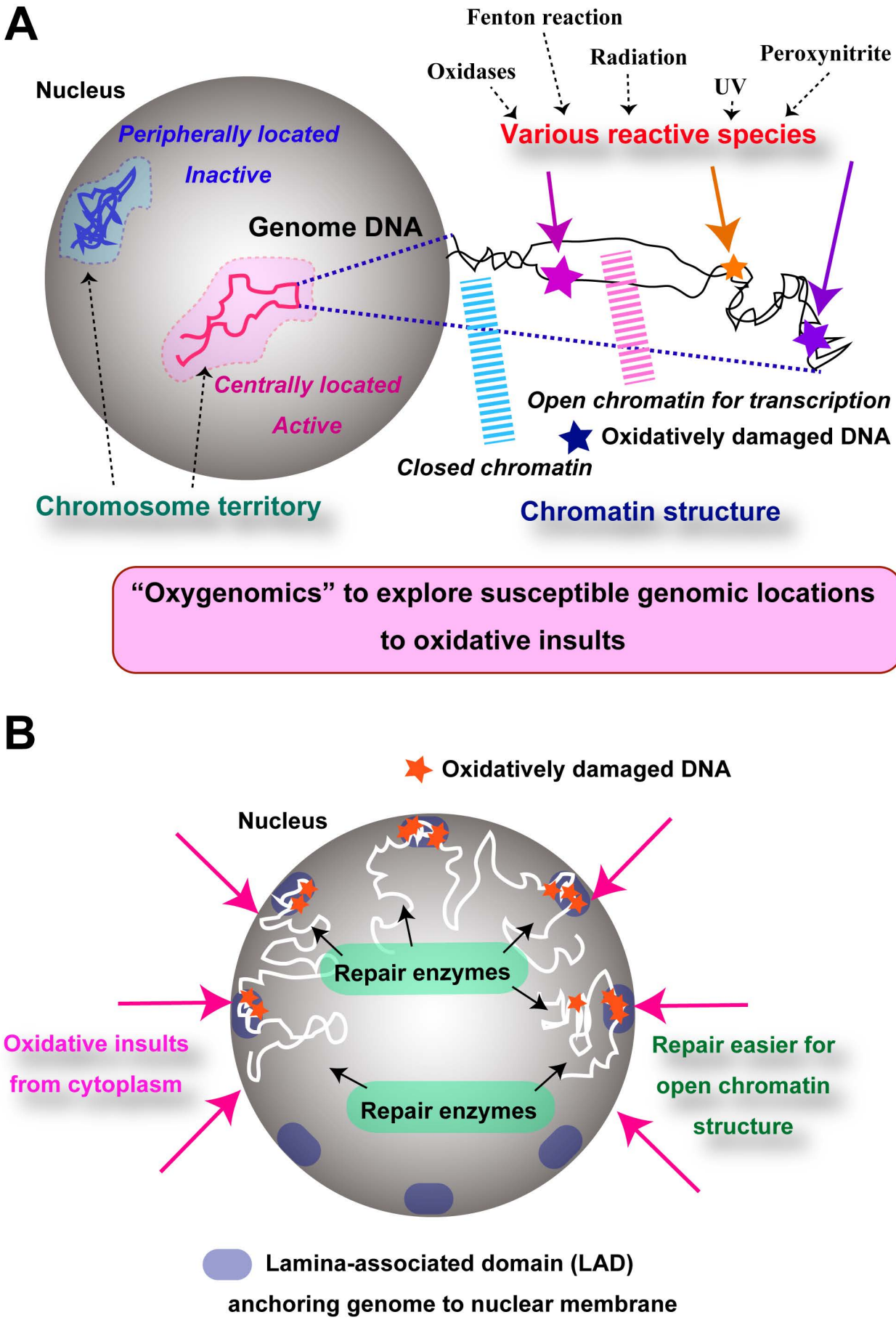
A
Fenton reaction
Oxidases
Radiation
UV
Peroxynitrite
Various reactive species
Nucleus
Peripherally located
Inactive
Genome DNA
Centrally located
Active
Chromosome territory
Open chromatin for transcription
Oxidatively damaged DNA
Closed chromatin
Chromatin structure
"Oxygenomics" to explore susceptible genomic locations to oxidative insults
B
Oxidatively damaged DNA
Nucleus
Repair enzymes
Repair enzymes
Oxidative insults from cytoplasm
Repair easier for open chromatin structure
Lamina-associated domain (LAD) anchoring genome to nuclear membrane

Figure 6

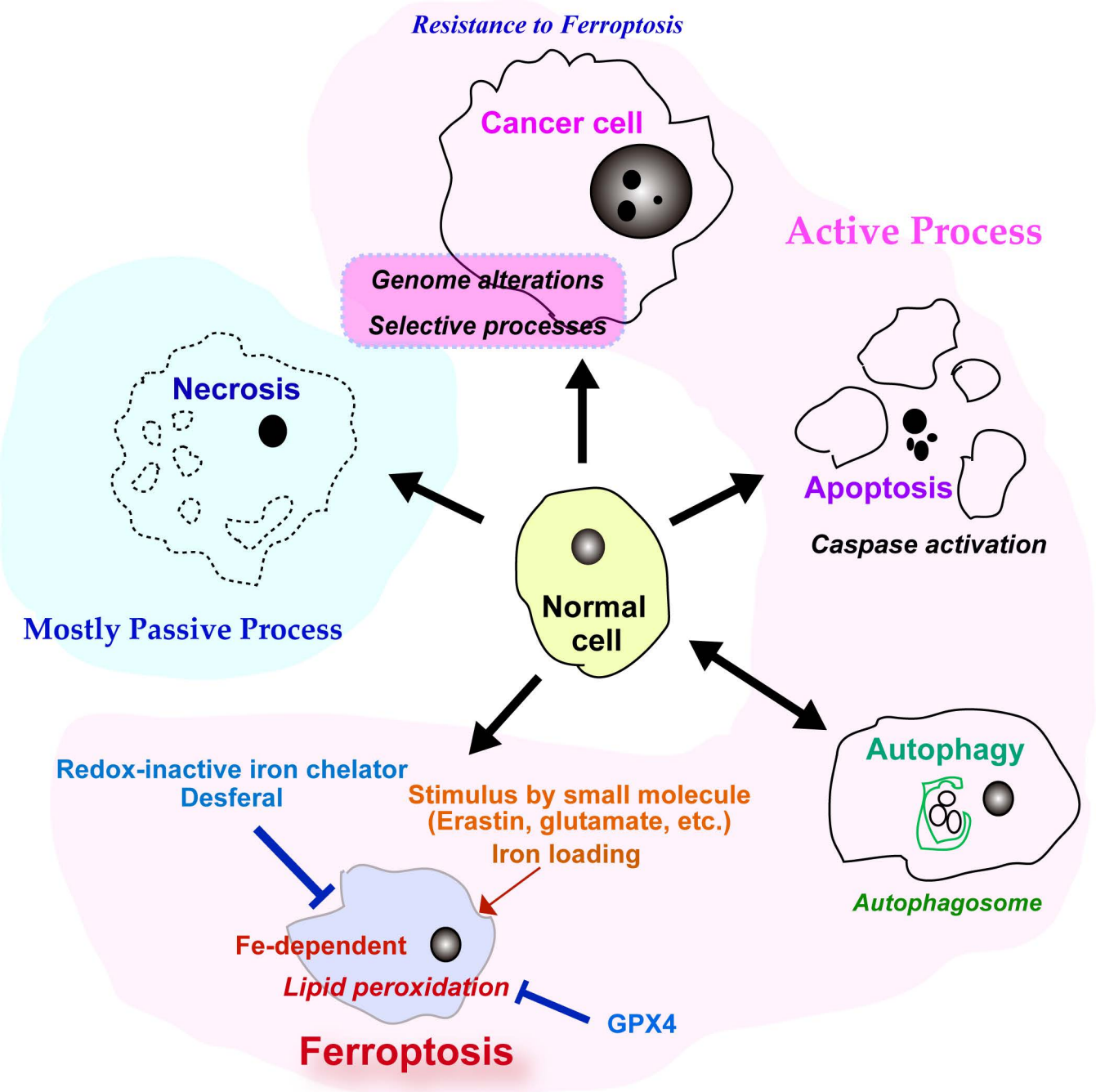
Resistance to Ferroptosis
Cancer cell
Active Process
Genome alterations
Selective processes
Necrosis
Apoptosis
Caspase activation
Mostly Passive Process
Normal cell
Redox-inactive iron chelator
Desferal
Stimulus by small molecule
(Erastin, glutamate, etc.)
Iron loading
Autophagy
Autophagosome
Fe-dependent
Lipid peroxidation
GPX4
Ferroptosis

Figure 7

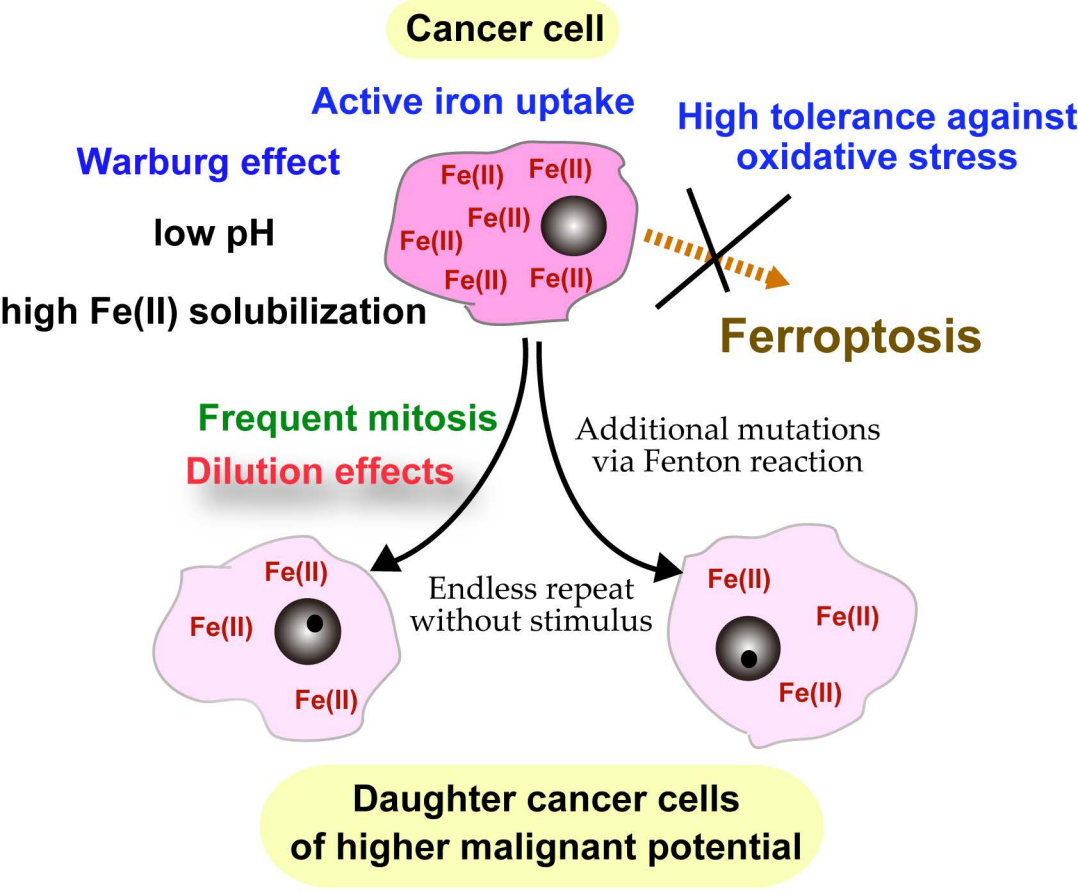
Cancer cell
Active iron uptake
Warburg effect
low pH
high Fe(II) solubilization
High tolerance against oxidative stress
Fe(II)
Ferroptosis
Frequent mitosis
Dilution effects
Additional mutations via Fenton reaction
Endless repeat without stimulus
Daughter cancer cells of higher malignant potential

Figure 8

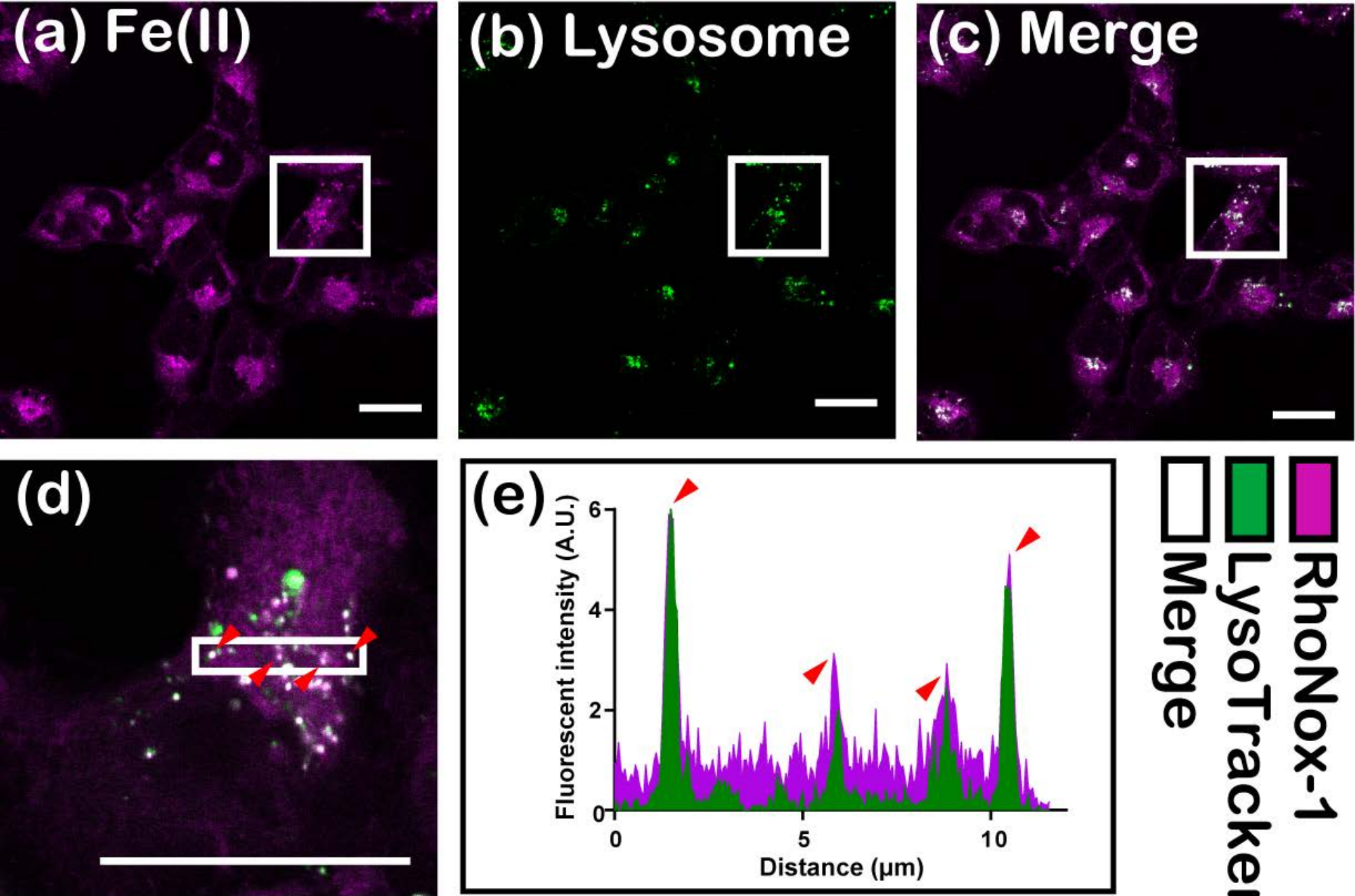
(a) Fe(II)
(b) Lysosome
(c) Merge
(d)
(e)
Fluorescent intensity (A.U.)
0
2
4
6
0
5
10
Distance (μm)
RhoNox-1
LysoTracker
Merge

Figure 9

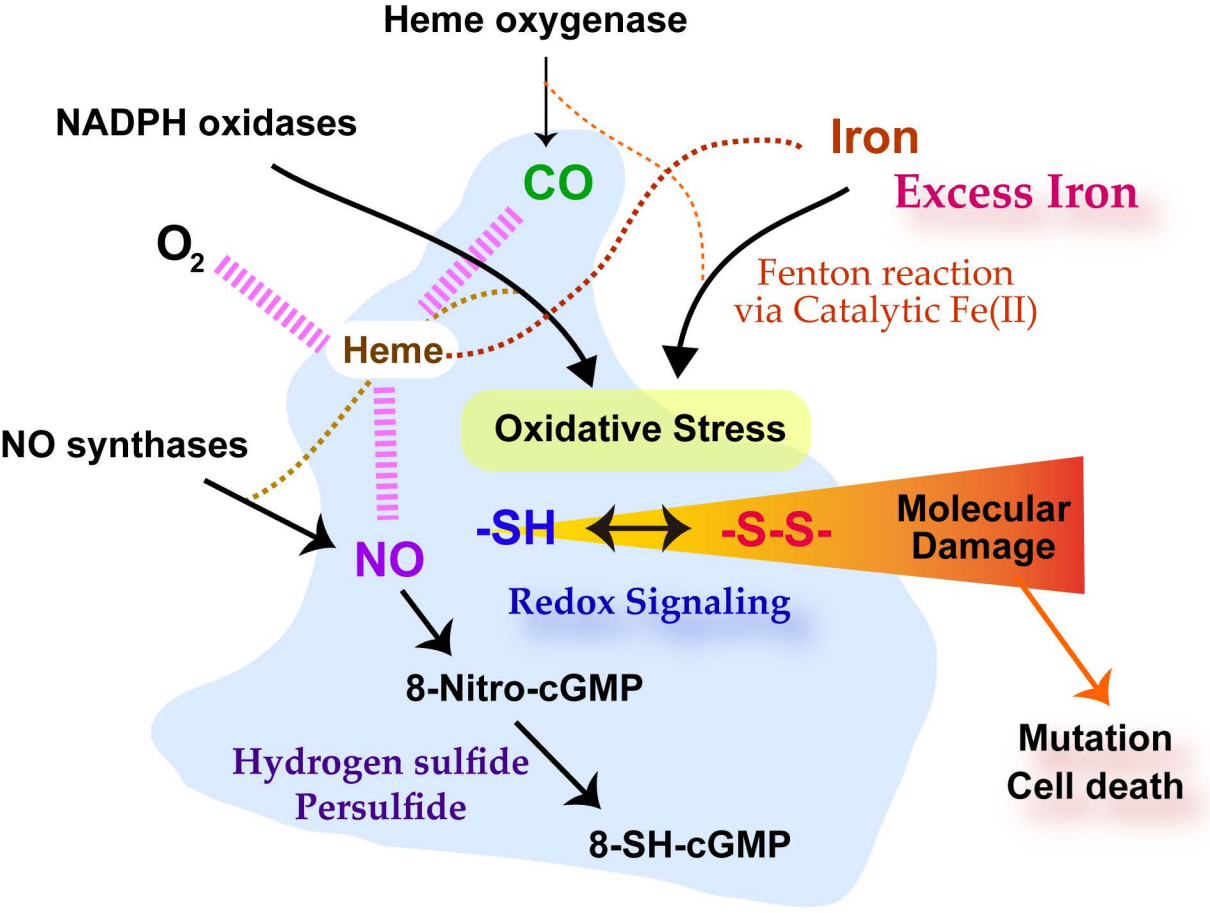
Heme oxygenase
NADPH oxidases
Iron
Excess Iron
CO
O_2
Fenton reaction
via Catalytic Fe(II)
Heme
Oxidative Stress
NO synthases
-SH
-S-S-
Molecular
Damage
NO
Redox Signaling
8-Nitro-cGMP
Hydrogen sulfide
Persulfide
Mutation
Cell death
8-SH-cGMP

Figure 10

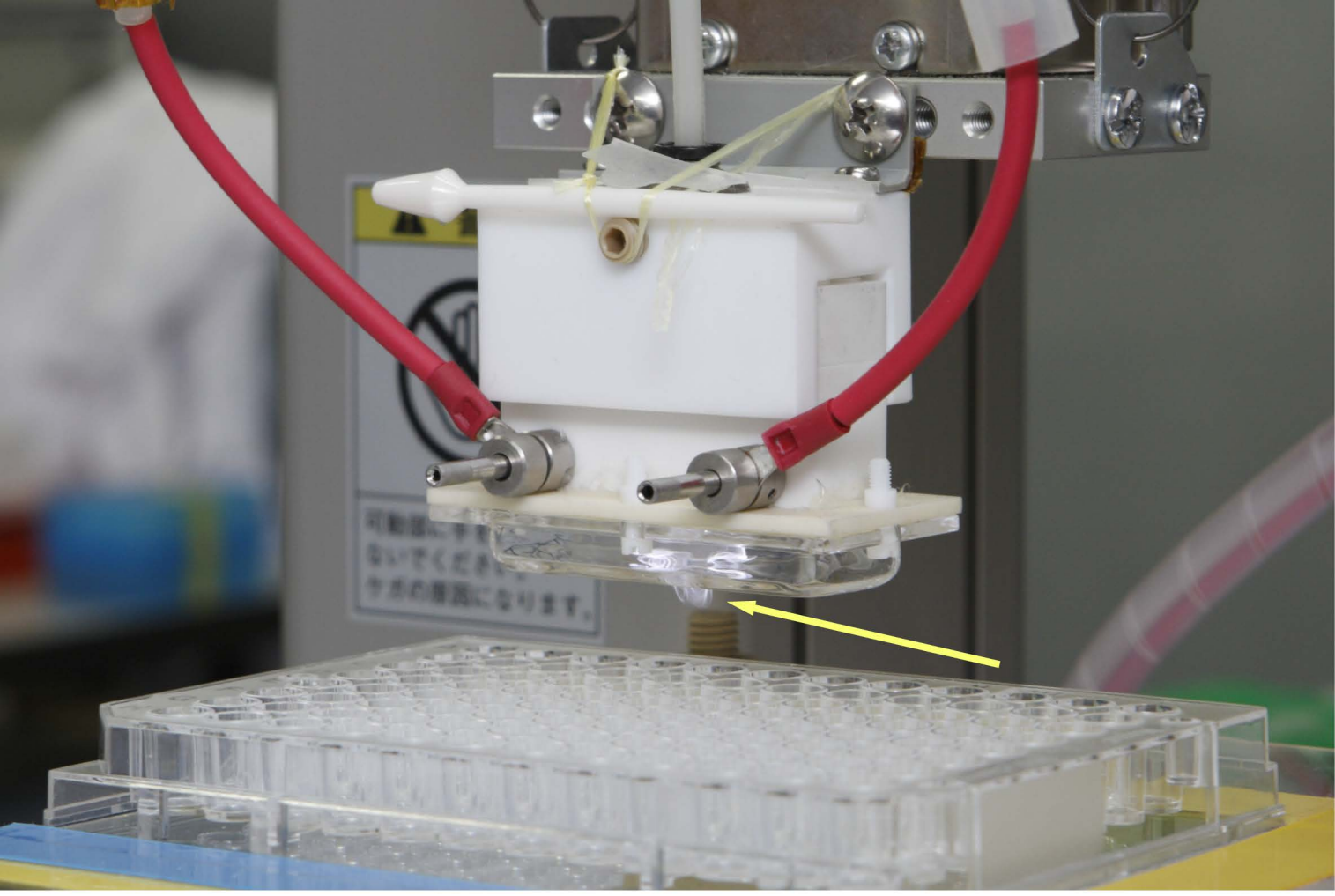

Figure 11