Ankersmit Laboratory
for Diagnosis & Regeneration in Thoracic Diseases & Applied Immunology

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

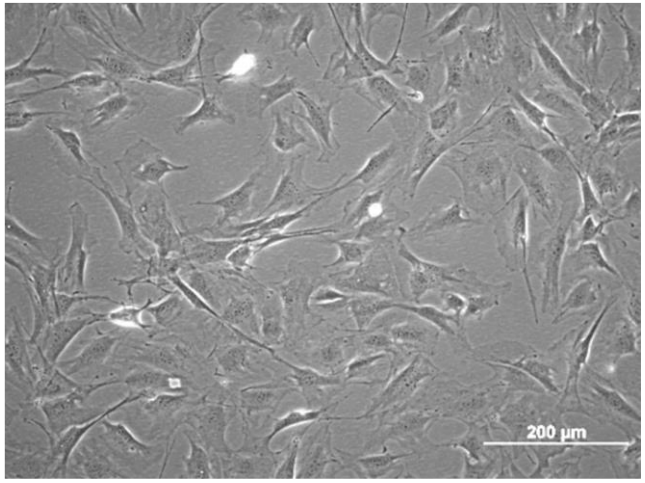
Gong *et al.*, Oncotarget. 2017 Apr 1.

Tanja Wagner

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

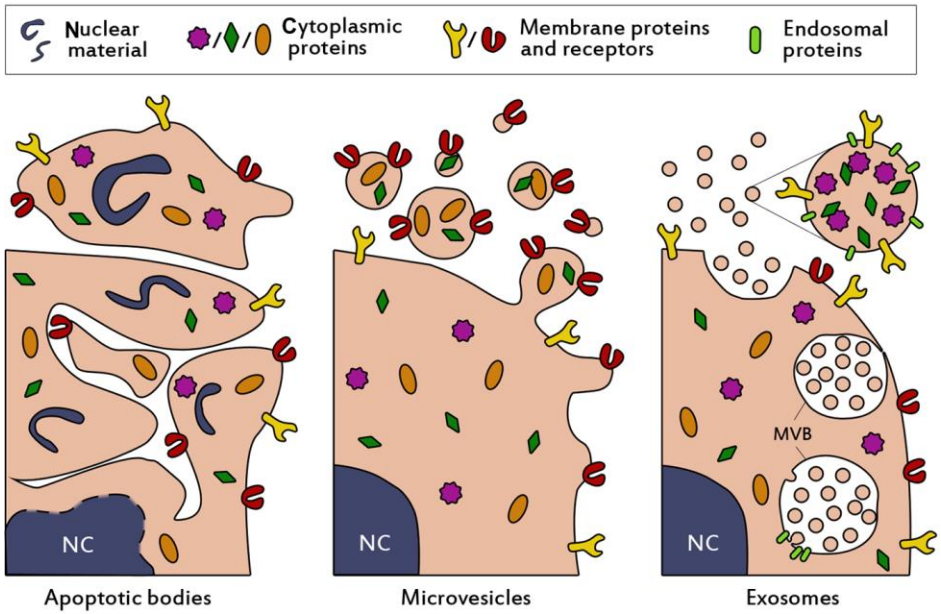
Gong *et al.*, Oncotarget. 2017 Apr 1.
Ullah *et al.*, Biosci Rep. 2015 Apr 28;35(2).

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

Gong *et al.*, Oncotarget. 2017 Apr 1.

Exosomes

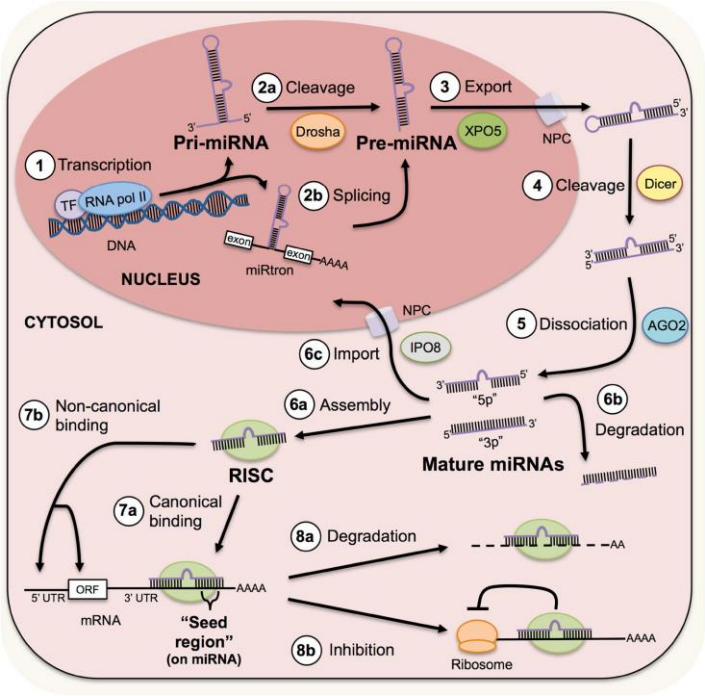
- Exist in almost **all biological fluids** including blood, urine, saliva, cerebrospinal fluid, and cell preconditioned medium

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

Gong *et al.*, Oncotarget. 2017 Apr 1.

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, hematopiesis, neurogenesis, aging, cancer and cardiovascular diseases
- miR-30 family targeted DLL4 in endothelial cells to promote angiogenesis



Gerlach and Vaidya, 2017

Gong *et al.*, Oncotarget. 2017 Apr 1.

Hypothesis

Whether MSC-derived exosomes shuttle various pro-angiogenic miRNAs and transfer these miRNAs to endothelial cells resulting in promoting angiogenesis

Gong *et al.*, Oncotarget. 2017 Apr 1.

Methods

Conditioned medium derived from MSCs

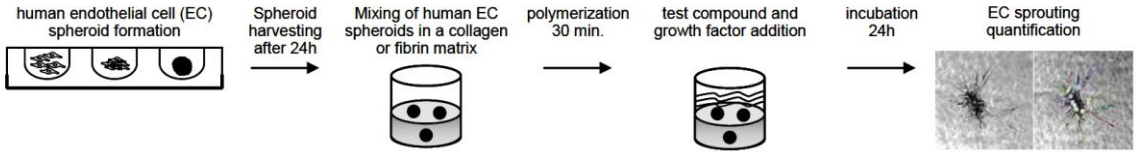
1. MSCs cultured in complete DMEM/F12 medium for 24h
2. Medium was replaced with 15 ml of **serum-free medium**
3. After 48 h culture the medium was collected and centrifuged to remove cell debris
4. Supernatant was filtered and centrifuged at 3200g at 4˚C for 45 minutes
5. Transferred into ultra-filtration 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

Gong *et al.*, Oncotarget. 2017 Apr 1.

Angiogenesis models

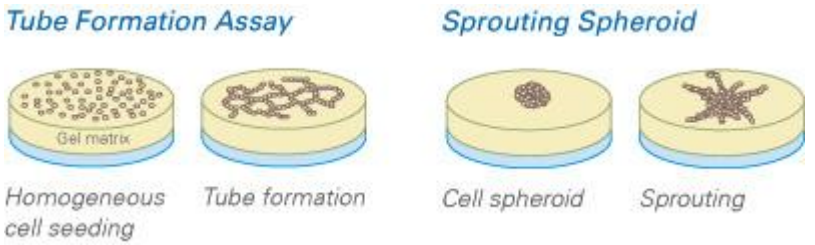
1. **Tube-like structure formation assay**
   - HUVECs were seeded on top of Matrigel
   - Treated with CdM or exosomes (100 µg/ml) for 16h
   - Images were taken

2. **Spheroid-based sprout assay**



www.proqinase.com

   - GFP+ HUVECs (500cells/spheroid) seed in non-adherent round bottom well plated overnight
   - Spheroids were generated and embedded into Matrigel for 16h in precence of CdM
   - Images were taken



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Gong *et al.*, Oncotarget. 2017 Apr 1.

Angiogenesis models

3. Matrigel plug assay

- Matrigel containing heparing 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

Gong *et al.*, Oncotarget. 2017 Apr 1.

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

Gong *et al.*, Oncotarget. 2017 Apr 1.

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

Gong *et al.*, Oncotarget. 2017 Apr 1.

Results

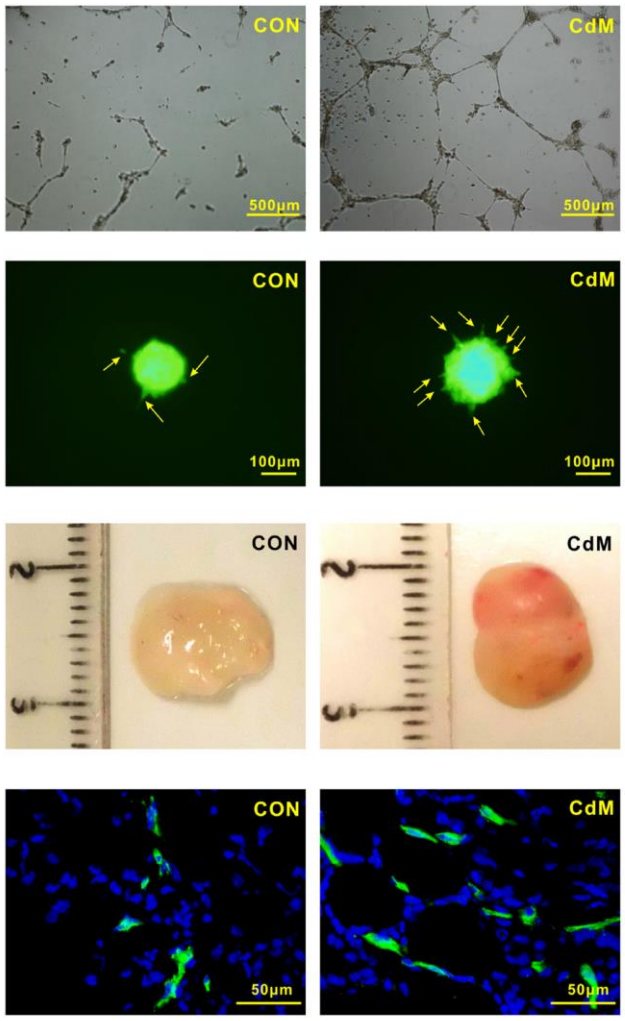
Conditioned medium derived from MSCs promotes angiogenesis

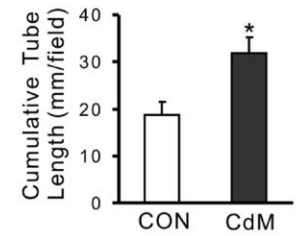
Tube-like structure formation of HUVECs

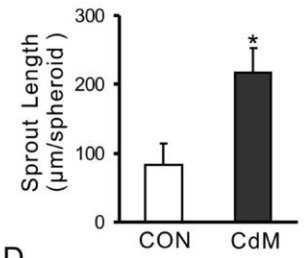
Spheroid-based sprouting of HUVECs

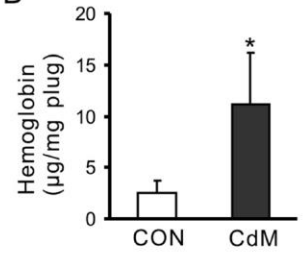
In vivo Matrigel plug assay

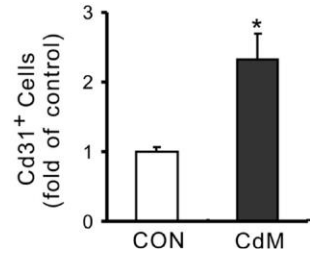
green:CD31 positve cells











→ Tube lenght and sprouth lenght per spheroid was significantly increased in HUVECs treated with CdM^{MSC}

<u>Matrigel plug contained **CdM^{MSC}** had a</u>:

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

→ significant higher number of CD31 positve cells

Gong *et al.*, Oncotarget. 2017 Apr 1.

Expression of pro-angiogenic miRNAs in CdM^{MSC} after adding into HUVECs culture for 48hours

Downregulated			Upregulated		
miRNA	**CdM^{MSC} $2^{(-\Delta Ct)}$**	**CdM^{MSC} with HUVECs $2^{(-\Delta Ct)}$**	**miRNA**	**CdM^{MSC} $2^{(-\Delta Ct)}$**	**CdM^{MSC} with HUVECs $2^{(-\Delta Ct)}$**
miR-424[#]	44.965 ± 5.542	10.725 ± 1.795*	miR-21	89.021 ± 9.117	187.956 ± 27.620*
miR-30c	6.420 ± 0.623	0.572 ± 0.140*	miR-10a	0.435 ± 0.040	10.160 ± 0.985*
miR-30b	5.877 ± 0.692	0.133 ± 0.012*	miR-126	0.045 ± 0.014	6.988 ± 0.933*
let-7f	4.592 ± 0.245	0.153 ± 0.003*	miR-10b	0.008 ± 0.002	5.869 ± 0.442*
			miR-19a	1.623 ± 0.063	3.380 ± 0.316*
			miR-19b	1.540 ± 0.116	2.950 ± 0.225*

(*$P < 0.05$ *vs* CdM^{MSC}).

[#]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^{MSC} was significantly **reduced** after adding into HUVECs culture

➔ indicating that extracellular **miRs transferred into HUVECs**

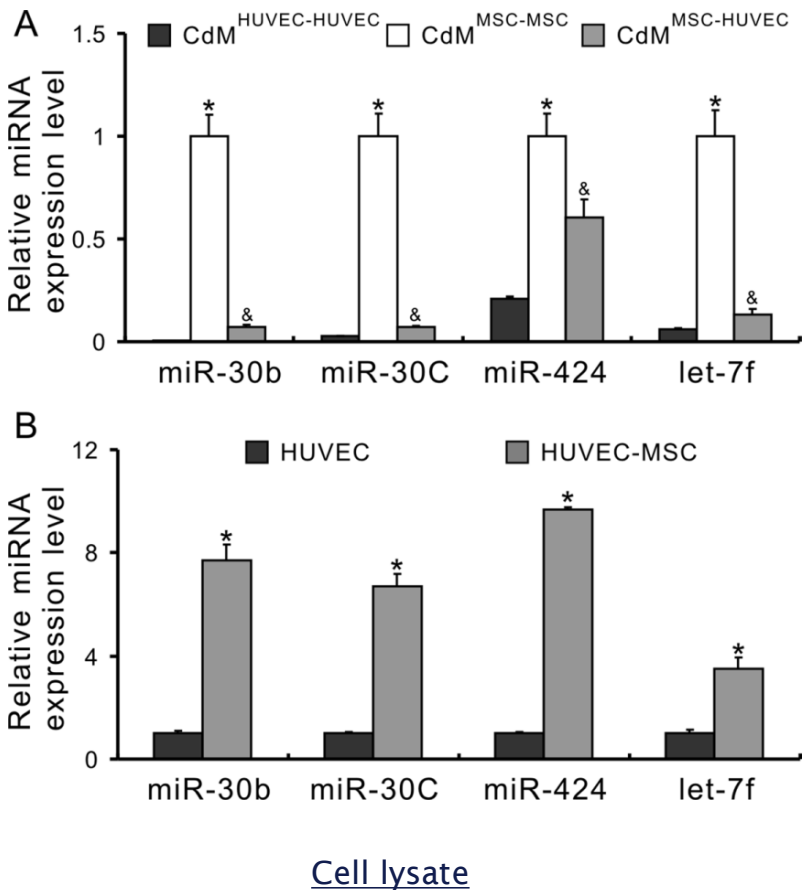
→ Expression of **miR-21, miR-10a, miR-126, miR-10b, miR-19a** and **miR-19b** was significantly **increased** after adding into HUVECs culture

➔ Suggesting that HUVECs might release these miRs

Gong *et al.*, Oncotarget. 2017 Apr 1.

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

Supernatant



Cell lysate

→ 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)

$CdM^{MSC-HUVEC}$ was low (grey bars)

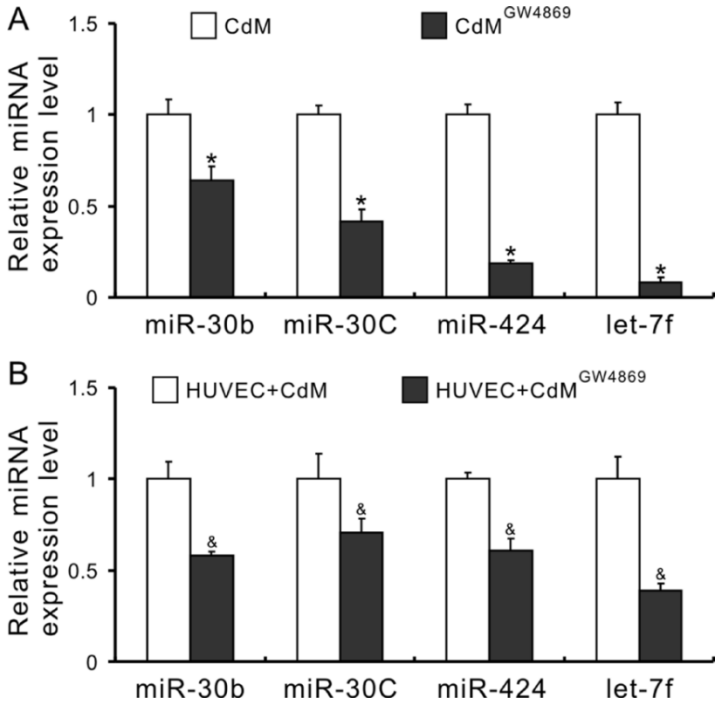
→ The expression of these **miRNAs in HUVECs** co-cultured with MSCs was significantly **higher** than in HUVECs without co-cultured with MSC

➔ **Demonstrating a transfer of these miRNAs into HUVECs**

Gong *et al.*, Oncotarget. 2017 Apr 1.

Exosomes derived from MSCs deliver pro-angiogenic miRNAs

Supernatant



Cell lysate

GW4869…an exosome release inhibitor

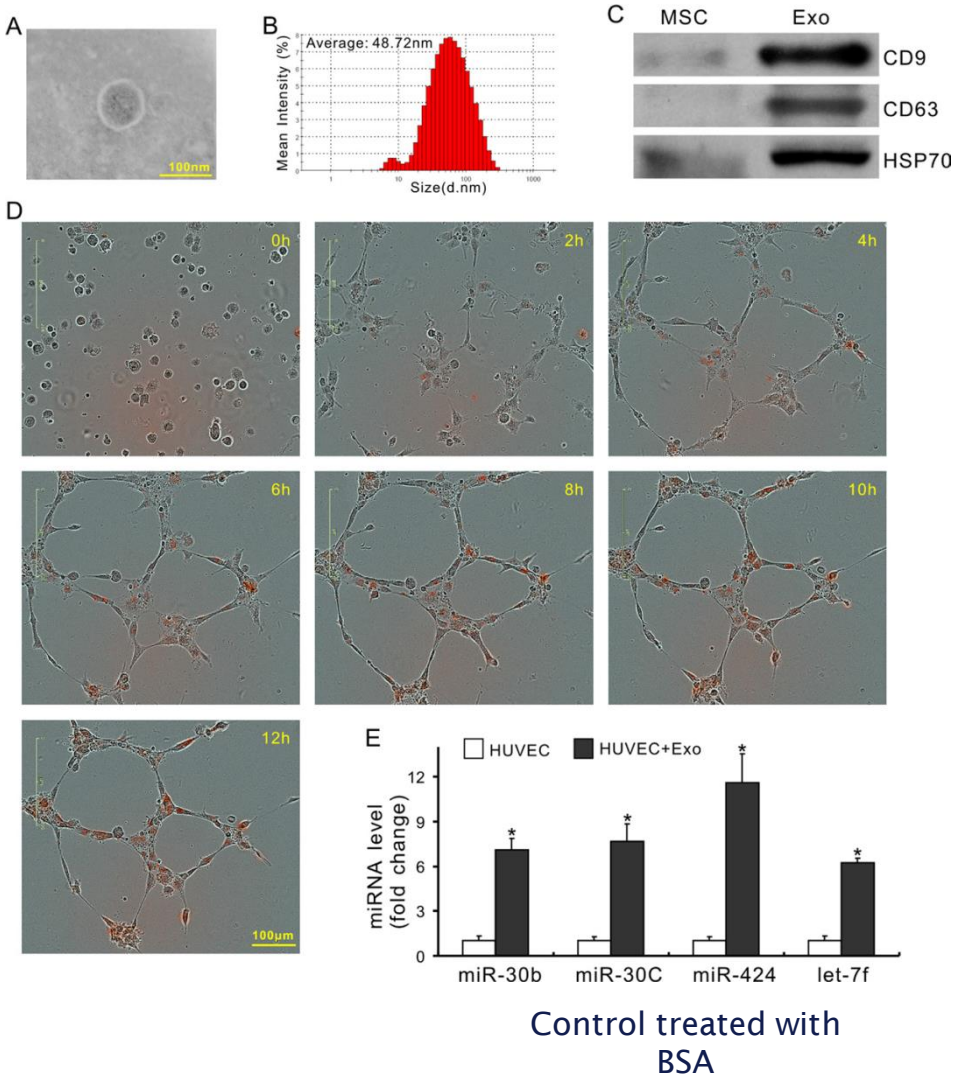
→ The expressoin of **miR-424, miR-30c, miR-30b** and **let-7f** in **CdM^{GW4869}** was significantly decreased (A: black bars)

→ The levels of these **miRs in HUVECs** treated with **CdM^{GW4869}** was significantly reduced (B: black bars)

➔ **Indicating that exosomes mediated miR transfer between MSC and HUVECs**

Gong *et al.*, Oncotarget. 2017 Apr 1.

Characterization of exosomes derived from MSCs



Control treated with BSA

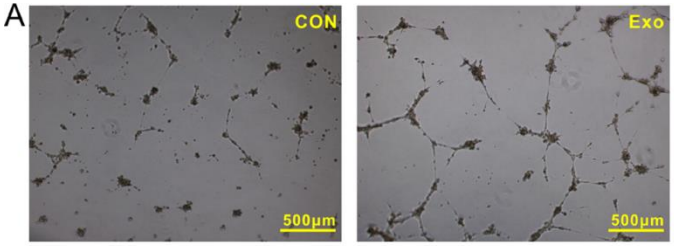
→ Internalization of exosomes pre-labeled with PKH26 (red fluorescence) by HUVECs reached its maxiumum after 10 h

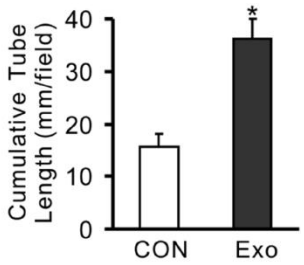
→ The expressoin of **miR-424, miR-30c, miR-30b** and **let-7f** in **HUVECs treated with exosomes** was significantly increased (black bars)

Gong *et al.*, Oncotarget. 2017 Apr 1.

Exosomes derived from MSCs promote angiogenesis

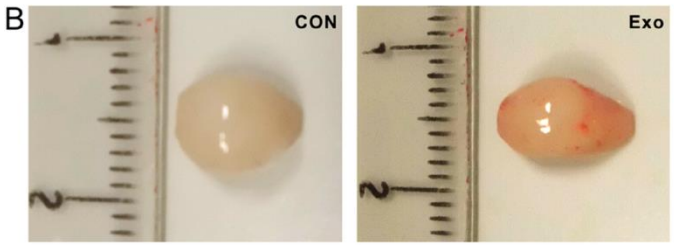
Tube-like structure formation of HUVECs

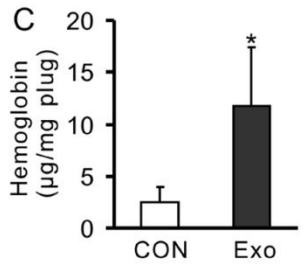




→ Tube lenght was significantly longer in HUVECs treated with exosomes

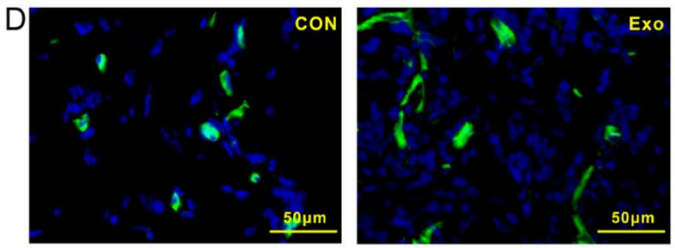
In vivo Matrigel plug assay

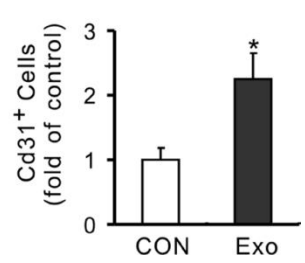




<u>Matrigel plug contained **exosomes** had a</u>:

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



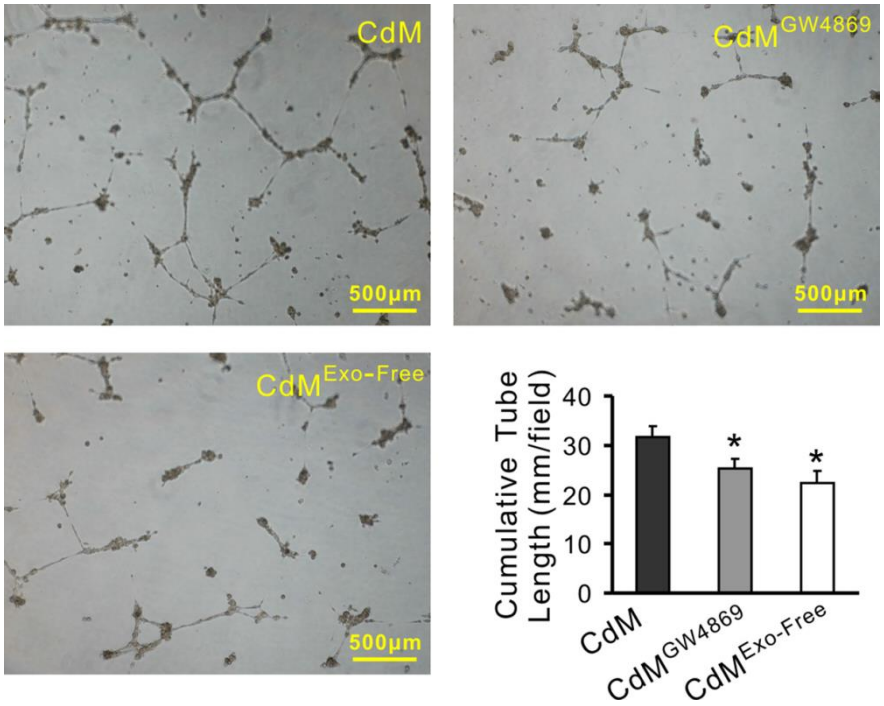


→ significant higher number of CD31 positve cells

Control treated with BSA (same protein amount)

Gong *et al.*, Oncotarget. 2017 Apr 1.

Exosomes derived from MSCs promote angiogenesis



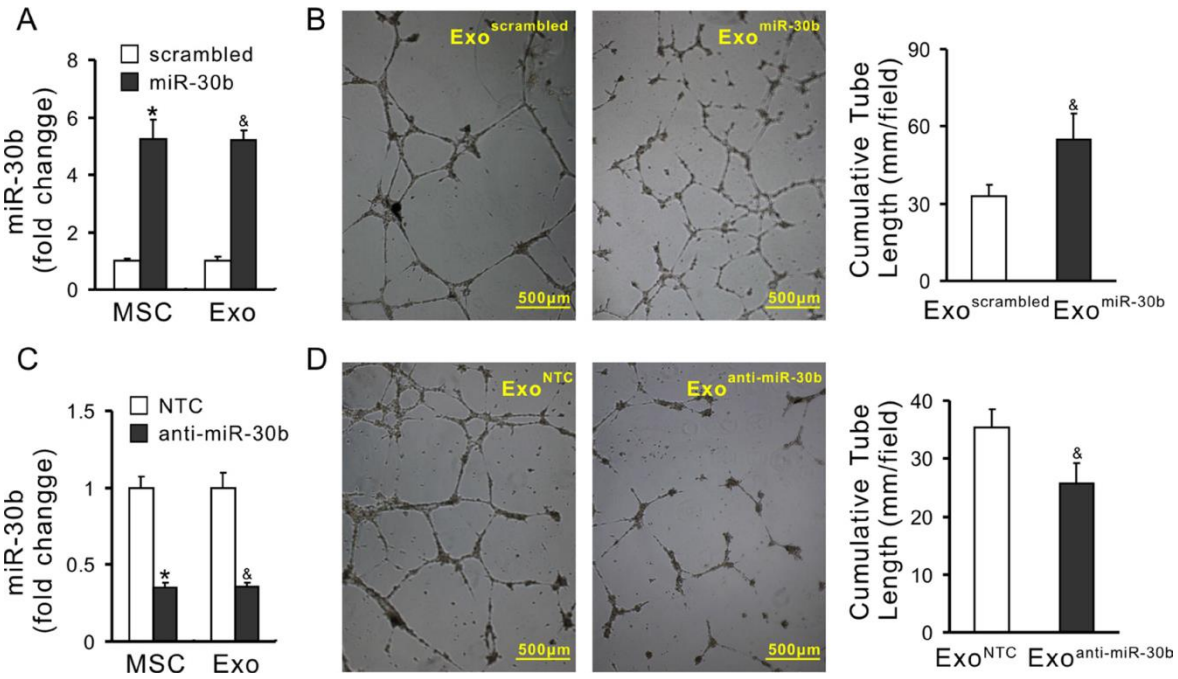
→ Pro-angiogenic capacity of CdM^{MSC} was reduced after inhibiting or depleting exosomes in the CdM

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$^{miR-30b}$** and **Exo$^{miR-30b}$** was increased

→ Tube lenght was increased in HUVECs treated with Exo$^{miR-30b}$

→ Expression of miR-30b in **MSC$^{anit-miR-30b}$** and **Exo$^{anit-miR-30b}$** was reduced

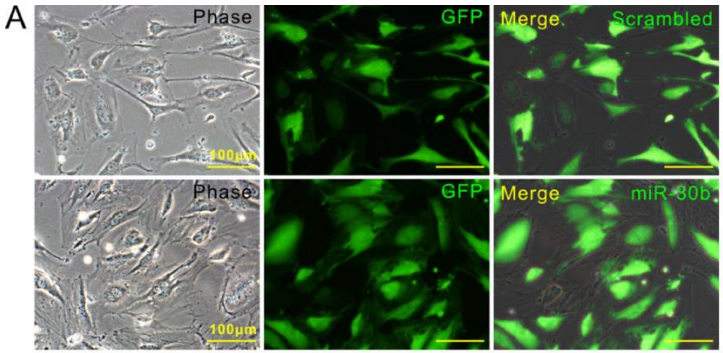
→ Tube lenght was reduced in HUVECs treated with Exo$^{anit-miR-30b}$

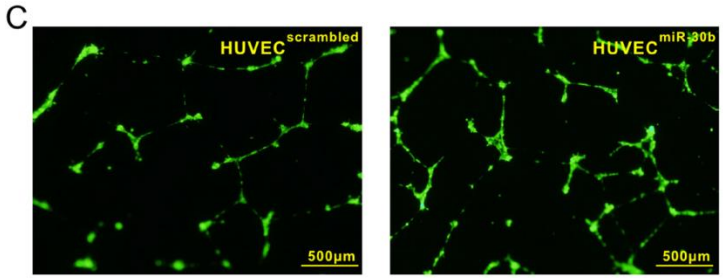
➔ **Indicating that overexpression of miR-30b enhanced and downregulation of miR-30b reduced the pro-angiogenic capacity of exosomes**

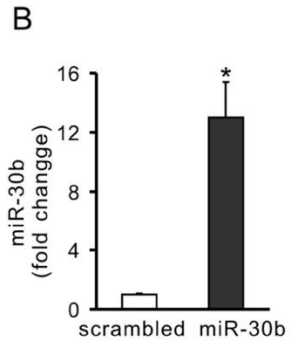
Gong *et al.*, Oncotarget. 2017 Apr 1.

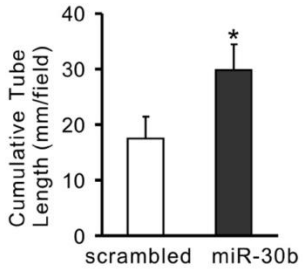
Pro-angiogenic properties of exosomes

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



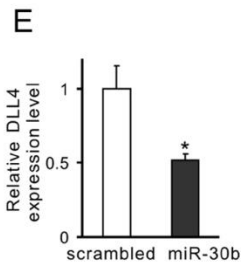


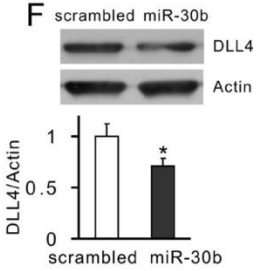




D

	predicted consequential pairing of target region (top) and miRNA (bottom)
Position 59-66 of DLL4 3' UTR	5' ...ACCUUCCUUCUGCAUUGUUUACA...
hsa-miR-30a	3' GAAGGUCAGCUCCUACAAAUGU
Position 59-66 of DLL4 3' UTR	5' ...ACCUUCCUUCUGCAUUGUUUACA...
hsa-miR-30b	3' UCGACUCACAUCCUACAAAUGU
Position 59-66 of DLL4 3' UTR	5' ...ACCUUCCUUCUGCAUUGUUUACA...
hsa-miR-30c	3' CGACUCUCACAUCCUACAAAUGU
Position 59-66 of DLL4 3' UTR	5' ...ACCUUCCUUCUGCAUUGUUUACA...
hsa-miR-30d	3' GAAGGUCAGCCCCUACAAAUGU
Position 59-66 of DLL4 3' UTR	5' ...ACCUUCCUUCUGCAUUGUUUACA...
hsa-miR-30e	3' GAAGGUCAGUUCCUACAAAUGU





→ Increased expression of miR-30b and tube length in **HUVECs**$^{\text{miR-30b}}$

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

→ Expression of DLL4 in **HUVECs**$^{\text{miR-30b}}$ was significantly reduced

Gong *et al.*, Oncotarget. 2017 Apr 1.

Discussion

- Conditioned medium of MSCs signigicantly 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 thier pro-angiomiRs cargo

Gong *et al.*, Oncotarget. 2017 Apr 1.

➔ MSCs promote angiogenesis through paracrine mechanisms

➔ Angiogenetic effects of MSCs may be related to the secretion of pro-angiomiRs and transfer of these miRs inot enothelial 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 angiogeneis

Gong *et al.*, Oncotarget. 2017 Apr 1.

➔ **MSC-derived exosomes could be considered for using in therapeutic angiogenesis especially for ischemic diseases**

Gong *et al.*, Oncotarget. 2017 Apr 1.

References

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Thank you for your attention!