Piastrine: genoma e trascrittoma

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“Workshop post-ISTH: novità dal meeting di Toronto 2015”
Bergamo, 29-30 gennaio 2016
1. Genetic regulation of platelet function in health and disease

- Genetic regulation of megakaryocytes and platelets
  Andy Weyrich (State of the art)

- Dicer, the key enzyme of RNA interference, is regulated by thrombin stimulation in human platelets
  Manni G et al. (Oral communication, Young Investigator Award)

- Diphosphorylated lipid A triggers tissue factor mRNA splicing in human platelets
  Krauel K et al. (Oral communication)

2. Platelet mRNAs and miRNAs in the interaction with other cells

- The role of circulating platelet transcripts
  Jane Freedman (State of the art)

- RNA signatures in platelets from cancer patients
  Thomas Wurdinger (SSC Platelet Physiology, lecture)

- Platelets release extracellular vesicles in an agonist dependent manner but release a consistent profile of microRNA
  Ambrose AR et al. (Oral communication, Young Investigator Award)
Genetic regulation of megakaryocytes and platelets

Andrew S. Weyrich
State of the art lecture

The genetic code of megakaryocytes and platelets in health

The transfer of genetic information in the form of coding mRNAs and regulatory miRNAs affects protein expression
Genetic regulation of megakaryocytes and platelets

The genetic code of megakaryocytes and platelets in disease

-Environmental Triggers
-Geneic Mutations
-Race/Age
-Other Factors

Endocytosis of mRNAs and miRNAs

The Megakaryocyte Genetic Code Changes

Megakaryocytes Transfer a Different Genetic Code Into Budding Platelets

Platelets Circulate With an Altered Genetic Code

Platelet Functional Responses Reflect Their Genetic Code

1) Altered Functions
2) Translation of Host and Viral RNAs
3) Transfer of mRNA and miRNA to Recipient Cells
Platelets synthesize new proteins

Activation-dependent pre mRNA-splicing starts protein synthesis

Examples:
• BCL-3
• IL-1β
• COX-1
• COX-2
• PAI-1
• TF
• TIMP-2

Weyrich A et al., J Thromb Haemost 2009, 7:241
Megakaryocytes differentially sort mRNA for MMPs/TIMPs into platelets
Variations in race, genetic mutations, or acute disease are associated with changes in the genetic code (i.e. mRNA and miRNA expression patterns) in megakaryocytes. Changes in the megakaryocyte genetic code are then transferred into developing platelets that are released into the circulation. **Alterations in the platelet transcriptome and corresponding proteins influence functional responses.**
Platelet transcriptome is different between STEMI or CAD patients

CD69

6.2 (2.3-29.8)  
<0.001

CD36 mRNA and MRP-14 mRNA

CD69

MPR-14

3.3 (1.5-9.0)  
0.002

Healy AM et al., Circulation 2006, 113:2278

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Platelet transcriptome is different between LES patients and healthy controls

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene title</th>
<th>Fold change</th>
</tr>
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<tbody>
<tr>
<td>IFI27</td>
<td>interferon, alpha-inducible protein 27</td>
<td>23.12</td>
</tr>
<tr>
<td>CD58*</td>
<td>CD58 antigen, (lymphocyte function-associated antigen 3)</td>
<td>13.04</td>
</tr>
<tr>
<td>PRKRA*</td>
<td>protein kinase, interferon-inducible double stranded RNA dependent activator</td>
<td>6.34</td>
</tr>
<tr>
<td>G1P3</td>
<td>interferon, alpha-inducible protein (clone IFI-6-16)</td>
<td>6.27</td>
</tr>
<tr>
<td>CD69*</td>
<td>CD69 antigen (p60, early T-cell activation antigen)</td>
<td>5.25</td>
</tr>
<tr>
<td>IFITM1*</td>
<td>interferon induced transmembrane protein 1 (9-27)</td>
<td>3.42</td>
</tr>
<tr>
<td>OAS1</td>
<td>2'-5'-oligoadenylate synthetase 1, 40/46kDa</td>
<td>2.87</td>
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<tr>
<td>STAT1</td>
<td>signal transducer and activator of transcription 1, 91kDa</td>
<td>2.58</td>
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<tr>
<td>LY6E</td>
<td>lymphocyte antigen 6 complex, locus E</td>
<td>2.43</td>
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<tr>
<td>IFNAR1</td>
<td>interferon gamma receptor 1</td>
<td>2.18</td>
</tr>
<tr>
<td>IFRD1</td>
<td>interferon-related developmental regulator 1</td>
<td>2.10</td>
</tr>
<tr>
<td>IFI44L</td>
<td>interferon-induced protein 44-like</td>
<td>2.04</td>
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<tr>
<td>OAS3</td>
<td>2'-5'-oligoadenylate synthetase 3, 100kDa</td>
<td>1.98</td>
</tr>
<tr>
<td>G1P2</td>
<td>interferon, alpha-inducible protein (clone IFI-15K)</td>
<td>1.98</td>
</tr>
<tr>
<td>PLSCR1</td>
<td>phospholipid scramblase 1</td>
<td>1.97</td>
</tr>
<tr>
<td>OAS2</td>
<td>2'-5'-oligoadenylate synthetase 2, 69/71kDa</td>
<td>1.92</td>
</tr>
<tr>
<td>IRF2BP2</td>
<td>interferon regulatory factor 2 binding protein 2</td>
<td>1.92</td>
</tr>
<tr>
<td>GBP1</td>
<td>guanylate binding protein 1, interferon-inducible, 67kDa</td>
<td>1.87</td>
</tr>
<tr>
<td>IFI16</td>
<td>interferon, gamma-inducible protein 16</td>
<td>1.83</td>
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<tr>
<td>IFITM3</td>
<td>interferon induced transmembrane protein 3 (1-8U)</td>
<td>1.80</td>
</tr>
<tr>
<td>IRF2</td>
<td>interferon regulatory factor 2</td>
<td>1.70</td>
</tr>
</tbody>
</table>
Platelets and miRNA

- Platelets contain miRNAs and the RNAi machinery
- miRNAs regulate gene expression in platelets
- miRNAs have a role in platelet activation

Platelet miRNAs as biomarkers of platelet reactivity

miRNA profiles resulted in 2 groups of subjects that appeared to cluster by platelet aggregation phenotypes.
- PAR4 response is higher in blacks
- mRNAs positively correlate with PAR4 response
- miRNAs negatively associate with PAR4 reactivity

Networks of miRNA and mRNA differentially expressed by age (A) and gender (B).


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Platelet miRNome in disease

Platelets from patients with sickle cell disease (SCD) exhibit an altered miRNA expression profile.

**Up-regulated miRNAs**
- miR-638
- miR-940
- miR-636
- miR-1238
- miR-1225-3p
- miR-1207-5p
- miR-33b
- miR-630
- miR-191

**Down-regulated miRNAs**
- miR-376a
- miR-381
- miR-409-3p
- miR-377
- miR-376c
- miR-495
- miR-376b
- miR-181c
- miR-487b
- miR-337-5p
Dicer, the key enzyme of RNA interference, is regulated by thrombin stimulation in human platelets

Giorgia Manni*, Loredana Bury, Alessandro Marturano, Elisa Piselli and Paolo Gresele

Dicer protein and mRNA are present in resting platelets. Thrombin induces Dicer synthesis.

- **DICER1**
  - HeLa
  - MKs
  - Plts

- **B2MG**
  - HeLa
  - MKs
  - Plts

- **Dicer 1**
  - HeLa
  - MKs
  - Plts

- **Tubulin**
  - HeLa
  - MKs
  - Plts

**Graphs:**
- OD (Arbitrary Unit) vs. Time (minutes) with thrombin concentration (Thr 0.1 U/ml) and binding index with puromycin 1 mM.
- S35 methionin (Fold Increase) over time (minutes) with thrombin concentration (Thr 0.1 U/ml) and puromycin 1 mM.
Dicer synthesis is associated with mir-223 maturation and target mRNA down-regulation.
Diphosphorylated lipid A triggers Tissue factor mRNA splicing in human platelets
Krauel K*, Thorack U, Schumacher A, and Schwertz H

- LPS is an agonist for platelet mRNA splicing of TF.
- LPS components are lipid A di- and mono-phosphate and 3-deoxy-D-manno-2-octulosonic acid (KDO).
- LPS and lipid A diphosphate, but not monophosphate or KDO, trigger mRNA splicing.
- TF mRNA required LPS activation for maturation, while IL-1β mRNA was already mature at baseline.
- Lipid A diphosphorylated is required to trigger TF mRNA-splicing in human platelets.
The role of circulating platelet transcripts

Jane E. Freedman
State of the art lecture
Platelet RNA transfer mechanisms

Platelet RNA transfer may affect vascular cell expression and homeostasis by:

• direct interaction of platelets with white blood and/or endothelial cells
• microparticles formation and subsequent interaction of microparticles with white blood and/or endothelial cells

Activated platelets transfer miRNA to endothelial cells in vitro

miR-39 in resting and activated platelets

miR-39 in endothelial cells cocultured with miR-39 transfected platelets

Gidlof O et al., Blood 2013;121:3908
Platelet mRNA transfer occurs *in vivo*

Risitano A et al., Blood 2012;119:6288
Platelets release extracellular vesicles in an agonist dependent manner but release a consistent profile of microRNA

Amrose AR, Pringle JH and Goodall AH

Platelets contain abundant microRNAs which can be packaged into extracellular vesicles (EV) and released into the circulation.

EVs contained between 57 and 79 different miRNAs depending on the stimulus.

miR-223 was expressed in all samples

These data suggest that the EVs released from platelets and their miRNA content are agonist-dependent.
Dengue virus pirates human platelets

Dengue virus (DENV) raids platelets and steals their translational machinery to replicate and produce infectious virus.

Simon AY et al., Blood 2015;126:378
RNA signatures in platelets from cancer patients

Thomas Wurdinger
SSC Platelet Physiology Lecture

«...splice events in response to external signals and the capacity of platelets to ingest spliced circulating mRNAs can provide tumor educated platelets with a highly dynamic mRNA repertoire...»
- 5003 different protein-coding and non-coding RNAs were detected in platelets of healthy donors and cancer patients
- 1453 mRNAs were increased and 793 decreased in TEPs
- Differentially-detected mRNAs distinguished two groups with minor overlap

Best MG et al., Cancer Cell 2015, 28:666
In addition to the pan-cancer diagnosis, the TEP mRNA profiles also distinguished healthy donors and patients with specific types of cancer, as demonstrated by the hierarchical clustering of differential platelet mRNA levels of healthy donors and all six individual tumor types.

NSCLC: non small cell lung  
CRC: colorectal  
GBM: glyoblastoma  
PAAD: pancreatic  
BrCa: breast  
HBC: hepatobiliary
# RNA-Seq of Tumor-Educated Platelets

## TRAINING COHORT

<table>
<thead>
<tr>
<th>Actual Class</th>
<th>Genes: 1072</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>131 (96%)</td>
</tr>
<tr>
<td>Healthy</td>
<td>5 (4%)</td>
</tr>
<tr>
<td><strong>Total (n)</strong></td>
<td>136 (100%)</td>
</tr>
</tbody>
</table>

### Accuracy:
95%, p < 0.01

## VALIDATION COHORT

<table>
<thead>
<tr>
<th>Actual Class</th>
<th>Genes: 1072</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>89 (97%)</td>
</tr>
<tr>
<td>Healthy</td>
<td>36 (92%)</td>
</tr>
<tr>
<td><strong>Total (n)</strong></td>
<td>92 (100%)</td>
</tr>
</tbody>
</table>

### Accuracy:
96%

### Pan-cancer TEP-test

<table>
<thead>
<tr>
<th></th>
<th>Correctly classified</th>
<th>Mean predictive strength (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized cancer</td>
<td>39/39 (100%)</td>
<td>0.99 (0.94-1.0)</td>
</tr>
<tr>
<td>Metastasized cancer</td>
<td>181/189 (96%)</td>
<td>0.96 (0.53-1.0)</td>
</tr>
<tr>
<td>Healthy donors</td>
<td>51/55 (93%)</td>
<td>0.87 (0.5-1.0)</td>
</tr>
<tr>
<td>Primary CNS tumors</td>
<td>33/39 (85%)</td>
<td>0.89 (0.54-1.0)</td>
</tr>
<tr>
<td>Adenocarcinomas</td>
<td>188/190 (99%)</td>
<td>0.98 (0.71-1.0)</td>
</tr>
</tbody>
</table>

Best MG et al., Cancer Cell 2015, 28:666
Blood platelets represent an advancement in blood-based “liquid biopsies”

- Tumor educated platelets (TEPs) provide a RNA biosource for pan-cancer, multiclass, and companion diagnostics
- Blood platelets are a potential all-in-one platform for blood-based cancer diagnostics, using the equivalent of one drop of blood.

Best MG et al., Cancer Cell 2015, 28:666
CONCLUSIONS

• The crucial role of platelets in hemostasis, inflammation, host defence has led to the evolutionary development of finely tuned mechanisms regulating platelet phenotype

• Alterations of platelet and megakaryocyte transcriptome/miRNome play a role in their participation in disease

• The expanding knowledge of these mechanisms may in perspective lead to the identification of novel therapeutic targets and/or to the use of platelets as therapeutic carriers
- Race
- Inherited mutations
- Acquired diseases
- Others

Determinants of the genomic response in megakaryocytes

Megakaryocytes transfer personalized genetic code into budding platelets

Platelets circulate with a unique genetic code

The platelet genetic code influences functional responses
Jane E. Freedman
The role of circulating platelet transcripts
State of the art

Platelet Transcriptome: Does it have a direct effect?

Genetic Variability (more information needed for platelets)
Atherothrombotic disease and risk factors
Other phenotypes

Platelet Transcripts
Reflect disease
RNA-miRNA transfer
Plasma/exRNA transfer?

Thrombosis
Vessel wall homeostasis
Infection-immunity
Atherosclerosis
Wound healing
Biomarkers, Targets for Treatment
**Andrew S. Weyrich**

**Genetic regulation of megakaryocytes and platelets**

**State of the art**

The genetic code of Megakaryocytes and Platelets Tells a Rich Story

<table>
<thead>
<tr>
<th>Feature identified by RNA-seq</th>
<th>Molecular function</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ cap and poly-A tail</td>
<td>Regulation of translation and RNA stability</td>
</tr>
<tr>
<td>Unspliced pre-mRNA and intron retention</td>
<td>Regulation of translation</td>
</tr>
<tr>
<td>Extended and alternate 5′ and 3′ UTR</td>
<td>Alternative regulatory motifs: uORFs, RNA binding proteins, and miRNA binding sites</td>
</tr>
<tr>
<td>Known and novel alternative splice variants</td>
<td>Regulation of translation, alternate functional protein isoforms, truncated proteins (i.e. secreted vs. membrane bound)</td>
</tr>
<tr>
<td>Non-coding RNA and antisense transcripts</td>
<td>Regulation of transcription and translation</td>
</tr>
<tr>
<td>Insertions, deletions, single nucleotide mutations</td>
<td>Altered functional proteins, altered protein expression</td>
</tr>
</tbody>
</table>

1. Insights into platelet function and phenotype
2. Biomarkers and regulators of disease
3. Insights into megakaryocyte development and function
RNA signatures in platelets from cancer patients

Thomas Wurdinger

(SSC Platelet Physiology)

Tumors “educated” platelets (TEPs) by altering the platelet RNA profile

Diagram showing the process of TEPs and their role in cancer diagnosis and identification of tumor type.
Protein synthesis by platelets

- Platelets do not contain DNA
- Platelets contain mRNA derived from megakaryocytes
- They are able of protein synthesis, constitutively or after stimuli