Mesenchymal stem cells release exosomes that transfer miRNAs to endothelial cells and promote angiogenesis

Gong et al., Oncotarget. 2017 Apr 1.

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Introduction
Mesenchymal stem cells (MSCs)

- non-haematopoietic, multipotent stem cells with the capacity to differentiate into mesodermal lineage such as osteocytes, adipocytes and chondrocytes as well ectodermal (neurocytes) and endodermal lineages (hepatocytes)
- Have a spindle-shaped fibroblast like morphology
- Can increase endothelial cell growth and enhance new blood vessel formation

Gong et al., 2017

Gong et al., Oncotarget. 2017 Apr 1.
Ullah et al., Biosci Rep. 2015 Apr 28;35(2).
**Exosomes**

- Cell-derived vesicles: diameter 30-100 nm

- Originate from budding into the limiting membrane of large endosomal structures (multivesicular bodies = MVB) in the cytosol

→ MVB are able to fuse with the plasma membrane, causing the release of exosomes into the extracellular space


Kooijmans *et al.*, 2012
Exosomes

- Exist in almost **all biological fluids** including blood, urine, saliva, cerebrospinal fluid, and cell preconditioned medium

- Shuttle **mRNAs, miRNAs and other molecular constituents** to achieve **cell-to-cell communication**
miRNAs

• Small non-coding RNAs (containing about 18-22 nucleotides)
• Regulate gene expression on the post-transcriptional level by binding to specific mRNA and inducing their
  ➢ degradation
  ➢ translational inhibition
• Play a role in biological and pathological processes including the cell cycle, hematopoesis, neurogenesis, aging, cancer and cardiovascular diseases
• miR-30 family targeted DLL4 in endothelial cells to promote angiogenesis

Gong et al., Oncotarget. 2017 Apr 1.

Gerlach and Vaidya, 2017
Hypothesis

Whether MSC-derived exosomes shuttle various pro-angiogenic miRNAs and transfer these miRNAs to endothelial cells resulting in promoting angiogenesis.
Methods
Conditioned medium derived from MSCs

1. MSCs cultured in complete DMEM/F12 medium for 24h
2. Medium was replaced with 15 ml of serum-free medium
3. After 48 h culture the medium was collected and centrifuged to remove cell debris
4. Supernatant was filtered and centrifuged at 3200g at 4°C for 45 minutes
5. Transferred into ultra-filtration conical tubes to concentrate medium to 100x
6. Exosomes were isolated from concentrated CdM using an ExoQuick-TC Exosome Precipitation Solution
7. Exosome pellets were resuspended with DMEM medium and stored at -80°C
Angiogenesis models

1. Tube-like structure formation assay
   - HUVECs were seeded on top of Matrigel
   - Treated with CdM or exosomes (100 µg/ml) for 16h
   - Images were taken

2. Spheroid-based sprout assay
   - GFP+ HUVECs (500 cells/spheroid) seed in non-adherent round bottom well plated overnight
   - Spheroids were generated and embedded into Matrigel for 16h in presence of CdM
   - Images were taken
Angiogenesis models

3. Matrigel plug assay

• Matrigel containing heparin was mixed with DMEM, CdM or exosomes (100 µg/plug)
• C57BL6 mice were anesthetized and then subcutaneously injected with Matrigel along the abdominal midline
• After 2 weeks: animals were sacrificed
Non-contact cell co-culture

- HUVECs were seeded onto the bottom of the plate
- MSCs were seeded and pre-cultured onto the insert (Corning Transwell; membrane cell culture insert)
- Next day:
  - insert was placed into the plate pre-cultured with HUVECs
  - cultured in serum-free DMEM medium for 48h
- Culture medium was cultured and concentrated 100x
Overexpression and knockdown of miR-30b in MSCs and HUVECs

Overexpression:
• miR-30b-copGFP expression plasmid and scramble-copGFP control plasmid were co-transfected into 293Ta cells (Lentiviral Packaging Cell Line)
  → for production of high titer lentiviral particles
• Then MSCs and HUVECs were infected with high titer lentiviral particles for 24h

Downregulation
• Synthetic anti-miR-30b was transfected into MSCs using Lipofectamine
• → to downregulate the expression of miR-30b in MSCs
Results
Conditioned medium derived from MSCs promotes angiogenesis

Tube-like structure formation of HUVECs

Spheroid-based sprouting of HUVECs

In vivo Matrigel plug assay

→ Tube length and sprout length per spheroid was significantly increased in HUVECs treated with CdM<sup>MSC</sup>

Matrigel plug contained CdM<sup>MSC</sup> had a:

→ significant increased hemoglobin content (a sign of increased new vessel formation)

→ significant higher number of CD31 positive cells

Gong et al., Oncotarget. 2017 Apr 1.
Expression of pro-angiogenic miRNAs in CdM<sub>MSC</sub> after adding into HUVECs culture for 48hours

<table>
<thead>
<tr>
<th>miRNA</th>
<th>CdM&lt;sub&gt;MSC&lt;/sub&gt; 2&lt;sup&gt;(ΔCt)&lt;/sup&gt;</th>
<th>CdM&lt;sub&gt;MSC&lt;/sub&gt; with HUVECs 2&lt;sup&gt;(ΔCt)&lt;/sup&gt;</th>
<th>miRNA</th>
<th>CdM&lt;sub&gt;MSC&lt;/sub&gt; 2&lt;sup&gt;(ΔCt)&lt;/sup&gt;</th>
<th>CdM&lt;sub&gt;MSC&lt;/sub&gt; with HUVECs 2&lt;sup&gt;(ΔCt)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-424&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.965 ± 5.542</td>
<td>10.725 ± 1.795*</td>
<td>miR-21</td>
<td>89.021 ± 9.117</td>
<td>187.956 ± 27.620*</td>
</tr>
<tr>
<td>miR-30c</td>
<td>6.420 ± 0.623</td>
<td>0.572 ± 0.140*</td>
<td>miR-10a</td>
<td>0.435 ± 0.040</td>
<td>10.160 ± 0.985*</td>
</tr>
<tr>
<td>miR-30b</td>
<td>5.877 ± 0.692</td>
<td>0.133 ± 0.012*</td>
<td>miR-126</td>
<td>0.045 ± 0.014</td>
<td>6.988 ± 0.933*</td>
</tr>
<tr>
<td>let-7f</td>
<td>4.592 ± 0.245</td>
<td>0.153 ± 0.003*</td>
<td>miR-10b</td>
<td>0.008 ± 0.002</td>
<td>5.869 ± 0.442*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>miR-19a</td>
<td>1.623 ± 0.063</td>
<td>3.380 ± 0.316*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>miR-19b</td>
<td>1.540 ± 0.116</td>
<td>2.950 ± 0.225*</td>
</tr>
</tbody>
</table>

(*P < 0.05 vs CdM<sub>MSC</sub>).

<sup>a</sup>The mouse homologue of miR-424 sequence from human is miR-322-5p.

→ Expression of **miR-424, miR-30c, miR-30b** and **let-7f** in CdM<sub>MSC</sub> was significantly reduced after adding into HUVECs culture
→ indicating that extracellular **miRs transferred into HUVECs**

→ Expression of **miR-21, miR-10a, miR-126, miR-10b, miR-19a** and **miR-19b** was significantly increased after adding into HUVECs culture
→ Suggesting that HUVECs might release these miRs

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Transfer of miRNAs between MSCs and HUVECs in a non-contact co-culture system

Supernatant

The levels of miR-424, miR-30c, miR-30b and let-7f in CdM_{HUVEC-HUVEC} was very low (black bars), CdM_{MSC-MSC} was very high (white bars) and CdM_{MSC-HUVEC} was low (grey bars).

The expression of these miRNAs in HUVECs co-cultured with MSCs was significantly higher than in HUVECs without co-cultured with MSC.

Demonstrating a transfer of these miRNAs into HUVECs.

Cell lysate

Gong et al., Oncotarget. 2017 Apr 1.
Exosomes derived from MSCs deliver pro-angiogenic miRNAs

- The expression of miR-424, miR-30c, miR-30b and let-7f in CdM\textsuperscript{GW4869} was significantly decreased (A: black bars)

- The levels of these miRs in HUVECs treated with CdM\textsuperscript{GW4869} was significantly reduced (B: black bars)

- Indicating that exosomes mediated miR transfer between MSC and HUVECs

GW4869...an exosome release inhibitor

Supernatant

Cell lysate

Gong et al., Oncotarget. 2017 Apr 1.
Characterization of exosomes derived from MSCs

- Internalization of exosomes pre-labeled with PKH26 (red fluorescence) by HUVECs reached its maximum after 10 h.

- The expression of miR-424, miR-30c, miR-30b and let-7f in HUVECs treated with exosomes was significantly increased (black bars).
Exosomes derived from MSCs promote angiogenesis

→ Tube length was significantly longer in HUVECs treated with exosomes

Matrigel plug contained exosomes had a:

→ significant increased hemoglobin content (a sign of increased new vessel formation)

→ significant higher number of CD31 positive cells

Gong et al., Oncotarget. 2017 Apr 1.
Exosomes derived from MSCs promote angiogenesis

→ Pro-angiogenic capacity of CdM^{MSC} was reduced after inhibiting or depleting exosomes in the CdM

Gong et al., Oncotarget. 2017 Apr 1.
Pro-angiogenic properties of exosomes

**Overexpression** of miR-30b in MSCs using lentiviral system

**Knockdown** of miR-30b using anti-miR-30b in MSCs

- Expression of miR-30b in MSC\textsuperscript{miR-30b} and Exo\textsuperscript{miR-30b} was increased
- Tube length was increased in HUVECs treated with Exo\textsuperscript{miR-30b}

- Expression of miR-30b in MSC\textsuperscript{antimiR-30b} and Exo\textsuperscript{antimiR-30b} was reduced
- Tube length was reduced in HUVECs treated with Exo\textsuperscript{antimiR-30b}

- **Indicating that overexpression of miR-30b enhanced** and **downregulation of miR-30b reduced** the pro-angiogenic capacity of exosomes

\[ \text{Gong et al., Oncotarget. 2017 Apr 1.} \]
Pro-angiogenic properties of exosomes

Overexpression of miR-30b in HUVECs using lentiviral system

→ Increased expression of miR-30b and tube length in HUVECs<sub>miR-30b</sub>

→ TargetScan shows that the 3' UTR of DLL4 contains the conserved miR-30 family binding sites

→ Expression of DLL4 in HUVECs<sub>miR-30b</sub> was significantly reduced

Discussion
• Conditioned medium of MSCs significantly increased tube-like structure formation, spheroid-based sprouting and neo-angiogenesis in Matrigel plug

• Exosomes derived from MSCs:
  → mediated the transfer of miRs from MSCs to HUVECs
  → promoted angiogenesis

• Gain-and-loss function of miRs in exosomes:
  → pro-angiogenic effect is dependent on their pro-angiomiRs cargo
MSCs promote angiogenesis through paracrine mechanisms.

Angiogenetic effects of MSCs may be related to the secretion of pro-angiomiRs and transfer of these miRs into endothelial cells.

Angiogenic effect of CdM was at least partly attributable to exosomes.

miR-30b carried by exosomes plays an important role in MSCs mediated angiogenesis.

Exosomes contain many growth factors, cytokines and chemokines, which may also participate in angiogenesis.
MSC-derived exosomes could be considered for using in therapeutic angiogenesis especially for ischemic diseases.
References

- www.ibidi.com
- www.proqinase.com
Thank you for your attention!