Stem Cell Microvesicles Transfer Cystinosin to Human Cystinotic Cells and Reduce Cystine Accumulation In Vitro

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Abstract

Cystinosis is a rare disease caused by homozygous mutations of the CTNS gene, encoding a cystine efflux channel in the lysosomal membrane. In Ctns knockout mice, the pathologic intralysosomal accumulation of cystine that drives progressive organ damage can be reversed by infusion of wildtype bone marrow-derived stem cells, but the mechanism involved is unclear since the exogeneous stem cells are rarely integrated into renal tubules. Here we show that human mesenchymal stem cells, from amniotic fluid or bone marrow, reduce pathologic cystine accumulation in co-cultured CTNS mutant fibroblasts or proximal tubular cells from cystinosis patients. This paracrine effect is associated with release into the culture medium of stem cell microvesicles (100–400 nm diameter) containing wildtype cystinosin protein and CTNS mRNA. Isolated stem cell microvesicles reduce target cell cystine accumulation in a dose-dependent, Annexin V-sensitive manner. Microvesicles from stem cells expressing CTNSRed transfer tagged CTNS protein to the lysosome/endosome compartment of cystinotic fibroblasts. Our observations suggest that exogenous stem cells may reprogram the biology of mutant tissues by direct microvesicle transfer of membrane-associated wildtype molecules.

Introduction

Cystinosis is a rare disorder caused by homozygous mutations of the CTNS gene on the short arm of chromosome 17q, encoding a ubiquitous cystine-selective transport channel in the lysosomal membrane [1]. Loss of this transporter prevents cystine efflux from the lysosome, causing massive accumulation of intralysosomal cystine in tissues throughout the body [2,3] and eventual apoptotic cell death [4]. Affected children may appear fairly well until the age of 4–6 months, when progressive dysfunction and atrophy of the proximal tubule cause renal Fanconi syndrome and failure to thrive [5]; by 10–12 years of age, dialysis or kidney transplantation is required to treat end-stage renal disease. Although the renal allograft is spared, lifespan is diminished by the inexorable dysfunction of other organs, including the thyroid gland, lungs, muscles, retina and brain [6].

In the late 1980’s, it was discovered that the pathologic accumulation of cystine could be substantially reduced by cysteamine therapy. This drug attacks the internal disulfide bond of cystine to form mixed disulfides that are able to efflux from the lysosome via alternative transport channels [2,7,8,9]. With the introduction of oral cysteamine therapy during infancy, the natural history of cystinosis was modified so as to delay the deterioration of the kidneys and other organs [10]. A cysteamine dose of 1.3 g/m2/day typically reduces leukocyte cystine levels to about 15% of baseline. However, the odour and gastric side-effects of oral cysteamine make adherence extremely difficult. Furthermore, even well-treated patients eventually require kidney transplantation and develop profound distal myopathy, cerebral atrophy and other complications as young adults [11]. Survival past the age of 30 years is uncharacteristic.

It was with great interest, therefore, that the medical community learned in 2009 of successful bone marrow stem cell therapy of Ctns–/– knockout mice [12]. Syres et al infused allogeneic wildtype bone marrow stem cells into Ctns–/– knockout mice after partial ablation of the bone marrow. The exogenous stem cells were progressively retained by cystinotic organs, resulting in 90% decrease in tissue cystine levels and, in the case of the kidney,
leading to normalization of organ dysfunction [12]. However, two aspects of these observations are puzzling. Firstly, the reduction in tissue cystine levels cannot be explained by a dilution effect produced by the arrival of wildtype stem cells; the investigators estimated that mutant organ Ctns transcript level rose to only 10% of normal [12,13]. Furthermore, infusion of GFP-tagged stem cells showed that the vast majority of exogenous cells took up a stromal position adjacent to the intrinsic nephrons and were only rarely integrated into the epithelia wall of renal tubules [12,13]. Thus, the profound decrease in whole-organ cystine level cannot be attributed to either stem cell transdifferentiation or fusion with resident mutant cells.

Stem cells are known to exert a number of useful paracrine effects, including the release of soluble factors that suppress inflammation or stimulate proliferation of endogenous cells following acute organ injury [14]. However, neither mechanism can be expected to correct the defective lysosomal transport channel in cystinosis. Recently, Al-Nedawi et al reported that cancer stem cells shed microvesicles from the cell surface and that endocytic uptake of the microvesicles transfers epidermal growth factor receptors to nearby endothelia; this induces proliferation of the normal endothelial cells, by rendering them responsive to local transforming growth factor-alpha [15,16,17]. We hypothesized that, in similar fashion, stem cell microvesicles could transfer wildtype cystinosin to membrane compartments of adjacent mutant cell targets. In this study, we use an in vitro co-culture model to demonstrate that human stem cells reverse cystine accumulation in cystinotic cells in paracrine fashion. We demonstrate that mesenchymal stem cells shed microvesicles containing wildtype cystinosin protein and CTNS mRNA into the culture medium and that uptake of isolated microvesicles transfers CTNS protein to the lysosomal compartment of cystinotic fibroblasts and reverses pathologic cystine accumulation.

Materials and Methods

Ethic Statement

The cells used in this study were obtained from the Cell Repository from the Montreal Children’s Hospital with ethical approval from the Montreal Children’s Hospital Research Ethics Board. For cell lines previously published, ethical approval was obtained and stated in the corresponding reference.

Vectors, RT/PCR and genotyping

CTNSRed was previously described [18]. pEGFP-C3 plasmid was purchased from Clontech (Clonetech, #6082-1). A lentiviral vector (LV) encoding the CTNS sequence linked to a triple repeat of the flag tag was constructed as follows. The CTNS cDNA sequence was amplified from the pCDNA3-CTNS T260I plasmid and inserted into a lentiviral transfer construct (pCMV8s-ires-NeoR). The resulting construct was sequence verified. This transfer construct encodes the 3F-CTNS under the control of the ubiquitous CMV promoter and also includes a bystanderic resistance gene to select the transduced cells. A second generation lentiviral vector was produced as previously described [19]. Total RNA was isolated with RNaseq kit (Qiagen). Purity and concentration of RNA were determined with a NanoDrop ND-1000 (NanoDrop Technologies, Inc). Transcripts were amplified (35 cycles) using: CTNS(1) 5'-gagtcaacggatttggtcgt-3', and CTNS(2) 5'-gcagtcacgctggtcaagta-3', GAPDH(1) 5'-gagtcagctctggtgaaga-3', GAPDH(2) 5'-ggtctgctgcttgagaatg-3'. Genotyping for CTNS mutation was performed with primers LDM1 flanking the common 57 kb deletion or CTNS Exon 4 [20].

Cell culture

Normal human amniotic mesenchymal stem cells (amMSC, passage 5–20) and adult bone marrow cells (bmMSC, passage 3–8) [21] were maintained in DMEM (Invitrogen) supplemented with 15–20% FBS (Wisent) and 1% Penicillin/Streptomycin (Invitrogen). Skin fibroblasts (Fib) from cystinotic patients and controls were maintained in DMEM with 10% FBS. Conditionally immortalized mutant proximal tubular epithelial cells (cPTEC) obtained with ethics approval from the urine of a cystinosis patient bearing the common homozygous 57 kb deletion were previously characterized [22].

Characterization of MSCs

One million cells were incubated for 30 minutes at 4°C with the following monoclonal antibodies: phycoerythrin-labeled HLA-DR (clone LN3) from eBioscience, allophycocyanin-labeled CD34 (clone 581), phycoerythrin-labeled CD44 (clone G44-26), phycoerythrin-labeled CD31 (clone WM59), phycoerythrin-labeled CD105 (clone 266), phycoerythrin-labeled CD45 (clone HI30), fluorescein isothiocyanate-labeled CD90 (clone SE10), phycoerythrin-labeled CD73 (clone AD2) from BD Biosciences (BD Biosciences Pharmingen, Mississauga, ON). Cells were analyzed with CellQuest Pro software on a FACSCalibur flow cytometer (BD Biosciences), using antibody isotype controls. In order to test for osteogenic differentiation of MSCs the cells were exposed to complete media supplemented with β-glycerol phosphate (10 mmol/l; Sigma-Aldrich), ascorbic acid 2-phosphate (50 μmol/l; Sigma-Aldrich), and dexamethasone (10−7 mol/l; Sigma-Aldrich) for 4 weeks and then stained with 1% Alizarin Red S (Sigma-Aldrich). In order to test for adipogenic differentiation of MSCs, cells were exposed to complete media supplemented with insulin (5 μg/ml), dexamethasone (1 μmol/l; Sigma-Aldrich), 3-isobutyl-methylxanthine (0.5 mmol/l; Sigma-Aldrich), and indomethacin (60 μmol/l; Sigma-Aldrich) for 14 days, and then stained with Oil Red O (Sigma-Aldrich).

Microvesicles (MV)

Microvesicles were isolated from conditioned culture medium as described by Al-Nedawi [16]. Briefly, the culture medium is subjected to centrifugation at 300 × g for 5 min at 4°C to remove debris and then ultracentrifuged at 100,000 g (Beckman Coulter Optima L-80 ultracentrifuge) for 2 hrs at 4°C, and resuspended in PBS after two washes with PBS each followed by ultracentrifugation at 100,000 g for 1 hr at 4°C. MV were analyzed with NanoSight LM20 (NanoSight, Amesbury, United Kingdom) using NTA2.1 software. MV protein was quantified (BCA protein assay kit, Calbiochem). Various amounts of MV were incubated for 24 hrs with target cells. Cell pellets were washed and assayed for protein and cystine content.

Cell cystine assay

For cystine assays, cells were grown in cystine-free culture medium for 24 hours. Cystine was measured by HPLC with homocysteine as an internal control [23]. Results are expressed as nmol half-cystine per mg cell protein.

Microscopy

CTNSRed tagged cells and microvesicles were inspected with a Zeiss Axioptih inverted microscope and analyzed using Axiosvision Rel v 4.7.1 software. Microvesicles isolated from non-transfected amMSC were used as a negative control for imaging. Confocal fluorescent live cell microscopy was performed using a Quorum Wave FX Spinning Disc confocal microscope (Quorum Technol-
Stem Cell Microvesicles Rescue Cystinotic Cells

amMSC cells reduce cystine accumulation in co-cultured cystinotic fibroblasts or cystinotic proximal tubular cells

To study the paracrine effects of wildtype stem cells on nearby adjacent mutant cell targets, we first isolated mesenchymal stem cells from bone marrow of healthy adults (bmMSC) (Figure 1a) and normal human amniotic fluid (18 weeks gestation) (amMSC) (Figure 1b). Each cell line was shown to express typical mesenchymal stem cell markers (CD14, CD73, CD90 and CD105), but not endothelial (CD31), hematopoietic (CD34, CD45) or MHC (HLA-DR) markers. Both mesenchymal stem cell lines could be induced to differentiate toward osteogenic and adipocyte fates (Figures 1a and 1b, right panels).

We then co-cultured amniotic mesenchymal stem cells (amMSC) with various proportions of mutant fibroblasts from a cystinosis patient with homozygous deletion of the CTNS gene (Figure 1c, left panel) containing high intracellular levels of cystine (Figure 1c, right panel). After 4 days in co-culture, we measured the cystine content of the mixed cell population and calculated the cystine attributable to the subset of mutant target cells. As seen in Figure 2a, mutant cell cystine was significantly reduced in proportion to the number of wildtype amMSC in the co-culture, demonstrating a paracrine effect of the stem cells on their cystinotic neighbors.

In a second set of experiments, we measured cystine content in GFP-tagged mutant conditionally-immortalized proximal tubular target cells (ciPTEC(−/−)GFP) after co-culture with a variety of donor cells. The target ciPTEC(−/−)GFP cells were from a cystinosis patient bearing the common homozygous 57 kb deletion of the CTNS gene [22]. After 4 days of co-culture, the GFP-tagged mutant target cells were isolated by fluorescence-activated cell sorting (FACS), following forward and then side scatter width/height gating to assure isolation of singlet cells only. The cells were then analyzed for cystine content. As seen in Figure 2b, co-culture with amMSC donors dramatically reduces cystine content in the ciPTEC(−/−)GFP targets compared to baseline. In another set of experiments, ciPTEC(−/−)GFP cell cystine was unaffected by co-culture with untagged mutant proximal tubular ciPTEC(−/−) donors; on the other hand, co-culture of ciPTEC(−/−)GFP with genetically corrected (transfected with wildtype CTNS) ciPTEC(−/−) cells again showed significant reduction of cystine content (Figure 2b). Taken together, these experiments identify a paracrine effect of wildtype donor cells on cystine content in nearby CTNS mutant target cells. The effect requires a wildtype CTNS gene in the donor but is not restricted to a unique donor cell type. Neither is the effect restricted to a specific cystinotic cell target.

MVC microvesicles reduce pathologic accumulation of cystine in mutant fibroblasts from cystinosis patients

Al-Nedawi et al suggested that an important characteristic of stem cells is that they shed plasma membrane-derived microparticles into the local environment and these are taken up by neighboring cells [17]. We reasoned that this mechanism might account for the paracrine effects of MSC on cystinotic cells. To address this hypothesis, monolayers of FACS-purified bmMSC were cultured for 48 hours and microparticles in the conditioned medium were isolated by ultracentrifugation [16]. Nanosight analysis of the suspended particles showed that over 95% had a diameter typical of microvesicles (100–400 nm) (Figure 3a).

To ascertain whether MVCs can reduce cystine content of CTNS mutant target cells, we isolated microvesicles from bmMSC or amMSC and transferred them to separate monolayer cultures of CTNS mutant fibroblasts (−/−) for 24 hours (1–2 mg microvesicles/mg fibroblast protein). The cells were then harvested and assayed for cystine content as above. As seen in Figure 3b, bmMSC microvesicles reduced mutant cell cystine level in a dose-dependent fashion. At the highest microvesicle dose studied, cell cystine was reduced even more than exposure to 1 mM cysteamine for 24 hours. A similar effect was noted with microvesicles isolated from amMSC (Figure 3c). Pre-treatment of microvesicles with annexin V (2 ug/150 ug microvesicle protein) completely blocked their ability to reduce mutant cell cystine accumulation, indicating that phosphatidylserine-dependent uptake of the microvesicles by target cells is necessary for the effect [24] (Figure 3d).

Stem cell microvesicles transfer wildtype cystinosin protein to mutant target cells

To test the possibility that microvesicles mediate direct CTNS protein transfer between donor and recipient cells, we stably transfected amMSC with CTNSRed (Figure 4a). We inspected red fluorescence in an aliquot of microvesicles obtained from culture media from the stably transfected amMSC with CTNSRed. The Red-tagged CTNS protein was evident in microvesicles shed into the conditioned medium (Figure 4b). We then co-cultured amMSC/CTNSRed with an equal number of CTNS−/−GFP fibroblast targets; there was no evidence of fusion between donor and target cells. As seen in Figure 4c, CTNSRed particles are visible in a single confocal plane within a cluster of amMSC (shown in the bright field microscopy image, Figure 4c inset) and within CTNS−/−GFP fibroblasts, indicating transfer of the tagged protein from amMSC to the target cell.

We also tracked the fate of CTNSRed vesicles (Figure 4d inset) applied directly to cultured CTNS−/−fibroblasts stained with a green-fluorescent endothosomal/lysosomal dye. As seen by confocal microscopy (Figure 4d), CTNSRed-tagged particles were again observed within the target cells. Furthermore, we were able to identify CTNSRed-tagged particles co-localizing with green fluorescence in intracellular structures, documenting transfer to the acidic intracellular endothosomal/lysosomal compartment of mutant target cells.
Figure 1. Characterization of mesenchymal stem cells and CTNS−/− mutant target cells. (a) FACS analysis (left) of bmMSC surface markers (blue) and isotype controls (red) and bmMSC differentiation from baseline (upper right) to Alizarin Red S-stained calcium-containing osteogenic (middle right) and Oil-Red O-stained adipocyte (bottom right) phenotypes. (b) same FACS and differentiation analysis of amMSC cells. (c) CTNS (-/-) fibroblasts with 5' upstream and CTNS deletion, showing cystine [mol/1000 mg of fibroblast protein] with WT and CTNS (-/-) with deletion and Exon 4.
Stem cell microvesicles also transfer CTNS mRNA to CTNS(−/−) fibroblasts

Other investigators have shown that microvesicles contain mRNA and miRNA as well as protein [25]. We were able to detect wildtype CTNS transcripts by RT/PCR in microvesicles isolated from amMSC (Figure 5a). We co-cultured CTNS(−/−)GFP fibroblasts with either amMSC or unlabelled CTNS(−/−) fibroblast donors for 48 hours and then separated the CTNS(−/−)GFP fibroblasts by FACS. As seen in Figure 5b, wildtype CTNS transcripts were detectable by RT/PCR in the mutant recipient target cell co-cultured with amMSC, but not with the mutant fibroblast donors.

Discussion

Long regarded as inactive cellular debris, microvesicles shed from the surface of viable cells are increasingly recognized as important vehicles for the transfer of complex biologic information between neighboring cells. Microvesicles arise either through vesiculation of the plasma membrane to generate 100–500 nm diameter particles exposing phosphatidyl serine residues shifted to the outer leaflet (ectosomes) or through redirection of endosomal structures to multivesicular bodies that disgorge their 40—80 nm diameter particles (exosomes) into the extracellular environment [24]. Unlike other intercellular communication mechanisms, such as release of neurotransmitters or soluble growth factors, endocytotic uptake of microvesicles allows transfer of membrane-associated protein complexes as well as certain cytoplasmic constituents such as mRNA and miRNA [24]. Proteomic analysis suggests that microvesicles carry about 500 hundred protein cargoes from the donor cell which could re-program the membrane biology of the recipient [26].

While many cells shed microvesicles, flux from the surface of stem cells and cancer cells is thought to be especially high and may exert important influence on the stem cell niche or on tumour invasiveness. Here, we show that mesenchymal stem cells shed microvesicles containing wildtype cystinosin protein and mRNA. Uptake of these microvesicles by mutant CTNS(−/−) fibroblasts or proximal tubule cells delivers CTNS to the endosomal/lysosomal compartment and reduces the pathologic accumulation of cystine. This provides a plausible mechanism to explain the clinical effects of bone marrow stem cell infusion on Ctns knockout mice [12,13]. In our studies, mutant fibroblast cystine accumulation was substantially reduced within four days when co-cultured (4:1) with a relatively small proportion of wildtype stem cells. We hypothesize that continuous microvesicle transfer between infiltrating stem cells and adjacent mutant epithelial cells reasonably accounts for the therapeutic effects accrued over several months in Syres’ experiments.

In the study by Syres et al, optimal clinical effect was obtained by infusion of 20 million wildtype hematopoietic stem cells (HSC) into Ctns knockout mice [12]. This resulted in excellent engraftment, increasing uptake of HSC in cystinotic tissues and striking (80%) reduction in tissue cystine content by four months. Although GFP-tagged HSC were occasionally incorporated into tubules, the vast majority of HSC took up an interstitial position in cystinotic organs. Similar studies were attempted with bone marrow-derivedstem cell microvesicles also transfer CTNS mRNA to CTNS(−/−) fibroblasts

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Mesenchymal stem cells (MSC), but technical issues restricted the infusion of MSC to only 2 million cells per mouse and MSC retention in cystinotic tissues diminished with time, making direct comparisons difficult. Nevertheless, initial engraftment of MSC was excellent and, at four months, tissue cystine was significantly reduced (50%) and serum creatinine was similar to that of the HSC-infused group [12]. Other groups have shown that exogenous MSC exert important paracrine effects in murine models of acute kidney injury, particularly when the cells are derived from amniotic fluid rather than adult bone marrow [27].

In our studies, paracrine reduction of cystine accumulation in CTNS mutant targets was seen with amniotic fluid MSC, adult bone marrow MSC and with genetically corrected, immortalized proximal tubular cells from a cystinosis patient. Although microvesicle shedding has not been examined in HSC or immortalized epithelial cells, the phenomenon is consistently seen in a wide range of stem and cancer cells [24]. We found that pre-treatment of amMSC microvesicles with annexin V completely abrogated their effect on cystine accumulation in mutant fibroblasts. This indicates that the rescue effect requires a membrane fusion event between microvesicles (ectosomes) and the target cell membrane rather than secretion of a soluble signalling molecule or an effect of exosomes.

Bruno et al have shown that MSC infused into mice with glycerol-induced renal injury ameliorate tissue damage by suppressing apoptosis and enhancing proliferation of endogenous cells [28]. In the setting of cystinosis, however, a similar effect on survival of mutant cells cannot explain the observed reduction in tissue cystine content. Whereas improved survival or proliferation of endogenous cells might sustain tubular integrity and function, the surviving mutant cells would nevertheless exhibit high levels of intralysosomal cystine. In the study by Bruno, it was also shown that infusion of isolated microvesicles could mimic the effect of MSC on acute tissue injury. However, the duration of this effect is likely to be short-lived and unsustainable as a repeated therapy for a congenital disease. This is in contrast to successful stem cell engraftment, where cystinotic cells would be exposed to continuous release of microvesicles containing wildtype cystinosin.

Figure 3. Analysis of stem cell microvesicles and their effects on cystine content of CTNS(−−) mutant fibroblasts. (a) diameter of bmMSC microparticles released into conditioned medium. (b) effect of increasing amounts of bmMSC microvesicles on cystine in mutant fibroblasts after 24 hour incubation, compared to the effect of 1 mM cysteamine. (c) diameter of amMSC microparticles released into conditioned medium. (d) effect of increasing amounts of amMSC microvesicles on cystine in mutant fibroblasts after 24 hour incubation, compared to the effect of 1 mM cysteamine and in the presence of annexin V. Statistical significance is indicated by the corresponding p-value.

doi:10.1371/journal.pone.0042840.g003
In our studies, transfection of amMSC with an expression vector containing Red-tagged CTNS generated microvesicles richly supplied with the tagged fusion protein. Wildtype cystinosin contains a 3'-sequence which targets most of the protein to the lysosomal membrane [1]. Nevertheless, some of this primary isoform is also detectable at the cell surface (albeit in smaller quantities), where it is presumably available for incorporation into microvesicles. Furthermore, Taranta et al. have reported a second cystinosin isoform (CTNSLKG), constituting about 15% of total cellular cystinosin, which lacks the lysosomal targeting sequence and readily appears at the plasma membrane [18]. Since CTNS expression is ubiquitous, it follows that any cell with an intact CTNS gene and the capacity to shed microvesicles should be able to reduce cystine content of adjacent mutant cells. Indeed, we found that mutant cell cystine accumulation was reduced by co-culture with either amMSC or genetically corrected proximal tubular cells and by microvesicles from either amMSC or bmMSC.

Microvesicles are known to contain proteins as well as mRNA and miRNA [25,29]. Although, we observed microvesicle transfer of CTNS mRNA, it is uncertain whether de novo CTNS synthesis in...
the mutant cell contributes to the rescue effect. It is likely that some cells shed more microvesicles than others and this could influence therapeutic efficacy. Furthermore, delivery of wildtype protein to the plasma membrane could limit the rate of protein transfer to the mutant cells. Steinherz showed that the efflux half-life of cystine dimethyl ester from heterogeneous cystinotic lysosomes is \(86.3^{+/-} 5\) vs normal \(43.1^{+/-} 3\) minutes [30], suggesting that heterozygous stem cells (from a sibling or parent) might generate less microvesicle cystinosin than a wildtype stem cell. Although cystinosin is clinically recessive, use of heterozygous donor cells should be carefully evaluated prior to use in clinical trials.

A wide range of lysosomal storage diseases have been treated with allogeneic hematopoietic stem cells derived from umbilical cord [31]. Following myeloablative chemotherapy, engraftment is effective, implying that soluble enzymes may be taken up by the mutant cell, independent of microvesicle transfer. On the other hand, infusion of recombinant enzyme is clinically effective, conceivability that enzyme associated with microvesicle membrane is transferred in similar fashion. Although cystinosis is clinically recessive, use of heterozygous mutant cell, independent of microvesicle transfer. This shifts the primary therapeutic consideration toward the ability of stem cells to shed microvesicles that transfer molecules to the mutant tissue rather than their transdifferentiation potential.

### Acknowledgments
We gratefully acknowledge Dr. Janusz Rak for the helpful discussions and technical advice on microvesicles and Dr. Brian Gillix for services provided for cystine measurements. We acknowledge Martina Ojeda and Elisabeth Fainstein for their excellent technical help.

### Author Contributions
Conceived and designed the experiments: DMI RE-K AT BF FE MB EL JT LVDH LC PG. Performed the experiments: DMI RE-K AT BF MB JT LVDH LC JZ YKY. Analyzed the data: DMI RE-K FE EL NE PG. Contributed reagents/materials/analysis tools: DMI RE-K AT BF FE MB EL JT LVDH LC PG. Performed the experiments: DMI RE-K AT BF FE MB JT LVDH LC JZ YKY NE PG. Wrote the paper: DMI RE-K PG.

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