Redox regulation of hematopoiesis and hematologic malignancy

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Redox regulation of hematopoiesis

- Hematopoietic stem/progenitor cells (HSPC);
- Bone marrow (BM) niche;
- Reactive oxygen species (ROS) and HSPC functions;
- ROS mediated signaling pathways in HSPC;
- Therapeutic potential;
- Achievements in the lab.
Hematopoietic stem/progenitor cells

- BM is the major site of adult hematopoiesis.
- HSPC
  - Most characterized stem cells in BM;
  - Produce all types of immune cells;
  - Maintain blood production for life.
Bone marrow niche

• HSPC function and fate (self-renewal, survival, differentiation, proliferation, engraftment and mobilization) are regulated by
  – Cell-intrinsic signaling;
  – Extrinsic signals mediated via specialized, regulatory microenvironment called “niche”.
Bone marrow niche

- Multiple cell types;
- Non-cellular components: secreted factors (angiopoietin-1, osteopontin, SCF, N-cadherin, SDF-1, VEGF), extracellular matrix, Ca^{2+}, oxygen/hypoxia, and ROS.
Two distinct BM niches

• **Osteoblastic niche** localized at the inner surface of the bone cavity might serve as a reservoir for long-term HSC storage in a quiescent state.

• **Vascular niche**, which consists of sinusoidal endothelial cell lining blood vessel, provides an environment for short-term HSC proliferation and differentiation.

• Both niches act together to maintain hematopoietic homeostasis.
Bone Marrow

Osteoblastic niche

Vascular niche

Quiescence
Self-renewal

Proliferation
Differentiation

Oxygen

In vivo evidence of the osteoblastic niche

Live-animal tracking of individual HSPC in their niche

Collage image of bone (blue), osteoblasts (green) and vasculature (red) acquired simultaneously with two-photon microscopy.
Cell- and niche-dependent HSPC localization.
Bone Marrow

Osteoblastic niche
Low HSC
Quiescence
Self-renewal

ROS

Vascular niche
High HSC
Proliferation
Differentiation

Peripheral blood

Oxygen

Calcium

ROS and HSPC function

• Clear correlation between ROS and HSPC function:
  – Low levels of ROS are involved in maintaining the quiescence of HSC;
  – Higher levels of ROS contribute to a greater proliferation, senescence or apoptosis.
ROS and its production

• ROS is a general term that refer to not only oxygen-centered free radicals but also reactive derivatives of oxygen:
  
  – Superoxide $\text{O}_2\cdot^- : \text{O}_2 + e^- \rightarrow \text{O}_2\cdot^-$, mainly produced by the reaction of $\text{O}_2$ with an escaped electron from mitochondria and NADPH oxidase (NOX);
  
  – Hydrogen peroxide $\text{H}_2\text{O}_2: 2\text{O}_2\cdot^- + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O}_2 + \text{O}_2$, an intermediate detoxification of $\text{O}_2\cdot^-$ by superoxide dismutase (SOD).
ROS generation and antioxidants

Nrf2, a master transcriptional activator of genes against oxidative stress
Nrf2 regulates hematopoietic stem cell function

• Nrf2 plays a regulatory role in several aspects of HSC homeostasis:

  ✓ Nrf2 deficiency results in an expansion of the HSPC compartment due to cell-intrinsic hyperproliferation, which was accomplished at the expense of HSC quiescence and self-renewal;
  ✓ Nrf2 governs both migration and retention of HSC in their niche;
  ✓ Nrf2 activates CXCR4 expression which contributes partially to the maintenance of HSC function.

Keap1-Nrf2 system regulates cell fate determination of hematopoietic stem cells.

Abstract
Nrf2 is a major transcriptional activator of cytoprotective genes against oxidative/electrophilic stress, and Keap1 negatively regulates Nrf2. Emerging works have also suggested a role for Nrf2 as a regulator of differentiation in various cells, but the contribution of Nrf2 to the differentiation of hematopoietic stem cells (HSCs) remains elusive. Clarifying this point is important to understand Nrf2 functions in the development and/or resolution of inflammation. Here, we established two transgenic reporter mouse lines that allowed us to examine Nrf2 expression precisely in HSCs. Nrf2 was abundantly transcribed in HSCs, but its activity was maintained at low levels due to the Keap1-mediated degradation of Nrf2 protein. When we characterized Keap1-deficient mice, their bone marrow cells showed enhanced granulocyte-monocyte differentiation at the expense of erythroid and lymphoid differentiation. Importantly, Keap1-null HSCs showed lower expression of erythroid and lymphoid genes than did control HSCs, suggesting granulocyte-monocyte lineage priming in Keap1-null HSCs. This abnormal lineage commitment was restored by a concomitant deletion of Nrf2, demonstrating the Nrf2-dependency of the skewing. Analysis of Nrf2-deficient mice revealed that the physiological level of Nrf2 is sufficient to contribute to the lineage commitment. This study unequivocally shows that the Keap1-Nrf2 system regulates the cell fate determination of HSCs.

Role of ROS in physiological and pathological cellular function

• Appropriate ROS production is required for physiological HSPC functions (neovascularization and tissue repair in response to ischemic injury);

• Excess ROS contribute to HSPC damage and apoptosis in pathological states (aging, diabetes, atherosclerosis, heart failure, hypertension and cancer).

Signaling pathway mediated by ROS in stem cells

- Jnk
- ↓PTEN
  - ↑PI3K/Akt → mTOR
  - ↓FoxO
- HIF-1α
- ↑ATM
- ↑FoxO
- ↑p53
- ↓ROS
- ↑p38MAPK
  - ↑↑p53
  - ↑p16\textsuperscript{ink4a}/Rb
  - Differentiation (Myeloid commitment)
  - Migration
  - Survival
  - Quiescence
  - Senescence

Therapeutic potential of redox regulation of HSPC

• Clinical trials of cell therapy using HSPC demonstrate its feasibility, safety, and benefit in patients with ischemic disease and heart failure, but needs to improve efficacy.

• Two different strategies have used to investigate the therapeutic potential.
Therapeutic potential of redox regulation of HSPC

• Two strategies:
  – Suppress excess oxidative stress
    • Administration of SOD mimetic attenuates the diabetes-related impairment of HSPC;
  – Stimulate progenitor cells with controlled pro-oxidant
    • Short-term pretreatment with low-dose H₂O₂ enhances efficacy of BM cells for therapeutic angiogenesis
Bone Marrow

Osteoblastic niche

- Low HSC
- Quiescence
- Self-renewal

ROS

Thiol

Vascular niche

- High HSC
- Proliferation
- Differentiation

Peripheral blood

Glutathione S-transferase Pi (GSTP)

- Phase II detoxification enzyme
- Antioxidant enzyme: Pr-SSG, Prx6, Nrf2 activation
- Regulates signaling pathways (p-JNK, p-ASK1)
- Strongly affects human susceptibility to cancers

Prx: peroxiredoxin; JNK: c-Jun N-terminal kinase; ASK1: apoptosis signal-regulating kinase 1.
GSTP knockout mice

Breed and develop normally, similar life span

More susceptible to tumorigenesis (skin, lung, colon cancer)

Altered drug sensitivity (acetaminophen, cyclophosphamide)

Increased HSPC numbers and myeloid proliferation and differentiation

Gstp1/p2^-/- mice
 GSTP and hematopoiesis

- *Telintra (TLK199)*, a GSH analogue, specifically inhibits GSTP;
- *TLK199* stimulates myeloproliferation in both rodents and man.

<table>
<thead>
<tr>
<th>GST isozyme</th>
<th>P1-1</th>
<th>A1-1</th>
<th>M1-1</th>
<th>M2-2</th>
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<tbody>
<tr>
<td>Inhibition constant (μM)</td>
<td>0.42</td>
<td>24.3</td>
<td>57.8</td>
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Effect of GSTP expression on haematopoiesis

Peripheral white blood cell counts of mouse

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<tr>
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<th>Control</th>
<th>Vehicle Control</th>
<th>TLK199</th>
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<tbody>
<tr>
<td>WT</td>
<td>7.7±2.8</td>
<td>6.9±1.9</td>
<td>11.8±2.5*</td>
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<tr>
<td>Gstp1/p2⁻/⁻</td>
<td>13.9±2.6*</td>
<td>17.0±2.6</td>
<td>16.4±3.7</td>
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CFU-GM from mouse bone marrow

<table>
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<tr>
<th>Single dose of TLK199 (mg/kg)</th>
<th>% Control</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td></td>
<td>Gstp1/p2⁻/⁻</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±4</td>
<td>100±6</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>156±8*</td>
<td>106±12</td>
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<tr>
<td>75</td>
<td>194±22*</td>
<td>107±9</td>
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**CFU-GM:** colony-forming unit-granulocyte, macrophage

* P< 0.02

Cell models

C57BL/6 wild type or Gstp1/p2−/− mice

Femurs and tibias

ACK Lysing buffer

Bone marrow derived dendritic cells (BMDC)

20-30 × 10⁶/dish

Bone marrow cells (BMC)
Lineage negative cells (Lin(−) cells)

4-5 × 10⁶/dish

GM-CSF

Day 0, 10 ml

Day 8, 20 ml
Increased cell proliferation in $Gstp1/p2^{-/-}$ bone marrow cell populations.

Decreased protein S-glutathionylation in \textit{Gstp1/p2}^{-/-} bone marrow cell populations.
Increased protein thiol and GSH levels in *Gstp1/p2*−/− bone marrow cells.
GSTP catalyzes SERCA glutathionylation and regulates intracellular Ca^{2+} dynamics.

SERCA: Sarco endoplasmic reticulum calcium ATPase.
Thapsigargin: specific inhibitor of SERCA.

GSTP catalyzes ERα glutathionylation through protein-protein interaction.
Gstp1/p2 depletion in mice causes higher ERα levels during BMDC differentiation
Estradiol/estrogen receptor alpha (ERα) signaling promotes GM-CSF-driven DC differentiation and yields DC with increased functional capacity.

IRF4: interferon regulatory factor 4
**Inducing factors**

- **Cytokines:** GM-CSF
- **Pathogens:** Lipopolysaccharide (LPS)

**Properties**

- **Antigen capture:**
  - High intracellular MHC
  - Endocytosis

- **Antigen presentation:**
  - High surface MHC
  - High CD80, CD86
  - Cytokine release
  - Glycolysis induction

**Bone marrow**

- **Immature DC (imDC):**
  - Day 7

- **Mature DC (mDC):**
  - Day 8

**T cells**

MHC: major histocompatibility complex
**Gstp1/p2−/− BMDC**

- Higher antigen endocytotic functions;
- Enhanced expression of the maturation markers was after LPS-treatment;
- Significantly increased secretion of the pro-inflammatory cytokine IL-12;
- Higher levels of T cell expansion;

Manuscript under preparation
Redox regulation of hematologic malignancy

• Multiple myeloma (MM);
• Elevated oxidative stress in MM patients;
• Mechanisms of proteasome inhibitors (Bortezomib (BTZ));
• Redox status and BTZ resistance;
• Achievements in the lab.
Multiple myeloma (MM)

- 2nd most frequent hematologic malignancy in USA.
- Essentially incurable malignancy of terminally differentiated B cells or plasma cells in the BM.
Multiple myeloma

- Myeloma develops when abnormal plasma cells in the BM begin to divide uncontrollably.
- Over time, the myeloma cells build up in the BM, which makes it hard for other blood cells to develop and work normally.
- Accumulation of malignant cells leads to osteolytic bone destruction and impaired hematopoiesis.
Elevated oxidative stress in MM patients

• Compared to healthy individuals, MM patients have lower antioxidants (SOD, GPx, GR, catalase, GSH, VC and VE) and higher oxidative stress markers (MDA, AOPPs, S-nitrosylated proteins);

• Depletion of antioxidants and increase of pro-oxidants are associated with MM progression;

• After induction therapy, there is significant increase of antioxidants in parallel with decreasing AOPPs and MDA levels in comparison with samples of MM patients before treatment.

MDA: malondialdehyde; AOPP: Advanced oxidation protein products.
Bortezomib (BTZ)

• Discovery of BTZ has dramatically improved the overall survival of MM patients.

• BTZ specifically inhibits the 26S proteasome, an enzyme complex regulating protein degradation in a controlled fashion.

• Proteins that are no longer required are tagged with ubiquitin which directs them to the proteasome.

Mechanisms of BTZ

• Inhibition of the breakdown of IkB/NFkB;
• ER stress and unfolded protein responses (UPR);
• Oxidative stress;
• Altered BM microenvironment;
• Decreasing the adhesion of myeloma to stromal cells.

Mechanisms of BTZ

- Inhibition of the breakdown of IκB/NFκB;

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Diagram:
- IKK Protein Kinase
- Phosphorylation of IκB
- Degradation of IκB
- Nuclear Translocation of NFκB
- Activation of Anti-apoptotic Genes
- Cell Cycle Progression: G1/S Transition
Functions of ER

Rough ER
- Protein synthesis on ribosomes of RER
- Glycosylation, folding and formation of disulfide bonds of newly synthesized proteins
- Transport of the proteins
- GSH/GSSG 3:1

Smooth ER
- Synthesis of lipid and steroid
- Detoxification
- A storage place for Ca^{2+}
Balance between ER protein load and the folding capacity to process this load.

ER status
ER stress and UPR

Non-stress condition

ER lumen

IRE1
Kinase domain-
RNase domain-

PERK
Kinase domain-

ATF6
bZip-
transcription factor domain

Caspase7/12

BiP

Unfolded protein
ER stress and UPR

Stress condition

ER lumen

ERAD machinery

Proteasome degradation

Cytosol

Translation

PERK

BiP

P

P

P

elf2a

ATF4

BiP

ATF4

XBP1

splicing

Golgi processing

Cleavage

Caspase7/12

Unfolded protein

Nuclear

Transcription of ER chaperones genes and ERAD molecules

BiP

ATF6 (N)

XBP1s

CHOP

Apoptosis
Proteasome Inhibitors and UPR

- PIs induce ER stress and cell death but suppress IRE1α-mediated XBP-1 mRNA splicing in MM Cells.

PIs induce a terminal UPR in MM cells

PIs specifically induce the expression of the transcription factor ATF4 in MM cells.

SOD1 and BTZ resistance

Up-regulation of SOD1 and GPx-1 was linked to BTZ resistance. The copper chelating drug disulfiram (DSF, Antabuse) could inhibit SOD1 activity, reverse BTZ resistance and increase BTZ cytotoxicity in MM.

Mitochondrial TrxR2 and BTZ resistance

BTZ induces oxidative stress before cell death.

Leukemia 2016; 30: 104–111.
Mitochondrial TrxR2 and BTZ resistance

TXNRD2 suppresses PI-dependent oxidative stress, ER stress and cytotoxicity in MM cells.

Leukemia 2016; 30: 104–111.
Mitochondrial TrxR2 and BTZ resistance

TXNRD2 suppresses PI-dependent oxidative stress, ER stress and cytotoxicity in MM cells.

Leukemia 2016; 30: 104–111.
Mitochondrial TrxR2 and BTZ resistance

TXNRD2 overexpression provided MM cell xenografts resistant to BTZ

TXNRD2 is upregulated in BTZ resistant MM cells.

Leukemia 2016; 30: 104–111.
GSTP is an ER resident protein, catalyzes ER protein glutathionylation, and regulate UPR.
## Cross resistance

<table>
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<th>IC50 (fold resistance)</th>
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<tbody>
<tr>
<td></td>
<td>Granta</td>
<td>Granta R</td>
<td>Mino</td>
<td>Mino R</td>
</tr>
<tr>
<td>BTZ (nM)</td>
<td>9</td>
<td>35 (~4)</td>
<td>13</td>
<td>45 (~3)</td>
</tr>
<tr>
<td>ThG (nM)</td>
<td>11</td>
<td>20 (~2)</td>
<td>4</td>
<td>40 (~10)</td>
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<tr>
<td>TuM (ng/mL)</td>
<td>7</td>
<td>50 (~7)</td>
<td>6</td>
<td>40 (~7)</td>
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<table>
<thead>
<tr>
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<th>IC50 (fold resistance)</th>
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<tbody>
<tr>
<td></td>
<td>BMDC Gstp^{+/+}</td>
<td>BMDC Gstp^{-/-}</td>
<td>MEF Gstp^{+/+}</td>
<td>MEF Gstp^{-/-}</td>
</tr>
<tr>
<td>BTZ (nM)</td>
<td>23.9 (~8)</td>
<td>2.97</td>
<td>33.4 (~4)</td>
<td>9.0</td>
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<tr>
<td>ThG (nM)</td>
<td>195.6 (~6)</td>
<td>30.4</td>
<td>1968 (~9)</td>
<td>226.8</td>
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<tr>
<td>TuM (ng/mL)</td>
<td>208.9 (~2)</td>
<td>91.8</td>
<td>2887 (~6)</td>
<td>507</td>
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Inhibition of GSTP sensitize the MM cells response to BTZ

IC50 values of BTZ (nM)

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>S+TLK199</th>
<th>R</th>
<th>R+TLK199</th>
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<tr>
<td>U266</td>
<td>5.4</td>
<td>3.2</td>
<td>27.9</td>
<td>10.1</td>
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<tr>
<td>MM1.s</td>
<td>5.3</td>
<td>4.1</td>
<td>26.5</td>
<td>9.8</td>
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S: BTZ sensitive MM cells; R: BTZ resistant MM cells
Resistant MM cells have increased Pr-SSG and inhibition of GSTP decreases Pr-SSG
BiP is subjected to S-glutathionylation at residual Cys41 and Cys420.

S-gluthathionylation of BiP decreases ATPase and enhances holdase activity.
Take home messages...

• HSC and progenitor cell function and fate are highly redox regulated (cell-intrinsic, cell-extrinsic signaling);
• Redox status are balanced between oxidants and antioxidants;
• Targeting the redox status (cell-intrinsic and cell-extrinsic) can have therapeutic potential.
Take home messages...

• Multiple myeloma is a hematologic malignancy of terminally differentiated plasma cells;
• Altered redox status is linked to MM progression and drug sensitivity;
• Targeting the redox status (cell-intrinsic and cell-extrinsic) can have therapeutic potential.