Secretagogin-dependent matrix metalloprotease-2 release from neurons regulates neuroblast migration

János Hanics\textsuperscript{a,b,1}, Edit Szodorai\textsuperscript{c,d,1}, Giuseppe Tortoriello\textsuperscript{e,2}, Katarzyna Malenczyk\textsuperscript{c,e}, Erik Keimpema\textsuperscript{c}, Gert Lubec\textsuperscript{d}, Zsófia Hevesi\textsuperscript{a,b}, Mirjam I. Lutz\textsuperscript{f}, Márk Kozserek\textsuperscript{b}, Zita Puskár\textsuperscript{b}, Zsuzsanna E. Tóth\textsuperscript{b}, Ludwig Wagner\textsuperscript{g}, Gábor G. Kovács\textsuperscript{f}, Tomas G. M. Hökfelt\textsuperscript{e,3}, Tibor Harkany\textsuperscript{c,e}, and Alán Alpár\textsuperscript{a,b,3}

PNAS. February, 2017 (IF\textsubscript{5years} : 10.3)

Thomas Haider

JC/TS Current Topics in Applied Immunology SS2017

March 6\textsuperscript{th}, 2017
Background
Central Nervous System

- Complex network

- Brain: 100 billion neurons (100 000 000 000)
  - Each neuron – connections (synapses) to up to 10,000 neurons
  - Estimated 1,000 trillion synapses! (1 000 000 000 000 000)

- During development high plasticity necessary
  - Learning
  - Memories

Front Neurosci. June 2015, Volume 8, Article 23.
Central Nervous System

Plasticity in adult brains?

“Once development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult center the nerve paths are something fixed and immutable, nothing may be regenerated.”

(S. Ramon y Cajal, 1928)
Central Nervous System

Plasticity in adult brains!

• 1960s and 1970s: damaged axons can grow
• 1990s: “adult neurogenesis” in primates and humans

• Regions of adult neurogenesis
  • Hippocampus (dentate gyrus) – Memories
  • Other areas controversially discussed

Central Nervous System

- In adults reduction of plasticity important
  - To protect developed circuits
  - To keep learned information
  - Avoid malignant formation

- Remaining plasticity tightly regulated
  - Deficits cause disease (Schizophrenia, Autism, Malignancies,...)

Front Neurosci. June 2015, Volume 8, Article 23.
Damage to the Central Nervous System

Caused by different events

- Ischemic (e.g. Stroke)
- Traumatic (e.g. Spinal cord/traumatic brain injury)
- Inflammatory (e.g. Multiple sclerosis)
- Haemorrhagic (e.g. aneurysma bleeding)
- Degenerative (e.g. Alzheimer disease, ALS)
- Neoplasms (e.g. Glioblastoma)
- Genetic (e.g. trisomy 21)
- Infectious (e.g. encephalitis spongiforme, Creutzfeldt-Jakob)
- Toxic-nutritive (e.g. ethanol abuse)

-> Problem: Limited regenerative capacity
Central Nervous System

Regulation of plasticity

- Regulation on multiple cellular levels

- Hormones (sexual hormones, stress hormones...)

- Inhibitors
  - MAG – myelin-neuron interaction, oligodendrocyte differentiation
  - NoGoA – inhibition of neurite growth
  - OMgp, CSPG,...
Rostral Migratory Stream

- Glia-enriched conduit of forward-migrating neuroblasts
- Target: olfactory bulb
- Regulation: Growth factors, ephrins, neuron-glia cell interactions
- Astroglial “tunnel” directs outgrowth
- Extensive neuroblast-glial interaction to modulate tunnel

Secretagogin

- First described in pancreatic beta-cells (2000)
- Calcium-binding protein
- Upon Ca-binding -> conformational change
- Interaction with SNARE among others
- Neuropeptide release from hypothalamic neurons
SNARE Protein complex

Nature Reviews | Molecular Cell Biology
Matrix Metalloproteinases

- Endoproteinases
- Degrade proteins of the extracellular matrix
- Regulation with specific inhibitors (TIMPs)
- Association with various diseases

- Lung – ventilation during CABG reduces MMP release

Beer et al., journal of surgical research 195 (2015)
Matrix Metalloproteinases in the CNS


Table 1 | Outcomes of adult MMP-null mice in CNS insults

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Reported outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2/−/−</td>
<td>Earlier onset and more severe EAE due to a compensatory increase in MMP9</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>No difference from wild type after focal ischaemia</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>Reduced glioma growth</td>
<td>117</td>
</tr>
<tr>
<td>MMP9/−/−</td>
<td>Better recovery from spinal cord injury</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Reduced apoptosis of retinal ganglion neurons after optic nerve ligation</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Less severe EAE disease course</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Improved histological and motor outcome in brain trauma</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Better histological outcome from ischaemic stroke</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Increased haemorrhage, neurological deficits and lethality after intracerebral haemorrhage</td>
<td>121</td>
</tr>
<tr>
<td>MMP12/−/−</td>
<td>Impaired remyelination after a spinal cord lesion</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Worse disease course in EAE</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Better recovery from spinal cord injury</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Better functional recovery from intracerebral haemorrhage</td>
<td>122</td>
</tr>
</tbody>
</table>

The varied outcomes highlight the influence of both the beneficial and detrimental properties of matrix metalloproteinases (MMPs) in the CNS. EAE, experimental autoimmune encephalomyelitis.
Elucidate mechanisms of communication between newborn neurons and a contingent of nonastroglial cells resident in the rostral migratory stream (RMS).

Identify and describe a novel subsets of neurons involved in migration of neuroblasts in the RMS.
Materials and Methods
Materials and Methods

• Fetal Tissue – Two male fetal brains with normal development (between gestational weeks 31–33) from Vienna brain bank
• Stereotactic removal of unilateral olfactory bulb
  • Wistar rats
  • Secretagogin−/− conditional knockout mice
• Immunohistochemistry
• Immunoprecipitation and Shotgun Proteomic Analysis
• qPCR
• Western blotting
• Secretagogin Silencing using siRNA
• In-vivo inhibition of MMP activity with Marimastat
Results
Secretagogin labels a distinct cell subset
Results I

• Secretagogin positive cells are present in the RMS

• Secretagogin positive cells are in close contact to chain-migrating neuroblasts

• Secretagogin positive cells are differentiated neurons!
Secretagogin compartmentalization

- Secretagogin compartmentalization associated with ER and mitochondria

Secretagogin staining in human fetal tissue

Are these secretagogin$^+$ cells resident in the RMS or specialized neuroblasts?
Turnover analysis
Secretagogin\(^+\) cells following bulbectomy

- Previous studies show RMS enlarges after bulbectomy
- 15 days after injury SCGN expression significantly increased
Secretagogin$^+$ cells following bulbectomy

- Induction of expression in resident cells rather than introduction of new contingent of cells

Secretagogin$$^{-/-}$$ mice

**Fig. 53.** Secretagogin$$^{-/-}$$ mice demonstrate successful loss of function. (A and A$_1$) Secretagogin$$^+$$ neurons in wild-type RMSs typically expressed calretinin (black arrowheads). The white arrowhead points to a secretagogin$$^{-/-}$$/calretinin$$^{-/-}$$ neuron. (B–C$_1$) Secretagogin$$^{-/-}$$ mice lacked secretagogin expression in both the olfactory bulb (B and B$_1$) and RMS (C and C$_1$). (Scale bars: 100 μm in C and C$_1$; 20 μm in B and B$_1$; 10 μm in A and A$_1$.)
Secretagogin−/− mice

- Altered RMS

- Bulbectomy (OBX) – no increase of BrdU+ cells in KO mice

  ➢ *bulbectomy-induced mobilization of neuroblasts toward the injury site is slowed in the absence of secretagogin*

Silencing of Secretagogin

Fig. 6. In vivo secretagogin silencing decreases DCX expression and slows neuroblast migration in the RMS. (A–A₂) Secretagogin (A₁ and A₁') but not scrambled siRNA (A and A') reduced DCX expression locally (arrowheads in A and A₁). This effect is independent of olfactory bulbectomy (Fig. S4). Note the reduced secretagogin expression upon using specific (A₁) but not scrambled (A') siRNA and compared with distal RMS free of silencing effect (A₂).

(B–B₂) The density of BrdU⁺ cells decreased rostral but increased caudal to the silencing site (B₂). P < 0.05, Student’s t test. Images in A, A₁, B, and B₁ were acquired using the tile-and-stitch function. (Scale bars: 200 μm in A, A₁, B, and B₁; 25 μm in A' and A₁'; 15 μm in B'–B₁...)

Silencing of Secretagogin in OBX rats

**Fig. S4.** Secretagogin expression in bullectomized rats following in vivo gene silencing. Secretagogin (A₁ and A₁) but not scrambled siRNA (A and A₁) reduced DCX expression locally (double arrowheads in A and A₁) in bullectomized rats. Images in A and A₁ were acquired using the tile-and-stitch function. (Scale bars: 200 μm in A and A₁; 25 μm in A₁ and A₁.)

*Proc Natl Acad Sci U S A. 2017 Feb 21.*
RMS explants

DCX expression is not directly regulated by secretagogin but instead that secretagogin expression regulates cell motility through an extracellular mechanism.
Functional aspects of Secretagogin?

• Micropunches of RMS -> Immunoprecipitation with Secretagogin then mass spectrometry

• 64 Secretagogin-specific proteins

• **AnnexinV** – known to be associated with MMP-2 release
Annexin V and MMP-2 in RMS

Fig. 8. Secretagogin regulates MMP-2 release. (A and B) RMS micropunch samples from bullectomized and control rats were immunoprecipitated with secretagogin in Ca²⁺-containing or Ca²⁺-free isolation buffers and were subjected to mass spectrometry. (B, ) Functional distribution of secretagogin-interacting proteins recruited from samples of bullectomized animals and homogenized in Ca²⁺-containing isolation buffer. (C) Western blotting verified DCX, secretagogin, fascin, annexin V, and collin expression in the RMS. (D–D‘) Post hoc immunohistochemistry resolved annexin V immunoreactivity in secretagogin⁺ neurons in the RMS (white arrowheads). The black arrowhead points to an annexin V⁺ secretagogin⁺ neuron. (E) In situ zymography revealed gelatinase activity in the RMS that was reminiscent of the distribution of secretagogin⁺ neurons. White and black arrowheads indicate profiles in the shell and axonal domains of the RMS, respectively. (F and F‘) MMP2⁺ profiles on the extracellular surface of secretagogin⁺ neurons in RMS explants (serial reconstruction, 700-nm thin optical slices, consecutive z-stack images). (G and G‘) Gel zymography from RMS micropunches showed increased proMMP-2 levels in both intracellular and membrane-bound fractions of secretagogin⁺ mice, but proMMP-2 levels decreased in the extracellular fraction. (H and H‘) Secretagogin silencing increased the expression of the proenzyme form of MMP-2 but not of MMP-9 in SH-SY5Y neuroblastoma cells in vitro. (I and I‘) Olfactory bullectomy increased the level of the active but not of the proenzyme form of MMP-2 in rat RMS. (J) Schematic overview of the secretagogin-regulated mechanism in the RMS. Secretagogin regulates the externalization of proMMP-2, thereby limiting the amount of the active form of MMP-2. MMP-2 degrades the extracellular matrix to promote neuroblast migration. P < 0.05, Student’s t test. AS, annexin V; Ctrl, control; OBX, bullectomized. (Scale bars: 40 μm in D; 10 μm in D‘ and D; and 5 μm in F.)

Inhibition of MMP-2

Fig. S7. Marimastat blocks forward neuroblast migration. (A–C) Focal administration of the dual MMP-2/MMP-9 inhibitor Marimastat (1 μM/0.1 μL) (Tocris) increased the diameter of the proximal RMS (B₂) but decreased the diameter of the distal RMS (B₃), compared with control (DMSO) application (A₂ and A₃, respectively) with secretagogin⁺ cells accumulating upstream the application site. The effect of Marimastat is quantified in C. (Scale bars: 100 μm in A and B; 20 μm in A₁, A₂, B₁, and B₂) *P < 0.05.
Conclusion
Conclusion

Identification of differentiated neurons in the mammalian RMS that express secretagogin to initiate a molecular cascade through annexin V to increase MMP-2 release, thus remodelling the extracellular matrix to aid neuroblast migration.

Discussion
Discussion

• Resident cells in the RMS

• Enzyme externalization mechanism hitherto only known in tumor biology

• Up-regulation of Secretagogin after injury

• Depletion of this cell-type irreversible?

• Molecular mechanism / interaction of Secretagogin and Annexin V?
Discussion

• Feedback mechanism?

• Different regions of the CNS?

• Involved in diseases?

• Previous work shows that secretagogin$^+$ cells are still present in olfactory tract in aged brains

• Potential therapeutic target after CNS injuries?
Thank you for your attention!
Results

Fig. S5. Secretagogin is coexpressed with the motility proteins fascin and coflin. (A–A‴) Fascin+secretagogin+ neurons were identified not only in the marginal region of the RMS in P1 rats (white arrowheads in A–A‴; black arrowheads point to fascin+/secretagogin− neurons) but also in groups leaving the RMS from its proximal limb transversally (white arrowheads in A, A‴; B–B‴) Coflin colocalized with secretagogin. The dotted line surrounds a coflin+/secretagogin− cell. (C–C‴) Secretagogin+ cells coexpress annexin V in the dorsal margin of the RMS. (D) In situ zymography revealed gelatinase activity in the RMS, which was reminiscent of the distribution of secretagogin+ neurons. (E and E‴) Knockdown of annexin V expression increased proMMP-2 expression. (Scale bars: 40 µm in A, A‴, B, and C; 200 µm in D; and, 10 µm in A″.)
Results

Fig. 56. The presence of extracellular matrix components and matrix metalloproteases in the RMS. (A) PCR amplicons from RMS micropunches indicate the presence of laminin, collagen types 1, 7, and 10, the link protein HAPLN-1, and tenasin-R. In contrast to MMP-2, MMP-9 mRNA expression was at the detection limit. White arrows indicate cDNA bands (or the lack of, in case of the RT lanes). (B–F) Corresponding in situ hybridization images from the open source database of the Allen Brain Atlas (experiment numbers are indicated in the figure images) demonstrate the presence of collagen types 1, 7, and 10, laminin, and HAPLN-1 (black arrowheads) in the adult mouse RMS. Col, collagen; HPLN-1, hyaluronan and proteoglycan link protein 1. (Scale bars: 300 μm for B, B′, B″, B‴, B‴‴, and B‴‴‴.)

Results

Fig. S8. Full lanes of Western blots and zymography gels. (A) Western blotting. (B) Gel zymography.
Results

Fig. 5B. In vitro, ex vivo, and in vivo secretagogin silencing (siRNA) does not affect cell survival or the expression of synaptic markers. (A–A’) Control experiments for secretagogin siRNA selectivity. The density of live cells as determined by counting Hoechst+ nuclei was identical after the application of scrambled or secretagogin-specific siRNA in P5 dissociated RMS cultures. (B and B’) Secretagogin silencing in SH-SY5Y neuroblastoma cells left the expression of the pre- and postsynaptic markers synaptophysin and PSD-95, respectively, unaltered. (C and C’) Synaptophysin expression did change after secretagogin silencing in P5 RMS explants. (D–D’) In vivo gene silencing did not affect the density of Hoechst+ cells at the application site immediately dorsal to the RMS (arrows within the dashed boxes). High-power images are shown in D0 and D1, inj, injection site; qu, quantification. (Scale bars: 20 μm in A, D0, and D1, 8 μm in D0.)
Role for RIP1 in mediating necroptosis in experimental intracerebral hemorrhage model both in vivo and in vitro

Haitao Shen, Chenglin Liu et al.
Anoikis

• cytoskeleton is composed of microtubules, microfilaments and intermediate filaments

• Adherent cells become detached from the extracellular matrix or neighbouring cells

• caspase-dependent cell death called anoikis

• cytoskeleton components can modulate mitochondria
Parthanatos

• Poly-ADP-Ribose Polymerase PARP synthesis is activated when DNA is fragmented in the presence of nuclear poly-ADP ribosylated proteins

• regulated necrosis in which PARP activation plays an important role

• PARP proteolysis facilitates nuclear disorganization and ensures irreversibility of the apoptotic process

• chromatin condensation and DNA fragmentation
Cell death

<table>
<thead>
<tr>
<th>Type of cell death</th>
<th>Morphological changes</th>
<th>Biochemical features</th>
<th>Common detection methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleus</td>
<td>Cytoplasm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Chromatin condensation; nuclear fragmentation; DNA laddering</td>
<td>Blebbing</td>
<td>Fragmentation (formation of apoptotic bodies)</td>
</tr>
<tr>
<td>Autophagy</td>
<td>Partial chromatin condensation; no DNA laddering</td>
<td>Blebbing</td>
<td>Increased number of autophagic vesicles</td>
</tr>
<tr>
<td>Mitotic catastrophe</td>
<td>Multiple micronuclei; nuclear fragmentation</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Necrosis</td>
<td>Clumping and random degradation of nuclear DNA</td>
<td>Swelling; rupture</td>
<td>Increased vacuolation; organelle degeneration; mitochondrial swelling</td>
</tr>
<tr>
<td>Senescence</td>
<td>Distinct heterochromatic structure (senescence-associated heterochromatic foci)</td>
<td>–</td>
<td>Flattening and increased granularity</td>
</tr>
</tbody>
</table>

CDK1, cyclin-dependent kinase 1; MDC, monodansylcadaverine; MPM2, mitotic phosphoprotein 2; SA-β-gal, senescence-associated β-galactosidase; RB, retinoblastoma protein.
Necroptosis

Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. Dimitry Ofengeim & Junying Yuan
Nature Reviews Molecular Cell Biology 14, 727–736 (2013) doi:10.1038/nrm3683
Necroptosis

Apoptosis

• Pyknosis

• Karyorrhexis

• Membrane blebbing

• Apoptotic bodies

Necroptosis

• Translucent cytoplasm

• Organelle swelling

• Membrane permeabilization

• Oncosis

Necroptosis

[Diagram showing the process of Necroptosis with labeled components including TNFα, TNFα receptor, RIP1, Caspase-8, FADD, zVAD, TAK1 complex, IKK complex, NF-κB activation, Cell survival, and Nec-1.]

https://yuan.med.harvard.edu/discoveries
Last date of access 16.10.16
Intracerebral Hemorrhage (ICH)

• Second largest type of bleeding (15%)

• Mortality rate of ca. 40% in 1 month

• Symptoms:
  • Paralysis
  • Aphasia
  • Nerve function damage

Intracerebral Hemorrhage (ICH)

- Primary brain injury
  - Hematoma mass effect
  - Mechanical damage to adjacent brain tissues

- Secondary brain injury
  - Nerve function damage
  - Cell death
  - Cerebral edema
  - Blood-brain barrier damage
  - Inflammatory response
  - Proteolytic enzyme and toxic effect

Necroptosis activation

- TNF-α forms trimer with receptors
- RIP-1 binds on the death domain of TNF-α
- Activation via ubiquitination
- Activation of RIP 1 leads to recruitment of RIP3, MLKL and caspase-8
- Formation of the necrosome
- Overproduction of Reactive oxygen species (ROS)
  - -> DNA damage, mitochondrial membrane permeability, lysosome damage, cell death

Materials and Methods

6-8 weeks old Sprague Dawley rats

- Experiment 1:
  - 9 groups with 6 rats

- Experiment 2:
  - 10 groups with 6 rats

- Experiment 3:
  - Six groups with 6 rats
Experimental design

Role for RIP1 in mediating necroptosis in experimental intracerebral hemorrhage model both in vivo and in vitro.
ICH model

- 100µl autologous blood collected from heart puncture
- Fixation in stereotaxic frame
- Drilling of a hole to right basal ganglia
  - 0.2mm anterior to the intersection between the coronal suture and sagittal midline and 3.5mm to the right sagittal suture)
- Microsyringe 5.5mm depth
- 100µl blood or NaCl injection
- Keeping the needle in the hole for 5 min
- Sealing of the hole with bone wax

Methods

• Primary neuron- and microglial cultures
  • Neuron-enriched cultures from brains of fetal rats
  • Microglial-enriched cultures from brains of 1 day old rats

• Transfection of siRNA and adenoviruses (overexpression)

• Nec-1 and zVAD application in 3µl DMSO injected in lateral cerebral ventricle (1-2h before ICH)
Methods

- Western blot
- Immunoprecipitation
- Immunofluorescent staining
- PI and TUNEL staining
  - PI was injected intraperitoneally (1µg/g)
- Brain edema
- Blood brain barrier injury

ICH model

<table>
<thead>
<tr>
<th>Category</th>
<th>Behavior</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appetite</td>
<td>Finished meal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Left meal unfinished</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Scarcely ate</td>
<td>2</td>
</tr>
<tr>
<td>Activity</td>
<td>Walk and reach three corners</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Walk with some stimulations</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Almost always lying down</td>
<td>2</td>
</tr>
<tr>
<td>Deficits</td>
<td>No deficits</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Unstable walk</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Impossible to walk</td>
<td>2</td>
</tr>
</tbody>
</table>
Results

Results

Results

Results

Results

Results

Results

Results

Results

Results

Discussion

• Dead necroptotic cells release danger associated molecular patterns (DAMPs) -> aggravation of secondary brain injury

• Necroptosis has an important role in neuronal dysfunction, brain edema and BBB permeability

• Necrostatin-1 can inhibit neuronal damage and BBB permeability

• Inflammatory factors secreted by microglia lead to necroptosis after ICH
Thank you for your attention!
Resident CD4+ T cells accumulate in lymphoid organs after prolonged antigen exposure

Milas Ugur¹, Olga Schulz¹, Manoj B. Menon², Andreas Krueger¹ & Oliver Pabst¹,³

Lukas Altenburger

Medical University of Vienna
T cell activation:
T cell activation:
CD62L

- CD62L: L selectine
- Leukocyte adhesion molecule (LAM)
- binds CD34, GlyCAM
- mediates rolling interactions with endothelium
- lymph-node homing receptor
- binds on HEV
- CD62L binds on HEV in lymph nodes
- Naïve T cells migrate to the T cell zone
- Scan for antigen presented on APC’s
- CD62L binds on HEV in lymph nodes
- Naïve T cells migrate to the T cell zone
- Scan for antigen presented on APC’s
- Clonal expansion into:
  - Effector (CD62L \(^{-/-}\))
  - Central memory (CD62L \(^{+/hi}\))
  - Effector memory (CD62L \(^{-/-}\))
CD62L on memory T cells
Peyer’s patch

- Oval lymphoid follicles in SI.
- Mediate first mucosal immune responses.
- Comprise T cells, B cells, DC’s, Macrophages.
- Activated cells pass to MLN’s which amplifies responses.
Photoswitchable fluorescent proteins

- Long-term protein tracking
- 450nm light irradiation
- Irreversible photoconversion
- Green – red fluorescence
- Histone 2B-Dendra fusion chimeras
- Long term tracking
Follicular helper CD4 T cells ($T_{FH}$)

New insights into the differentiation and function of T follicular helper cells
Follicular helper CD4 T cells ($T_{FH}$)

Provide help for B cells

Germinal center formation

..and maintenance
Follicular helper CD4 T cells ($T_{FH}$)

New insights into the differentiation and function of T follicular helper cells.
S1PR1 on cell surface
S1P1 gradient guides T cells
Out of SLO´s.

Most resident cells in PP were CD69^{hi}.

CD69 on cell surface binds to S1PR1 – emigration?

WT/CD69 / mixed H2B-Dendra2 BM chimeras
a) NS

b) % FITC^+

c) S1pr1 expression (AU)

CD62L^hi vs CD62L^lo: **

ND
**T cell receptor**

Are T cells generated in every PP and become resident?

Seed CD4⁺ memory/effector T cells other PP’s?

T cell receptor analysis.

Va8 (TRAV12) family

Morisita–Horn index (MHI)

1.0 – 100% similarity

0.0 – 0% similarity
RAG-sufficient OT-II transgenic mice

Most CD4⁺ T cells express the OT-II TCR.

Consists of Va2 and Vb5 chains.

Specific for Ovalbumin (OVA).

Some CD4⁺ T cells express other TCR’s.

- Endogenous recombination events.
TCR signaling is important for resident T cell generation

Generation of resident OT-II in PP’s.

Adoptive transfer of congenially marked (CD45.1+) OT-II cells

WT mice immunized with OVA + cholera toxin
Conclusion

TEM cells are CD62Llo CCR7⁻ - home to non-lymphoid tissues

TCM cells are CD62Lhi CCR7⁺ - circulate through lymphoid tissues

First evidence of antigen-experienced CD4⁺ T cells retained in lymphoid tissues.

Phenotype - CD62L lo - make up to 50% of effector/memory.

Reside for 7 days.

...longer duration?
JC/TS Current Topics in Applied Immunology SS 17

04.03.2017
Propionate Ameliorates Dextran Sodium Sulfate-Induced Colitis by Improving Intestinal Barrier Function and Reducing Inflammation and Oxidative Stress

Ling-chang Tong¹², Yue Wang¹³, Zhi-bin Wang², Wei-ye Liu², Sheng Sun², Ling Li², Ding-feng Su² and Li-chao Zhang¹

¹ Department of Pharmacy, Shanghai Municipal Hospital of Traditional Chinese Medicine, Shanghai, China, ² Department of Pharmacology, College of Pharmacy, Second Military Medical University, Shanghai, China, ³ Department of Pharmacy, Ningxia Medical University, Yinchuan, China

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Sapienza University of Rome, Italy

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ORIGINAL RESEARCH
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Inflammatory bowel disease (IBD)

Multifactorial disorder

• gene susceptibility, immune dysregulation, microbial flora, environmental factors

Two principal types

• Crohn’s disease (entire gastrointestinal tract)
• Ulcerative colitis (colon, rectum)
DSS

- Induction of experimental IBD with Dextran Sulfate Sodium (DSS)

- Loss of tight junction proteins → loss of colonic integrity → inflammation

- DSS binds to Medium-Chain-Length Fatty Acids forming a complex (~200nm) → able to fuse with colonocyte membranes

Common therapy

- Glucocorticoids, sulfasalazine, immunosuppressive drugs
- Clinical application of these substances is limited → adverse effects
Sodium propionate

- Short chain fatty acid
- Produced by anaerobic fermentation
- Reducing the production of pro-inflammatory cytokines
- Enhancing intestinal barrier function
- Inhibition of oxidative stress
Methods: DSS induced colitis in Animals

- C57BL/6J male mice

- 40 mice randomized to four groups:
  - Control group (drinking water for 14 days)
  - Propionate group (1% in ddH2O for 14 days)
  - DSS group (d1-d6 drinking water, d7-d14 3% DSS in ddH2O)
  - DSS/Propionate group (d1-d6 1% propionate from d7-d14 supplemented with 3% DSS)
Methods: Histopathological assessment

- Measurement of colon length
- Paraffin embedded → cross-sectioning → HE-stain
- Histopathological evaluation
  - 0: no obvious inflammatory reaction
  - 1: the presence of low-level inflammatory reaction with a few scattered inflammatory cells
  - 2: the presence of moderate inflammatory infiltration
  - 3: the presence of severe inflammatory reaction in the colon tissue as represented by increased vascular density and thickness
  - 4: the presence of large amounts of inflammation cell infiltration and rupture of goblet cell mass.
Methods:

*In vivo* Intestinal Permeability

- Mice were fastened o/n
- FITC-dextran delivered via gavage
- Scarification 4h after administration
- Serum levels of FITC (480 and 520nm microplate fluorometer)
Methods:
RNA Isolation and Quantitative RT-PCR

• RNA extraction from colon tissue

• Inflammatory factors:
  • TNFα
  • IL-1β
  • IL-6
Methods:
Immunoblotting

• Protein extraction

• Antibodies used:
  • Anti-ZO-1
  • Anti-occludin
  • Anti-E-cadherin
  • Anti-STAT3
  • Anti-p-STAT3
Methods:
Measurement of Myeloperoxidase (MPO) Level in Colon and Serum

• MPO can modulate hydrogen peroxide

• Measurement of MPO activity

• MPO activity was defined as the quantity of enzyme degrading 1 mmol/ml of peroxide at 37°C
Methods:
Assessment of Macrophages in Colonic Mucosa by Immunofluorescence

- Immunofluorescence of colonic tissue
- Anti-CD68-antibody
Results
Results
Results
Results
Results
Results
Discussion

• Sodium propionate inhibits down-regulation of ZO-1, occluding, E-cadherin

• Sodium propionate reduces the expression of pro-inflammatory cytokines: inflammatory factors TNF-α, IL-1β, and IL-6 mRNA

• Sodium propionate reduces CD68 expression in colonic tissue ↓ macrophages infiltration

• Sodium propionate inhibits oxidative stress reduces MPO activity and enhances SOD and CAT activities in serum

• Sodium propionate inhibits phosphorylation of STAT3
Regeneration of fat cells from myofibroblasts during wound healing


Science

Volume 355(6326):748-752

February 17, 2017
Background
Scars

• 100 million people acquire scars every year, approximately 11 million keloid scars and 4 million burn scars

• In the USA, there is an estimated market of 12 Billion Dollars annually on the treatment of skin scarring, and 25 billion dollars were spent related to the treatment of wounds in general in 2015

Background

Wound healing and scar formation

- Coagulation
- Early inflammation
- Late inflammation
- Proliferation
- Remodelling

The phases of cutaneous wound healing

The Bratlie Research Group, Department of Materials Science and Engineering, Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011 © 2011 Kaitlin Bratlie
Background
Wound healing and scar formation

Scars:
• Excess collagen
• Lack of hair follicles
• Less subcutaneous fat

**Background**

**Scar formation**

- Scarless wound healing until the end of second trimester
- Differences scar formation-scarless healing:
  - No inflammation
  - Decreased fibrogenic and pro-angiogenic factors
  - Collagen I vs. Collagen III
  - TGF-beta 1 vs. TGF-beta 3

---

Background
BMP & TGF-beta

• BMP = bone morphogenic protein

• TGF-beta = transforming growth factor beta
  • TGF –beta 1-3, Activine, Inhibine
  • BMP= Subgroup of TGF-beta superfamily
  • critical roles in mesoderm formation, heart development, cartilage development and postnatal bone formation.

• Recombinant BMP-2 and 7 used clinically for interventions such as non-union fractures and spinal fusion
Sonia Villapol, Trevor T. Logan and Aviva J. Symes (2013). Role of TGF-β Signaling in Neurogenic Regions After Brain Injury, Trends in Cell Signaling Pathways in Neuronal Fate Decision, Dr Sabine Wislet-Gendebien (Ed.), InTech, DOI: 10.5772/53941.
Methods

• **Smart-seq2**: improved single-cell RNA-sequencing, provides expression profile of individual cells

• **Staining of adipose tissue**: Oil Red O dye, skin is viewed from the undersurface in all pictures

• **Meta-analyses**. Transcriptome-wide meta-analyses was performed on microarray and RNA-seq datasets from skin-derived precursors

• FACS, immunostaining, qRT-PCR,

• Mouse adipogenic cell culture, human adipogenic cell culture

• Human scar cell isolation and culture
Results

New adipocytes only regenerate around new hair follicles during wound healing

- Wound healing in humans and in mice: scar with excess collagen and absence of hair follicles
- Large skin wounds in mice regenerate hair follicles via Wnt/FGF pathways

Fig. S1: Wound induced new hair follicles and new fat. (A-B) View from skin surface.
Results
New adipocytes only regenerate around new hair follicles during wound healing.

Fig. 1A New adipocytes (orange) only regenerate around new hair follicles (blue) during wound healing.

“New adipocytes are undistinguishable in size, density, and depth from skin surface.”
Results
New adipocytes only regenerate around new hair follicles during wound healing

Fig. S2: Maturation of new adipocytes.

Mature adipose tissue cells:
- Resistin
- Adiponectin
- lacZ-positive cells

„Are hair follicles necessary to establish adipocyte precursors?“
Results
New adipocytes only regenerate around new hair follicles during wound healing.

Fig. 1B, C:
- dermal cells from wounds with regenerated hair follicles differentiated into adipocytes
- Cells from wounds without hair follicles did not differentiate into adipocytes
Results
New adipocytes originate from wound myofibroblasts.

Fig 2A:
- Myofibroblasts appear in wounds on day 5, abundant in scar by day 12
- No expression of smooth muscle actin by day 12
Results
New adipocytes originate from wound myofibroblasts.

**Fig. 2B, C:** Cellular origin of adipocytes?
- SM22Cre and SMACre are not activated in normal white fat in SM22Cre and SMACreER Mice: new adipocytes in wounds express LacZ
- LacZ in adipocytes indicates myofibroblast origin
Results

New adipocytes originate from wound myofibroblasts.

Fig 2D, E:
• Deletion of Ppareγ in myofibroblasts resulted in near-complete loss of new adipocytes
• normal cutaneous adipocytes at the wound edge remained intact.

Lineage tracing experiments: myofibroblasts are the source for new regenerating adipocytes.
Results
Molecular profiling and functional studies of adipocyte regeneration reveal that ZFP423 and BMP signaling are necessary for adipocyte regeneration.

Fig. 3A: myofibroblast transcriptome reveals distinct changes across four postwounding time points
Fig. 3B: 4120 differentially expressed genes are expressed in 5 clusters
- Upregulated: Regulators of adipocyte lineage (Zfp 423, Cerbl2, Stat5b)
- Downregulated: chondro/osteogenic: Sox9, Runx1/2,...
Results
Molecular profiling and functional studies of adipocyte regeneration reveal that ZFP423 and BMP signaling are necessary for adipocyte regeneration.

Fig 3C: temporal changes of gene expression in myofibroblasts.
Results
Molecular profiling and functional studies of adipocyte regeneration reveal that ZFP423 and BMP signaling are necessary for adipocyte regeneration.

**Fig 3D:** Zfp 423 mutant mice fail to regenerate fat completely but show no difference of adipocytes during development.

**Fig 3E:** Noggin = soluble BMP-antagonist. K14-mice overexpress Noggin; they fail to regenerate adipocytes but form hair follicles.
Results
Molecular profiling and functional studies of adipocyte regeneration reveal that ZFP423 and BMP signaling are necessary for adipocyte regeneration.

**Fig 3F:** deletion of BMP-receptor leads to lack of new adipocytes, but does not impede hair growth.

**Fig 3G:** inhibition of SMAD-1/5/8 prevents adipocyte regeneration in hair-bearing wounds.
Results
4 BMP drives reprogramming of mouse myofibroblasts and human keloid fibroblasts into adipocytes.

Fig 4A: human keloid scar cells treated with BMP4 induce conversion to adipocytes

Fig 4B: mouse dermal wound cells treated with BMP2 activate adipocyte-specific genes
Results
4 BMP drives reprogramming of mouse myofibroblasts and human keloid fibroblasts into adipocytes.

Fig 4C: Treatment of cultured human keloid scar cells with human recombinant BMP4 induces reprogramming into adipocytes

Fig 4D: BMP4-induced activation of adipocyte-specific genes
Results
4 BMP drives reprogramming of mouse myofibroblasts and human keloid fibroblasts into adipocytes.

**Fig 4E:** human scalp hair follicles induced adipogenic conversion of human keloid scar cells

**Fig 4F:** increase in adipocyte genes in coculture with hair follicles
Discussion

• new hair follicles in a wound reprogram myofibroblasts to an adipocyte fate by activation of the BMP-ZFP423 pathway
• observed conversion of myofibroblasts to adipocytes demonstrates lineage reprogramming in vivo in an adult mammal
• Window of opportunity after wounding to influence regeneration rather than scarring?
• Hair follicles grow independently of fat
• BMP plays a key role for regenerating hair follicles
Discussion

- Regenerating hair follicles could benefit all patients with disorders due to lack of fat
  - Scars
  - Lipodystrophies
  - Keloids
  - Ageing

Comments

• Very complex paper- majority of information provided in supplementary material only

• Major part of scar tissue is in dermis, not subcutaneously

• Does improvement of fat regeneration really improve scar quality?

• Significance in human model?
  • Less hair follicles in human skin than in mouse
Questions?
Thank you!
Journal Club SS17

Sonja Hager, Msc
Institute of Cancer Research
In situ activation of platelets with checkpoint inhibitors for post-surgical cancer immunotherapy

Chao Wang¹,², Wujin Sun¹,², Yanqi Ye¹,², Quanyin Hu¹,², Hunter N. Bomba¹ and Zhen Gu¹,²,³*
Introduction

• Surgery – the main treatment option for most solid tumors

• But: residual microtumors & circulating tumor cells (CTCs) → and may also induce promotion of cancer metastasis

• Immunotherapy may kill residual cancer cells
Immune checkpoint therapy – PD-L1

PD-L1 binds to PD-1 and inhibits T cell killing of tumor cell

Blocking PD-L1 or PD-1 allows T cell killing of tumor cell
Platelets

• No nucleus
• Derived from megakaryocytes
• Important in hemostasis
• Involved in thrombosis
• Modulated inflammation
• Platelet transfusion in thrombocytopenia
Emerging role of platelets in cancer

Platelets and the Hallmarks of Cancer

- Supporting cancer stem cells
- Metastasis and evading immune detection
- Inducing angiogenesis
- Resisting cell death
- Sustaining proliferative signals

Platelets at the interface of thrombosis, inflammation, and cancer
Aime T. Franco, Adam Corken, and Jerry Ware, Blood. 2015 Jul 30; 126(5): 582–588.
Prepublished online 2015 Jun 24. doi: 10.1182/blood-2014-08-531582
Platelets as drug carriers

- For increased efficacy
- Longer live span
- Migrate to the surgical wound
- Interact with circulating tumor cells (CTCs)
- Enhances immune response
- Upregulates PD-L1

Platelets: at the nexus of antimicrobial defence
doi:10.1038/nrmicro3269, Published online 16 May 2014
Aim of the study

• Targeting residual as well as circulating tumor cells by binding a PD-L1 antibody to the surface of platelets
Materials & methods

- Cell lines: mouse melanoma B16F10, mouse mammary carcinoma 4TI (both expressing luciferase and GFP)
- Mice: C57BL/6 and BALB/c (6-10 weeks old)
- Platelet preparation (P-aPDL1): Isolation from whole blood, conjugation of aPDL1 via a maleimide linker, Platelet activation with thrombin
- ELISA for antibody and cytokine detection (aPDL1, IL-1β, TNFα, IL-6, sCD40L)
- TEM
- Fluorescence microscopy and flow cytometry
- In vivo bioluminescence and imaging of tumors and antibody
- Tail bleeding assay
Materials & methods

- *In vivo* experiments

- I. Therapy model of incomplete resection
- II. Therapy model of incomplete resection and metastasis
- III. Therapy model of recurrent triple negative 4T1 tumor
Results – aPDL1 binding to platelets
Results – Efficiency, Stability, Surface proteins

(a) Conjugated aPDL1 (pg/Platelet cell) vs. Added aPDL1 (pg/Platelet cell)

(b) Cell viability at T=0h and T=24h

(c) Loading percentage over time

CD9, CD41, CD61, CD62P, CD40L (Platelet and P-aPDL1) histograms

Intensity

Counts
Results – Platelet activation I

![Images of platelet activation](images)

- **Calcein** and **aPDL1** images showing different activation states of platelets.
Platelet activation II

Figures:

b) Diagram showing the process of platelet activation.

P-aPDL1 → Activated P-aPDL1 → PMPs-aPDL1

Transwell images:

Unactivated P-aPDL1
Activated P-aPDL1

Graphs:

c) Graph showing released pPDL1 (%) over time.

d) Graph showing TGFα (pg/mL) over time.

e) Graph showing IL1 (pg/mL) over time.
Transwell experiment

a

Upper compartment
Platelets
Lower compartment
1.0 μm microporous membrane
Cancer cells

b

Unactivated P-aPDL1

Activated P-aPDL1
Pharmacokinetics

(a) Intensity at different wavelengths.

(b) Distribution of P-aPDL1 and free aPDL1 in different organs.

(c) Mean intensity of P-aPDL1 and free aPDL1 in various tissues.

Haemostatic effect

(b) Images showing the haemostatic effect of P-aPDL1.

(c) Bleeding time comparison between PBS, Platelets, and P-aPDL1.
I. *In vivo* therapy of incomplete resection
Local and distant inflammation and immune infiltration

6h post injection

tumor
14 days post surgery
14 days post surgery
II. Therapy model of incomplete resection and metastasis
<table>
<thead>
<tr>
<th>Day 8</th>
<th>Day 12</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="PBS Images" /></td>
<td><img src="image2" alt="PMP-aPDL1 Images" /></td>
<td><img src="image3" alt="aPDL1 (higher dose) Images" /></td>
</tr>
</tbody>
</table>

![Graph a](image4)  
![Graph b](image5)  
![Graph c](image6)

- **Graph a**: Tumor Size (mm$^3$) vs. Time (day)
- **Graph b**: Survival (%) vs. Time (Day)
- **Graph c**: Comparison of PBS, PMP-aPDL1 (1mg/kg), and aPDL1 (higher dose, 2mg/kg)
III. Therapy model of a recurrent triple negative 4T1 tumor
Conclusion

- Residual tumor cells after surgery were greatly reduced by platelets conjugated to aPDL1
  - Effective and stable conjugation of platelets to aPDL1
  - Activation of platelets and release of aPDL1 in surgical wound
  - Reduction of residual tumor cells as well as metastasis
Neuroprotective pentapeptide CN-105 is associated with reduced sterile inflammation and improved functional outcomes in a traumatic brain injury mouse model

Daniel T. Laskowitz et al.
Department of Neurobiology, Duke University School of Medicine, Durham, NC, USA

published on 21\textsuperscript{th} April 2017
in Nature Scientific Reports

Gerrit Lewik, 15.05.2017
Contents

• Background information
• Aim of the study
• Material and Methods
• Results
• Discussion
Apo E

Monocyte

Differentiation
↑ apo E production

Macrophage / foam cell

Sterol enrichment, TGFβ, LXRα, LXRβ

LPS
GM-CSF
IFNγ
TNFα
IL-1β

Peroxidation of lipids

Platelet aggregation

NO

Lymphocyte activation & proliferation

SMC proliferation

Remnant formation

HDL-E (γLpE, preβ-LpE, αLpE)

LRP1

Liver

Apo Al

Apo Al + cholesterol

Reverse cholesterol transport

HDL

SRB-1

Cholesterol clearance (bile)

SR-A

LDL → OxLDL

↑ apo E
Apo E

- Apolipoprotein E
  - Lipoprotein: transport of triglyceride acids and cholesterol in blood
- Polymorphism on Chr. 19
  - apoE2: reduced binding affinity to LDL-receptor, atherosclerosis,
  - apoE3: the „neutral“ Apo E genotype
  - apoE4: associated with atherosclerosis, alzheimers disease, cerebrovascular disease
- Function:
  - found to reduce glial activation and inflammatory cytokine release in vitro
  - extend to mouse model,
  - but: Apo E holoprotein does not cross the blood brain barrier and would not be suitable for peripheral administration
- CN-105:
  - smaller peptide
  - Designed modeling the polar receptor binding face of the helical apoE receptor binding region (Ac-VSRRR-amide)
Aim of the study

- to investigate the therapeutic potential of CN-105 in a murine model of closed head injury

Hypothesis: intravenous administration of CN-105 dampens neuroinflammatory responses and thus possibly improves the functional outcomes
Material and Methods

• Closed head injury model
  • 12-14 weeks old male mice
  • anesthesia induction, tracheal intubation and lungs were mechanically ventilated
  • secured in a stereotactic device on acrylic cast to allow 3mm of space below the head for acceleration/deceleration; no ear bars to avoid basilar skull fracture
  • metallic disc was adhered at the skull, immediately caudal to bregma
  • 2.0-mm diameter pneumatic impactor → single midline impact
  • sham mice were treated identically except for absence of impact

• Drug administration
  • Animals were placed in restrainer
  • A) single i.v. dose of 100μL drug was administered by tail vein
  • B) vehicle treated animals received 100μL of normal saline
Material and Methods

• **Immunohistochemistry**
  - to assess effect of CN-105 on inflammation, neuronal injury etc.
  - immunohistochemical staining performed using the F4/80 antibody and Fluoro-Jade B stain on days 10 and 1, respectively, after TBI
  - IHC was performed on separate cohorts of mice from those used in neurobehavioral tests
  - for histological assessment: secondary antibody, biotinylated goat anti-mouse IgG (1:3000), ABC, DAB (all from vector laboratories)

• **Cell quantification and image analysis**
  - A) F4/80 quantification:
    - brains of 5 TBI treated and 6 TBI vehicle treated mice were counted
    - sections of dorsal hippocampus was analyzed by stereoinvestigator software
    - immunopositive microglia identified with 20x objective and total number estimated by optical fractionator method
Material and Methods

- B) Fluoro-Jade B:
  - Brain sections of 6 TBI treated and 6 TBI vehicle treated mice were counted
  - dorsal hippocampus was examined for degenerating neurons using epifluorescence microscope

- Testing for functional deficits
  - Automated Rotarod to assess vestibulomotor function
  - one day before TBI: clinical training trial at accelerating rotational speed for 200 seconds and then three additional test trials (n=11-12 per group)
  - → average time to fall from cylinder was recorded as baseline latency
  - Mice were tested on consecutive days post-injury and received three consecutive daily trials with accelerating rotational speed
Material and Methods

• **Morris Water Maze** to assess spatial learning and memory
  • submerged platform placed in a pool with 105cm diameter, opaque water
  • Four trials /day for 4 consecutive days: days 28-31 after TBI (n=11-12 per group)
  • Mice were introduced to varying quadrants of the pool
  • Probe trial on day 4 (=last day) of the experiment: platform was removed and mice were allowed to swim freely for 60 sec → percent of time the mice spent in the platform quadrant was quantified

• **RNA extraction and RT-PCR**
  • brain tissue was processed for RNA extraction from a separate cohort of treated and untreated mice on day 1 post injury (CN-105, n=4; vehicle, n=3; control (sham-operated), n=3)
  • gene expression was measured using the **Mouse Inflammatory Response and Autoimmunity PCR Array** (profiles the expression of 84 inflammation and autoimmunity related genes)
Material and Methods

- **Gene expression data analysis:**
  - inflammatory and autoimmunity related genes (84 genes)

- **Pharmacokinetic analysis:**
  - to assess CNS penetration: quantitative whole body autoradiography analysis (QWBA) was performed and areas of interest in blood and brain were compared
Results

Assessment by Rotarod (CN-105: 2h post TBI)

Assessment by MWM (CN-105: 2h post TBI)

* $p < 0.05$ and ** $p < 0.01$
Results

Probe trial on day 4 of MWM experiment

Swim speed during whole MWM experiment

*C 35

%Time spent in platform quadrant

**

0 5

10

15

20

25

30

35

vehicle CN-105 (0.05mg/kg)

*p < 0.05 and ** p < 0.01

D 20

Speed (cm/sec)

0 2 4 6 8 10 12 14 16 18 20

vehicle CN-105 (0.05mg/kg)
Results

Assessment by Rotarod (CN-105: 4h post TBI)

Assessment by MWM (CN-105: 4h post TBI)

* $p < 0.05$ and ** $p < 0.01$
**Results**

Probe trial on day 4 of MWM experiment (CN-105: 4h post TBI)

Swim speed during whole MWM experiment (CN-105: 4h post TBI)

* $p < 0.05$ and ** $p < 0.01$
Results

Comparison of activated F4/80 immunostained microglia in hippocampus (10 days post TBI)

A, C, E, G → treated by vehicle
B, D, F, H → treated by CN-105

C+D: CA3 region (cornu ammonis mit Pyramidenzellen)

E+F: polymorphic region (with marked microglia cells)

G+H: periventricular region (corpus callosum and fimbria)
Results

Comparison of activated F4/80 immunostained microglia in hippocampus (10 days post TBI)

→ significant reduction in microgliosis

***p = 0.0002
Results

Fluoro-Jade B stained brain slices 24h post-TBI: degenerating neurons in dorsal dentate gyrus

vehicle treated

CN-105 treated

***p <0.0001

→ significant reduction of neuron degeneration
Results

Assessment of gene expression
(24h post sham or TBI injury)

→ using a pathway array specific for inflammatory and immune responses (84 genes)

4 cohorts:
• Sham + vehicle (S-v)
• Sham + CN-105 (S-CN-105)
• TBI + vehicle (TBI-v)
• TBI + CN-105 (TBI-CN-105)

→ 57 genes were upregulated,
12 were downregulated, 10 were unchanged

* mRNA expression was not detectable
** Relative values could not be calculated because one of the components was not detectable
Results

Assessment of gene expression
(24h post TBI with CN-105)

→ inflammatory gene expression of CN-105 relative to vehicle treated group

* mRNA expression was not detectable
Results

CN-105 ameliorates changes in inflammatory gene expression (24h post TBI)

→ upregulated/downregulated expression of genes relative to sham vehicle
Results

TLR signalling is downregulated by CN-105
(24h post TBI)
Results

Pharmacokinetic studies of CN-105 demonstrate CNS bioavailability

Percent of radioactivity contributed to blood in the brain

concentration of C14-radioactivity in plasma and CNS following intravenous dose of radiolabeled CN-105 peptide
Results

CN-105 is associated with:

• A) improved behavioral function
  • by Rotarod (better vestibulomotor performance)
  • by MWM (better preserved spatial learning memory)
  • no significant motor differences
  • pretreatment of mice 30min prior to TBI had no additional effects („data not shown“)

• B) reduced microgliosis and neuronal injury following TBI
  • esp. in CA3 und polymorphic region of hippocampus
  • degenerating neurons are significantly reduced

• C) changes in inflammatory gene expression patterns following TBI
  • 57 of 84 inflammatory genes were upregulated in TBI mice (esp. TLR-signalling pathway)
  • TBI-CN-105 lead to reduced inflammatory gene upregulation compared to TBI-v

• D) penetration into the CNS compartment
  • progressive increase of radioactivity in brain as compared to blood (3,6% at 5 minutes, 170% at 24h)
• ApoE and ApoE-mimetic peptides decrease neuroinflammatory responses and secondary cell death
  → Already shown in several animal studies of acute brain injury
• mRNA levels of inflammatory cytokines and chemokines return to pre-injury levels by 24 hours or more?
• Difficulty to characterize endogenous microglia
  • Primitive macrophage entering embryonic brain vs. hematogenous macrophage that are recruited into brain following to injury
  • But number and activation status is decreased after CN-105 treatment
• Not all indicators are decreased by CN-105 treatment at 24 hours post injury
  • TNF alpha increases
Discussion

• MyD88 has recently shown to be significantly increased after TBI in several mouse model experiments

• Mechanisms by which CN-105 (ApoE) modulate inflammatory response is completely undefined
  • Via specific receptor interaction
  • Connection to LRP-1 receptor? NMDA-receptor via PSD-95?

• Gene expression suggests key role of BCL6 in inflammation process
  • Early changes of NF-kB, chemokines and cytokines
  • Central key repressor in TLR signalling pathway?

• CN-105 may directly effect the blood brain barrier (tight junctions)
Discussion

• Administered up to 4 hours following injury
  → Temporal window should be further extended

• Limitations of study:
  • ApoE polymorphism → modulate receptor binding via allosteric effects
  • Differential gene assay only focused on expression of inflammatory markers

• Rodent models:
  • Brains have reduced ration of white: grey matter
    → Not ideal to model the diffuse axonal injury
  • Early changes of NF-κB, chemokines and cytokines
  • Central key repressor in TLR signalling pathway?

• CN-105 may directly effect the blood brain barrier (tight junctions)

→ promising therapeutic strategy in treatment of acute brain injury
Thank you for your attention!
Any questions?
Dysbiosis and zonulin upregulation alter gut epithelial and vascular barriers in patients with ankylosing spondylitis

Annals of Rheumatoc Diseases, 06 2017

Irina Gessl JC Translational Immunology
BACKGROUND
ankylosing spondylitis (AS)

- =Bechterew's disease
- Chronic inflammatory disease
- Prevalence: 0.1- 2 %
- Men > women
- Seronegative spondylarthritis
- Mainly affects spine, sacroiliacal joints
- Peripheral joints, eyes, bowel involvement
- Genetic (HLA-B27) and environmental factors
ankylosing spondylitis (AS)

ankylos = stiffening, spondylos = vertebra
zonulin

Dysbiosis

Tight junction proteins
claudin 1
claudin 4
occludin
zonula occludens 1
AS and the gut

- Dysbiosis
- Subclinical gut inflammation
- Cause or consequence?
Study aim

- tissue localization of bacteria in the gut of patients with AS
- gut-epithelial barrier and gut vascular barrier (GVB) integrity.
- Role of zonulin
  - modulating intestinal permeability
  - Monocyte activation
- Associations with systemic inflammation?
METHODS
patients

- Ileal biopsies
  - 50 patients with AS
  - 20 healthy controls
- Interleukin (IL)-8 in the tissue
- Histologically divided:
  - normal
  - Acute inflammation
  - Chronic inflammation
- RT-PCR
- Lactulose/ mannitol ratio test for gut permeability
bacteria

- Ileal biopsies from AS patients and controls
- Cultures for aerobic and facultative anaerobic bacteria
- Bacteria isolated from 5 AS patients
- Incubated with Caco-2 epithelial cells
- Modulation of zonulin mRNA assessed by RT-PCR
Sera

- Levels of lipopolysaccharide (LPS), LPS- binding protein (BP), intestinal fatty acid-BP (iFACBP) and zonulin proteins
- Analysed in sera of all AS patients and controls
- In vitro effects of recombinant human zonulin
  - on human umbilical vein endothelial cells (HUVECs)
  - On peripheral monocytes
Human leukocyte antigen (HLA)-B27 TG rats

- 5 HLA-B27 TG rats, 5 WT
- Ileal samples
RESULTS
Intestinal gut inflammation in AS

• IL-8 overexpressed in AS with chronic inflammation

• 50 AS patients
  • N=20 no gut inflammation
  • N=11 acute gut inflammation
  • N=19 chronic gut inflammation
Intestinal bacteria

- Adherent and invading bacteria present in AS (35/50), not in controls
RT-PCR ileal samples

Downregulation of tight junction proteins in AS
Zonulin in ileal samples

(G) Elevated Zonulin levels in AS chronic (RT-PCR); (H) AS, (I) control
Zonulin in ileal samples

(K) The number of zonulin positive cells was significantly and directly correlated with the number of IL-8 positive cells.
Caco-2 cells incubated with AS- bacteria
Gut vascular barrier (GVB) proteins in ileal samples

Reduced relative m-RNA levels of VE-cadherin (A), junctional adhesion molecule (JAM)-1 (B) and PV1 (C) were assessed by RT-PCR in AS.
Serum levels of zonulin were evaluated in 20 patients with AS and 20 controls (C) and correlated with LA/MA ratio (D).
In vitro effects of zonulin on human umbilical vein endothelial cells (HUVECs)

Downregulation of occludin (A) and VE-cadherin after zonulin treatment
In vitro effects of zonulin on peripheral monocytes

zonulin: CD163 binding motif

percentage of CD163+c-MAF+ cells increased after incubation with AS PBMCs = M2 polarised macrophages
Elevated serum levels of lipopolysaccharide (LPS) (A), LPS-binding protein (BP) (B) and intestinal fatty acid-BP (iFABP) (C) in AS.
AS and CD14+

CD14= on monocytes/macrophages, involved in LPS binding

High LPS downregulates CD14

Percentages of CD14+ cells is reduced in peripheral blood mononuclear cells (PBMCs) from patients with AS.
AS and HLADR+

Percentages of CD14+ HLADR+ cells is reduced in peripheral blood mononuclear cells (PBMCs) from patients with AS.
AS and CD14+

Effects of monocyte stimulation with LPS alone, sCD14 alone or sCD14+LPS on CD14+ and CD14- monocytes
HLA-B27 rats ileal samples

- Increased IL-23 expression
- Occludin downregulation
- Presence of adherent bacteria

→ Antibiotic treatment
Discussion

• Adherent and invading bacteria in ileum of AS patients associated with alteration of epithelial barrier and the GVB

• Zonulin-dependent leaky epithelium and endothelium in AS ileum

• → translocation of zonulin and bacterial products into the bloodstream

• → inducing modulation of innate immune system in AS

• E. coli, Prevotella spp.
My opinion

Pros:

• Assessed several aspects- descriptive, functional
• Human samples

Cons:

• Tipping errors, incorrect legends
• Bacterial score?

• Link bacteria- zonulin?
• CD14+HLADR+/ LPS??
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
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**

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 or 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

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 \textit{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. \textbf{Exosomes} were isolated from concentrated CdM using an ExoQuick-TC Exosome Precipitation Solution
7. \textbf{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

green:CD31 positive cells

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

Matrigel plug contained CdM MSC 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<sup>MSC</sup> after adding into HUVECs culture for 48 hours

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

(*P < 0.05 vs CdM<sup>MSC</sup>).
<sup>*</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<sup>MSC</sup> 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
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\textsuperscript{HUVEC-HUVEC} was very low (black bars), CdM\textsuperscript{MSC-MSC} was very high (white bars), and CdM\textsuperscript{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

GW4869...an exosome release inhibitor

Supernatant

Cell lysate

→ The expression 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

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).

Control treated with BSA

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

Tube-like structure formation of HUVECs

In vivo Matrigel plug assay

Control treated with BSA (same protein amount)

→ 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<sup>miR-30b</sup> and Exo<sup>miR-30b</sup> was increased
- Tube length was increased in HUVECs treated with Exo<sup>miR-30b</sup>

- Expression of miR-30b in MSC<sup>anit-miR-30b</sup> and Exo<sup>anit-miR-30b</sup> was reduced
- Tube length was reduced in HUVECs treated with Exo<sup>anit-miR-30b</sup>

⇒ 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

- 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

---

**Table:**

<table>
<thead>
<tr>
<th>miRNA</th>
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</tr>
</tbody>
</table>

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**Gong et al., Oncotarget. 2017 Apr 1.**
Discussion
• Conditioned medium of MSCs significantly increased tube-like structure formation, spheroid-based sprouting and neo-angiogenesis in Matrigel plug

• Exosomes derived from MSCs:
  \(\rightarrow\) mediated the transfer of miRs from MSCs to HUVECs
  \(\rightarrow\) promoted angiogenesis

• Gain-and-loss function of miRs in exosomes:
  \(\rightarrow\) pro-angiogenic effect is dependent on their 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 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!
CRISPR-Cpf1 correction of muscular dystrophy mutations in human cardiomyocytes and mice

Yu Zhang, Chengzu Long, Hui Li, John R. McAnally, Kedryn K. Baskin, John M. Shelton, Rhonda Bassel-Duby, Eric N. Olson

Science Advances, April 2017
Introduction

• Duchenne muscular dystrophy (DMD) is an X-linked recessive disease caused by mutations in the gene coding for dystrophin

• dystrophin: large cytoskeletal protein essential for the integrity of muscle cell membranes

• DMD causes progressive muscle weakness, premature death by the age of 30 (cardiomyopathy)

• no effective treatment for this disease

• treatments: delivery of truncated dystrophin, utrophin by recombinant adeno-associated virus (AAV), skipping of mutant exons with antisense oligonucleotides and small molecules
Introduction

• these approaches cannot correct DMD mutations / permanently restore dystrophin expression

• new genome editing method as therapy?: **CRISPR-Cas9 system** (clustered regularly interspaced short palindromic repeats) for the correction of diverse genetic defects

  • adaptive immune system in bacteria that defends against phage infection

  • endonuclease (Cas9) guided to specific genomic sequences by guide RNA (gRNA) resulting in DNA cutting near a protospacer adjacent motif (PAM)

  • large size of SpCas9 reduces efficiency of packaging and delivery in AAV vectors

  • smaller CRISPR enzymes would facilitate precision gene editing

  • new RNA-guided endonuclease Cpf1 (CRISPR from *Prevotella* and *Francisella* 1)

https://www.addgene.org/crispr/guide/
Methods

- generation of pLbCpf1-2A-GFP and pAsCpf1-2A-GFP plasmids: LbCpf1 (*Lachnospiraceae bacterium ND2006*) and AsCpf1 (*Acidaminococcus sp. BV3L6*)

- human iPSCs from DMD patient fibroblasts (Riken HPS0164, deletion of exons 48 to 50, introducing a STOP codon within exon 51): nucleofection with plasmids, GFP+ and GFP- cells were sorted, single clones from GFP+ cells → sequencing, iPSCs induced to differentiate into cardiomyocytes (chemical induction)

- genomic DNA isolation (mouse fibroblasts, human DMD iPSCs)

- reverse transcription PCR (primers flanking DMD exons 47 and 52; amplified from WT cardiomyocytes, uncorrected cardiomyocytes and exon 51-skipped cardiomyocytes)

- dystrophin Western blot analysis

- dystrophin immunocytochemistry and immunohistochemistry
Methods

• mitochondrial DNA (mtDNA) copy number quantification (qPCR) → functional analysis of DMD iPSC-derived cardiomyocytes

• cellular respiration rates: oxygen consumption rates (OCR) determined in human iPSC-derived cardiomyocytes using XF24 Extracellular Flux Analyzer (Seahorse Bioscience)

• in vitro transcription of LbCpf1 mRNA and gRNA

• single-stranded oligodeoxynucleotide: ssODN used as a HDR template (template for correction of mutations)

• CRISPR-Cpf1-mediated genome editing by one-cell embryo injection in mdx mice

• PCR amplification of genomic DNA

• T7E1 assay (mismatch-specific T7 endonuclease I assay)

• Tse I RFLP analysis (restriction fragment length polymorphism analysis)

• mouse forelimb grip strength test, serum CK measurement
Results – Figure 1

Correction of DMD mutations by Cpf1-mediated genome editing

A

Reframing Cpf1 Exon skipping

47 48 49 50 51 52 53

B

Splice donor Exon GT (T/C)_n AG Splice acceptor Exon

Cpf1 PAM: TTTTN

C

Target-DMD-Ex51

5’.. tatatttagCTCTACTCAGACTGTACTCTGGTG..3’

3’.. ataaactcGAGGATGAGTCTGACAATGAGACC..5’

Exon 51

PAM

E

293T cells Riken51 iPSCs (del 48–50)

LbCpf1 AsCpf1 LbCpf1 AsCpf1

GFP: - + - + - +

M bp

-600 400 200
Results – Figure 2

DMD iPSC-derived cardiomyocytes express dystrophin after Cpf1-mediated genome editing by reframing

A

B

C

Katrin Zlabinger
JC/TS Current Topics in Applied Immunology and Tissue Regeneration
SS2017
Results – Figure 2

DMD iPSC-derived cardiomyocytes express dystrophin after Cpf1-mediated genome editing by reframing

DMD iPSC-derived cardiomyocytes express dystrophin after Cpf1-mediated genome editing by reframing.
Results – Figure 3

DMD iPSC-derived cardiomyocytes express dystrophin after Cpf1-mediated exon skipping.

F

Exon 47

Uncorrected

Exon 51

Ile, Ala, Thr, Arg, Leu, Leu, Leu, Leu, Trp, Stop

Exon 51 skipped

Ile, Ala, Thr, Arg, Leu, Leu, Glu, Glu, Arg

G

RT-PCR

WT CMs

Uncorrected CMs

LbCpf1-edited CMs g1+g2 (mixture)

bp

M

WT

Uncorrected

g1+g2 (mixture)

Dystrophin

αMHC
Results – Figure 4

A. Diagram of the Dmd gene with exons 22 to 25 marked.

B. gRNAs g1, g2, and g3 are shown with g1 and g2 overlapping.

C. Gel electrophoresis showing the effect of LbCpf1 and AsCpf1 with markers (M) and bands at different distances.

D. Sequence analysis showing the target site on Dmd with PAM and gRNA sequences.
Results – Figure 5

CRISPR-LbCpf1–mediated *Dmd* correction in *mdx* mice

**A**
Donor mutant

**B**
_HDR-mediated correction

**C**

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<th>Litter 3</th>
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<td>HDR (%)</td>
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In-frame deletion of premature stop codon
CRISPR-LbCpf1–mediated Dmd correction in mdx mice

D

WT

G

T

C

T

G

A

A

A

A

G

C

A

A

A

G

C

A

A

A

T

G

C

T

C

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C

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G

Ser

Ser

Leu

Lys

Glu

Gln

Gln

Asn

Gly

Phe

Asn

Tyr

Leu

mdx

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G

Ser

Ser

Leu

Lys

Glu

Gln

Gln

Asn

Gly

Phe

Asn

Tyr

Leu

E

WT

mdx

mdx-C

TA

H&E

GFP

F

WT

mdx

mdx-C

TA

H&E

GFP

Dystrophin
Results – Supplement 6

Immunohistochemistry of skeletal muscles, heart, and brain from WT, \textit{mdx}, and LbCpf1-edited mice (\textit{mdx-C})
Results – Supplement 7

H&E staining of skeletal muscles, heart, and brain from WT, *mdx*, and LbCpf1-edited mice (*mdx-C*)
Western blot analysis of skeletal muscles, heart, and brain from WT, *mdx*, and LbCpf1-edited mice (*mdx*-C)
Table 1. Serum CK measurement and forelimb grip strength of WT, *mdx*, and *LbCpf1*-corrected *mdx*-C mice. M, male; F, female.

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<tr>
<th>Mouse no.</th>
<th>Percent correction by HDR</th>
<th>Sex</th>
<th>Weight (g)</th>
<th>CK (U/liter)</th>
<th>Forelimb grip strength (grams of force)</th>
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Discussion

• newly discovered CRISPR-Cpf1 nuclease can efficiently correct DMD mutations in patient-derived iPSCs and *mdx* mice, allowing for restauration of dystrophin expression

• robustness and efficiency of Cpf1 in mouse genome editing → HDR-mediated correction, ORF of mouse *Dmd* gene was completely restored → fibrosis, inflammatory infiltration rescued

• two different strategies: reframing (only one gRNA needed) and exon skipping

• but differences in dystrophin expression level, mtDNA quantity, OCR in different edited clones → reframed dystrophin may not be structurally or functionally identical to WT dystrophin
Discussion

• use of one or two gRNA?
  • two are more effective for disruption of splice acceptor site
  • removes deleterious “AG” nucleotides → pseudosplice acceptor site generation eliminated
  • both gRNAs should cleave simultaneously which may not occur
  • one gRNA → uncertainty of length of INDELs?
• unique T-rich PAM sequence (G-rich PAM sequence in SpCas9)
• LbCpf1 is 140 AA smaller → enhances packaging and delivery by AAV
• LbCpf1 and AsCpf1 had high genome-wide targeting efficiency and high specificity comparable to those of SpCas9
• new powerful approach to permanently eliminate genetic mutations and rescue abnormalities associated with DMD and other disorders
Pro-Cons

• interesting new approach to eliminate genetic mutations, safe therapy? possible risks?

• iPSCs from just one patient? why not generating more iPSC clones from different patients?

• out of 24 pups born only 5 carried corrected alleles with different correction rates → just 8 to 50%, no 100% correction? and 50% only in one mouse

• for me personally difficult to read because of genetic engineering science vocabulary
Thank you for your attention!