Fanconi Anemia and Aldehyde Degradation Deficiency Syndrome: metabolism and DNA repair protect the genome and hematopoiesis from endogenous DNA Damage

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Abstract

We have identified a set of Japanese children with hypoplastic anemia caused by combined defects in aldehyde degrading enzymes ADH5 and ALDH2. Their clinical characteristics overlap with a hereditary DNA repair disorder, Fanconi anemia. Our discovery of this disorder, termed Aldehyde Degradation Deficiency Syndrome (ADDS), reinforces the notion that endogenously generated aldehydes exert genotoxic effects; thus, the coupled actions of metabolism and DNA repair are required to maintain proper hematopoiesis and health.

Introduction

Inherited Bone Marrow Failure Syndrome (IBMFS) is a rare, but devastating, hereditary condition that is often accompanied by an increased incidence of leukemia and solid tumors [1]. Fanconi anemia (FA) is a typical IBMFS disease with a variable constellation of developmental defects and severe clinical manifestations that include hematopoietic stem cell failure, leukemia, and malignant tumors due to defects in the FA pathway [2]. The FA pathway consists of DNA damage repair genes (including 22 genes currently known to be mutated in FA patients) that maintain genome stability by protecting stalled replication forks and repairing DNA interstrand crosslink (ICL) damage. At the cellular level, FA is characterized by a high sensitivity to DNA crosslinking agents such as mitomycin C (MMC), and elevated levels of chromosomal breaks are observed after MMC treatment. This anomaly was discovered by Dr. Masao S. Sasaki [3] and defines FA as a disease [2]. It is a hallmark that is still used for clinical diagnosis of FA, commonly referred to as a "chromosome breakage test".

Our lab has been focusing on the pathophysiology of FA. We have also conducted molecular epidemiological studies to identify genes mutated in FA patients in Japan and elsewhere. Thus, we reported patients with mutations in *FANCT/UBE2T* [4] and *FANCW/RFWD3* [5,6] and the spectrum of mutated genes among FA patients in Japan [7] and India [8]. Meanwhile, we noticed that the JCRB Cell Bank, which was established at the National Institute of Biomedical Innovation, Health, and Nutrition (Osaka, Saito), stores deposited clinical specimens (mostly cultured fibroblasts) from a set of patients with pediatric aplastic anemia of unknown cause. Strikingly, these cells displayed very high levels of sister chromatid exchange (SCE). Because Bloom syndrome was already excluded as the diagnosis, this finding immediately opened up the possibility that these samples may have originated from patients with an unknown genetic etiology. We requested a set of cultured fibroblasts from the repository. Analyses of these and subsequent samples established that seven teenage patients with hypoplastic anemia carried mutations resulting in deficiencies in both the formaldehyde-degrading enzyme ADH5 and acetaldehyde-degrading enzyme ALDH2 [9]. The *ADH5* gene was mutated in a biallelic manner, while the *ALDH2* mutation was heterozygous (*ALDH2*2*, a.k.a. rs671, encoding an E504K missense mutation, commonly referred to as the A-type allele).

Ubiquitously expressed, ADH5 is the most efficient intracellular caretaker for the removal of formaldehyde with help from an important co-factor glutathione as well as Esterase D/ Formylglutathione hydrolase (Figure 1A) [10]. The *ADH5* gene, one of 8 ADH family members, is well conserved throughout evolution, probably reflecting its crucial function in removing formaldehyde resulting from basic biological processes. On the other hand, *ALDH2* is one of 19 (human) or 21 (mouse) ALDH family members [11]. ALDH2 plays a major role in the decomposition of acetaldehyde produced after alcohol consumption [12]. In East Asia, a substantial fraction of the population (~40% in Japan) carries the heterozygous *ALDH2*2* mutation, which acts in a dominant-negative fashion (Figure 1C) because the ALDH2 holoenzyme is a tetramer. Roughly 10% of the

Japanese population are homozygous for the A allele (AA), and usually cannot drink alcohol at all. It is well known that heterozygous carriers of the A allele develop hot flushes and hangovers after drinking alcohol, and they have a very high lifetime risk of esophageal cancer if they habitually drink [13]. The *ALDH2*2* mutation originated somewhere in ancient China [14] and spread to East Asian countries including Japan, where it was positively selected [15]. Why the variation conferred a selective advantage remains unexplained and its whole impact on human physiology awaits further exploration. It is interesting to note that both ADH5 and ALDH2 enzymes are highly expressed in human hematopoietic stem cells (Figure 1D), though some other family members are also expressed at quite high levels.

We propose to refer to the disease caused by a combined deficiency of ADH5/ALDH2 as Aldehyde Degradation Deficiency (ADD) syndrome [16][17]. This disorder is also referred to as "AMeD syndrome" (anemia, mental retardation, dwarfism) [18]; however, we think ADD syndrome is a better name since it directly indicates the pathophysiology. In this brief review, we summarize the research history leading to the discovery of ADD syndrome and discuss how genotoxic aldehydes affect hematopoiesis in ADD syndrome and FA.

Endogenous aldehyde metabolism and hematopoietic failure

Dr. Jun Nakamura (then affiliated with the University of North Carolina at Chapel Hill, USA, now at Osaka Metropolitan University) was the first to report that FA cells show strong sensitivity to treatment with formaldehyde [19]. Formaldehyde may generate DNA interstrand crosslinks (ICLs) as well as protein-DNA crosslinks (DPCs), and the FA pathway was suggested to play a crucial role to remove them. The report provoked considerable interest in the field, with the corollary hypothesis that endogenous aldehydes constitute the primary cause of bone marrow failure in FA. Several other reports later confirmed the requirement of the FA pathway and some other DNA repair genes for alleviating formaldehyde toxicity [20–22]. Importantly, Ketan J. Patel's group at Cambridge University (UK) then made a seminal discovery: as opposed to mouse models of FA that are generally known to be phenotypically very mild, double-knockout mice with deletions of *FANCD2* and *ALDH2* or *ADH5* developed early hematopoietic failure and leukemia [23,24]. Stimulated by these findings, we genotyped *ALDH2* in Japanese FA patients and reported that the presence of the variant A allele strongly accelerates the progression of bone marrow failure (BMF) [25]. In particular, we identified three FA cases carrying homozygous ALDH2 A alleles, and all of them showed very early development of BMF as well as MDS/leukemia. Interestingly, we also found no significant effects of the *ADH1B* variant, which greatly increases the rate of ethanol conversion to acetaldehyde [26], on FA phenotypes among our patient cohort (Hira et al. unpublished). Thus, infant FA patients are unlikely to be affected by alcohol exposure (e.g., *in utero* or by dietary consumption) or endogenous ethanol generation (such as ethanol fermentation by intestinal flora, the auto-brewery syndrome [27]). Our findings confirmed

that the accumulation of DNA damage caused by endogenous aldehydes plays a major role in the pathogenesis of BMF in FA patients. It is important to note that not all of the FA phenotypic features are similarly affected by the *ALDH2* A allele, hinting that the other types of endogenous DNA damage, such as R-loops, potentially contribute to the FA phenotype [28–30]. For example, the body size at birth or the total number of physical abnormalities are not affected by the *ALDH2* status [25].

History leading to the discovery of ADD syndrome

Professor Emeritus Sasaki collected the patient samples mentioned above during his tenure and deposited them in the JCRB cell bank at the time of his retirement (year 2000). Personal information was redacted when the samples were deposited because it is a public repository; thus, the only currently available clinical information is limited to the clinical diagnosis (i.e., aplastic anemia), gender, and age. However, Dr. Sasaki and his co-workers noticed very high levels of SCEs in PHA-stimulated lymphocytes from these cases; surprisingly, they also found that this anomaly was absent in fibroblasts from the same patients (later described in [31][9]). The SCE testing on a clinical specimen such as PHA-stimulated lymphoblasts requires a high degree of experimental skill, and Dr. Sasaki and his group were expert cytogeneticists. Nevertheless, this surprising finding was largely overlooked. The degree of the SCE increase was comparable to that of Bloom syndrome, for which high SCE levels are a hallmark, but they had previously confirmed that these were not Bloom syndrome patients.

SCEs arise from crossover events between sister chromatids that occur when Holliday junctions during homologous recombination (HR) are resolved by the structure-specific nucleases GEN1, MUS81-SLX4, or the BLM helicase complex [32,33]. A replication fork is often stalled by replication stress (e.g., DNA damage) and can be restarted by HR-mediated rescue [34]. Therefore, the number of SCE events reflects (1) the number of DNA lesions, (2) the overall activity of HR, (3) the activity of repair pathways that antagonize HR, and (4) the sub-pathway selection within HR. The high SCE incidence in Bloom syndrome is due to the absence of BLM helicase, which is responsible for non-crossover HJ resolution and is therefore understood to be caused by the lack of one HR sub-pathway option [35]. We suggest that the high SCE levels in ADD syndrome cases reflect the high number of DNA lesions (see below).

Around 2007, we became aware of the existence of these unique samples in JCRB and started analyzing them. At first, we expected to find defects in subunits of the BLM helicase complex or FANCM complex (FANCM interacts with BLM [36], and its deficiency has been associated with increased SCE levels [37]) but could not obtain any results supporting this hypothesis. More recently, next-generation sequencing (NGS) was provided by the Ministry of Health, Labor and Welfare's group research project to overcome intractable diseases (Kojima group, Ito group). We were able to include these samples for NGS analysis as a part of the effort to develop a molecular diagnosis for childhood

BMF cases in Japan. To our surprise, exome sequencing using NGS showed biallelic mutations in the *ADH5* gene in these cases [9]. Because we were working on the role of the *ALDH2* gene in Fanconi anemia patients, and both ADH5 and ALDH2 are aldehyde degradation enzymes, we also tested the *ALDH2* genotype in the samples. They were all GA type heterozygotes, suggesting the involvement of the *ALDH2* variant in their pathophysiology.

To search for more cases with *ADH5* mutations, we then sought cooperation from our collaborators Drs. Miharu Yabe and Hiromasa Yabe of Tokai University Hospital, who had extensive experience in treating childhood hypoplastic anemia, including FA. They sent us samples from six cases with BMF whose clinical diagnoses were unclear, despite an extensive workup (FA and the other conventional IBMFSs were already excluded). Much to our surprise, we readily detected two cases with *ADH5/ALDH2* mutations. In addition, we were able to discover two additional cases with *ADH5/ALDH2* mutations from the samples that were referred to us with suspected FA based on clinical features (chromosome breakage testing was not carried out in these cases).

The identified mutations in the patients and the family trees are summarized in Figure 2 [9]. All in all, we identified four *ADH5* mutations: two missense mutations, one single nucleotide deletion (leading to frameshift truncation), and one splice-site mutation. All of them are very rare, with an allele frequency less than 0.001. The majority of ADDS patients carried *ADH5* biallelic mutations in a compound heterozygous manner (Table 1). The ADH5 holoenzyme is a dimer and the missense mutations locate in the dimer interface. Our experiments suggested that the missense mutations disrupt the dimer formation leading to the loss of protein stability [9]. Thus, the ADDS patient-derived cells did not express a detectable amount of ADH5 protein. The *ALDH2* mutation is also a missense mutation; however, it is known to affect the protein conformation and enzymatic activity but not the protein stability [12].

Common clinical findings of these four patients included short stature, mild mental retardation, aplastic anemia, myelodysplastic syndrome (MDS), and leukemia, and all had already undergone hematopoietic stem cell transplantation at the time of referral. Therefore, the patients with ADD syndrome are clinically/phenotypically similar to FA (Figure 3). The notable difference is that the mild mental retardation seen in ADD syndrome is rarely seen in FA, and the high frequency of skeletal abnormalities in FA is not observed in these patients (Figure 3). A recent report indicated that formaldehyde is generated in the brain, and the resulting DNA damage requires repair by transcription-coupled nucleotide excision repair (e.g., CSB) [38] rather than the FA pathway, which acts in a replication-coupled manner. To more clearly differentiate the clinical findings between the two disorders, it is necessary to accumulate a large number of cases with long-term follow-up. Not surprisingly, the chromosomal breakage test after MMC addition is normal, implying that the DNA repair ability is not impaired in ADD syndrome.

To clarify the relationship between *ADH5/ALDH2* gene abnormalities and elevated levels

of SCEs, we tested the effects of ADH5 inhibitor treatment [39] on SCE levels in the PHA-stimulated lymphocytes of healthy volunteers with different *ALDH2* genotypes [31]. We confirmed that ADH5 inhibition stimulated SCE levels more robustly in individuals with an *ALDH2* heterozygous mutation (E504K, type A allele) compared to *ALDH2* wild-type individuals. Interestingly, the addition of the ADH5 inhibitor to cultures of lymphocytes that were homozygous for the ALDH2 variant inhibited cell proliferation, precluding any SCE evaluation.

The formaldehyde-degrading function of ALDH2

Although we assumed that impairment of both ALDH2 and ADH5 is responsible for the defective hematopoiesis in ADDS patients, it was unclear what kind of endogenous aldehyde is ineffectively catabolized by ALDH2 in the ADDS pathogenesis. Since ADH5 is the main enzyme for formaldehyde catabolism and has not been reported to be active on 4-HNE and the other aldehydes [10], it seems that formaldehyde toxicity should contribute in large part to the pathogenesis of ADD syndrome. Previous studies indicated that, besides acetaldehyde, ALDH2 degrades aldehydes such as 4-hydroxy-2-nonenal (4-HNE) [40]. However, it remained unclear whether ALDH2 can significantly catalyze formaldehyde degradation or not. To investigate the role of ADH5 and ALDH2 in the suppression of formaldehyde toxicity, we used CRISPR/Cas9 to generate several *ADH5/ALDH2* single- and double knockout (KO) cell lines from HCT116, K562, and HAP-1 (derived from chronic myelogenous leukemia) cells. These model cell lines derived from both blood and non-blood cells were examined for formaldehyde sensitivity.

First, we examined the number of SCEs per cell as an indicator of genome damage [9][31]. There was no difference in SCE levels between KO cells and wild-type cells in the unstimulated state. This contrasted with the patient-derived lymphocytes but was consistent with the SCE data in fibroblasts. However, when a small amount of formaldehyde (0.5 μM) was added to cells in culture, more pronounced SCE induction was observed in *adh5/aldh2* double KO cells than in single-KO cells. Previous reports indicate that the blood formaldehyde concentration in humans is around 50-100 μM [19]. These results may suggest that little formaldehyde is produced during normal cell growth, even in blood cells. Alternatively, it can be sufficiently removed by minor degradative systems other than ADH5 and ALDH2. In any case, the PHA-stimulated lymphocytes might be different and generate higher levels of formaldehyde that necessitate ALDH2/ADH5 activity to suppress SCE levels (see below for discussion).

Importantly, in a formaldehyde sensitivity assay, we found that *ALDH2* played a crucial backup role in cell survival, likely functioning to catabolize formaldehyde when ADH5 was deficient [31]. Purified ALDH2 was also proficient to enzymatically degrade formaldehyde [9]. The dominant role of ADH5 over ALDH2 in formaldehyde degradation in cells may be related to the distribution of the former in the cytoplasm and the latter in the mitochondria. In addition, according to Dingler et al.

[9], the respective blood formaldehyde concentrations in *adh5/aldh2*-deficient model mice were as follows: aldh2 deficiency, 9 μM; adh5 deficiency, 11 μM; adh5/aldh2 combined deficiency, 44 μM; by contrast, wild type mice had only 4 μM. These results suggest that ALDH2 is also an important protector against formaldehyde toxicity.

Recapitulating ADDS *in vitro* with iPS model cells

To investigate whether human *ADH5/ALDH2* mutations are the cause of hematopoietic failure, we established model iPS cells by plasmid-based reprogramming of primary fibroblasts from two patients with ADD syndrome [31]. Additionally, using CRISPR/Cas9-based genome editing, we introduced a doxycycline (DOX)-inducible ADH5 expression cassette into the ROSA26 locus of the patient-derived iPSCs. ROSA26 is considered to be a safe harbor locus [41]. In a collaborative research effort, these cells were subjected to an *in vitro* hematopoietic differentiation assay developed by Dr. Akira Niwa and Megumu Saito (Center for iPS Cell Research and Application, Kyoto University (CiRA)) [42]. The model iPSCs were serially treated with cocktails of cytokines and chemicals, and the CD34+KDR+ hematopoietic stem/progenitor fraction was sorted into MethoCult medium on a OP9 feeder layer (Figure 4A). Hematopoietic colonies were barely formed from the patient-derived iPS cells, but the number of colonies was remarkably increased by the induction of ADH5 following the addition of DOX (Figure 4B) [31].

Regarding ALDH2, given that variant ALDH2 acts in a dominant-negative manner, we decided to test the effect of enhancing ALDH2 activity by drugs rather than by genetic complementation [31]. We observed that the addition of a newly developed ALDH2 activator compound called C1 increased the number of hematopoietic colonies, albeit modestly. Immunohistochemical analysis of hematopoietic progenitor cells isolated by CD34+KDR+ flow cytometric sorting during hematopoietic differentiation revealed dot-like relocalization (foci) of DNA damage markers such as FANCD2 and γH2AX in the nucleus, suggesting that DNA damage accumulates during hematopoietic differentiation. More recently, using CRISPR-based prime editing [43], we successfully converted the *ALDH2* A allele to the G allele in the iPSCs and obtained consistent results (Anfeng Mu, unpublished).

To further test the effects of *ADH5* and *ALDH2* mutations in an otherwise normal genetic background, single- and double-KOs of *ALDH2* and *ADH5* were generated using CRISPR/Cas9-based gene targeting in the 201B7 strain, which is an iPS cell line derived from a normal human (obtained from the RIKEN cell bank)[31]. When these model iPS cells were differentiated into the hematopoietic lineage in a monolayer culture system, the CD34-positive double-KO hematopoietic progenitor cells were blocked in differentiation and proliferation, in contrast to the single-KO and the wild type cells. The above results indicate that *ADH5/ALDH2* double-KO iPS cells grow without overt problems under normal maintenance culture conditions, but when induced to differentiate into hematopoietic

cells, they accumulate DNA damage and stop growing. The level of DNA damage at this time is thought to exceed the amount that can be processed by DNA repair via the normal FA pathway. For example, *ADH5/ALDH2* double-KO HAP1 cells exposed to 20 μM formaldehyde accumulated more DNA damage than *FANCD2*-deficient HAP1 cells under the same conditions [31]. At this concentration of formaldehyde, the genome could be protected by the ADH5/ALDH2-mediated degradation pathway more than the FA DNA repair pathway. It is also notable that ADH5/ALDH2 double-KO mice displayed phenotypes comparable to the ADDS patients [9,18,44].

Collectively, we proposed that impaired formaldehyde metabolism is the cause of ADD syndrome (Figure 5). Similarly, FA may result from high levels of DNA damage due to the defective repair of any remaining DNA adducts after formaldehyde removal by functional ADH5/ALDH2. Unfortunately, our experiments have so far failed to directly detect formaldehyde itself or formaldehyde-specific DNA adducts during hematopoietic differentiation of iPSCs. However, since the DNA damage detected during hematopoietic differentiation of iPS cells is suppressed by ADH5/ALDH2, it is reasonable to assume that it is due to formaldehyde production. We could not identify an individual carrying *ADH5* biallelic mutations and wild-type *ALDH2*, and therefore it is unclear how single ADH5 deficiency affects human health. By contrast, we believe the requirement of the *ALDH2* variant for the ADDS phenotype is definite, for the following reasons: (1) all of the identified ADDS patients carried heterozygous *ALDH2* mutations, which is statistically very strong evidence against the argument that an *ADH5* mutation alone is sufficient for ADDS phenotypes; (2) our iPSC models showed an *ADH5* mutation alone did not strongly impair the hematopoietic colony formation *in vitro*; (3) the *ADH5/ALDH2* single- vs double-KO model mice phenotypes also supported our conclusion [9,18,44].

Possibility of other genotoxic aldehydes contributing to human disorders

Although we have argued in favor of formaldehyde as the chief villain in ADDS, we cannot exclude the additional contribution of aldehydes other than formaldehyde in FA; for example, since if *ADH5* is normal, ALDH2 may play only a minor role in formaldehyde catabolism. The severe form of FA caused by additional *ALDH2* mutations may reflect the role of ALDH2 targeting the other genotoxic aldehydes [25]. Vakoc et al. observed that *ALDH2* was under-expressed in acute myeloid leukemia (AML)-derived human cell lines, and cell proliferation was dependent on the FA pathway [45]. This means that either the *ALDH2* or the FA pathway is required for normal leukemic cell proliferation. In line with this study, it also has been reported that defects in *BRCA1* or *BRCA2* together with *ALDH2* deficiency display a synthetic lethal interaction in human cancer cell lines [46]. *BRCA1* and *BRCA2* are critical regulators of HR and are *bona fide* FA genes, *FANCS* or *FANCD1,* respectively. Exactly which aldehydes (or possibly ROS) are the critical substrate of ALDH2 in a wild-type ADH5 background remains to be determined.

A recent study carried out a metabolism-focused drop-out CRISPR screen in *FANCD2* KO cells derived from the Jurkat human leukemia cell line, and identified ALDH9A1 and ADH5, but not ALDH2, as being synthetic lethal with the Fanconi pathway [47]. The relevant endogenous aldehyde targeted by *ALDH9A1* is also suggested to be 3-aminopropanol or acrolein based on a suppressor CRISPR screen. Compared to the synthetic phenotype caused by loss of *ALDH2* in FA, it seems *ALDH9A1* deficiency impaired FA mice only mildly. Nonetheless, this study potentially expands the scope of genotoxic aldehydes and corresponding metabolic protection by these enzymes in FA.

Based on our observation that the *ALDH2* variant strongly affects FA disease progression and given the functional similarity in FA and *BRCA* genes in HR/ICL repair/fork protection, we were interested to test whether a genetic interaction between *BRCA1* or *BRCA2* with *ALDH2* can be observed in breast cancer development among hereditary breast and ovarian cancer syndrome (HBOC) patients [48]. HBOC is a disorder with dominant inheritance. Breast cancer development in an individual with a heterozygous mutation in either BRCA1 or BRCA2 invariably involves the loss of the wild-type *BRCA* allele (loss of heterozygosity), leading to loss of HR activity [49]. We hypothesized that the *ALDH2* variant may affect breast cancer development in HBOC since *BRCA* loss and the *ALDH2* variant are synthetic lethal. We collected ~100 Japanese HBOC patients with verified *BRCA1* or *BRCA2* pathogenic mutations and carried out *ALDH2* genotyping. However, we could not detect any clear effects of the *ALDH2* variant on any clinical parameters including the age at first diagnosis of breast cancer [48]. Interestingly, another group reported a similar observation that a common *ALDH2* variant in European ancestry (rs10744777) did not affect breast cancer development in those with pathogenic *BRCA2* mutations, though breast cancers in carriers with *BRCA2* p.K3326* which is a c-terminal small deletion with a questionable clinical impact (Variant of Unknown Significance, VUS), were significantly accelerated [50]. Thus, the synthetic lethal relations between FA or *BRCA* genes and *ALDH2* might be context-dependent or limited to some organs like hematopoietic stem/progenitor cells or cultured cell lines.

What mechanisms induce formaldehyde production during hematopoietic differentiation?

During hematopoietic differentiation in humans, massive histone methylation and demethylation may occur over the genome because of transcriptional reprogramming of genes [51,52]. We speculate that this methyl group (CH3-) is converted to large amounts of formaldehyde upon demethylation [53]. Formaldehyde produced during hematopoietic differentiation is removed if the degradation system by ADH5 and ALDH2 functions well, but DNA damage caused by some remaining formaldehyde could be the cause of BMF in FA. A recently published study by Lei Li et al. detected strong transcriptional activation and formaldehyde production associated with the induction of hematopoietic differentiation in cell lines, supporting this hypothesis [54,55]. We speculate that the lymphocyte-specific elevation of SCEs in ADD syndrome reflects the induction of transcriptional reprogramming and robust cell

proliferation in the process of PHA-stimulated lymphocyte blastogenesis. This needs to be confirmed in future studies.

Conclusions and future perspective

Bone marrow transplantation is currently the only curative therapy for BMF of FA and ADD syndrome in clinical practice. Efforts by researchers in this area, including ours, have suggested that endogenous aldehydes appear to be the major cause of hematopoietic failure in FA and ADD syndrome. The identification of endogenous aldehydes as a potential source of DNA damage in FA or ADD syndrome might open a new avenue to the development of novel therapies to ameliorate the progression of BMF in these disorders. In principle, the efficient removal of excess formaldehyde from the body would be expected to become an effective therapeutic method. Formaldehyde could be targeted by a quencher drug [56] or the mechanism that generates formaldehyde might be inhibited by histone demethylase inhibitors. ADH5/ALDH2 activator drugs might be an exciting possibility for FA patients. To test these possibilities, the human iPSC model with the *in vitro* hematopoietic differentiation assay might be valuable for screening chemical or CRISPR libraries and to further confirm the efficacy of candidates in preclinical testing.

Although hematopoietic stem cell transplantation is effective against BMF and MDS/leukemia, it does not solve systemic problems, and carcinogenesis after bone marrow transplantation is an important clinical problem with FA [57]. It is currently unclear whether ADDS patients show an increased incidence of cancer after hematopoietic stem cell transplantation. This should be kept in mind since ADH5 knockout mice show an increased incidence of hepatocellular carcinoma [58], and the ADDS model mice develop liver tumors as well [9]. Strict follow-up is essential after identifying a larger number of ADD patients, because additional problems may occur in the clinical course of ADD syndrome. We suggest measuring and comparing blood formaldehyde levels before and after hematopoietic stem cell transplantation in new ADDS patients. This may reveal to what extent hematopoietic differentiation contributes to overall endogenous formaldehyde generation in the body and can be helpful in considering the long-term prognosis of ADDS patients. Notably, ADH5 has another enzymatic function as S-nitrosoglutathione reductase (GSNOR) and it is unclear how loss of this function affects phenotypes in ADDS patients. Finally, we caution that there may be downsides to the clinical use of an ADH5/GSNOR inhibitor [59], particularly for East Asian populations.

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Table1. Summary of ADDS patients and their phenotypes.

Case #: ID	Age (years) Gender	*ADH5* mutations	*ALDH2* genotype	Treatment and outcome	Birth weight and stature	Other clinical features
P1: AP39P	10/F	c.564+1G>A: p.L188PfsX4 c.832G>C: p.A278P (Het)	G/A	Not known	Not known	Not known
P2: AP57P	13/M	c.966delG: p.W322X c.832G>C: p.A278P (Het)	G/A	Not known	Not known	Not known
P3: FA50P	19/F	c.966delG: p.W322X (Hom)	G/A	Not known	Not known	Not known
P4: TKFA-	1/F	c.966delG: p.W322X c.932G>A: p.G311D (Het)	G/A	HSCT at age 11 years, ongoing remission at 59 months post-HSCT.	Birthweight, 2616g, -1.48SD; short stature, -4.9SD at 138 months.	Skin pigmentation, café au lait spots, mild mental retardation.

P5: TKFB-09	15/M	c.966delG: p.W322X c.832G>C: p.A278P (Het)	G/A	HSCT at age 16 years, died 60 months post-HSCT.	Birthweight, 2784g, -1.31SD; short stature, -3.4SD at 182 months.	Skin pigmentation, vitiligo, mild mental retardation.
P6: KDFA-08	16/F	c.966delG: p.W322X (Hom)	G/A	1st and 2nd HSCT at age 18 and 19 years, ongoing remission at 6 months post 2nd HSCT.	Birthweight, 2730g, -0.37SD; short stature, -2.4SD at 194 months.	Short left 4th toe, microcephaly, mild mental retardation.
P7: KDFA-13	4/F	c.966delG: p.W322X c.832G>C: p.A278P (Het)	G/A	HSCT at age 4 years, ongoing remission.	Birthweight, 2935g, -0.34SD; short stature, -2.0SD at 49 months.	Skin pigmentation, café au lait spots, microcephaly, mild mental retardation.

F, female; M, male; HSCT, hematopoietic stem cell transplant; SD, standard deviation from local population median.

Figure 1. Canonical biochemical functions of ADH5 (A) and ALDH2 (B) enzymes in aldehyde catabolism. GSH, Glutathione. ESD, esterase D/ Formylglutathione hydrolase. The image was created using BioRender (https://www.biorender.com/). C. Summary of the ALDH2 genotypes and phenotypes. D. Microarray expression of members of ADH or ALDH family in human hematopoietic stem/progenitor cells. CB, cord blood. BM, bone marrow. HSC hematopoietic stem cells. The data were derived from Gene Expression Commons (https://gexc.riken.jp/).

Figure 2. The identified mutations in ADDS patients (A) and the family pedigrees (B). N.T., not tested. Variations in ADH5 and ALDH2 genes in the family members are shown. The genotype data from the ADDS patients (P4-P7) are in Table 1. These panels are adapted from Dingler et al. [9], with modifications.

Figure 3. Comparison of the clinical features between ADDS and FA. The image was created using BioRender.

Figure 4. *In vitro* hematopoietic differentiation assay of ADDS model iPSCs. A. Simplified outline of the protocol to induce hematopoietic differentiation from iPSCs. During the HSC induction period, the cells were serially stimulated with cocktails of various cytokines and chemicals. At day 7, the induced cell population was stained with anti-CD34 and anti-KDR, and the double-positive fraction was sorted into Methocult colony forming medium on OP9 feeder cells. B. A representative image of the induced hematopoietic colonies. The iPSCs derived from an ADDS patient AP39P were induced for 7 days and allowed to form colonies in Methocult for 12 days. DOX treatment was applied to induce ADH5 from the expression cassette, which was integrated into the safe harbor locus using CRISPR genome editing.

Figure 5. Our proposed model for pathophysiology of ADD syndrome and Fanconi anemia compared with the situation in a healthy individual. HCHO, formaldehyde. HSPCs, hematopoietic stem and progenitor cells.

References

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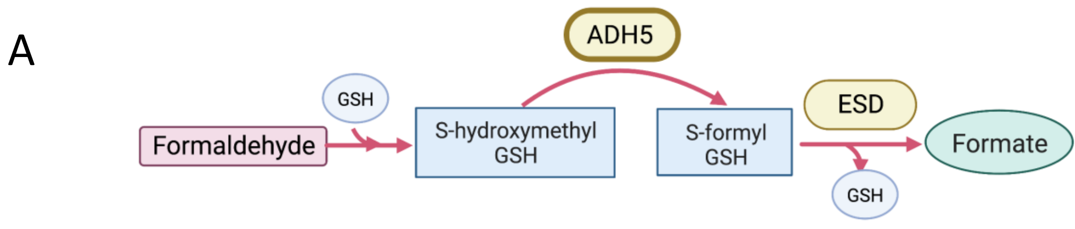
A



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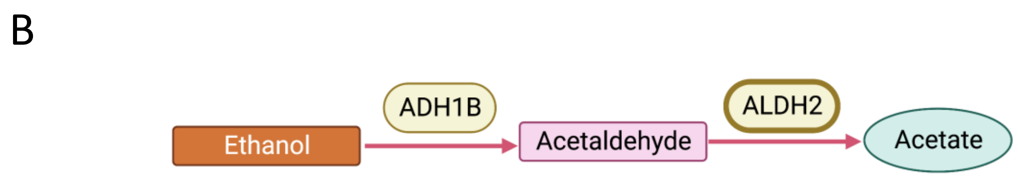


C

ALDH2 genotype	Amino acids	Enzymatic activity	Alcohol flushing	Frequency among Japanese
GG type	E504	normal	-	~50%
GA type	E504/K504	~10%	+	~40%
AA type	K504	deficient	++	~10%

D

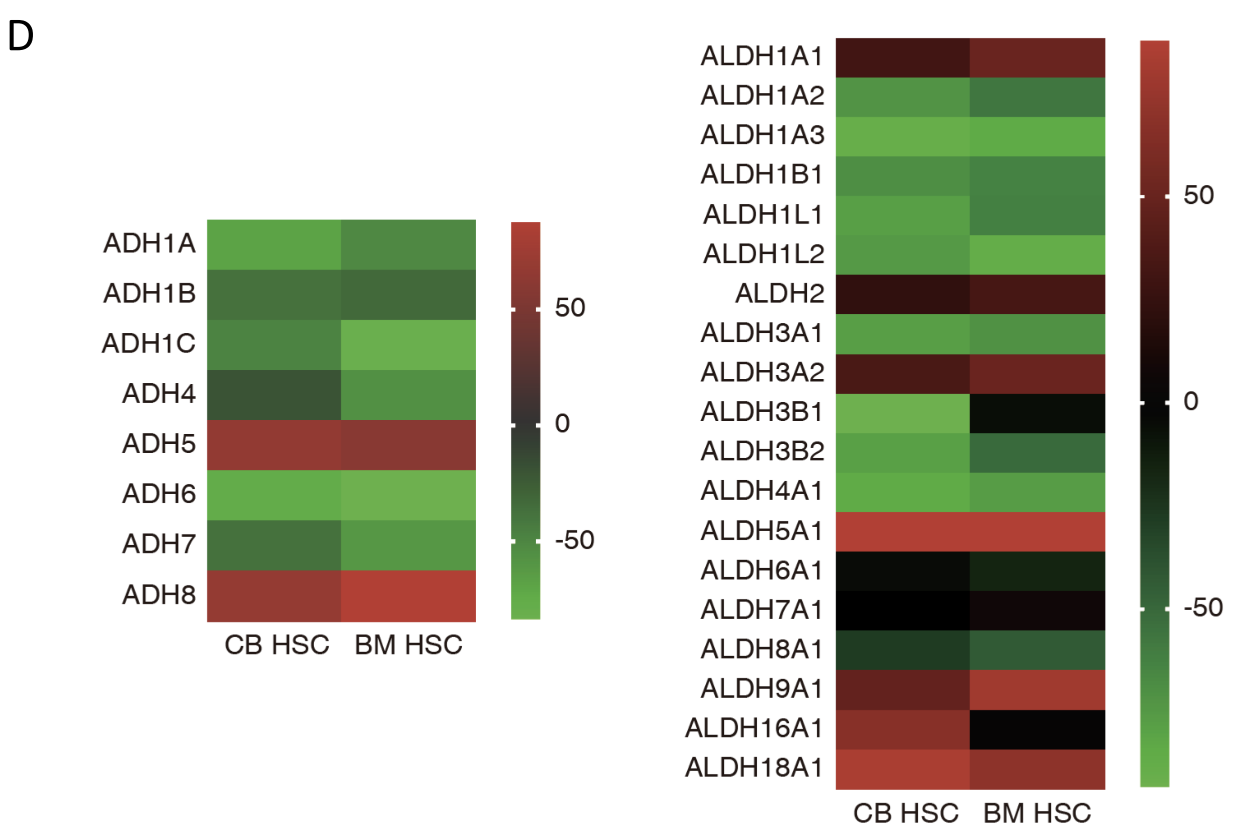
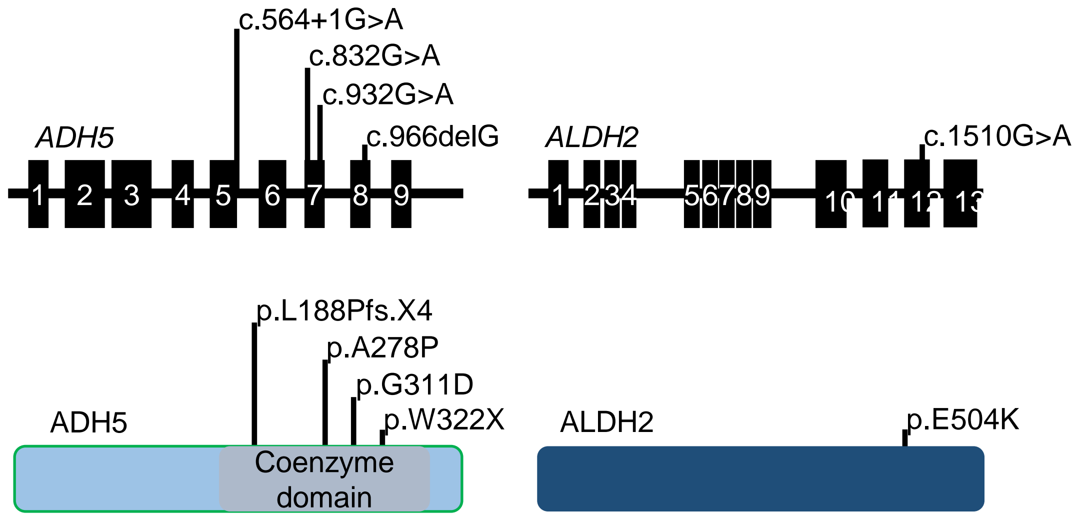


Figure 1.

A



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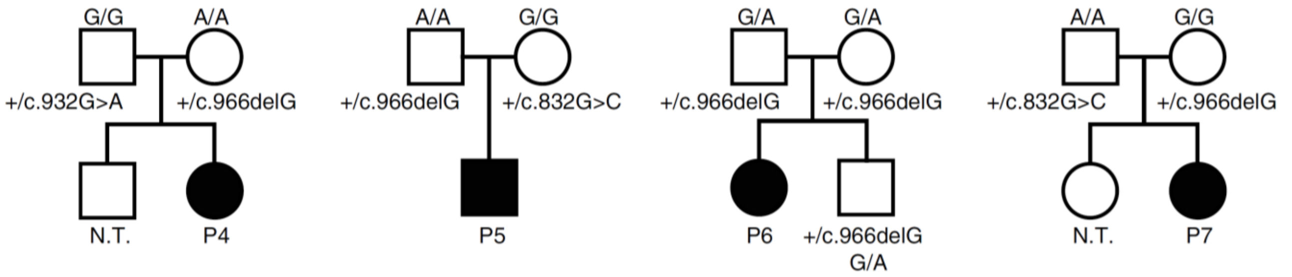


Figure 2.

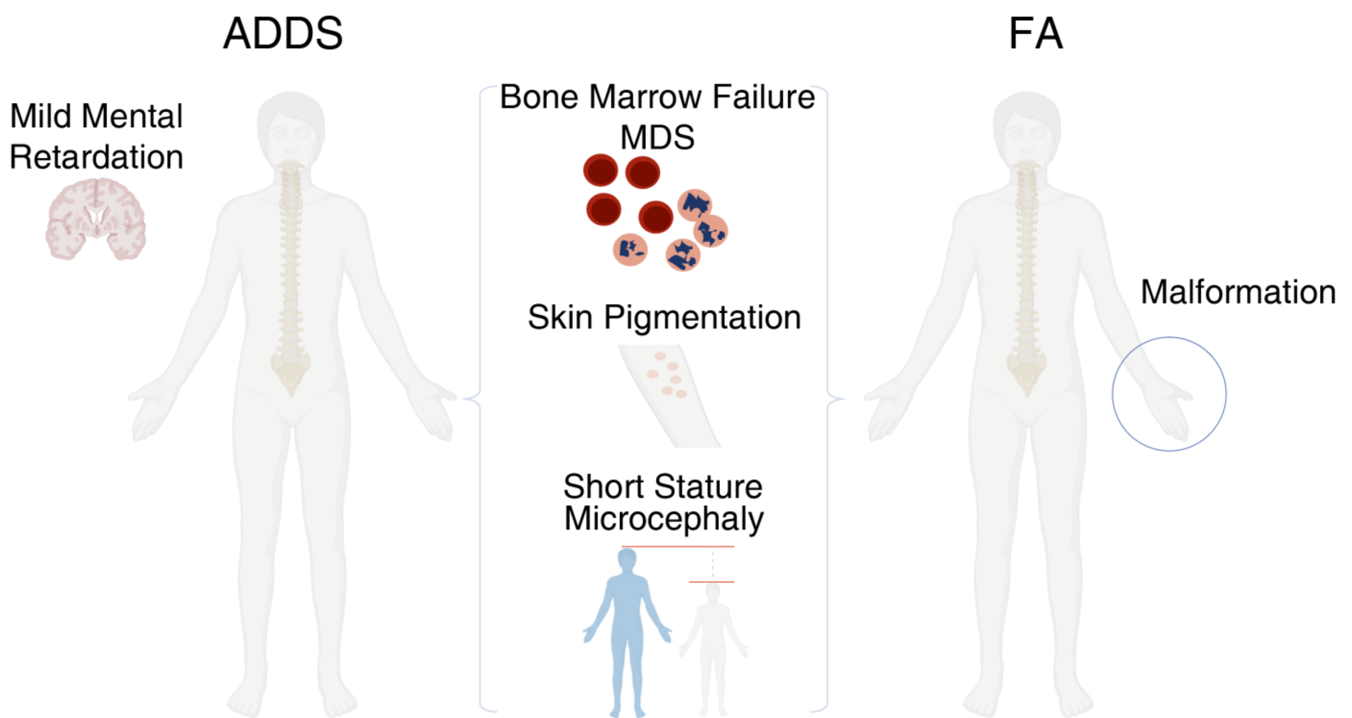
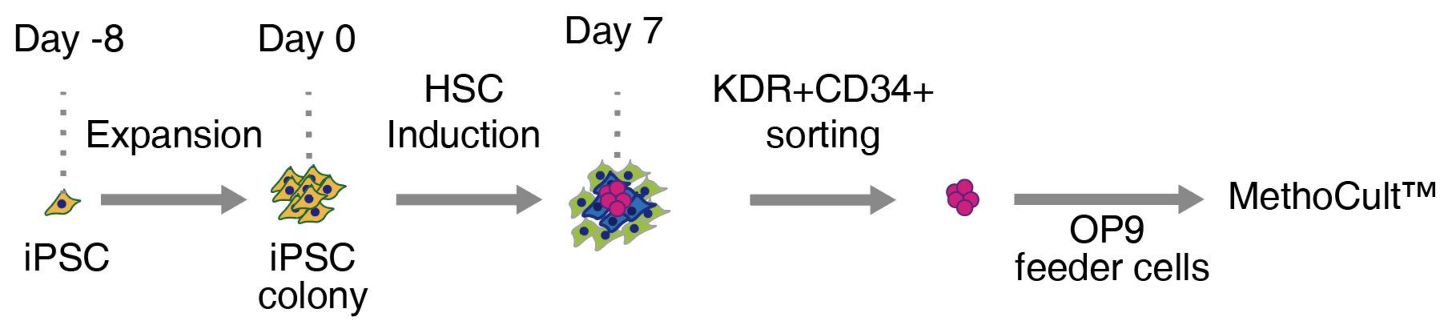
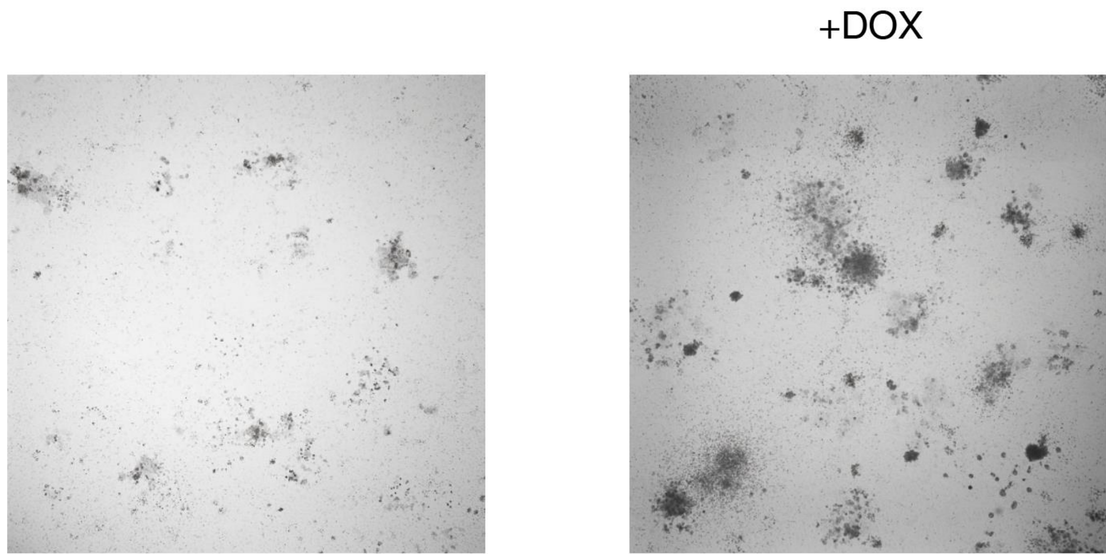


Figure 3.





AP39P-iPSC (12 days in MethoCult)

Figure 4.

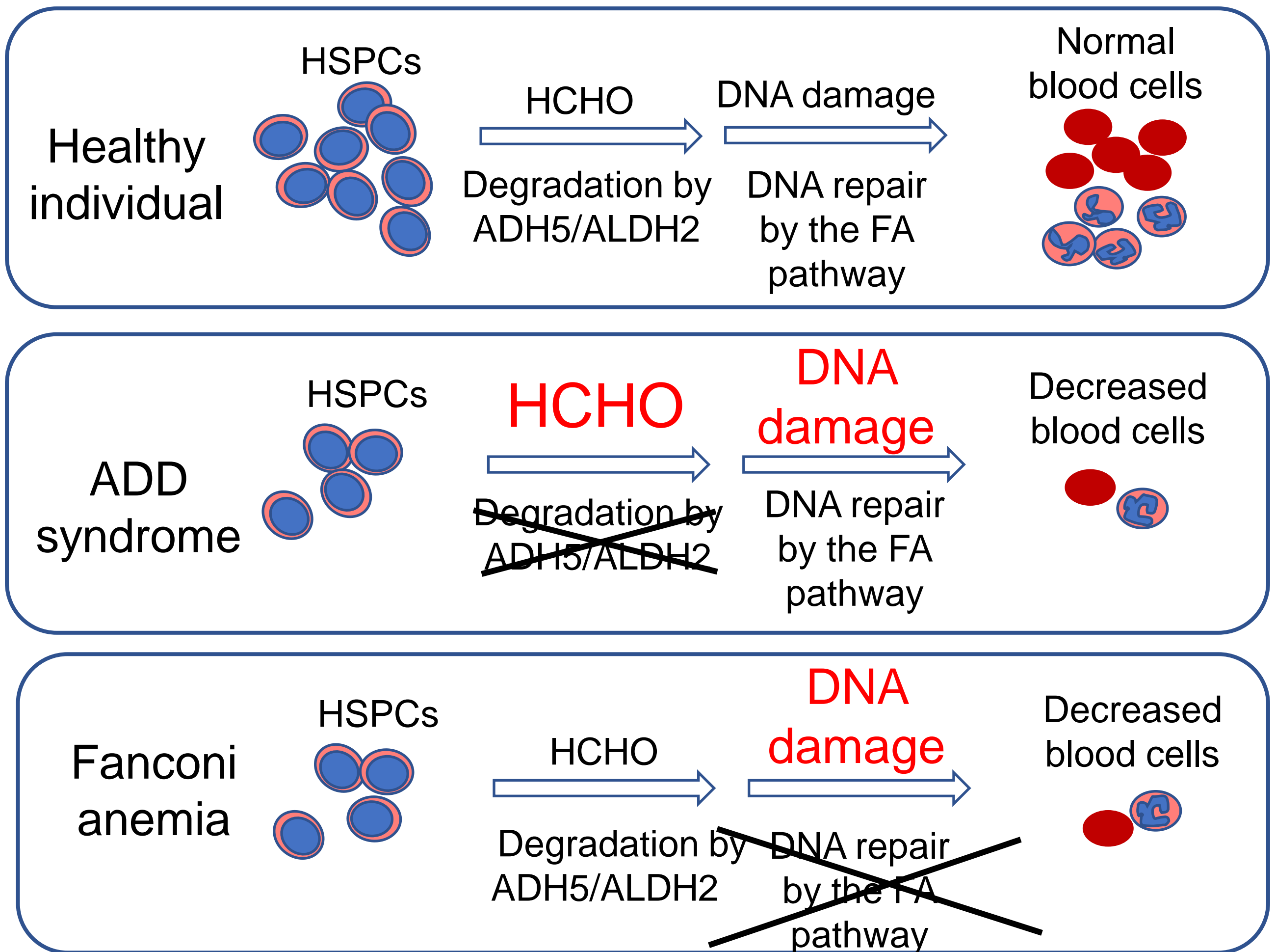


Figure 5.