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Chk'ing p53-deficient breast cancers

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Commentary

Loss or functional impairment of p53 occurs in many human cancers, and its absence is often associated with a poor response to conventional chemotherapy. Hence, much effort is currently devoted to developing novel treatments for p53-deficient malignancies. One approach is to target pathways that are selectively required for the survival of p53-deficient cancer cells, thus exploiting a synthetic lethal interaction. Previous studies have demonstrated that inhibition of the ataxia telangiectasia and Rad3-related (ATR) and checkpoint kinase 1 (Chk1) pathway in p53-deficient cells can induce such a synthetic lethal outcome. In this issue of the *JCI*, Ma et al. take these findings a step closer to the clinic by demonstrating that highly specific inhibitors of Chk1 synergize with chemotherapy to stem progression of p53-deficient triple-negative breast cancers in a xenotransplant model of this disease. Together with other recent studies, this report highlights the promise of ATR and Chk1 inhibitors in targeted cancer treatment.

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1. Goetz SC, Anderson KV. The primary cilium: a signalling centre during vertebrate development. *Nat Rev Genet*. 2010;11(5):331–344.
2. Zaghloul NA, Katsanis N. Mechanistic insights into Bardet-Biedl syndrome, a model ciliopathy. *J Clin Invest*. 2009;119(3):428–437.
3. Rachel RA, et al. Combining *Cep290* and *Mkks* ciliopathy alleles in mice rescues sensory defects and restores ciliogenesis. *J Clin Invest*. 2012; 122(4):1233–1245.
4. Gerdes JM, Davis EE, Katsanis N. The vertebrate primary cilium in development, homeostasis, and disease. *Cell*. 2009;137(1):32–45.
5. Moser JJ, Fritzler MJ, Ou Y, Rattner JB. The PCM-basal body/primary cilium coalition. *Semin Cell Dev Biol*. 2010;21(2):148–155.
6. Wiszniewski W, et al. Potential involvement of more than one locus in trait manifestation for individuals with Leber congenital amaurosis. *Hum Genet*. 2011;129(3):319–327.
7. den Hollander AI, Roepman R, Koenekoop RK, Cremers FP. Leber congenital amaurosis: genes, proteins and disease mechanisms. *Prog Retin Eye Res*. 2008;27(4):391–419.
8. McEwen DP, et al. Hypomorphic CEP290/NPHP6 mutations result in anosmia caused by the selective loss of G proteins in cilia of olfactory sensory neurons. *Proc Natl Acad Sci U S A*. 2007;104(40):15917–15922.
9. Fath MA, et al. Mkks-null mice have a phenotype resembling Bardet-Biedl syndrome. *Hum Mol Genet*. 2005;14(9):1109–1118.
10. Betleja E, Cole DG. Ciliary trafficking: CEP290 guards a gated community. *Curr Biol*. 2010; 20(21):R928–R931.
11. Coppieters F, Lefever S, Leroy BP, De Baere E. CEP290, a gene with many faces: mutation overview and presentation of CEP290base. *Hum Mutat*. 2010;31(10):1097–1108.
12. Kim J, Krishnaswami SR, Gleeson JG. CEP290 interacts with the centriolar satellite component PCM-1 and is required for Rab8 localization to the primary cilium. *Hum Mol Genet*. 2008;17(23):3796–3805.
13. Tsang WY, et al. CP110 suppresses primary cilia formation through its interaction with CEP290, a protein deficient in human ciliary disease. *Dev Cell*. 2008;15(2):187–197.
14. Craige B, et al. CEP290 tethers flagellar transition zone microtubules to the membrane and regulates flagellar protein content. *J Cell Biol*. 2010; 190(5):927–940.
15. Murga-Zamalloa CA, et al. Accumulation of the Raf-1 kinase inhibitory protein (Rkip) is associated with Cep290-mediated photoreceptor degeneration in ciliopathies. *J Biol Chem*. 2011; 286(32):28276–28286.
16. Seo S, et al. BBS6, BBS10, and BBS12 form a complex with CCT/TRiC family chaperonins and mediate BBSome assembly. *Proc Natl Acad Sci U S A*. 2010;107(4):1488–1493.
17. Nachury MV, et al. A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. *Cell*. 2007; 129(6):1201–1213.
18. Cao H, Molday RS, Hu J. Gene therapy: light is finally in the tunnel. *Protein Cell*. 2011;2(12):973–989.
19. Ashtari M, et al. The human visual cortex responds to gene therapy-mediated recovery of retinal function. *J Clin Invest*. 2011;121(6):2160–2168.
20. Smith AJ, Bainbridge JW, Ali RR. Gene supplementation therapy for recessive forms of inherited retinal dystrophies. *Gene Ther*. 2012;19(2):154–161.

Chk'ing p53-deficient breast cancers

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Loss or functional impairment of p53 occurs in many human cancers, and its absence is often associated with a poor response to conventional chemotherapy. Hence, much effort is currently devoted to developing novel treatments for p53-deficient malignancies. One approach is to target pathways that are selectively required for the survival of p53-deficient cancer cells, thus exploiting a synthetic lethal interaction. Previous studies have demonstrated that inhibition of the ataxia telangiectasia and Rad3-related (ATR) and checkpoint kinase 1 (Chk1) pathway in p53-deficient cells can induce such a synthetic lethal outcome. In this issue of the *JCI*, Ma et al. take these findings a step closer to the clinic by demonstrating that highly specific inhibitors of Chk1 synergize with chemotherapy to stem progression of p53-deficient triple-negative breast cancers in a xenotransplant model of this disease. Together with other recent studies, this report highlights the promise of ATR and Chk1 inhibitors in targeted cancer treatment.

Breast cancers are a heterogeneous group of tumors that can be classified into several subtypes based on histological observations and molecular profiling. Each subtype can vary in epidemiology, response to treatment, and risk of progression and recurrence. Triple-negative breast cancer (TNBC) is defined by the loss of estrogen receptor and progesterone receptor expression as well as the lack of human epidermal growth factor receptor 2 (*HER2*) amplification (1). Management of patients with these cancers can represent a serious challenge, as TNBCs are generally very aggressive and unresponsive to the standard molecularly targeted therapy (HER2 interference and hormonal therapy). Hence, there is much interest, and recent preliminary success, in identifying and manipulating other targets for the treatment of this disease (2). Notably, the p53 pathway is often disrupted in TNBC. In this issue of the *JCI*, Ma et al. report data from a human-in-mouse model of TNBC that highlight the promise of checkpoint kinase 1 (Chk1) inhibitors as targeted therapy for p53-deficient TNBCs (3).

Targeting Chk1 in an advanced experimental model of TNBC

The ataxia telangiectasia and Rad3 related (ATR) and Chk1 kinases function in a linear pathway that serves as a "shock absorber" to perturbations to DNA replication. Specifically, activation of the ATR/Chk1 pathway during replication stress both prevents collapse of troubled replication forks into DNA double-strand breaks and inhibits cell-cycle progression into M phase. Previous culture-based studies have demonstrated that suppressing the G_2-M phase checkpoint through ATR and Chk1 inhibition is particularly toxic when combined with loss of G_1-S checkpoint function via p53 deficiency (4–9). This dual loss produces a checkpoint short circuit (Figure 1A). These observations, together with the fact that TNBCs frequently harbor mutations in *TP53*, led Ma and colleagues to hypothesize that p53-deficient TNBCs might be sensitive to selective inhibition of Chk1 (3).

To best model TNBC, Ma et al. grafted cancerous tissue obtained from patient

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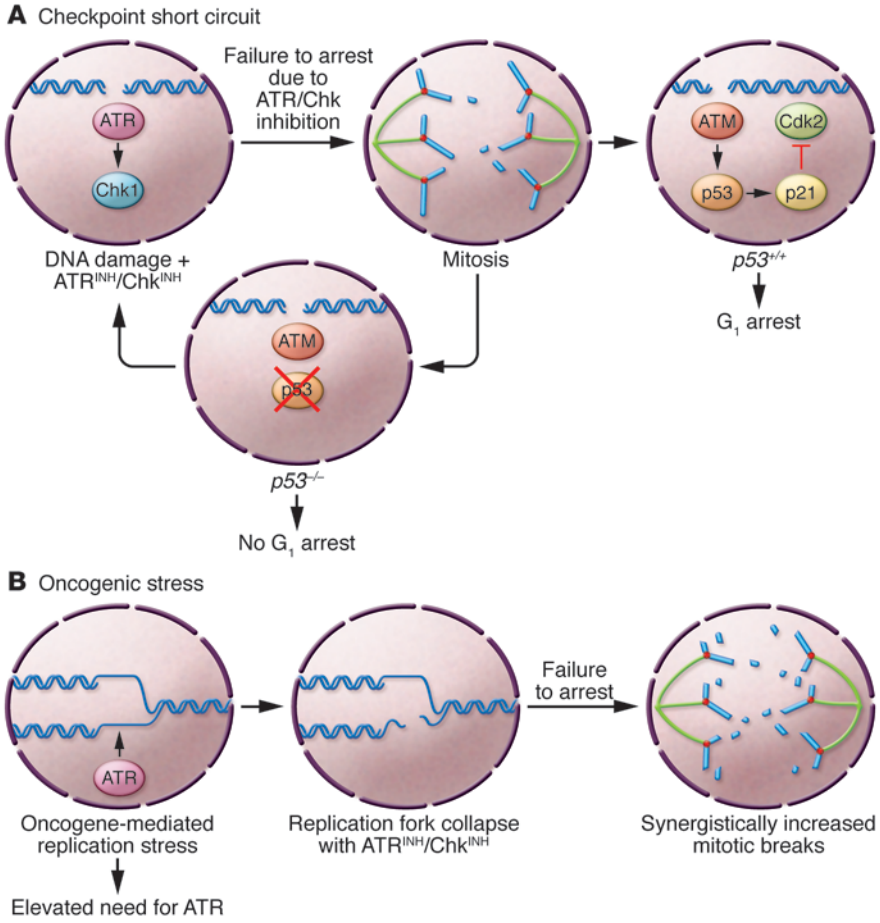

Figure 1

Proposed means by which p53 deficiency and oncogenic stress sensitize cancer cells to ATR/Chk1 inhibition. (**A**) Checkpoint short circuit. Loss of control of both the G_2-M and G_1-S checkpoints through ATR/Chk1 pathway inhibition and p53 loss, respectively, leads to increased damage through reiterative cycles into S phase and through mitosis, ultimately resulting in cell death. (**B**) Oncogenic stress. Oncogenic stress produces an increased reliance on the ATR/Chk1 pathway to prevent replication fork collapse into DNA double-strand breaks. Synergistic increases in DNA damage following ATR/Chk1 inhibition in oncogene-transformed cells are generated within individual cell cycles (13–17).

biopsies directly into humanized mammary fat pads of NOD/SCID mice (3). This approach represents one of the major strengths of the study, as it avoids the well-recognized genetic and epigenetic alterations that occur following the passage of tumor cells in culture. Furthermore, Ma et al. confirmed that the engrafted TNBCs remained similar to the original human tumor by performing gene-expression profiling and went on to classify the tumors into breast cancer subtypes prior to treatment. This overall strategy certainly holds great promise for modeling therapy-resistant human malignancies and, moreover, provides a means to predict sensitivity to targeted treatments on an individualized basis prior to clinical intervention.

Consistent with results obtained using culture-based systems (4–9), cotreatment of a p53-mutant TNBC with chemotherapy and a Chk1 inhibitor substantially delayed cancer progression and improved survival relative to chemotherapy alone (3). Notably, p53-mutant tumors contained many cells with DNA damage that were also in mitosis, suggesting that checkpoint bypass was the underlying mechanism of synthetic lethality following chemotherapy and Chk1 inhibitor treatment. Inappropriate progression into M phase through Chk1 inhibition was associated with an increase in apoptosis in p53-mutant tumors, but not their p53 WT counterparts. Overall, the study by Ma et al. employs a robust model of TNBC with direct clinical relevance. It also substantially reaffirms the conclusion that p53-deficient TNBC cells are exquisitely sensitive to G_2-M checkpoint abrogation following chemotherapy treatment. These results will certainly incite further interest in the development of Chk1 inhibitors and other compounds that target kinases controlling the G2-M checkpoint.

Renewing interest in ATR/Chk1 targeting

Although potential of ATR/Chk1 inhibition for cancer therapy has been known for over a decade, initial clinical trials using the relatively nonspecific Chk1 inhibitor UCN-01 in a variety of human cancers have been somewhat disappointing (10). These largely negative outcomes have been attributed to a number of factors, ranging from an unfavorable pharmacokinetic profile to general toxic effects, which may be related to both on-target and off-target actions of this drug (10). However, some partial tumor responses have been observed in certain trials, and these have, in select situations, correlated with p53 deficiency (11). Notably, by using a highly specific inhibitor of Chk1 (AZD7762), Ma et al. avoided many of the problems associated with UCN-01 (3). Moreover, their results suggest that the conflicting outcomes produced by UCN-01 treatment may indeed be the product of off-target toxicities and thus have reintroduced Chk1 as a bona fide target for the treatment of p53-deficient cancers. As p53 is mutated or otherwise inactivated in many human malignancies, ATR/Chk1 inhibitors may indeed find their place as a substantial therapeutic option, especially for cancers refractory to conventional treatment modalities.

Understanding the synthetic lethality induced by ATR/Chk1 inhibition

The effective short-circuiting of both cell-cycle checkpoints and genome maintenance regulation has been proposed as one mechanism driving the synthetic lethal interaction between p53 deficiency and ATR/Chk1 pathway inhibition (4–9). The compound effects of G_1-S checkpoint loss conferred by p53 deficiency and S/G_2-M abrogation via ATR/Chk1 suppression are thought to allow for continued cell cycling and the accrual of additional DNA damage during repeated S phases, ultimately leading to cell death (Figure 1A). Initial work in this area demonstrated that p53-deficient cells are more sensitive to ionizing radiation when treated with either caffeine or UCN-01, compounds that can suppress the S/G_2-M

checkpoint through inhibition of multiple kinases, including ATR and Chk1 (4–9). These reports indicate the particular importance of ATR and Chk1 in maintaining the survival of p53-deficient cells under chemotherapeutic treatment.

Additional support and expansion of this model have subsequently been provided by a number of groups (9, 10). Recently, Yaffe and colleagues demonstrated that suppression of MAPK-activated protein kinase 2 (MK2), a component of the G_2-M checkpoint pathway that operates in concert with Chk1, induces selective lethality in p53-deficient cells through a similar checkpoint-bypass mechanism (12). Although the preferential sensitivity of p53-deficient cells to G_2-M phase checkpoint inhibition appears to be mediated at least partly through such a checkpoint short circuit (Figure 1A), it remains possible that this synthetic lethal interaction is also attributable to distinct mechanisms, including direct genome-destabilizing effects in the S and G_2 phases of the cell cycle. It therefore may be that the usefulness of ATR/Chk1 inhibitors extends beyond the scope of p53 deficiency to situations in which maintenance of genome integrity in S and G_2 is made tenuous through other cancer-associated alterations.

Accordingly, recent work from several laboratories has demonstrated that p53 deficiency is not the only condition under which ATR/Chk1 inhibitor–based therapies will be particularly effective. These studies have shown that high levels of oncogenic stress, such as those generated by overexpression of oncogenic Ras mutants or c-Myc, sensitize cells to ATR and Chk1 inhibition (Figure 1B). This sensitization does not require the addition of DNA-damaging chemotherapy, but instead relies on the inherent genome-destabilizing effect of ATR/Chk1 pathway inhibition when it is combined with oncogene-mediated replicative stress (13–17). Such replication stress causes aberrant DNA replication progression and the activation of the ATR pathway (18, 19). Therefore, oncogenic stress appears to create an increased reliance on ATR and Chk1 to prevent double-strand breaks during S phase. Thus, inhibition of ATR or Chk1 in combination with oncogenic stress causes greater-than-additive increases in genomic instability within individual cell cycles (Figure 1 B and refs. 13–17). Although the mechanisms underlying the synthetic lethal interaction between oncogene expression and ATR/Chk1 pathway disruption are largely unknown, they are likely associated with an increased dependence on the ATR/Chk1 pathway to maintain replication fork stabilization in the face of oncogene-associated metabolic imbalances and accelerated entry into S phase (13).

Like ATR/Chk1 inhibition and p53 deficiency, the synthetic lethal relationship between oncogenic stress and ATR/Chk1 pathway suppression has recently been associated with a robust therapeutic response in a wide variety of frank malignancies (15–17). Interestingly, when normalized by cell-cycle number, the elevated genomic instability produced by ATR suppression in transformed cells correlates better with oncogene expression than p53 deficiency alone (17). These studies demonstrate that oncogenic stress, like p53 deficiency, is a key determinant of cancer cell susceptibility to ATR/Chk1 inhibition. Elucidating the specific mechanisms by which oncogenic stress increases dependence on the ATR/Chk1 pathway and precisely how these mechanisms are distinct from those produced by p53 deficiency will provide valuable insights into the genetic indicators of therapeutic responses to ATR/Chk1 inhibition.

Further developing the therapeutic potential of ATR/Chk1 inhibition

Although the preclinical findings described herein hold great promise for cancer treatment in humans, caution is still warranted in regard to the potential general toxicities of ATR/Chk1 inhibition (3, 20, 21). However, in one recent study, a genetic approach was used to compare the overall toxicity of ATR suppression with its therapeutic potential (17). In this model, ATR expression was systemically suppressed to 10% of normal levels. Despite this substantial reduction in ATR expression, the functions and cellularity of the bone marrow and intestines remained largely intact, indicating that a 90% reduction in ATR expression is well tolerated under normal proliferative stimuli. However, this level of ATR suppression strongly suppressed the growth of MLL-ENL and N-ras^{G12D}–driven acute myeloid leukemias (AML) and H-ras^{G12V}–expressing fibrosarcomas (17). These findings again verify the selectivity of ATR/Chk1 suppression toward cancerous tissues and demonstrate that tolerable levels of ATR/Chk1 inhibition are possible in regard to normal tissue function.

The findings in Ma et al. in this issue of the *JCI* (3), together with the recent findings described above (13–17), reignite the promise of ATR/Chk1 pathway inhibition as a means of targeting a broad range of cancers. Notably, the genetic alterations that selectively sensitize cells to ATR/Chk1 inhibition (e.g., p53 deficiency and high levels of oncogene activation) are common in a variety of cancers and are often associated with poor responses to conventional treatment. In addition, because the ATR/Chk1 pathway plays a central role in the response to a variety of cellular stresses, it is conceivable that other common characteristic in cancers, such as ATM deficiency, will also predict responsiveness to ATR/Chk1 inhibition (22). Thus, the careful genetic characterization and individualized targeting of cancers that have a predicted sensitivity to ATR/Chk1 inhibitor–based therapies will be a more effectual approach for future clinical trials. In aggregate, these studies highlight the importance of the individualized application of cancer treatments to optimize efficacy and, ultimately, save lives.

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1. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med.* 2010;363(20):1938–1948.
2. Carey L, Winer E, Viale G, Cameron D, Gianni L. Triple-negative breast cancer: disease entity or title of convenience? *Nat Rev Clin Oncol.* 2010;7(12):683–692.
3. Ma CX, et al. Targeting Chk1 in p53-deficient triple-negative breast cancer is therapeutically beneficial in human-in-mouse tumor models. *J Clin Invest.* 2012;122(4):1541–1552.
4. Powell SN, et al. Differential sensitivity of p53(–) and p53(+) cells to caffeine-induced radiosensitization and override of G2 delay. *Cancer Res.* 1995; 55(8):1643–1648.
5. Russell KJ, Wiens LW, Demers GW, Galloway DA, Plon SE, Groudine M. Abrogation of the G2 checkpoint results in differential radiosensitization of G1 checkpoint-deficient and G1 checkpoint-competent cells. *Cancer Res.* 1995; 55(8):1639–1642.
6. Wang Q, Fan S, Eastman A, Worland PJ, Sausville EA, O'Connor PM. UCN-01: a potent abrogator of G2 checkpoint function in cancer cells with disrupted p53. *J Natl Cancer Inst.* 1996;88(14):956–965.
7. Nghiem P, Park PK, Kim Y, Vaziri C, Schreiber SL. ATR

inhibition selectively sensitizes G1 checkpoint-deficient cells to lethal premature chromatin condensation. *Proc Natl Acad Sci U S A*. 2001;98(16):9092–9097.
8. Koniaras K, Cuddihy AR, Christopoulos H, Hogg A, O'Connell MJ. Inhibition of Chk1-dependent G2 DNA damage checkpoint radiosensitizes p53 mutant human cells. *Oncogene*. 2001;20(51):7453–7463.
9. Zhou BB, Bartek J. Targeting the checkpoint kinases: chemosensitization versus chemoprotection. *Nat Rev Cancer*. 2004;4(3):216–225.
10. Ma CX, Janetka JW, Piwnica-Worms H. Death by releasing the breaks: CHK1 inhibitors as cancer therapeutics. *Trends Mol Med*. 2011;17(2):88–96.
11. Fracasso PM, et al. A Phase 1 study of UCN-01 in combination with irinotecan in patients with resistant solid tumor malignancies. *Cancer Chemother Pharmacol*. 2010;67(6):1225–1237.
12. Reinhardt HC, Aslanian AS, Lees JA, Yaffe MB. p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. *Cancer Cell*. 2007;11(2):175–189.
13. Gilad O, et al. Combining ATR suppression with oncogenic Ras synergistically increases genomic instability, causing synthetic lethality or tumorigenesis in a dosage-dependent manner. *Cancer Res*. 2010;70(23):9693–9702.
14. Toledo LI, et al. A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. *Nat Struct Mol Biol*. 2011;18(6):721–727.
15. Ferrao PT, Bukczynska EP, Johnstone RW, McArthur GA. Efficacy of CHK inhibitors as single agents in MYC-driven lymphoma cells. *Oncogene*. 2012;31(13):1661–1672.
16. Murga M, et al. Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors. *Nat Struct Mol Biol*. 2011;18(12):1331–1335.
17. Schoppy DW, et al. Oncogenic stress sensitizes murine cancers to hypomorphic suppression of ATR. *J Clin Invest*. 2012;122(1):241–252.
18. Bartkova J, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*. 2005;434(7035):864–870.
19. Gorgoulis VG, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature*. 2005;434(7035):907–913.
20. Brown EJ, Baltimore D. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev*. 2000;14(4):397–402.
21. Ruzankina Y, et al. Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell*. 2007;1(1):113–126.
22. Reaper PM, et al. Selective killing of ATM- or p53-deficient cancer cells through inhibition of ATR. *Nat Chem Biol*. 2011;7(7):428–430.

Hemolysis and cell-free hemoglobin drive an intrinsic mechanism for human disease

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Blood transfusion represents the first and most prescribed cell-based therapy; however, clinical safety and efficacy trials are lacking. Clinical cohort studies have suggested that massive transfusion and/or transfusion of aged stored blood may contribute to multiorgan dysfunction in susceptible patients. In this issue of the *JCI*, Baek and colleagues report that aged stored blood hemolyzes after massive transfusion in a guinea pig model. Hemolysis led to vascular and kidney injury that was mediated by cell-free plasma hemoglobin and prevented by coinfusion of the specific hemoglobin scavenger protein, haptoglobin. These studies support an expanding body of research indicating that intravascular hemolysis is a pathological mechanism in several human diseases, including multiorgan dysfunction after either massive red blood cell transfusion or hemoglobin-based blood substitute therapy, the hemoglobinopathies, malaria, and other acquired and genetic hemolytic conditions.

Blood transfusion and the storage lesion

Blood transfusion is one of the first and most prescribed cell-based therapies. Despite the frequency with which blood transfusion is prescribed, the timing, dose, and established placebo-adjusted benefits of this "drug" have not been established. Blood transfusion is clearly beneficial in a multitude of clinical conditions, such as massive traumatic and surgical hemorrhage, critical anemia, and anemia associated with ischemic heart disease; it is also clearly beneficial as a supportive and exchange therapy for hemoglobinopathies. However, an increasing number of studies suggest that massive transfusion — defined as the transfusion of approximately one complete blood volume within the first 24 hours of resuscitation — may increase the risk of multiorgan dysfunction, respiratory and renal insufficiency, and death (1–5).

Studies in patients who have undergone cardiac surgery or experienced trauma or critical illness have suggested that the age of the transfused blood may relate to the risk of adverse clinical outcomes (1). The combined effects of storage on red blood cells have been termed the "red blood cell storage lesion." These effects modulate membrane integrity (e.g., reducing deformability and increasing rigidity), alter rheological properties, and cause hemolysis and have been proposed to contribute in some way to the risk of transfusion of aged blood. However, the epidemiologically established relationship between age of transfused blood and risk of adverse clinical outcomes is confounded by a number of important variables. First, transfusion of multiple units of blood increases the probability that an older unit is given, creating uncertainty about the role of massive transfusion as opposed to that from a storage lesion mechanism (2). Second, sicker patients receive more units (3). Finally, O blood group units are more rare and are more rapidly depleted from the inventory, so that patients who have type O blood are more likely to receive fresh blood, leading to fundamental differences among groups in published case-controlled cohort studies that may influence outcomes (4).

In order to address the major variables confounding the assessment of risk of transfusing blood stored for a long time, NIH-funded well-controlled transfusion studies in preclinical animal models and human placebo-controlled clinical trials are being performed. While the clinical trials are underway, because of safety and ethical considerations, these trials evaluate only modest ranges of storage time as

Conflict of interest: Mark T. Gladwin and Daniel B. Kim-Shapiro are named as co-inventors on an NIH government patent for the use of nitrite salts for cardiovascular conditions.

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