Review

Iron as spirit of life to share under monopoly

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Any independent life requires iron to survive. Whereas iron deficiency causes oxygen insufficiency, excess iron is a risk for cancer, generating a double-edged sword. Iron metabolism is strictly regulated via specific systems, including iron-responsive element (IRE)/iron regulatory proteins (IRPs) and the corresponding ubiquitin ligase FBXL5. Here we briefly reflect the history of bioiron research and describe major recent advancements. Ferroptosis, a newly coined Fe(II)-dependent regulated necrosis, is providing huge impact on science. Carcinogenesis is a process to acquire ferroptosis-resistance and ferroptosis is preferred in cancer therapy due to immunogenicity. Poly(*rC*)-binding proteins 1/2 (PCBP1/2) were identified as major cytosolic Fe(II) chaperone proteins. The mechanism how cells retrieve stored iron in ferritin cores was unraveled as ferritinophagy, a form of autophagy. Of note, ferroptosis may exploit ferritinophagy during the progression. Recently, we discovered that cellular ferritin secretion is through extracellular vesicles (EVs) escorted by CD63 under the regulation of IRE/IRP system. Furthermore, this process was abused in asbestos-induced mesothelial carcinogenesis. In summary, cellular iron metabolism is tightly regulated by multi-system organizations as surplus iron is shared through ferritin in EVs among neighbor and distant cells in need. However, various noxious stimuli dramatically promote cellular iron uptake/storage, which may result in ferroptosis.

***Key Words*: iron, carcinogenesis, extracellular vesicles, ferroptosis, iron chaperone**

The origin of our laboratory starts in 1982, when Shinya Toyokuni started research activity as a medical student under the guidance of Dr. Shigeru Okada and Prof. Osamu Midorikawa in the Department of Pathology, Faculty of Medicine, Kyoto University, Kyoto, Japan. At that time, researchers of experimental pathology have been trying to generate disease models by administering a variety of chemicals to animals via different routes, when genome information and genetically engineered animals were not available. The department's interest had been the role of metals in diseases. Affected by the academic environments, Shinya Toyokuni became interested in the role of chelators in modifying the biological effects of heavy metals *in vivo*, and published the first work as a PhD student on a copper toxicosis model by repeated intraperitoneal administration of cupric nitrilotriacetate, in which pathology of Wilson disease was reproduced.[1] Shinya Toyokuni thereafter started the work on rodent renal carcinogenesis model by ferric nitrilotriacetate (Fe-NTA), which was discovered with serendipity in 1982 by Dr. Shigeru Okada and Prof. Osamu Midorikawa.[2–8] This renal carcinogenesis model attracted Shinya Toyokuni so much that our laboratory still works on this model even 40 years after the discovery. This model has produced at least 64 papers thus far in our laboratory since Shinya Toyokuni started his own laboratory in 1992. Importantly, this model has generated and is still generating numerous novel concepts by the members of our laboratory as described below.

Fe-NTA-induced Renal Carcinogenesis Model as Ferroptosis-resistance

The value of this model generated in *wild-type* rodents may be still underestimated even now. It has taken several decades for us to understand the entire molecular mechanisms of carcinogenesis, which is summarized in Fig. 1 and Table 1. Intraperitoneal injection of Fe-NTA (molecular weight 243.96) causes Fenton reaction specifically in the renal proximal tubules, which depends on the complete filtration through the glomeruli and protein-deficient reductive intraluminal renal tubular environment.[9] The most important point is the adaptive response of the somatic cells, here renal proximal tubular cells, against repeated oxidative stress, which forces to make decision on a delicate balance between the individual death due to renal failure or cellular evolution to overcome persistent oxidative stress. Kidney is a vital organ which selectively excrete toxic metabolites and modulate water and salt mass in the body. We have recognized that the rodent individuals unconsciously select cellular evolution rather than immediate death even if the evolution may eventually kill the host.

Intraperitoneal (*ip*) injection of Fe-NTA induces renal proximal tubular necrosis via Fenton reaction, which starts as early as 30 min after the injection.[10–13] Fe-NTA has been used to load Fe(III) to transferrin, a major Fe(III) transporting protein in the serum.[14] NTA is an aminopolycarboxylic acid like ethylenediamine tetraacetic acid (EDTA) and can generate an iron chelate, which is soluble at neutral pH[15] and is still redox-active.[16–18] Fe-NTA is indeed a most potent Fenton reagent at the near physiological conditions.[16,19] After the *ip* administration, most of the Fe-NTA is excreted through urine within 3 h[20] (Fig. 1). However, after repeated daily *ip* administration of 3 weeks, we rarely observe necrosis but finds numerous atypical proximal tubular cells with huge bizarre nucleus, which we call karyomegalic cells.[3,4,10,21] We now know that the cells which survived 3 weeks' severe oxidative stress through Fenton reaction already exhibit genetic alterations, including deletion of *p16*^{Ink4a} tumor suppressor gene.[22–24] Of note, at this subacute stage, renal tubular cells accumulate iron in the cytoplasm.[2]

In the 1980's and 1990's, we performed many morphological and functional studies on this model (Fig. 1 and Table 1). Especially, the acute model was extremely reproducible as an animal model,[21,25–28] and major type of cell death mode in the renal proximal tubules was necrosis.[10,11] We recognized in 2014 that renal tubular necrosis by Fe-NTA must be classified as ferroptosis after the proposal of a novel cell death mode designated as ferroptosis.[29] There was the first International Conference on ferroptosis in the Banbury Center, Cold Spring Harbor Laboratory on April 2–5, 2017 as a closed meeting, where Shinya Toyokuni was invited and presented the Fe-NTA-

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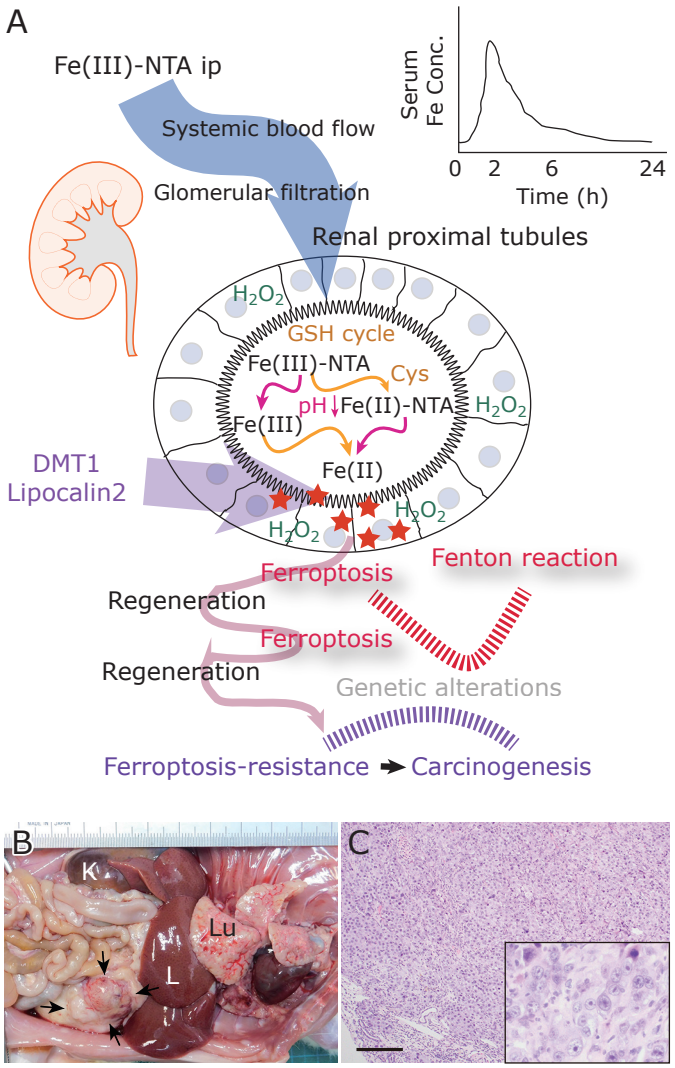

Fig. 1. Ferric nitrilotriacetate (Fe-NTA)-induced renal carcinogenesis. (A) Summary of molecular mechanisms how repeated intraperitoneal (*ip*) administration of Fe-NTA causes specifically renal cell carcinoma. DMT1, divalent metal transporter 1 (Slc11A2). Refer to text for details. (B) Macroscopic view of a representative case of renal cell carcinoma in a rat induced by Fe-NTA. Arrows show the renal cell carcinoma originating in the left kidney and invading the surrounding tissue. K, right kidney; L, liver; Lu, lung with pulmonary metastasis. (C) Histology of the same renal cell carcinoma (Fuhrman grade 4[150]; bar = 200 μm, 50 μm in the inset).

induced renal carcinogenesis model.[30] No independent life on earth can survive without iron, which constitutes a basis for the persistent electron flow through the organelles, cytosol and the entire cells. On the other hand, sulfur or sulfhydryls work as antioxidants, where iron and sulfhydryls are usually competing each other except for Fe-S cluster.[7,8,31] The definition of ferroptosis is catalytic Fe(II)-dependent regulated necrosis accompanied by lipid peroxidation.[30] Our revised understanding of ferroptosis is simpler in that an sharply increased ratio of catalytic Fe(II) to sulfhydryls leads to necrotic form of cell death associated with lipid peroxidation.[32] In this way, Fe-NTA-induced renal carcinogenesis generated a condition of ferroptosis-resistance.[32,33]

Screening of Oxidative Stress Biomarkers through the Acute Phase of Fe-NTA Model

Since the 1990's, we have been using the acute phase of Fe-NTA-induced renal carcinogenesis (3 h after *ip* administration) to screen for practical oxidative stress biomarkers. Among the oxidative DNA base modifications, 8-hydroxy-2'-deoxyguanosine (8-OHdG) was the most sensitive[25] and we have produced a monoclonal antibody against 8-OHdG (N45.1), thus specific for the DNA form.[27] N45.1 recognizes not only the hydroxyl (−OH)/keto(=O) structure at C8 of 8-OHdG but also 2'-deoxy structure of 2'-deoxyribose, which can differentiate RNA form of 8-OH-guanosine in immunohistochemistry and immunoprecipitation[34] (Fig. 2). The latter in association with the genome information opened up a novel research area called oxygenomics,[35–39] which is still growing insidiously.[40,41] Oxygenomics indeed provides us with a variety of genomic information, such as intranuclear location and expression of genes linked with oxidative stress (Fig. 2). We believe that oxygenomics approach would be more recognized with the advancement of artificial intelligence.

Oxidized lipids as lipid peroxidation products were also good candidates for oxidative stress biomarkers. Final products of lipid peroxidation are aldehydes in most of the reactions *in vitro* and *in vivo*. We found that 4-hydroxy-2-nonenal (HNE) was the most sensitive as a maker through the screening of the Fe-NTA model.[26,42] Whereas aldehydes themselves including HNE are not retained in the formalin-fixed paraffin-embedded (FFPE) specimens because of their lipophilicity, HNE was reactive enough to initiate Michael addition reaction with His/Lys/Cys residues of various proteins to generate specific hemiacetal structure,[43–45] which could be fixed in FFPE specimens based on large molecular weight with relative hydrophilicity.[42,46] Thus, we could produce 5 clones of mouse monoclonal antibodies against HNE-modified proteins, which showed distinct recognition of the epitopes.[47,48] We used HNE-modified albumin in 1995 for the screening of the most sensitive clone, which was HNEJ-2 with a specificity to the His adducts (Fig. 3). HNE-2 has been commercialized and contributed a lot to the understanding of pathologies in various diseases (Table 2) because immunohistochemical analyses under microscope can locate the target cells for oxidative stress among a variety of cells of more than 200 different kinds. In the 1990's we did not expect at all that other clones than HNEJ-2 would be helpful for the detection of ferroptosis in 2021.[13]

Mysterious Link Between p16 and Carcinogenesis

Cancer is one of the present-day leading causes of human mortality worldwide as well as in Japan since 1981 (https://ganjoho.jp/public/qa_links/report/statistics/2021_en.html). For a long time till the 1950's, tuberculosis, a bacterial infectious disease, was the top cause of death all over the world, which was successfully interrupted by the discovery of antibiotics, such as streptomycin[49] and isoniazid.[50,51] However, we have not succeeded in decreasing cancer incidence thus far, and advanced-stage cancers are still difficult to be cured even with the latest treatment strategies.

In the textbook we see a long list of etiology of cancer, which usually starts from smoking and include excess alcohol drinking, excess red meat, specific virus/bacterial infections, obesity and insufficiency of fruits/vegetables and exercise. Are there really fully responsible for all the cancers? Cancer has been recognized from the Greek Era at the latest. We now think that even the long list is just the tip of iceberg.[52] We are now proposing that carcinogenesis is at least responsible from the long use of iron and oxygen for the average of 80 years.[33] Iron and oxygen present a high affinity each other.[53] No life on the earth can survive without iron.[54]

Table 1. Seminal findings associated with Fe-NTA-induced renal carcinogenesis

1971	Bates and Wernicke	Use of Fe-NTA to load iron to transferrin[14]
1979	Awai *et al.*	Use of Fe-NTA *ip* injection in rats/rabbits as a model of hemochromatosis[20]
1982	Okada and Midorikawa	Discovery of Fe-NTA-induced renal carcinogenesis in rats (in Japanese)[2]
1985	Hamazaki *et al.*	Renal tubular injury after single *ip* administration of Fe-NTA at the acute phase[10]
1986	Ebina *et al.*	Fe-NTA-induced renal carcinogenesis in rats[3]
1987	Li *et al.*	Fe-NTA-induced renal carcinogenesis in mice[4]
1987	Okada *et al.*	TBARS increased after after *ip* administration of Fe-NTA in rats, which was prevented by pre-administration of vitamin E[114]
1990	Toyokuni *et al.*	Males mice are more susceptible to renal lipid peroxidation by Fe-NTA than females[12]
1992	Toyokuni and Sagripanti	Fe-NTA as the most efficient catalyst for Fenton reaction at neutral pH to cause DNA single/double strand breaks[16]
1994	Toyokuni *et al.*	HNE-modified detected in the renal tubules by immunohistochemistry[42]
1994	Toyokuni *et al.*	8-OHdG as the most increased oxidative DNA modification 3 h after single *ip* administration of Fe-NTA[25]
1995	Toyokuni *et al.*	Monoclonal antibodies against HNE-modified proteins established[47]
1997	Toyokuni *et al.*	HNE as the most increased aldehydes 3 h after single *ip* administration of Fe-NTA[26]
1997	Toyokuni *et al.*	Monoclonal antibody against 8-OHdG established[27]
1999	Tanaka *et al.*	*p16*^{Ink4a} identified as a major target tumor suppressor gene in Fe-NTA-induced carcinogenesis with genetic analysis[22]
2002	Hiroyasu *et al.*	Allelic loss of *p16*^{Ink4a} occurs 3 weeks after the start of Fe-NTA-induced carcinogenesis protocol[23]
2006	Akatsuka *et al.*	Concept of oxygenomics established[34]
2012	Akatsuka *et al.*	aCGH analysis of Fe-NTA-induced RCCs revealed similarity of genomic alterations to those of human cancer[24]
2022	Cheng *et al.*	Mouse strain difference in susceptibility to Fe-NTA-induced renal carcinogenesis depends on ferroptosis-resistance[112]
2022	Kong *et al.*	Rat *Brca1*(L63X/+) provides promotional effect on carcinogenesis through chromosomal amplification and ferroptosis-resistance[151]

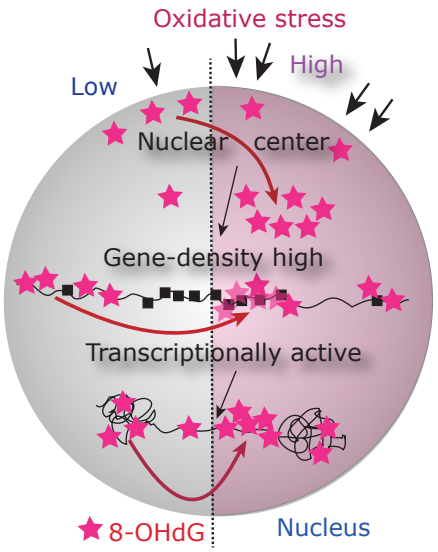

Fig. 2. 8-Hydroxy-2'-deoxyguanosine (8-OHdG) and oxygenomics. Summary of the recent results on the 8-OHdG distribution *in vivo* in the renal tubular cells in the untreated normal condition and under oxidative stress after Fe-NTA administration in association with intranuclear localization, gene density and transcriptional activity. Refer to text for details.

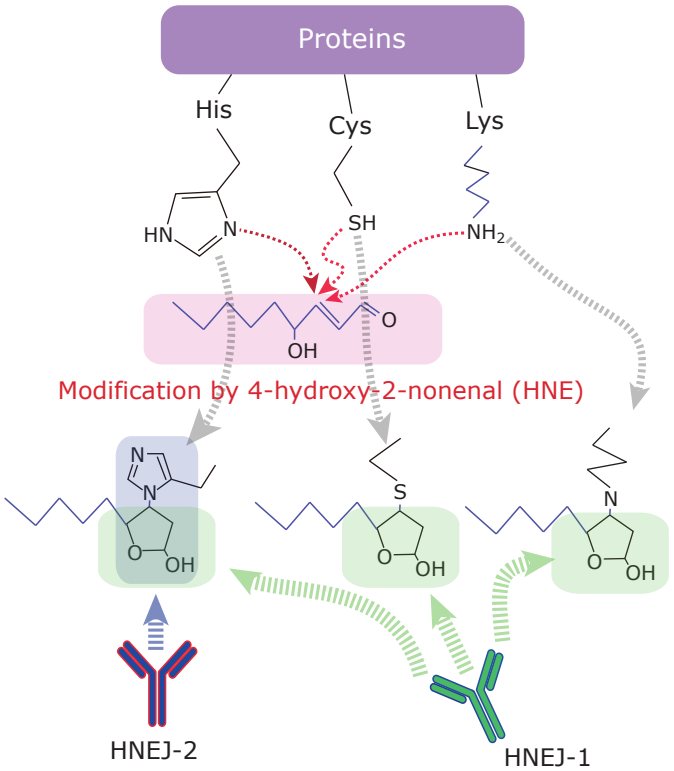

Fig. 3. Monoclonal antibodies against 4-hydroxy-2-nonenal (HNE)-modified proteins. A major lipid peroxidation end product, HNE, still can react with amino acid residues, histidine, cysteine and lysine, in proteins to generate Michael adducts. We have produced several mouse monoclonal antibodies to recognize this structure of Michael adducts. Whereas HNEJ-2 recognizes specifically histidine adducts, HNEJ-1 shows high affinity for all the histidine, cysteine and lysine adducts, which we recently found appropriate to detect ferroptosis in formalin-fixed paraffin-embedded sections.[13]

In this context, Fe-NTA-induced renal carcinogenesis as already described is intriguing as a carcinogenesis model purely by repeated Fenton reaction.[2–7,55] We evaluated the induced renal cell carcinoma (RCC) with genetic analysis and later with array-based comparative genome hybridization, which revealed that homozygous deletion of *p16*^{Ink4a} tumor suppressor gene and amplification of *c-Met* oncogene (receptor for hepatocyte growth factor) are the common mutations.[22,24] These two genes are

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Table 2. HNE as a marker of oxidative stress in formalin-fixed paraffin-embedded (FFPE) specimens of various pathologies

1994	Toyokuni *et al.*	Fe-NTA-induced renal proximal tubular injury in rats[42,48]
1994	Okamoto *et al.*	Human renal cell carcinoma[115]
1994	Uchida *et al.*	Atherosclerosis[116]
1997	Ma *et al.*	Long-Evans Cinnamon rat, Cu-induced liver injury[117]
1998	Ohhira *et al.*	Human alcoholic liver disease[118]
1998	Minamiyama *et al.*	Endotoxemic hepatic injury in rats[119]
1999	Um *et al.*	Ischemia-reperfusion of rat island skin flap[120]
1999	Ihara *et al.*	Pancreatic β-cells in rat type 2 diabetes mellitus model (Goto-Kakizaki rat)[121]
1999	Kondo *et al.*	Human colorectal carcinoma[122]
2000	Kageyama *et al.*	Chronic hepatitis C[123]
2000	Yamamoto *et al.*	CCl_4-induced liver injury in rats[124]
2000	Kawamura *et al.*	Liver of primary biliary cirrhosis[125]
2000	Toyokuni *et al.*	Serum albumin in human type 2 diabetes mellitus[126]
2000	Yamagami *et al.*	Ischemia-reperfusion in rat liver[127]
2002	Nakamura *et al.*	Human myocardial biopsy from dilated cardiomyopathy[128]
2004	Schäbitz *et al.*	Rat focal cerebral ischemia[128]
2014	Okazaki *et al.*	Direct exposure of non-thermal plasma to liver[129]
2016	Tsuzuki *et al.*	Human term placenta[130]
2021	Zheng *et al.*	Embryonal erythropoiesis and aging in rats[13]

Selected findings are described.

among the most popular targets in various human cancers.[56,57] Regarding the allelic loss of *p16*^{Ink4a} tumor suppressor gene, this phenomenon was frequently observed in many cell lines which were cultured over a long period in the 1990's. It was thus once thought as an artifactual mutation.[58,59] However, homozygous deletion of *p16*^{Ink4a} tumor suppressor gene was found to be frequently observed in malignant mesothelioma of human cases (epithelioid subtype ~60%, sarcomatoid subtype ~100%), which established the biological role in human carcinogenesis.[60,61] Of note, the pathogenesis of asbestos-induced malignant mesothelioma is highly iron-dependent.[62] Furthermore, melanoma-prone kindreds were reported, which identified *p16*^{Ink4a} tumor suppressor gene as responsible.[63,64]

When we first recognized that *p16*^{Ink4a} tumor suppressor gene is one of the responsible genes in Fe-NTA-induced renal carcinogenesis, we thought that this is a mysterious link.[65] The gene locus of *p16*^{Ink4a} tumor suppressor gene encodes two genes, thus two proteins by alternative splicing, INK4A and ARF, which is a cell cycle brake and apoptosis promoter, respectively. This is probably one of the most important portions of the genome, which release the cell cycle brake with no apoptotic pathways, promoting carcinogenesis two steps with one stone, if this portion is homozygously deleted.[66] Accordingly, we believe that some fraction of human cancer is caused by the long use of iron and oxygen, where iron becomes excess with aging.[33,52] The association of *p16*^{Ink4a} tumor suppressor gene deletion with iron-induced carcinogenesis has been a mysterious link for a long time. Now we have at least reached a hypothesis that Fe-NTA-induced renal carcinogenesis represent a model of usual human carcinogenesis as a process to acquire ferroptosis-resistance.[32]

Iron Chaperones

Regarding the chemical character, Fe(II) is an initiator of Fenton reaction $[Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^{-}]$ to generate hydroxyl radicals, and thus is an indispensable but dangerous molecule.[53] However, Fe(II) has to go through cellular cytosol to its final destination organelles. Therefore, how Fe(II) is transported through cytosol was a long-time mystery. The first important finding was that poly repeated cytidine (*rC*) binding protein 1 (PCBP1) can chaperone Fe(II) to load iron to ferritin core eventually as Fe(III).[67] Fe(III) is almost insoluble at neutral pH and so is a safe iron for storage.[53]

Izumi Yanatori performed a series of experiments in the 2010's to screen a major cytosolic Fe(II) chaperone by the use of yeast two-hybrid system to identify poly repeated cytidine (*rC*) binding protein 2 (PCBP2). Indeed, PCBP2 can accommodate Fe(II) from divalent metal transporter 1 (DMT1, SLC11A2)[68] or heme oxygenase 1/cytochrome p450 complex[69] and pass Fe(II) to ferroportin (SLC40A1), a sole iron exporter from the cell.[70] These findings, including ours, hold a huge biological significance in that DMT1 takes out delivered iron to cytosol through transferrin receptor/endosome/lysosome system and heme oxygenase 1/cytochrome p450 complex metabolizes recovered heme from hemoglobin of aged red blood cells or wornout proteins retaining heme cofactor to retrieve iron. Figure 4 shows the current understanding of iron metabolism in higher species. Thus, cytosolic Fe(II) transport system has been established.[71,72]

As the name suggests PCBP1/2 have been discovered to play multiple roles in the nucleus such as translation regulation[73,74] and recognition of heavily oxidized RNAs.[75,76] PCBP1, one exon gene, shares ~80% homology in amino acids to PCBP1, suggesting that PCBP1 is derived from PCBP2 by retrotransposition. Both PCBP1 and PCBP2 accommodate 3 molecules of Fe(II), which would be redox inactive.[72,77–79] As already mentioned, affinity of PCBP1/2 to other iron metabolism-associated proteins are antagonizing. Namely, PCBP1 transports Fe(II) to ferritin for storage[67] whereas PCBP2 collects and transports Fe(II) for use at organelles or to ship out extracellularly. It is interesting to mention here that PCBP2 play a role as oncogene[80,81] whereas PCBP1 as tumor suppressor gene[80,82] in various human cancers (Table 3). It is understandable in that cancer cells require and use a large amount of iron for endless proliferation, invasion and metastasis and that current endpoint for cancer therapy includes ferroptosis.[81,83,84]

Ferritinophagy

Ferritin cores consist of 24 building-block units consisting of ferritin heavy chains (FTH) and ferritin light chains (FTL). Single ferritin core can store iron as Fe(III) hydroxide/phosphate up to ~4,200 molecules, and thus is a huge storage for safe redox-

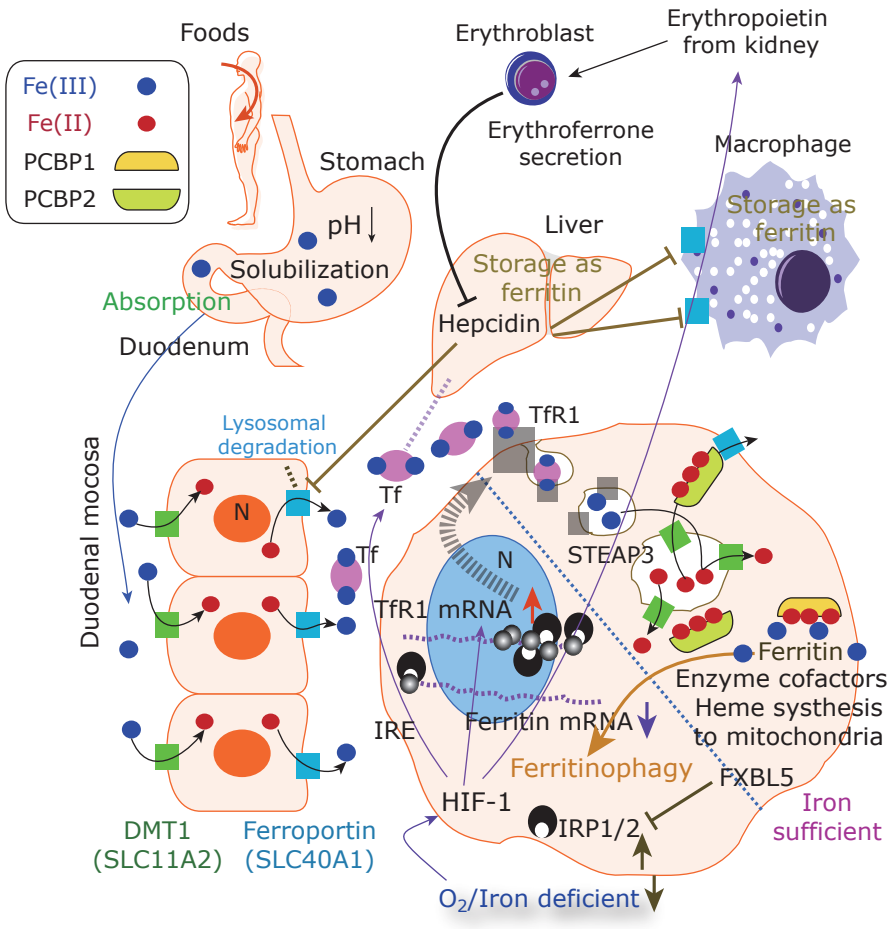

Fig. 4. Recent understanding of iron metabolism in higher species. Iron is absorbed from duodenal mucosa. Note that there is no active pathway to excrete iron to outside of the body except for cell loss from skin and gastrointestinal tract and bleeding. N, nucleus; Tf, transferrin; TfR1, transferrin receptor 1; IRE, iron-responsive element on mRNA; IRP, iron-responsive protein; HIF, hypoxia-inducible factor; FBXL5, F-box/LRR-repeated protein 5, working for ubiquitination of IRP-2/IREB2. STEAP3, six-transmembrane epithelial antigen of prostate 3; PCBP, poly r(C) binding protein.

inactive iron.[84] Only FTH can oxidize Fe(II) transported via PCBP1 to Fe(III).[67,85,86] Ferritin is also a commonly used serum marker to evaluate iron storage status. In this case, protein portion of ferritin is detected by specific antibodies, and it is generally recognized that iron content in serum ferritin is low.[87] In excess of cellular iron, the master posttranscriptional regulatory system, iron responsive element/iron regulatory protein (IRE/IRP) system, senses the iron condition through Fe-S cluster, namely mitochondrial status (IRP1), or oxidation status (IRP2), increasing ferritin and decreasing transferrin receptor and DMT1.[88] However, how cells retrieve iron from ferritin cores were unknown till 2014.

Autophagy is a common process in cells, sometimes physiological and sometimes pathological.[89,90] Basically, this "eating itself" generates essential molecules to live by digesting preexisting larger molecules, whether aged or sometimes newly synthesized. In comparison, proteasomes need ubiquitin ligation through specific ubiquitin ligases and are more specific for single molecules. Withdrawal of deposited iron is performed by a specific adaptor protein, nuclear receptor coactivator 4 (NCOA4) and autophagic process,[91] which merges with lysosomes where retrieved Fe(III) is reduced to Fe(II) via six-transmembrane epithelial antigen of prostate 3 (STEAP3) metalloreductase. This process is now called ferritinophagy, and is regulated by iron status.

As mentioned in the previous section, carcinogenic process of malignant mesothelioma is dependent on iron excess via asbestos exposure.[62,92,93] Thus, mesothelioma cells can hold a larger amounts of catalytic Fe(II) in the cytosol in comparison to non-tumorous cells.[94–96] This means the inaugulation of ferroptosis-resistance. We have reported that high expression of carbonic anhydrase IX is one of those processes.[97,98] Not the least, abundant catalytic Fe(II) in the cytosol can be a common characteristics of cancer cells in general because they have to utilize iron quickly for persistent proliferation. DNA replication (ribonucleotide reductase), oxidative phosphorylation (cytochrome oxidase) and antioxidative function (catalase) all need iron as cofactors. This abundant Fe(II) can be the target for therapy directed for cancer cell-specific ferroptosis. Non-thermal plasma activated lactate Ringer's solution (PAL) is one of them at the preclinical stage. PAL causes ferroptosis specifically in mesothelioma cells in comparison to mesothelial cells. During this process, we observed autophagic process with nitric oxide-associated oxidants in lysosomes. This autophagy, presumably lysophagy, is eventually pathologic, leading to ferroptosis.[96]

Iron and Extracellular Vesicles

In 2021 we reported a finding of the association between iron metabolism and extracellular vesicles (EVs). It is established that various kinds of cells secrete cellular contents as EVs.[99] EVs are classified by their diameter and the formation mechanism into

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Table 3. Contrasting role of poly (rC) binding proteins (PCBPs) in cancer

PCBP1		
2010	Zhang *et al.*	Inhibits invasion of human hepatoma cell line HepG2[131]
2012	Shi *et al.*	Downregulation of PCBP1 correlates with malignant transformation of hydatidiform mole[131]
2015	Wagener *et al.*	Recurrently mutated in Burkitt lymphoma[132]
2015	Zhang *et al.*	HOTAIR long non-coding RNA promotes gastric cancer metastasis through suppression of PCBP1[133]
2015	Liu *et al.*	High expression of PCBP1 with better prognosis of non-small cell lung cancer through preventing EMT[134]
2015	Chen *et al.*	Central to maintenance of prostate cancer stem cells[135]
2016	Horiguchi *et al.*	miR-7977 in extracellular vesicle suppress PCBP1 in myeloid neoplasms to cause hematopoietic dysfunction[136]
2016	Zhang *et al.*	Negative regulator of thyroid carcinoma[137]
2018	Zhang *et al.*	Functions as a tumor suppressor gene in prostate cancer[137]
2022	Lin *et al.*	C12orf48 inhibits gastric cancer growth via PCBP1 upregulation[138]
2022	Lee *et al.*	PCBP1 represses ferritinophagy-mediated ferroptosis in head and neck cancer[138]
PCBP2 (hnRNP E2)		
2002	Perrotti *et al.*	C/EBPalfa is suppressed at the translational level by PCBP hnRNP E2 in BCR-ABL chronic myelogenous leukemia[139]
2010	Eiring *et al.*	miR-328 antagonize hnRNP E2 to impair survival of leukemia[140]
2015	Tang *et al.*	miRNA-214 targets PCBP2 to suppress growth of glioma cells[141]
2015	Xia *et al.*	PCBP2 regulates hepatic insulin sensitivity via HIF-1alpha and STAT3 pathway in HepG2 cells[142]
2016	Wan *et al.*	PCBP2-dependent *c-myc* expression as a binding partner of β2-adrenergic receptor in pancreatic ductal adenocarcinoma[143]
2016	Ye *et al.*	Promotes progression of squamous cell carcinoma[144]
2016	Zhang *et al.*	Overexpression contributes to poor prognosis of human hepatocellular carcinoma[145]
2020	Wen *et al.*	LINC02535 co-functions with PCBP2 to regulate DNA damage repair in cervical cancer[146]
2021	Li *et al.*	Silencing normalizes desmoplastic stroma and chemoresistance in pancreatic cancer[147]
2021	Hou *et al.*	circRNA GRHPR interact with PCBP2 to promote proliferation in non small-cell lung cancer[147]
2021	Ma *et al.*	LincRNA AC104958.2 stabilized by PCBP2 promotes proliferation and invasion of hepatocellular carcinoma[148]
PCBP4		
2015	Ito *et al.*	Suppression reduced cisplatin resistance in human maxillary cancer cells[149]

Selected findings are described.

three classes of exosomes (30–120 nm), microvesicles (100–1,000 nm) and apoptotic bodies (800–5,000 nm).[100] EVs are the scientific basis for the diagnosis of cancer from one droplet of blood, which is already in clinical use. We here refer to EVs as exosomes and some small portion of microvesicles. We found that a typical exsome marker CD63 is under the regulation of IRE/IRP posttranscriptional system by the use of human fibroblast cell IMR90 (Fig. 5).[101] Loading of iron as Fe(III) ammonium citrate significantly increased CD63 protein with IRE-IRP system, where de-repression of CD63 translation started. At the same time significantly increased EVs containing iron-loaded ferritin were released to the media. The IRE sequence in 5' untranslated region was identified in all the higher primates including humans, but not necessarily all the species such as mice and rats. This is an *in vitro* analysis, so further study is necessary on the ferritin section to the serum. However, we believe that this is an important process for the cells to share excess iron among neighbor and distant cells only of the single individual with a safe form of iron as ferritin. Receptors for these EVs containing iron-loaded ferritin have not been unequivocally identified yet.

However, this iron sharing system using EVs may cause some unexpected outcomes to provide the surrounding population of cells with deleterious effects, such as in the case of asbestos exposure. Macrophages are important phagocytic cells, born in the bone marrow as monocytes, recognize foreign antigens with phagocytosis, pass the antigenic information to lymphocytes and work also as a scavenger of iron left by aged or dead cells. Thus, macrophage is located also in the center of iron metabolism in addition to hepatocytes where the iron metabolism is a semi-closed system during the entire life.[32,66]

The target cells in asbestos-induced carcinogenesis are mesothelial cells. There are many reports, including our own, on the direct effect of asbestos.[62,93,102–107] We recently found an indirect effect of asbestos mediating macrophages to mesothelial cells. When asbestos comes to the mesothelium, submesothelial macrophages intrinsically collect most of the exposed asbestos fibers. However, the macrophages cannot digest asbestos fibers, leading to ferroptosis with massive iron inside.[108,109] At this stage, the macrophages emit EVs, which we coined as ferroptosis-dependent EVs (FedEVs). FedEVs contain a high amount of iron-loaded ferritin and of note are taken up by the mesothelial cells present at the surface of somatic cavities, eventually causing oxidative DNA damage.[110] Thus, iron sharing system may lead to harmful effects under the situation of monopoly, where the individual tries not to release any subtle amount of iron to the other infected species or their equivalents.[111]

Physiological Ferroptosis

Starting from the early 2018, we reevaluated the five clones (HNEJ-1~5) of monoclonal antibody against HNE-modified proteins.[42,47] We had a strong belief that some of the clones may be more useful to visualize ferroptosis in FFPE specimens. In our experience, immunohistochemistry is a very strong method to localize and understand responsible pathologies in *in vivo* situations.[7,46] One of our interests in recent years has been to define physiological ferroptosis if present. The five clones showed distinct characteristics and affinity to HNE-associated Michael adducts.[47,48] Thus, we have used many models of ferroptotic as well as non-ferroptotic cell death including apoptosis, and reached the conclusion that HNEJ-1, equally reacting to Cys-, His- and Lys-Michael adducts, is the best to

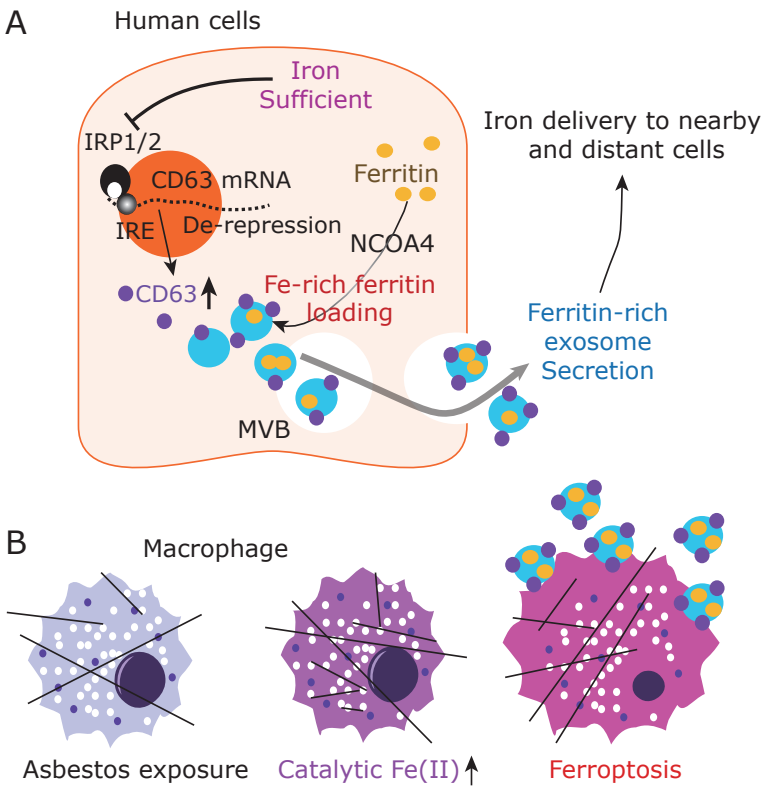

Fig. 5. Extracellular vesicles and iron metabolism. (A) Human cells hold IRE sequence at the 5' region of mRNA for CD63, which is a major marker molecule of exosomes. In case of iron sufficiency, human cells secrete exosomes loaded with untranslated iron-filled ferritin to share the excess iron with the nearby or distant cells of the same individual. IRE, iron-responsive element on mRNA; IRP, iron-responsive protein; MVB, multivesicular body; NCOA4, nuclear receptor coactivator 4. (B) Asbestos exposure to macrophages causes ferroptosis as a pathological condition. During this process, exosomes loaded with iron-filled ferritin are secreted, which causes iron overload in the mesothelial cells, the target of asbestos-induced carcinogenesis. Refer to text for details.

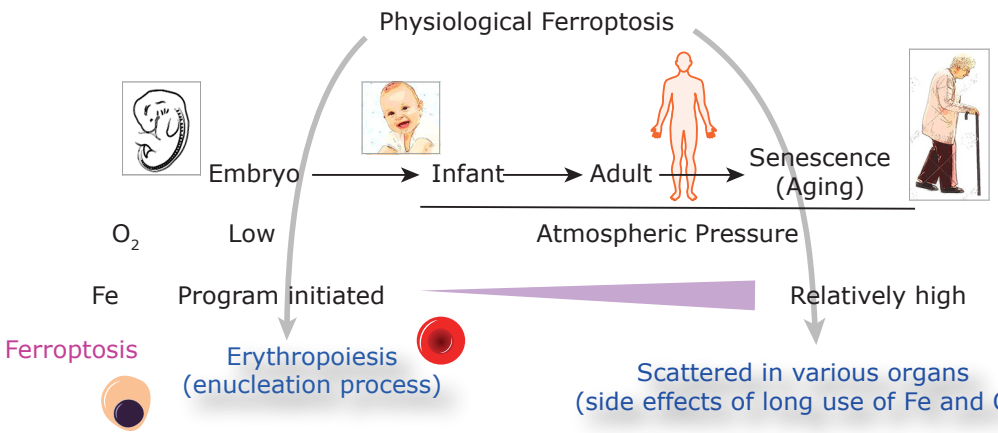

Fig. 6. Ferroptotic process in physiological contexts. We recently found ferroptotic process in embryonal erythropoiesis and aging in rats, which appear to be associated with iron and oxygen metabolisms. Refer to text for details. This figure is partially hypothetical.

visualize ferroptotic process in FFPE specimens.[13] The only weak point of this antibody is of mice origin. Therefore, careful interpretation would be necessary for the application of HNEJ-1 to the cases of *wild-type* and genetically engineered mice models.

Recently, we have performed a series of rat experiments using HNEJ-1 to define physiological ferroptosis (Fig. 6). We observed that ferroptotic cells are increased with aging in various organs, including kidney, spleen, ovary, uterus, and skin. The other interesting finding was that ferroptotic process was involved in embryonic hematopoiesis.[13] Ferroptotic process was observed in the endodermal component of visceral yolk sac at E9.5 of rats and in the nucleated erythrocytes at E13.5 and E15.5. Of note, prevention of ferroptosis by lipoxstatin caused the significant retention of nucleated erythrocytes with anemia. These observations demonstrate the existence of physiological ferroptotic processes.[13]

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Conclusion

Our laboratory started from investigating Fe-NTA-induced renal carcinogenesis model in the 1980's. This model using *wild-type* rodents has been solid enough to mimic human carcinogenesis and contributed tremendously to the concept of carcinogenesis as a process to gain ferroptosis resistance.[7,32,33] Recently, we showed using various strains that ferroptosis resistance determines the susceptibility to Fe-NTA-induced renal carcinogenesis in mice.[112] These findings support the idea of excess iron as a risk for cancer.[66,113] In humans, risk factors associated with carcinogens are identified for certain cancers, such as smoking with lung/laryngeal cancer and asbestos with malignant mesothelioma, but for a major portion of them are not identified. Cancer in the latter category may owe largely to the side effects of long use of iron and oxygen.[33]

No life on earth can survive without iron. Because of this preciousness of iron, each individual has no active pathway to damp iron outside in higher animals. Each cell tries to keep as much iron as possible with various mechanisms (monopoly) when other species invade (infection and inflammation).[32] In the peaceful period, cells within the same individual can share iron via EVs containing iron-loaded ferritin. We for the first time reported that *CD63* encoding a major marker of exosome is under the regulation of IRE/IRP posttranscriptional system specific for iron metabolism.[101] Physiological ferroptosis is observed during embryonal hematopoiesis and aging.[13] We sincerely hope that this review article would stimulate interest in iron metabolism and redox biology of the young investigators worldwide.

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Abbreviations

DMT1	divalent metal transporter 1
EDTA	ethylenediaminetetraacetic acid
EV	extracellular vesicle
FedEVs	ferroptosis-dependent extracellular vesicles
Fe-NTA	ferric nitrilotriacetate
FFPE	formalin-fixed paraffin-embedded
FTH	ferritin heavy chain
FTL	ferritin light chain
HNE	4-hydroxy-2-nonenal
ip	intraperitoneal(ly)
IRE	iron-responsive element
IRP1/2	iron regulatory protein 1/2
NCOA4	nuclear receptor coactivator 4
NTA	nitrilotriacetate
8-OHdG	8-hydroxy-2'-deoxyguanosine
PAL	non-thermal plasma activated lactate Ringer's solution
PCBP1	poly repeated cytidine (*rC*) binding proteins 1
PCBP2	poly repeated cytidine (*rC*) binding proteins 2
RCC	renal cell carcinoma
STEAP3	six-transmembrane epithelial antigen of prostate 3

Conflict of Interest

No potential conflicts of interest were disclosed.

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