FUJIFILM
Value from Innovation

Wako

TIM4-affinity methods targeting phosphatidylserine for isolation or detection of extracellular vesicles (PS-affinity methods)

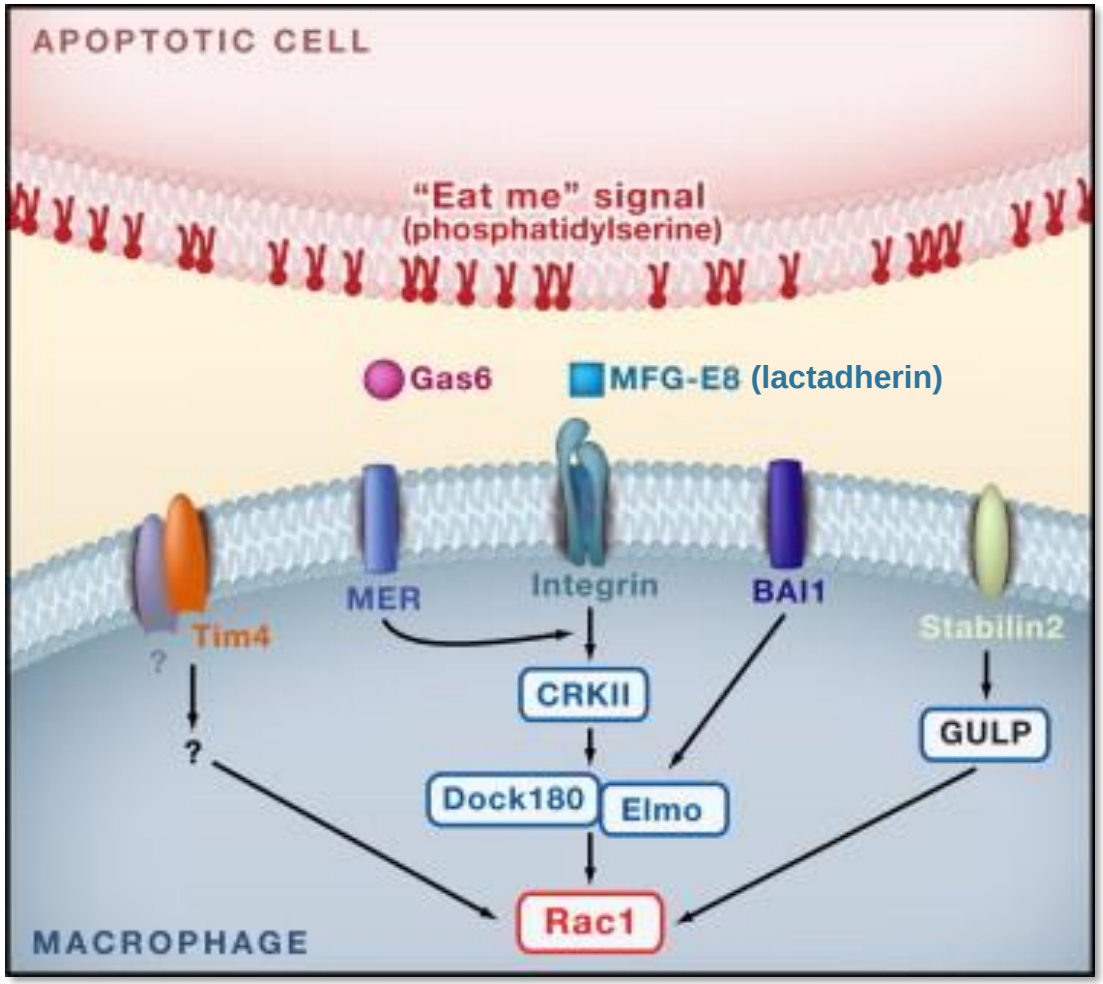
Rikinari HANAYAMA

WPI Nano Life Science Institute
KANAZAWA UNIVERSTIY

NanoLSI
WPI KANAZAWA UNIVERSITY

ISEV2022
Platinum Sponsored Session
Fujifilm-Wako Seminar

Identification of phosphatidylserine receptors

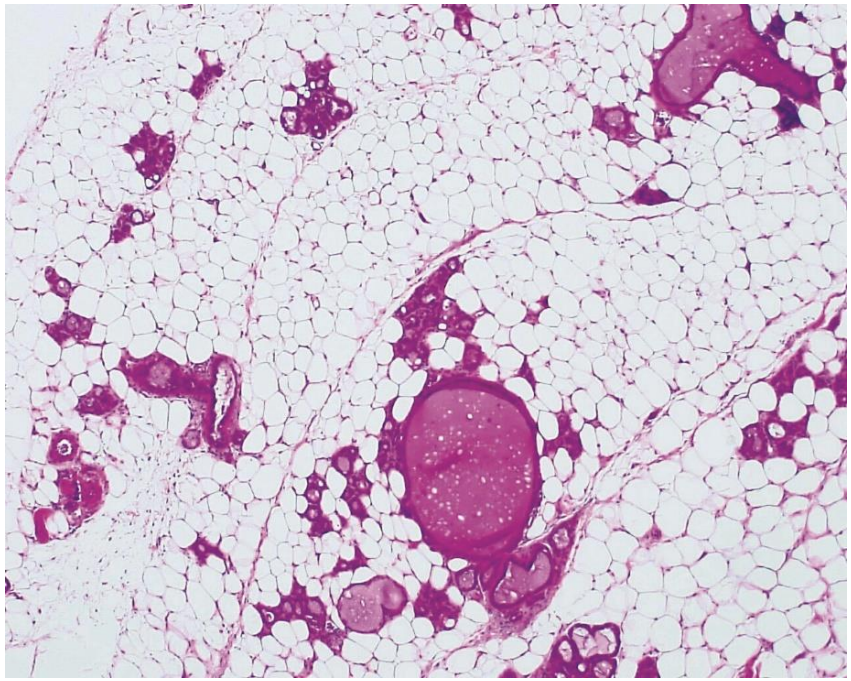


Nagata S, Hanayama R et al., Cell. (2010)
Miyanishi et al., Nature (2007)
Hanayama et al., Science (2004)
Hanayama et al., Nature (2002)

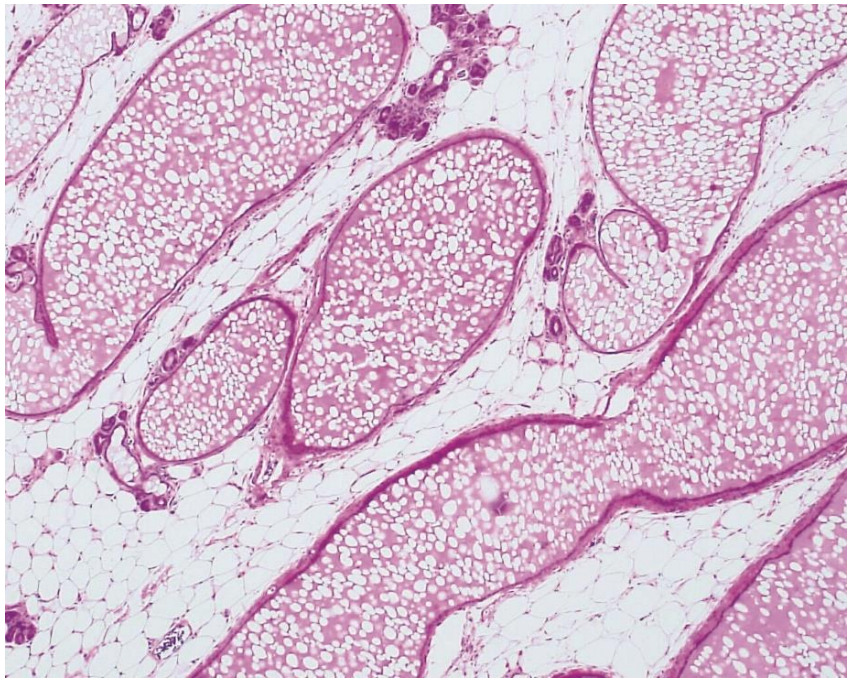
Milk fat globules are cleared by MFG-E8

Upon weaning (lactation ends),
the developed mammary gland undergo involution

WT



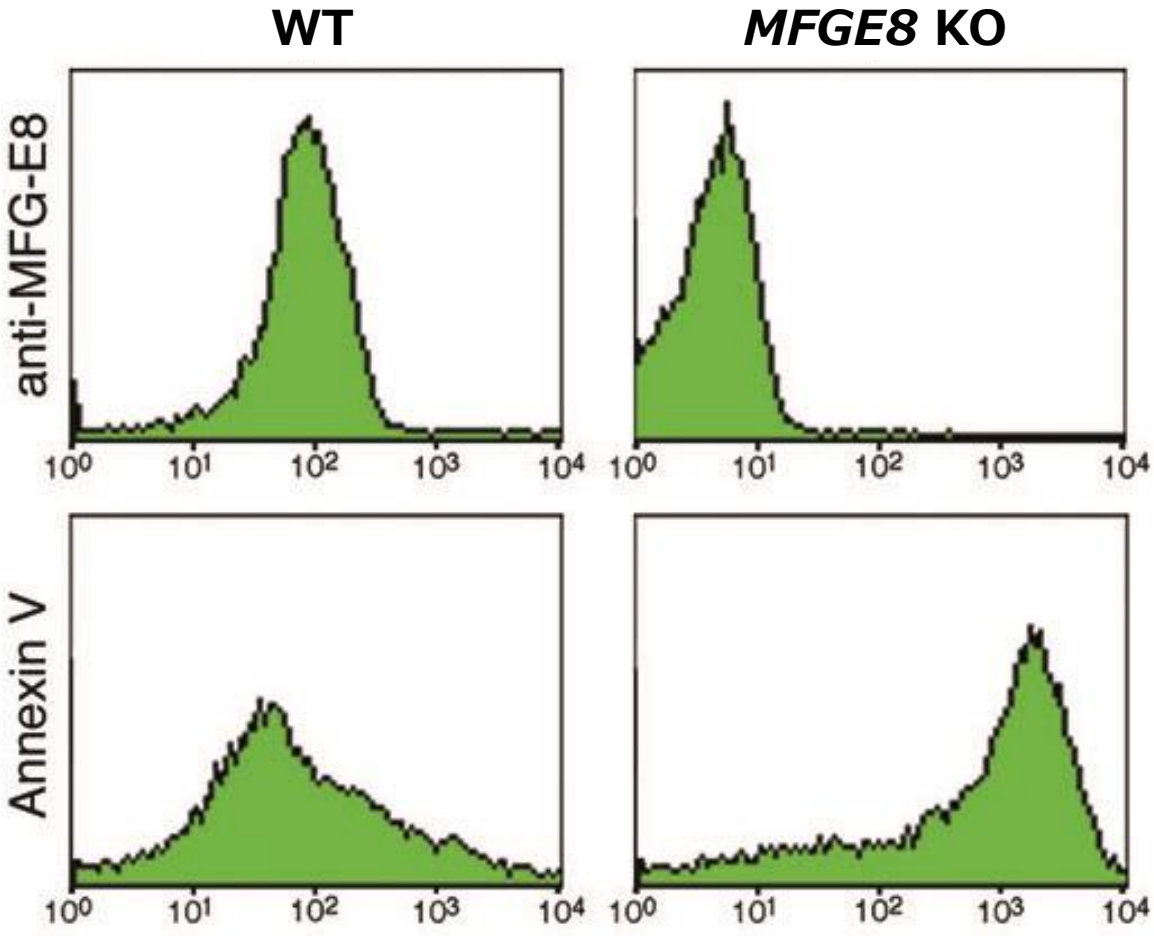
***MFGE8* KO**



Hanayama et al.,
PNAS (2005)

Milk fat globules expose phosphatidylserine (PS)

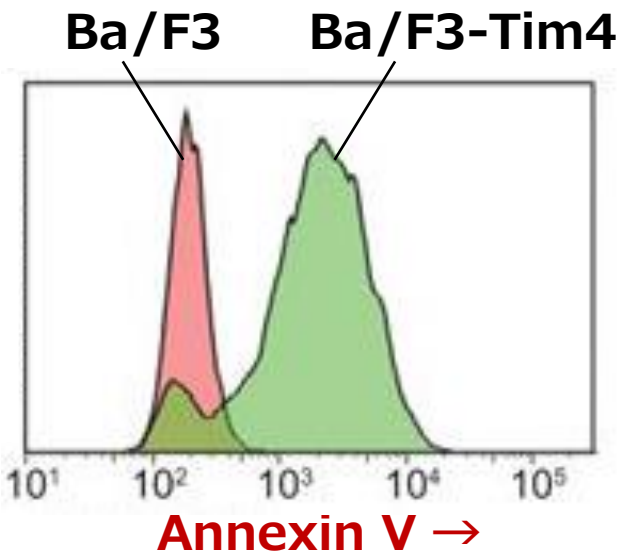
Flow cytometric analysis of single vesicles



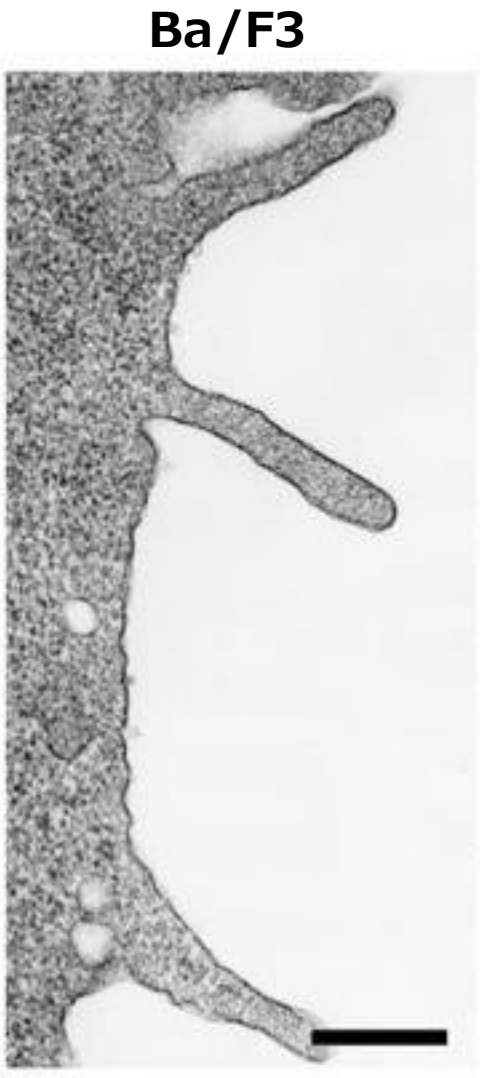
Masking of phosphatidylserine by MFG-E8

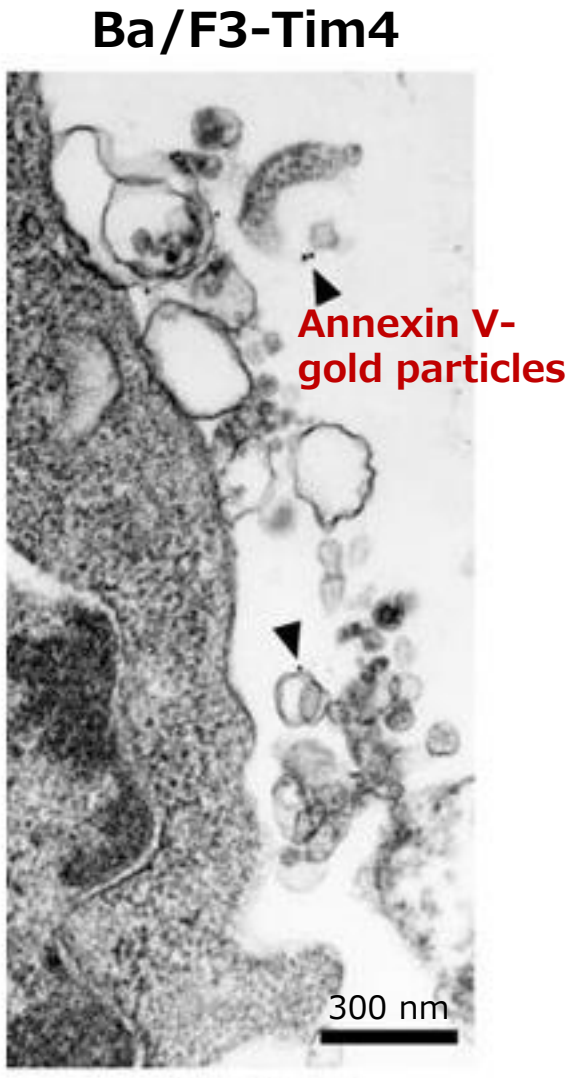
Hanayama et al., PNAS (2005)

Tim4-associated EVs expose phosphatidylserine (PS)

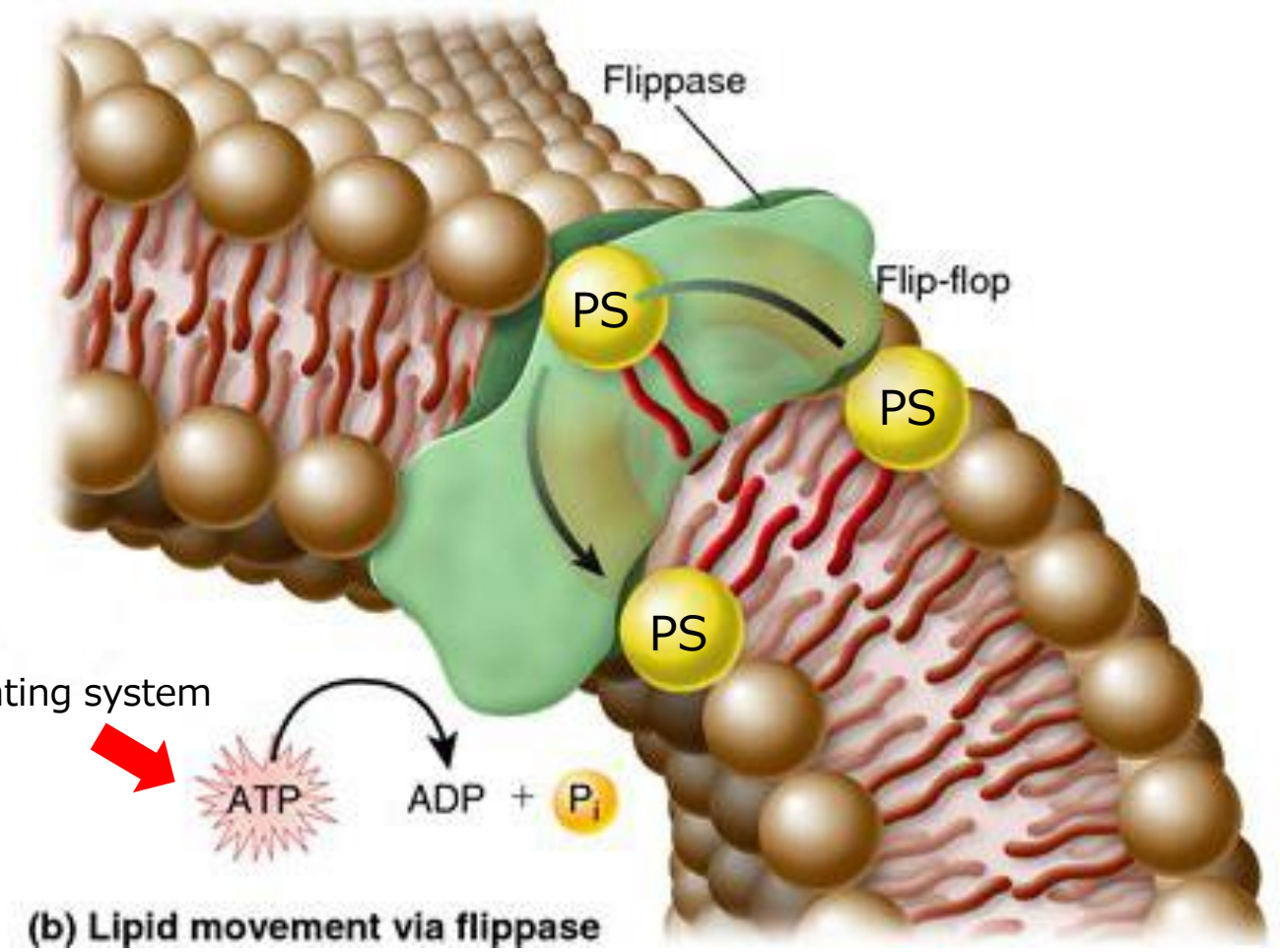


Miyanishi et al., Nature (2007)





Exhaustion of ATP might be cause of PS exposure



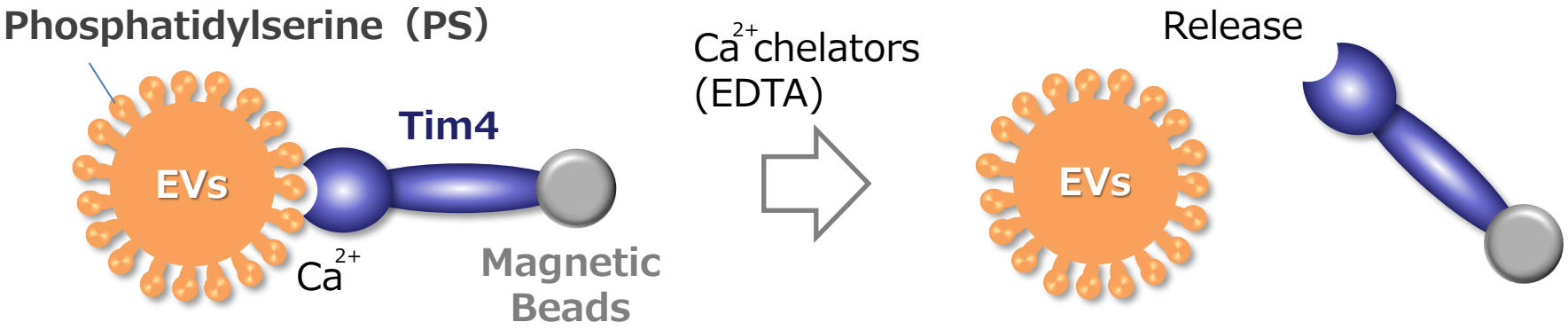
Type IV P-type ATPase

Segawa et al., Science (2014)

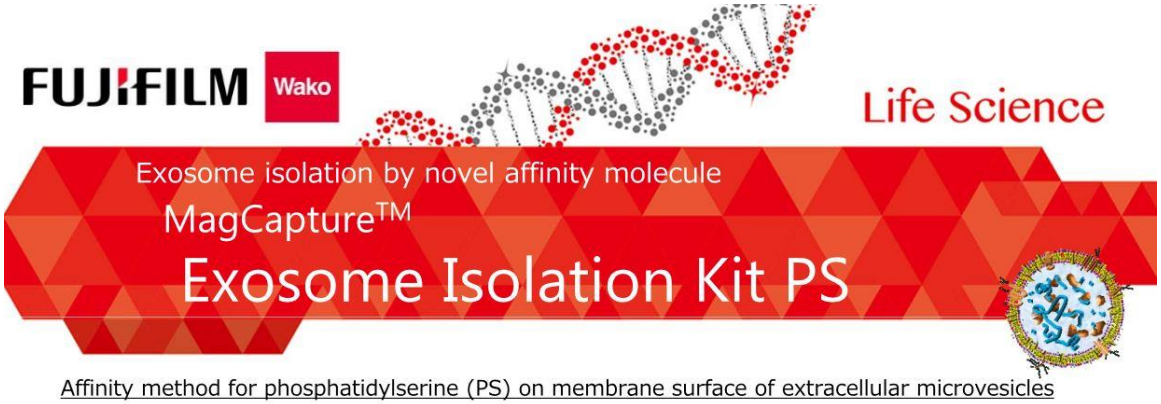
From Biology Forum Gallery

A high purity isolation method of EVs using Tim4

A novel affinity-based EV isolation method

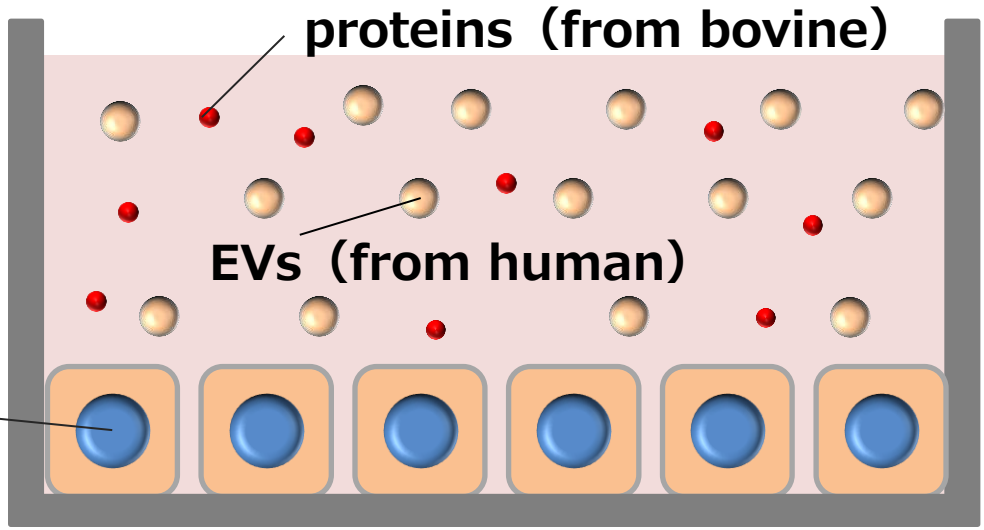


Nakai & Hanayama, Scientific Reports (2015)



Isolation of small EVs from human cells

Cultured in medium containing **5% FBS (EV-depleted by UC and PEG prep)**



The conditioned medium

↓300 x *g*, 10min
↓2,000 x *g*, 20min
↓10,000 x *g*, 30min
↓**10K supernatants**, 0.22um filtration

sEVs

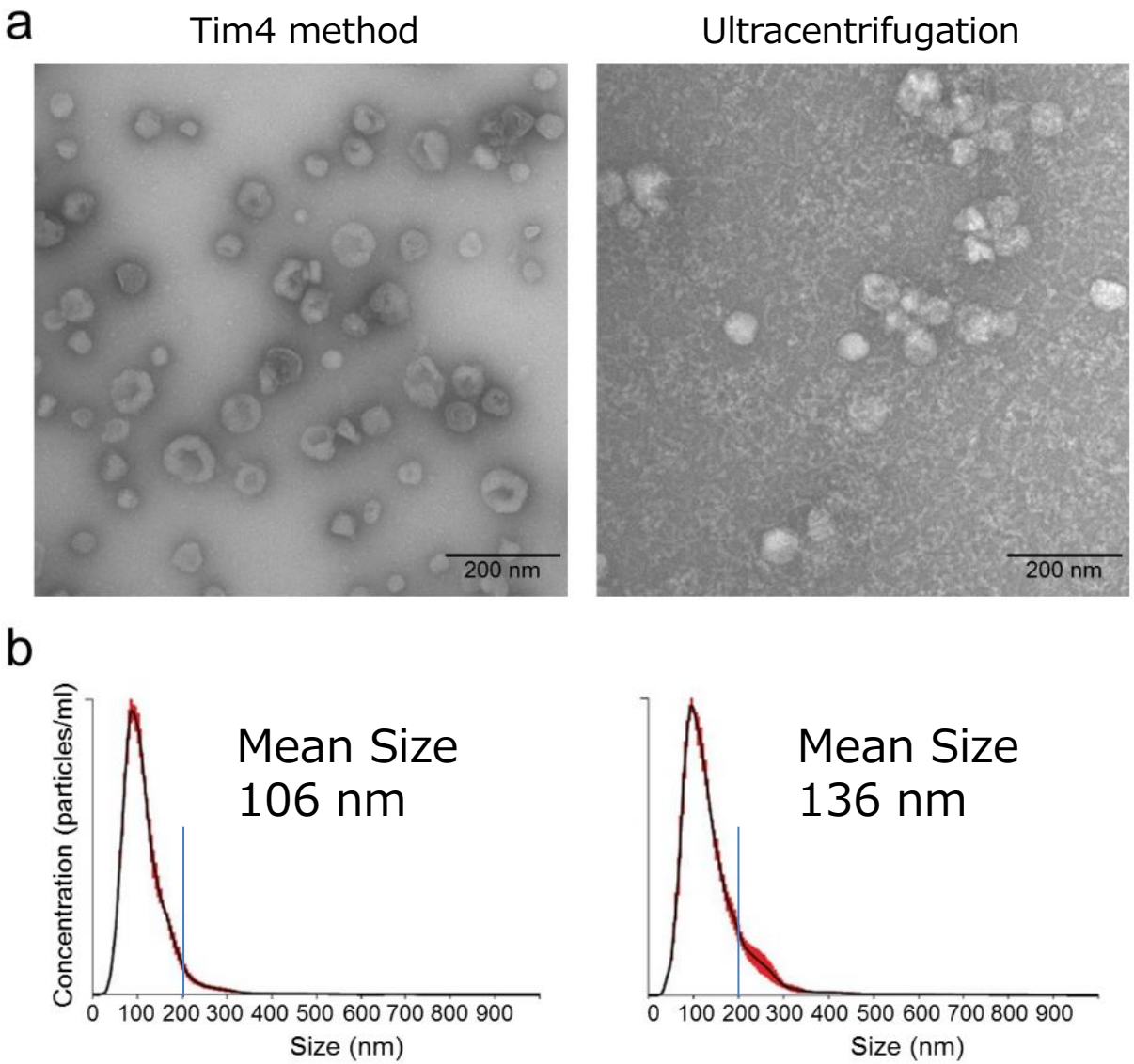
①Tim4 method

MagCapture
Exosome Isolation Kit PS

②Ultracentrifugation

↓100,000 x *g*, 120min
↓PBS wash
↓100,000 x *g*, 120min

TEM and NTA analyses of isolated sEVs

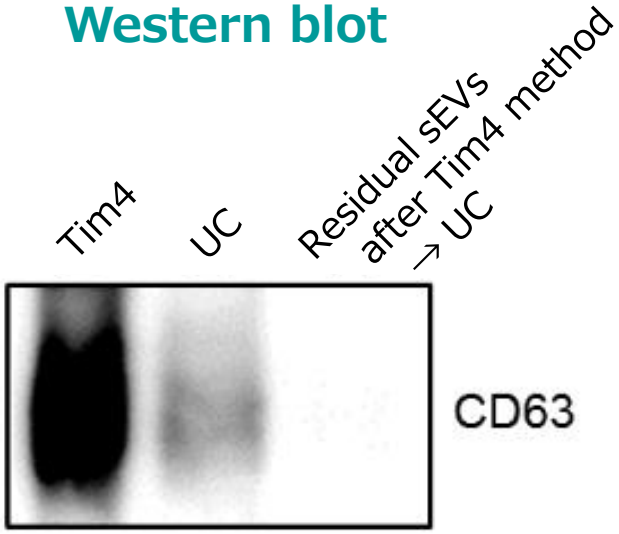


Comparison of the purity of sEVs

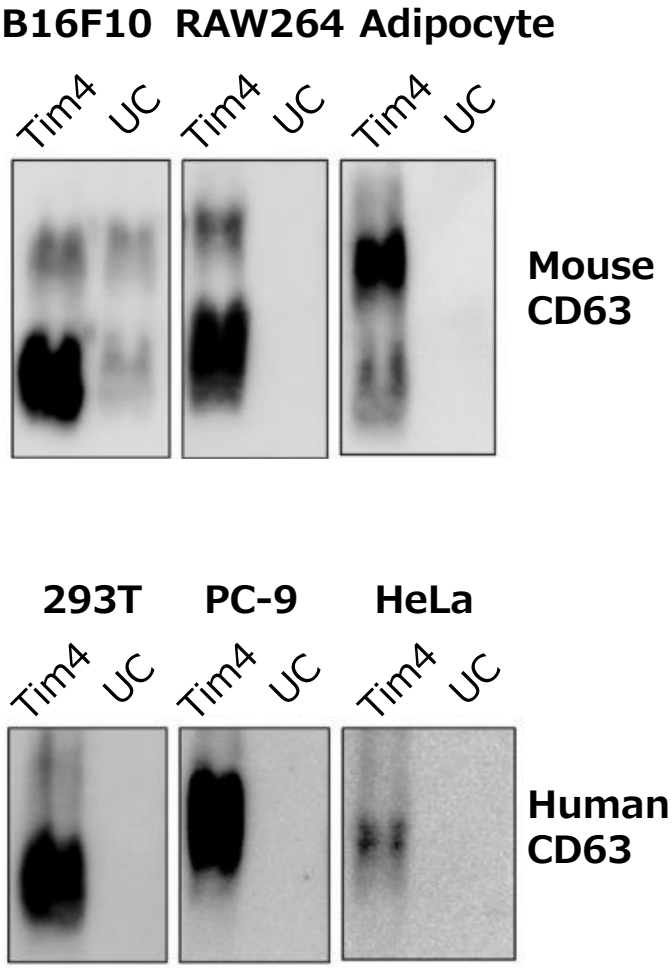
sEVs from K562

(100 ng/lane)

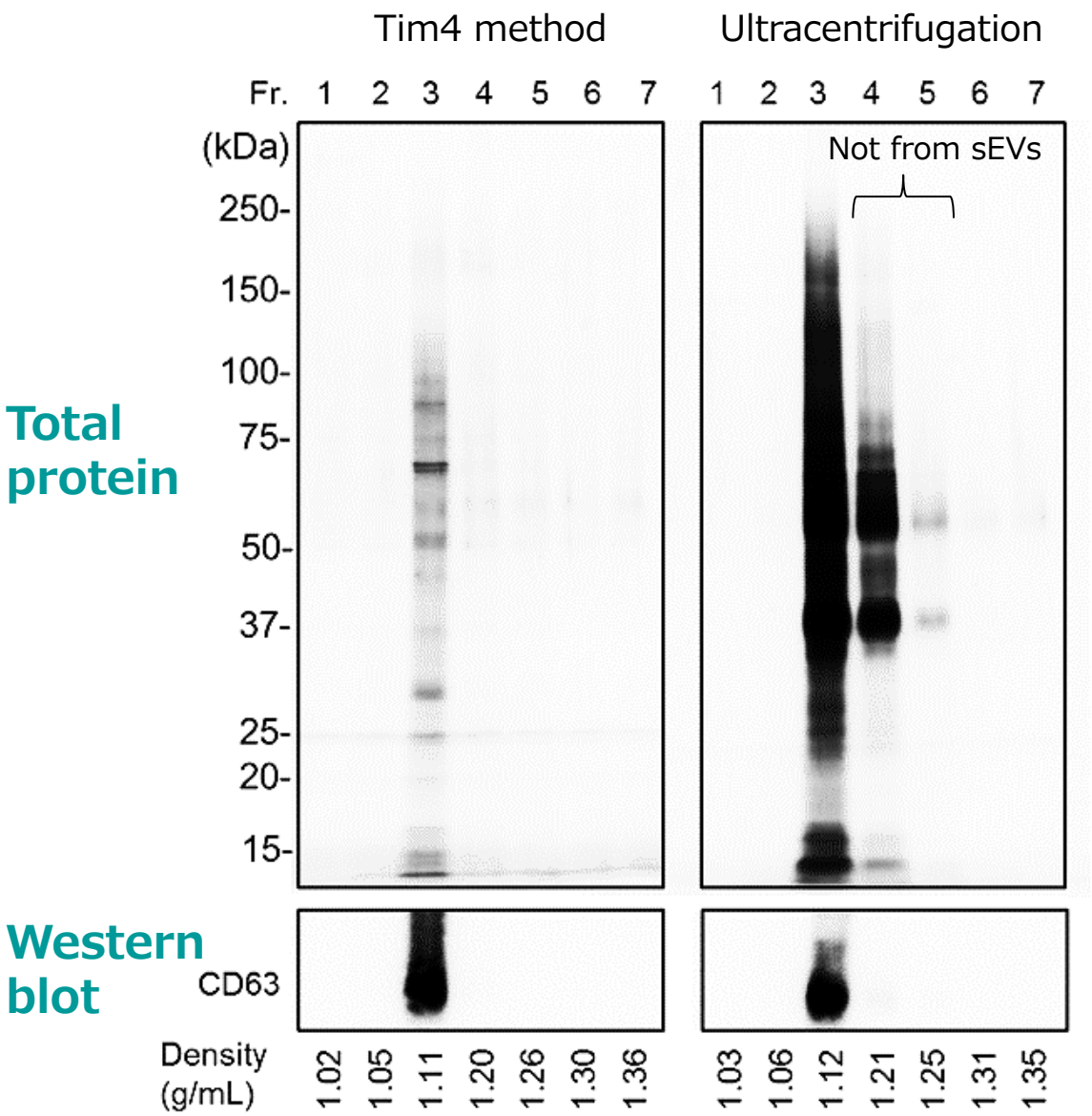
Western blot



sEVs from various cell types



Density gradient fractionation



Top 15 proteins identified in the isolated sEVs

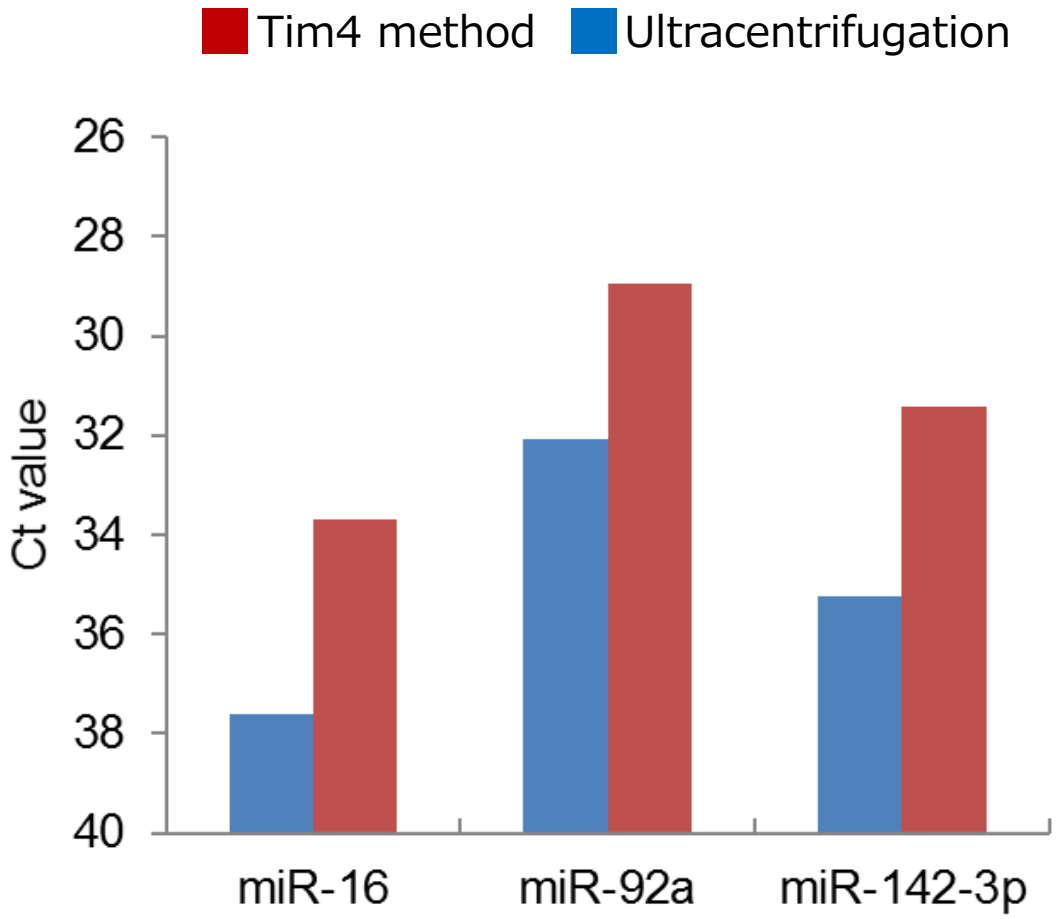
White columns: human proteins (from sEVs)
Gray columns: bovine proteins (from FBS)

	Tim4 method	Ultracentrifugation
1	Heat shock cognate 71 kDa protein	**DNA-PK catalytic subunit**
2	**Annexin A6**	Transferrin receptor protein 1
3	Transferrin receptor protein 1	Serum albumin
4	V-type proton ATPase subunit A	**ATP-dependent RNA helicase A**
5	**Flotillin-2**	Tubulin beta-5 chain
6	Programmed cell death 6	Heat shock cognate 71 kDa protein
7	4F2 cell-surface antigen heavy chain	Fatty acid synthase
8	**Annexin A1**	4F2 cell-surface antigen heavy chain
9	Kinase D-interacting substrate	**U5 small nuclear RNP helicase**
10	**Annexin A2**	Tubulin beta-4B chain
11	**Flotillin-1**	**Ribonucleoprotein M**
12	V-type proton ATPase subunit B	Hemoglobin fetal subunit beta
13	**Annexin A11**	Clathrin heavy chain 1
14	**Annexin A7**	Fibronectin
15	**Syntenin-1**	Tubulin alpha-1B chain

Known sEV markers **Nuclear proteins**

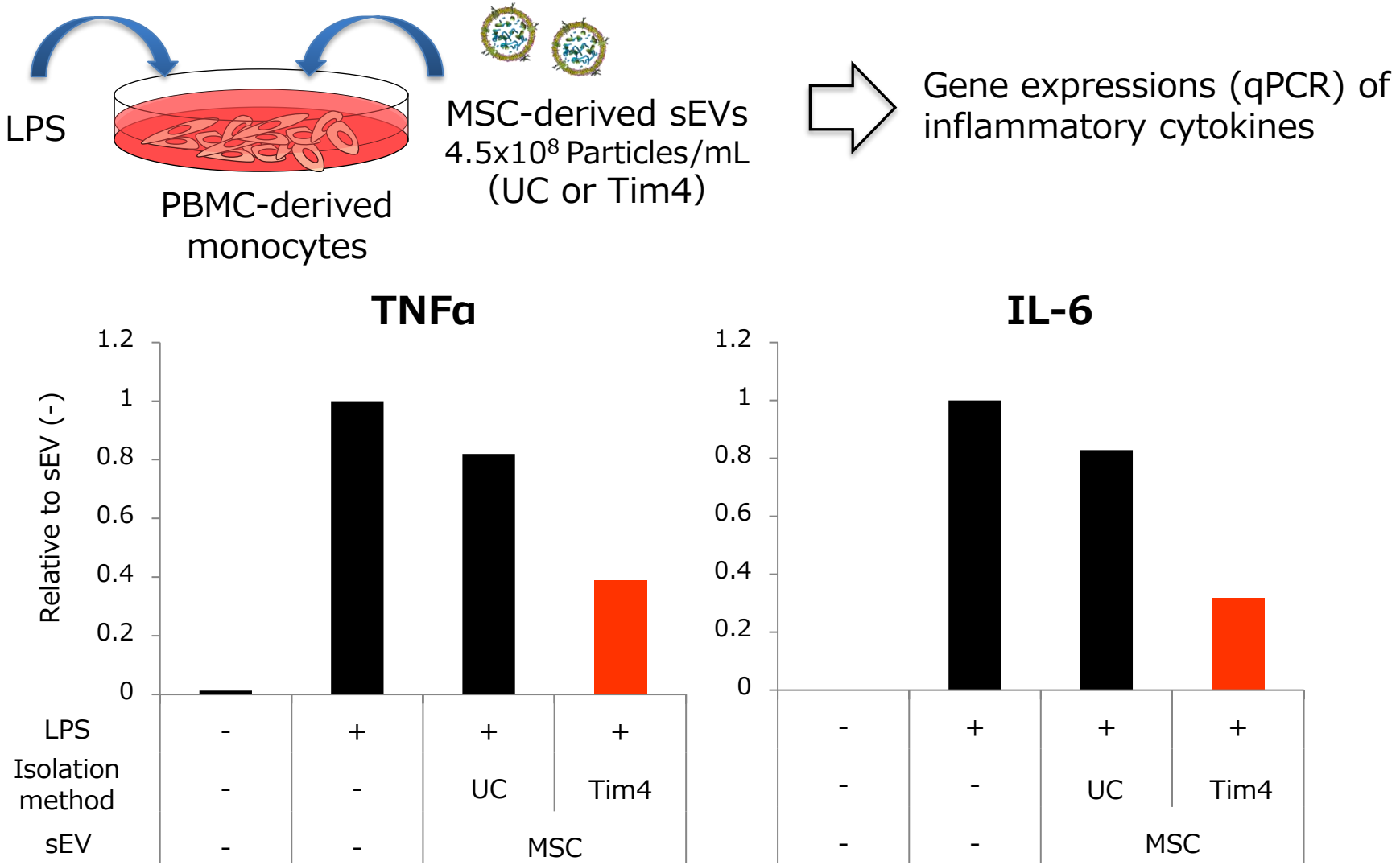
Comparison of enrichment of sEV microRNAs

qPCR analyses in the isolated sEV fractions

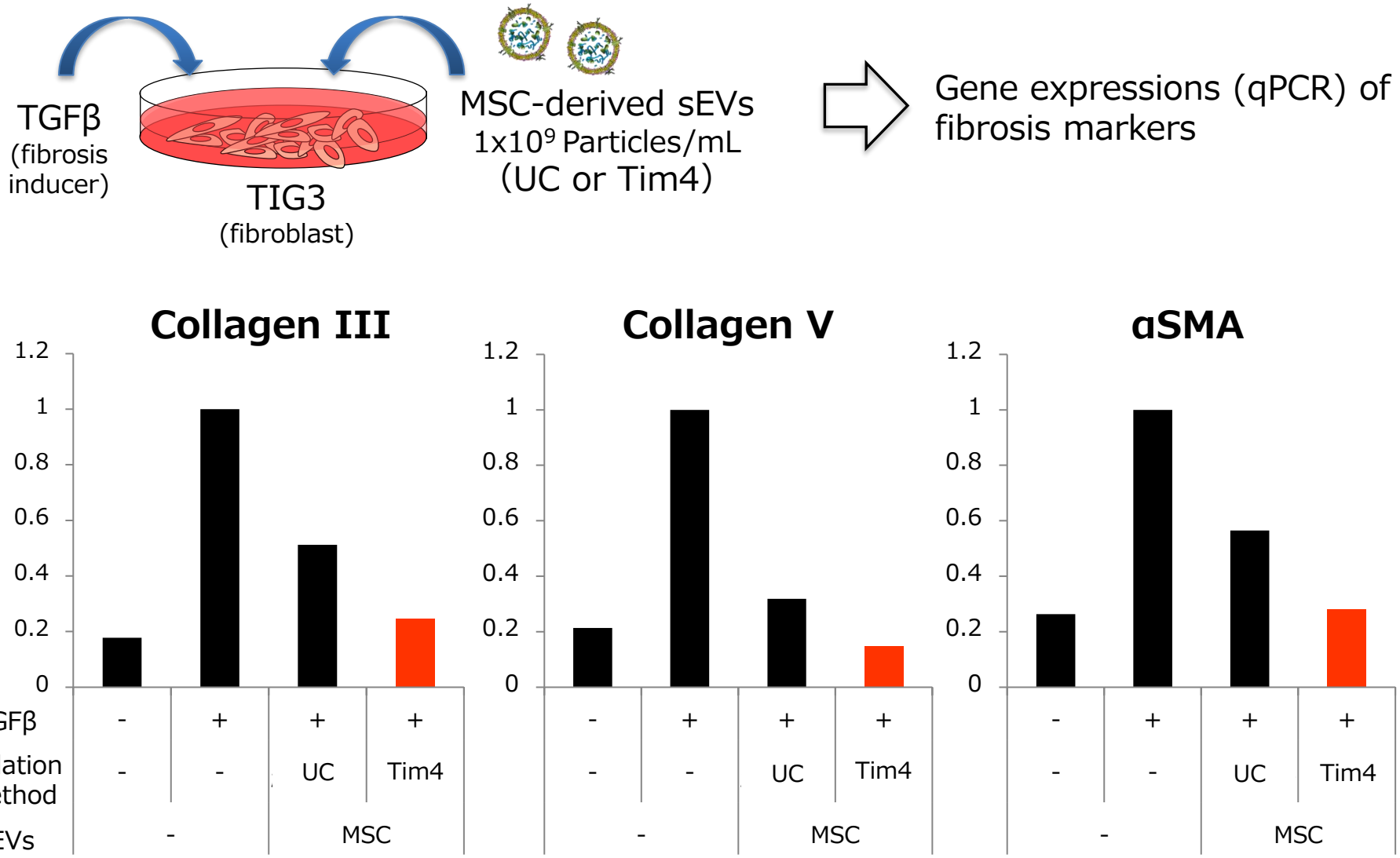


10 times more sEV RNAs were isolated by Tim4 method

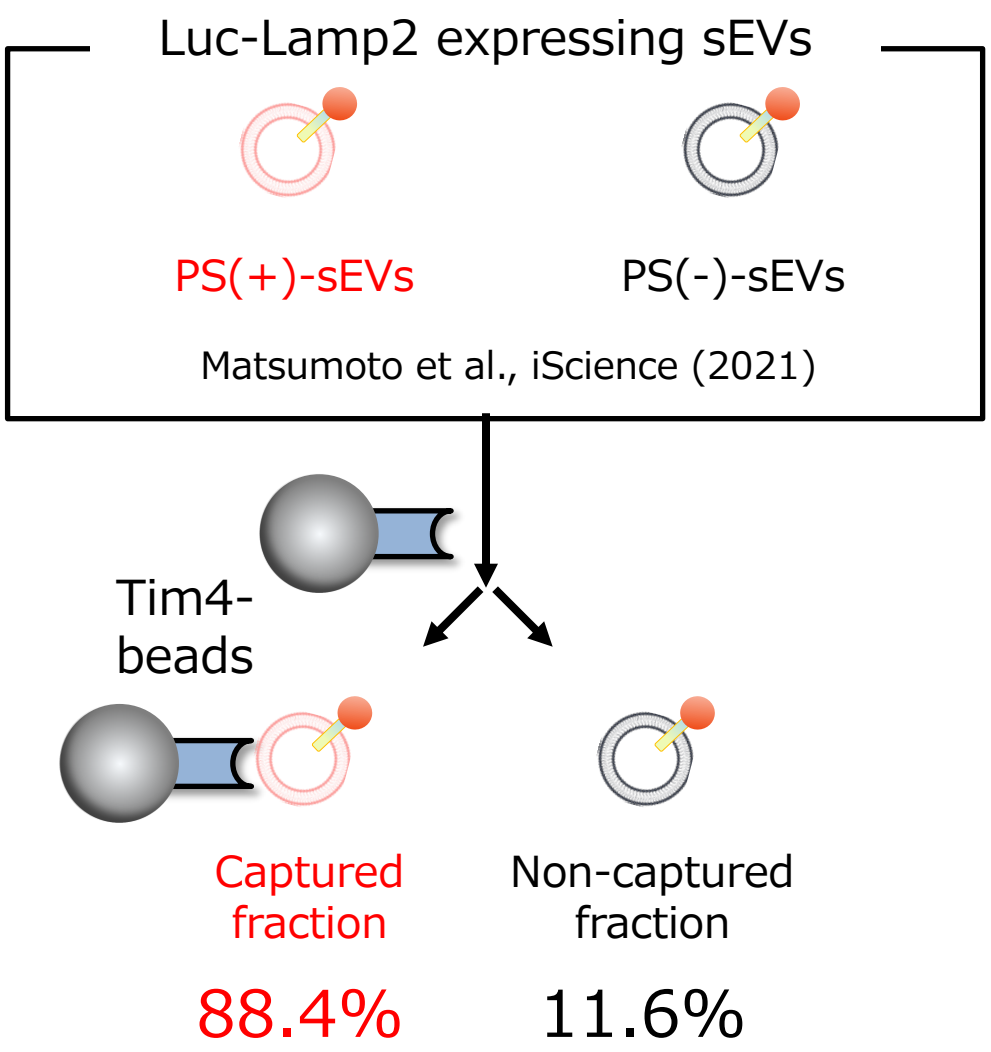
Comparison of anti-inflammatory activity of sEVs



Comparison of anti-fibrotic activity of sEVs

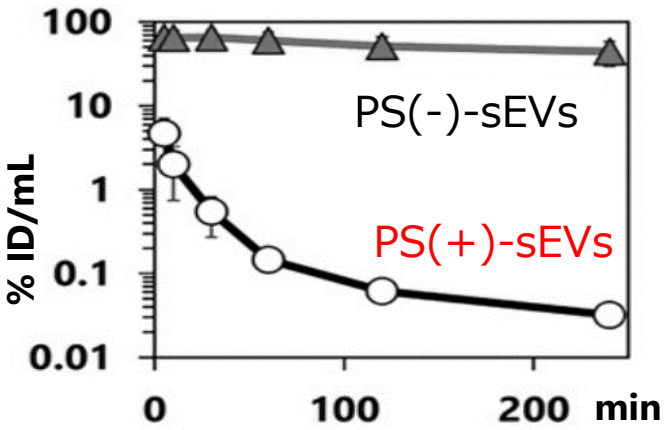


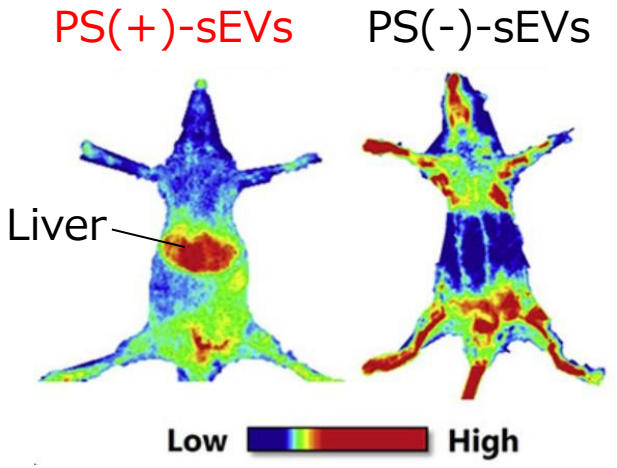
What % of sEVs expose PS & can be isolated by Tim4?



Mass balance (% of Luc recovered rate)

Clearance in serum after iv injection

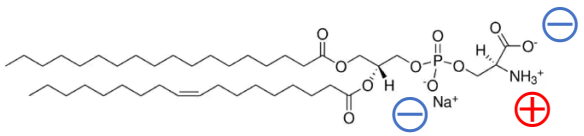




For EV-based DDS

Which subpopulations could be enriched?

Phosphatidylserine



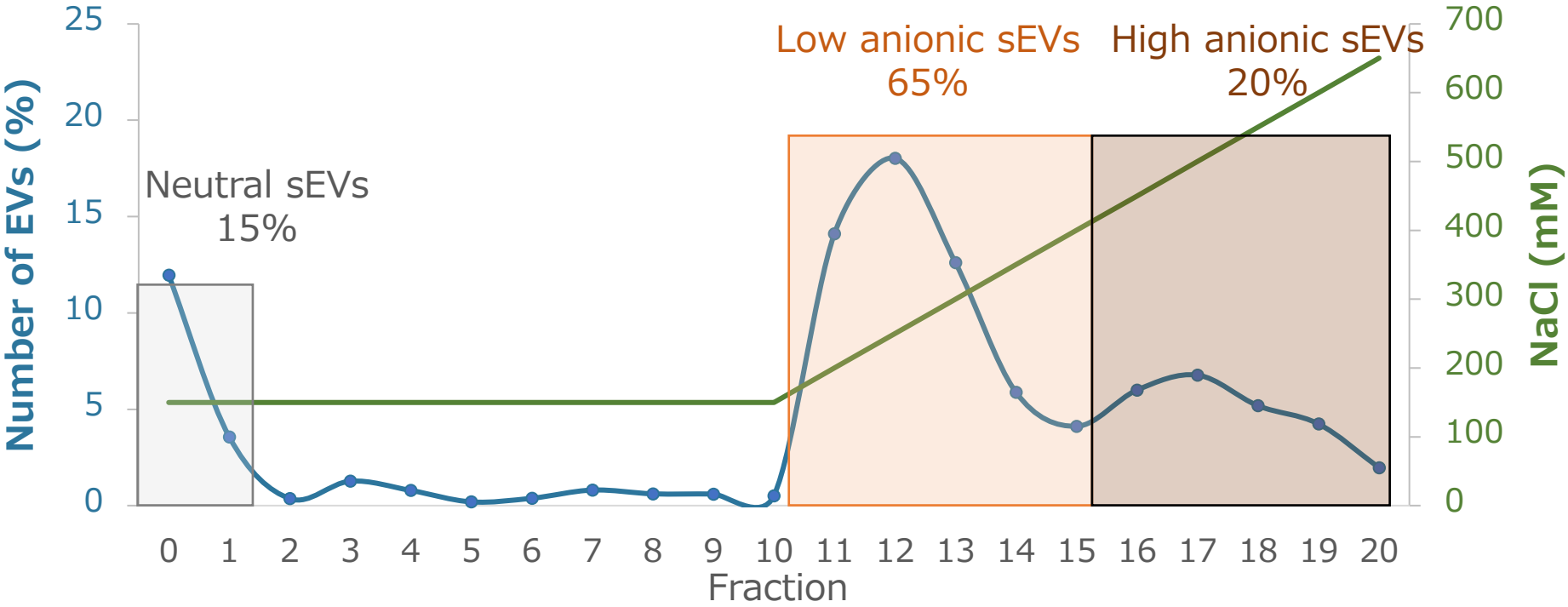
Bulk sEVs (isolated by UC)

Anion exchange column
(DEAE-Sepharose)

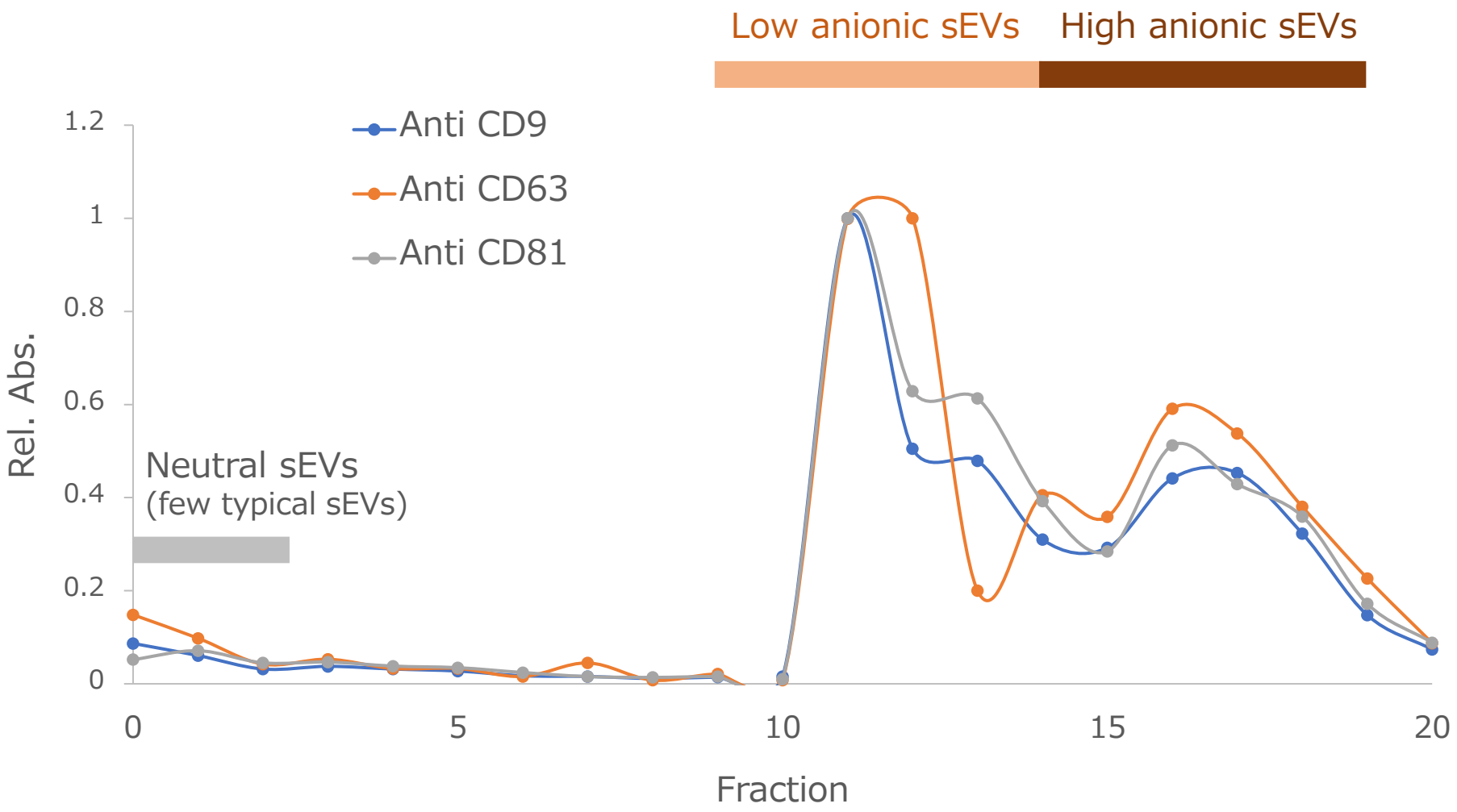
High anionic sEVs

Low anionic sEVs

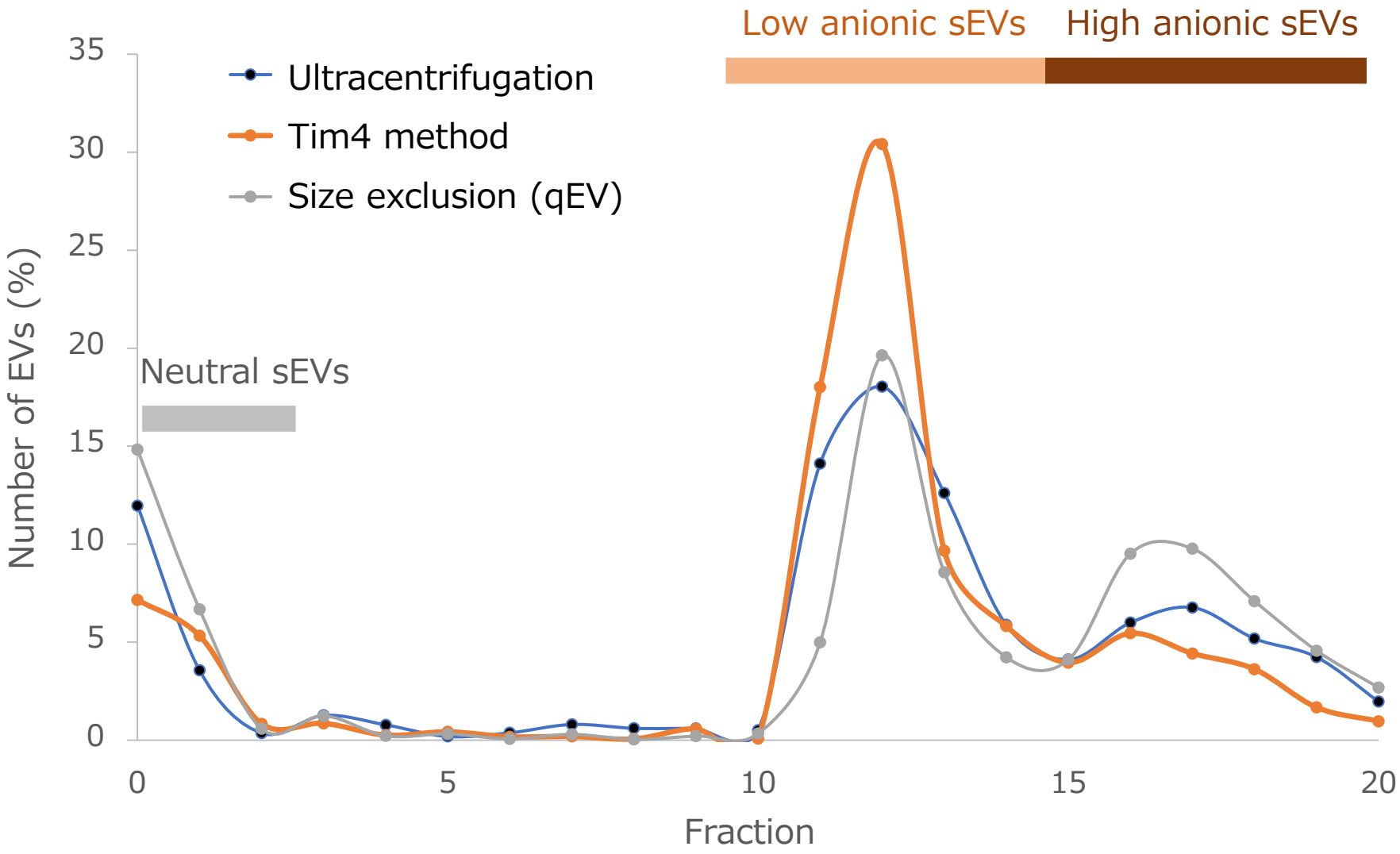
Neutral sEVs



Anti-tetraspanin Abs binding to each fraction

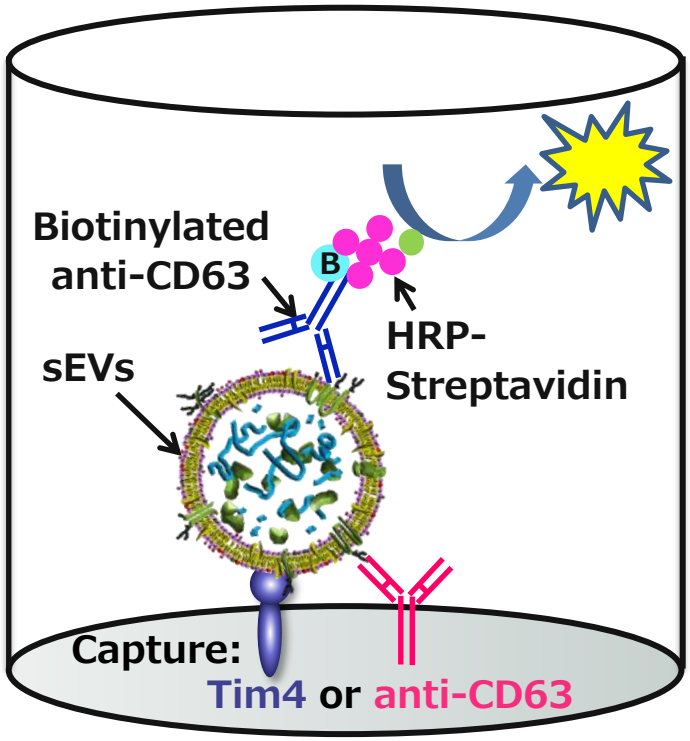


Comparison of the number of sEV subpopulations



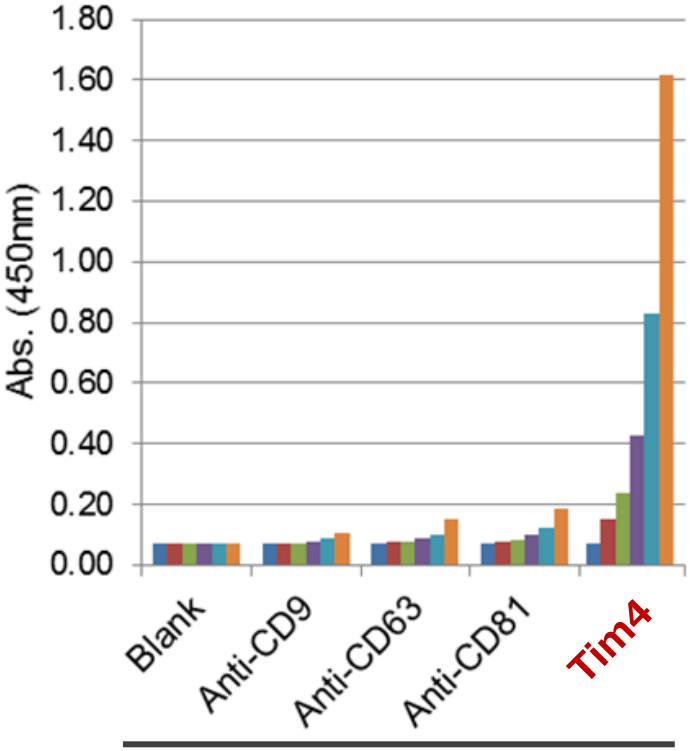
Sensitive quantification of EVs by ELISA using Tim4

Sensitive quantification of EVs by Tim4-ELISA



1000 times more sensitive

(limit: 16ng vs 11pg)



Proteins coated on a microtiter plate

Concentrations of sEVs ◺ 0 ← 2μg/ml (1/2 dilution)

FUJIFILM Wako

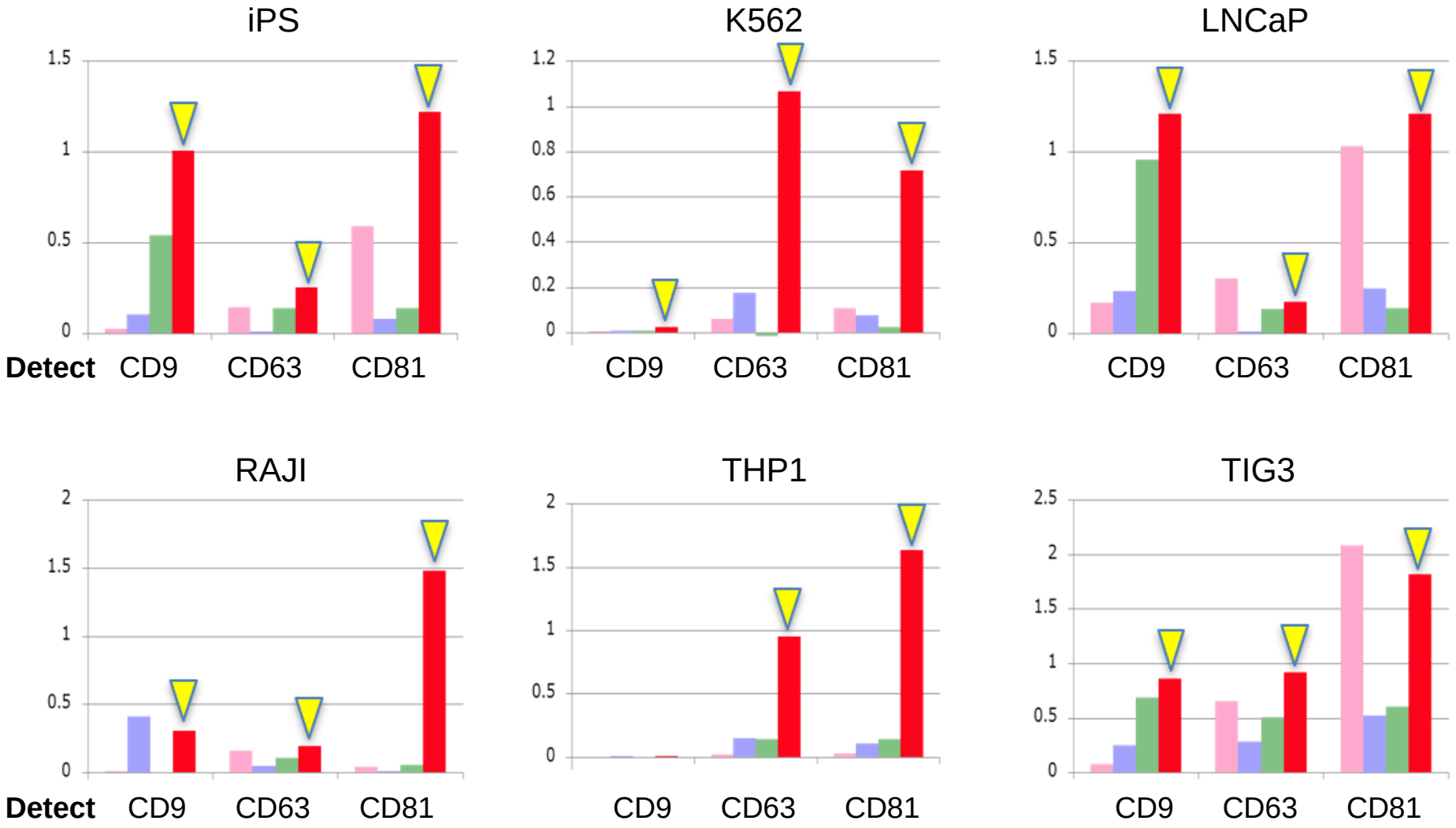
A novel tool for qualitative and quantitative analysis of extracellular vesicles in sample of cell culture supernatant

PS Capture™

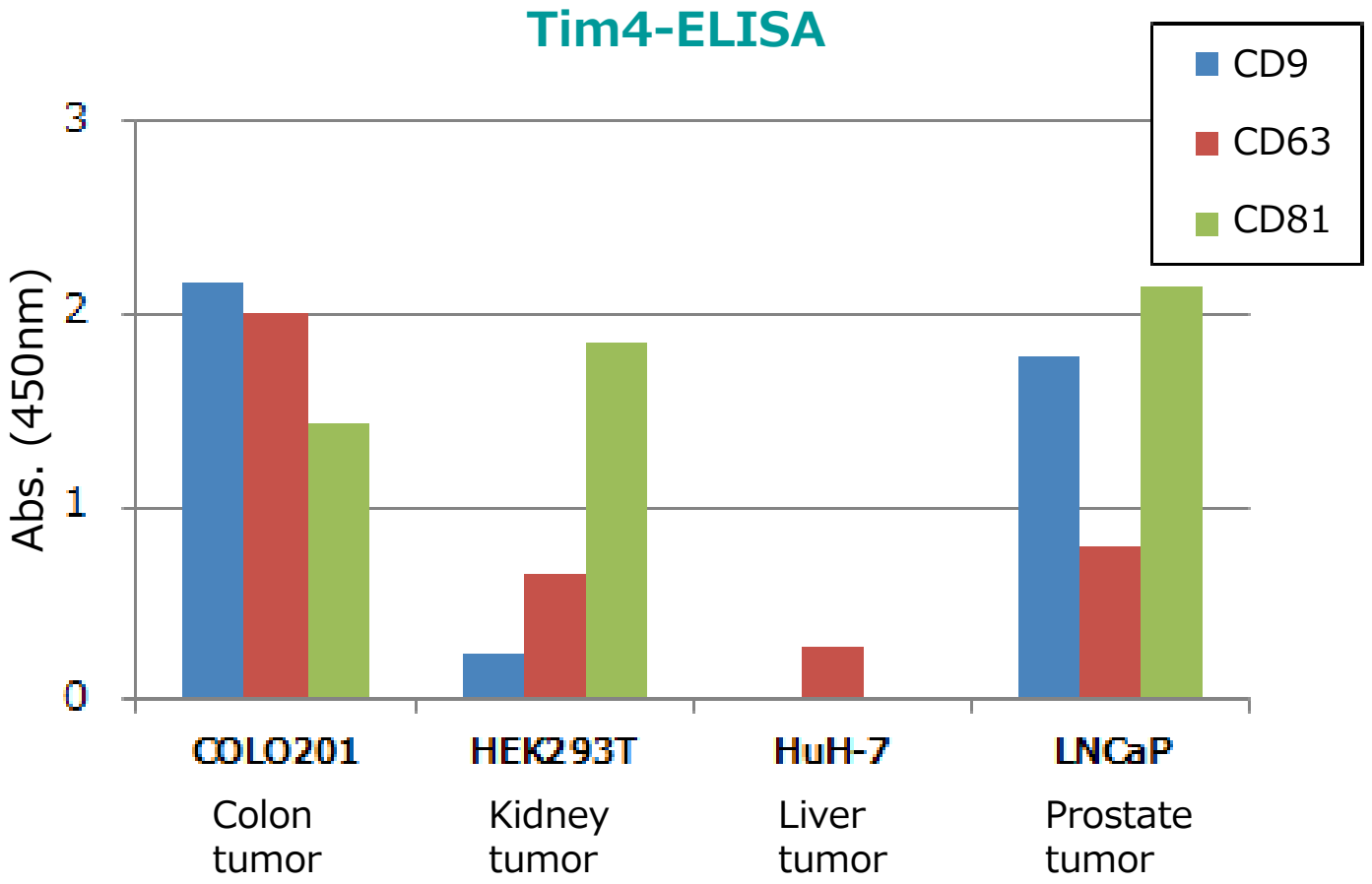
Exosome ELISA Kit (Streptavidin HRP)

Sensitive quantification of sEVs from various cell types

Capture : CD9 Ab CD63 Ab CD81 Ab Tim4



Expression patterns of tetraspanins



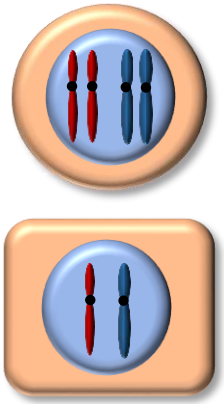
Tim4-ELISA is more sensitive and unbiased method to analyze various sEV subpopulations than using anti-tetraspanin capturing antibodies

Screening for regulators of sEV secretion

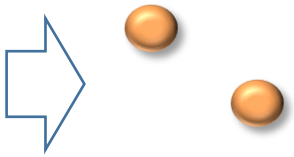
Genetic screening

K562 cells
+ shRNA library

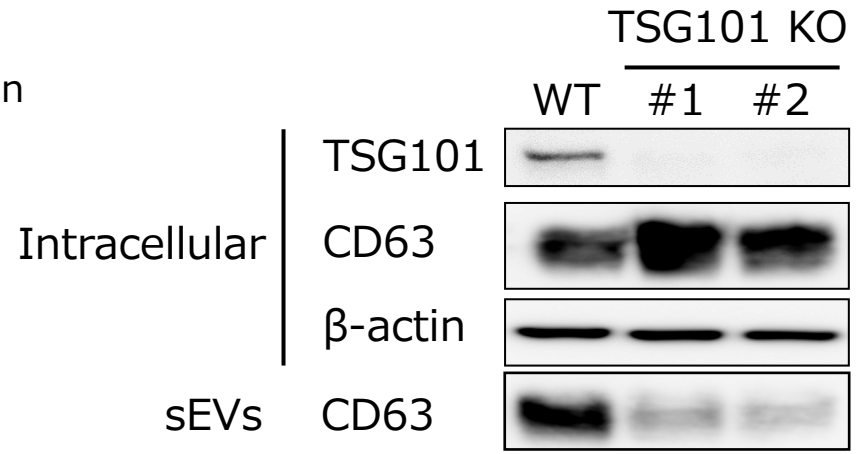
HAP1 cells
+ gene-trap vector

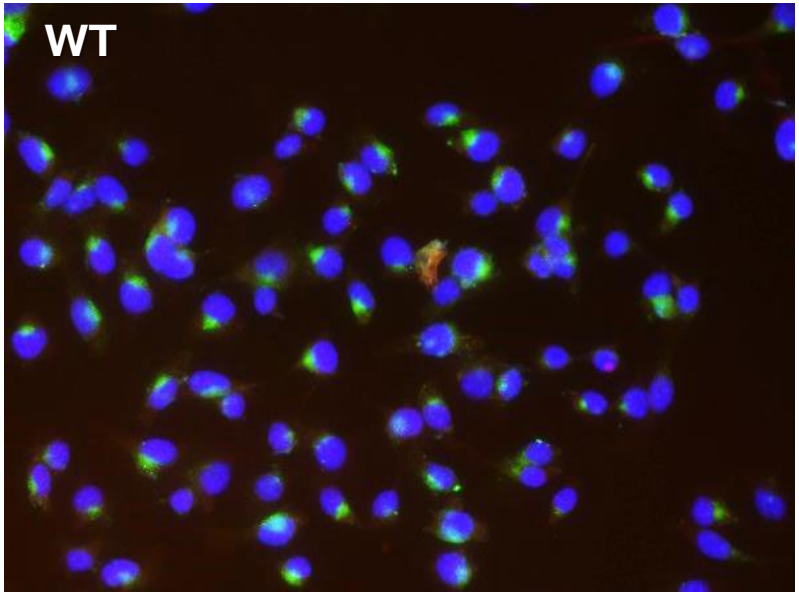


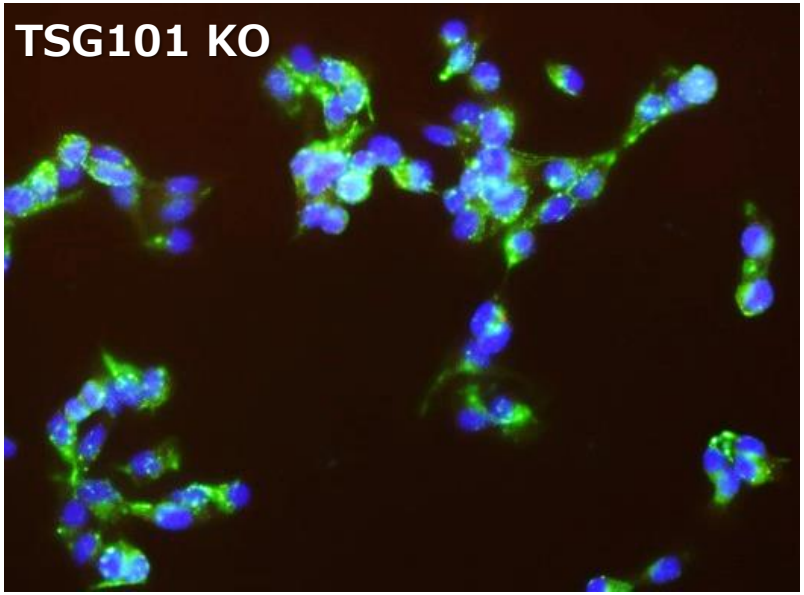
Examining changes in the amount of sEVs



Tim4-ELISA

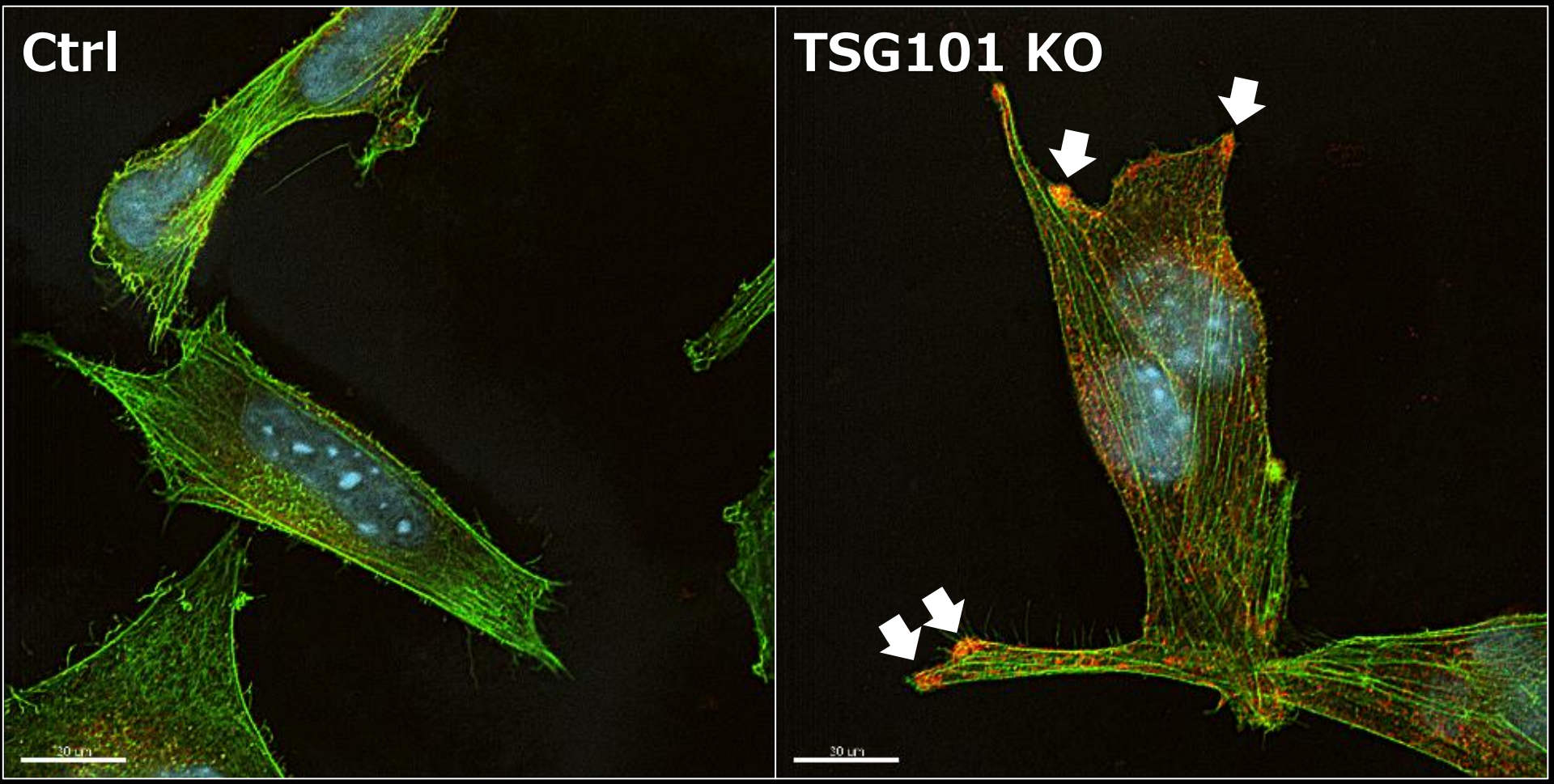






DAPI (Nucleus) ／ CD63 (Exosomes)

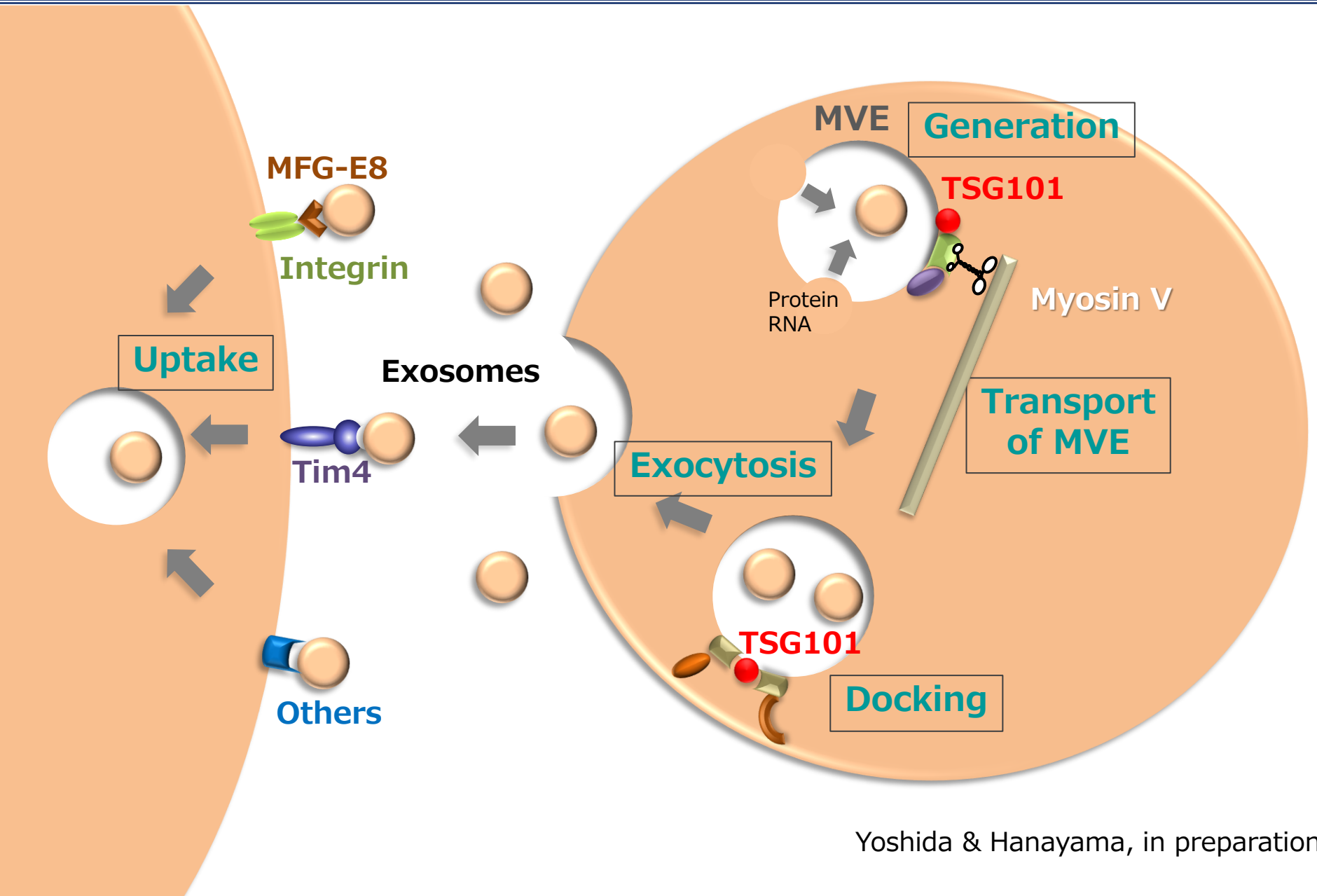
Accumulation of non-secreted vesicles in TSG101-KO



DAPI (Nucleus) Phalloidin (Actin) CD63 (ILVs)

B16F10 cell

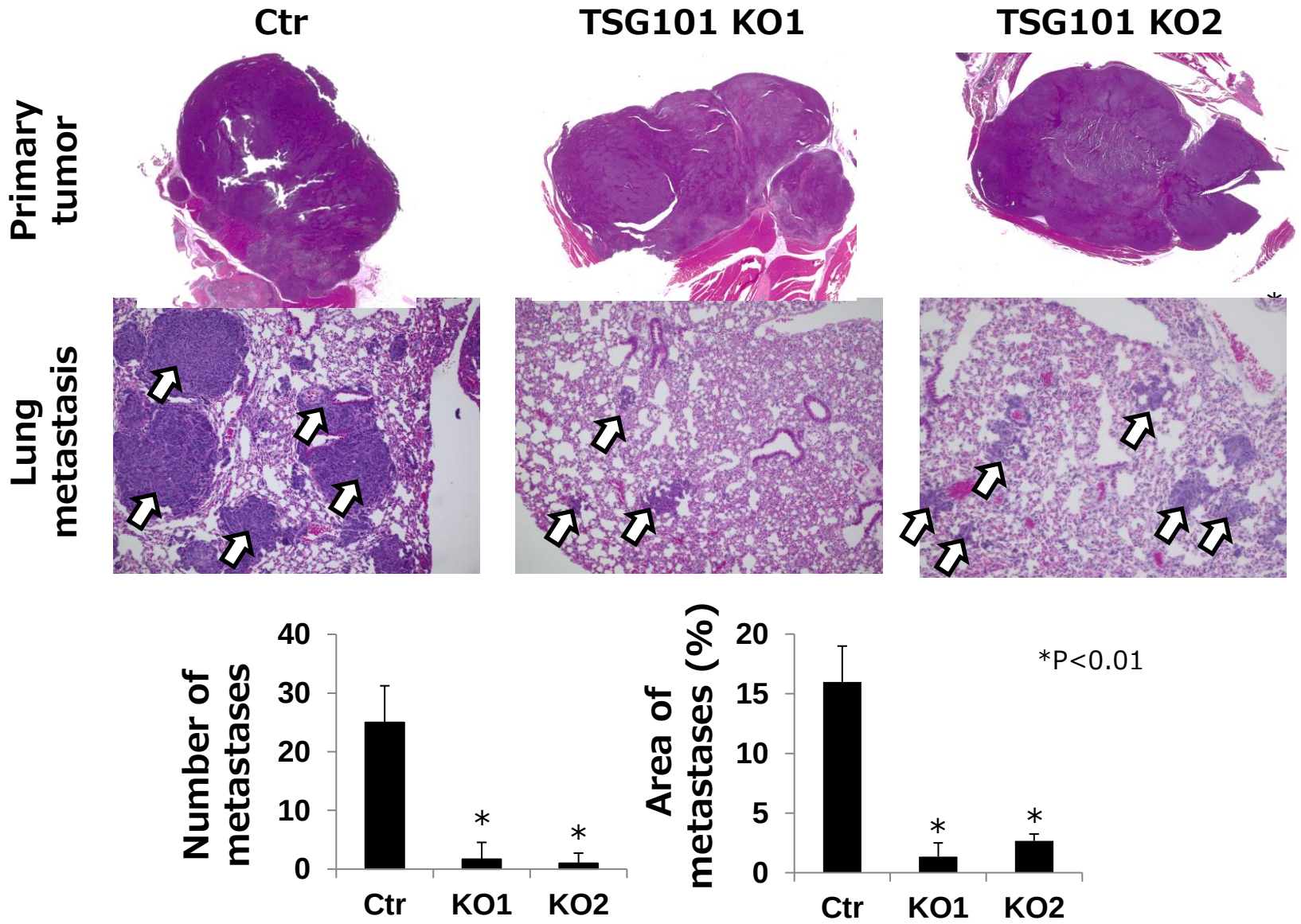
A working model for exosome secretion and uptake



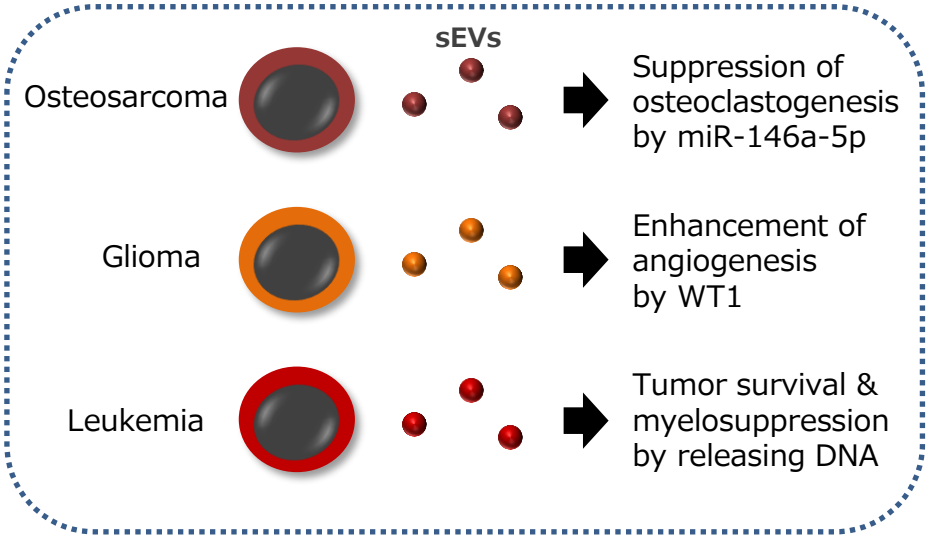
Yoshida & Hanayama, in preparation

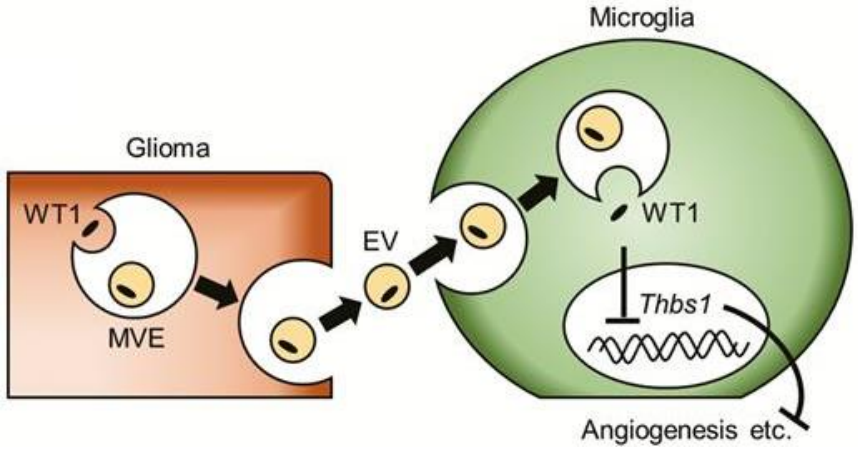
TSG101 KO reduces tumor metastasis

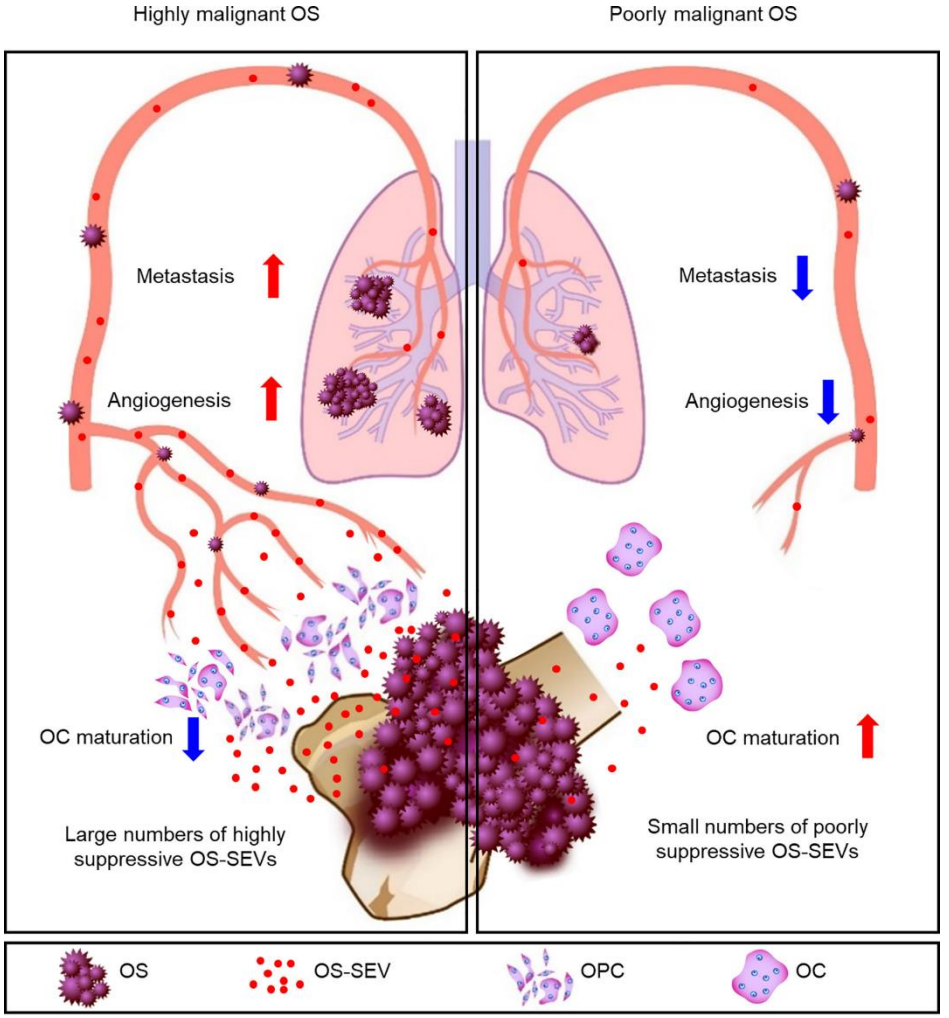
Osteosarcoma



Mechanisms that promote tumor progression by sEVs







Tsutsui & Hanayama, Carcinogenesis (2020)

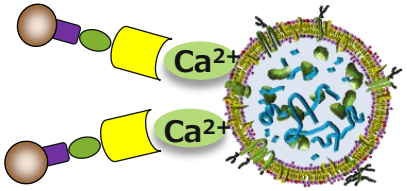
Baba, Hanayama, Mukaida, Cell Death Dis (2021)

Araki & Hanayama, Front Oncol (2021)

EV research products using Tim4

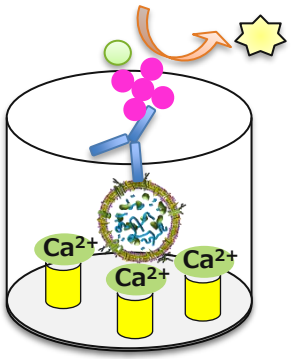
Tim4

MagCapture Exosome Isolation Kit PS



High purity & intact EVs (small EVs, large EVs, enveloped viruses)

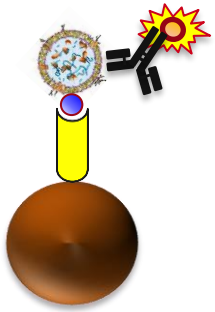
PS Capture Exosome ELISA Kit



High-sensitive qualitative and quantitative analyses

Direct detection without purification

PS Capture Exosome Flow Cytometry Kit



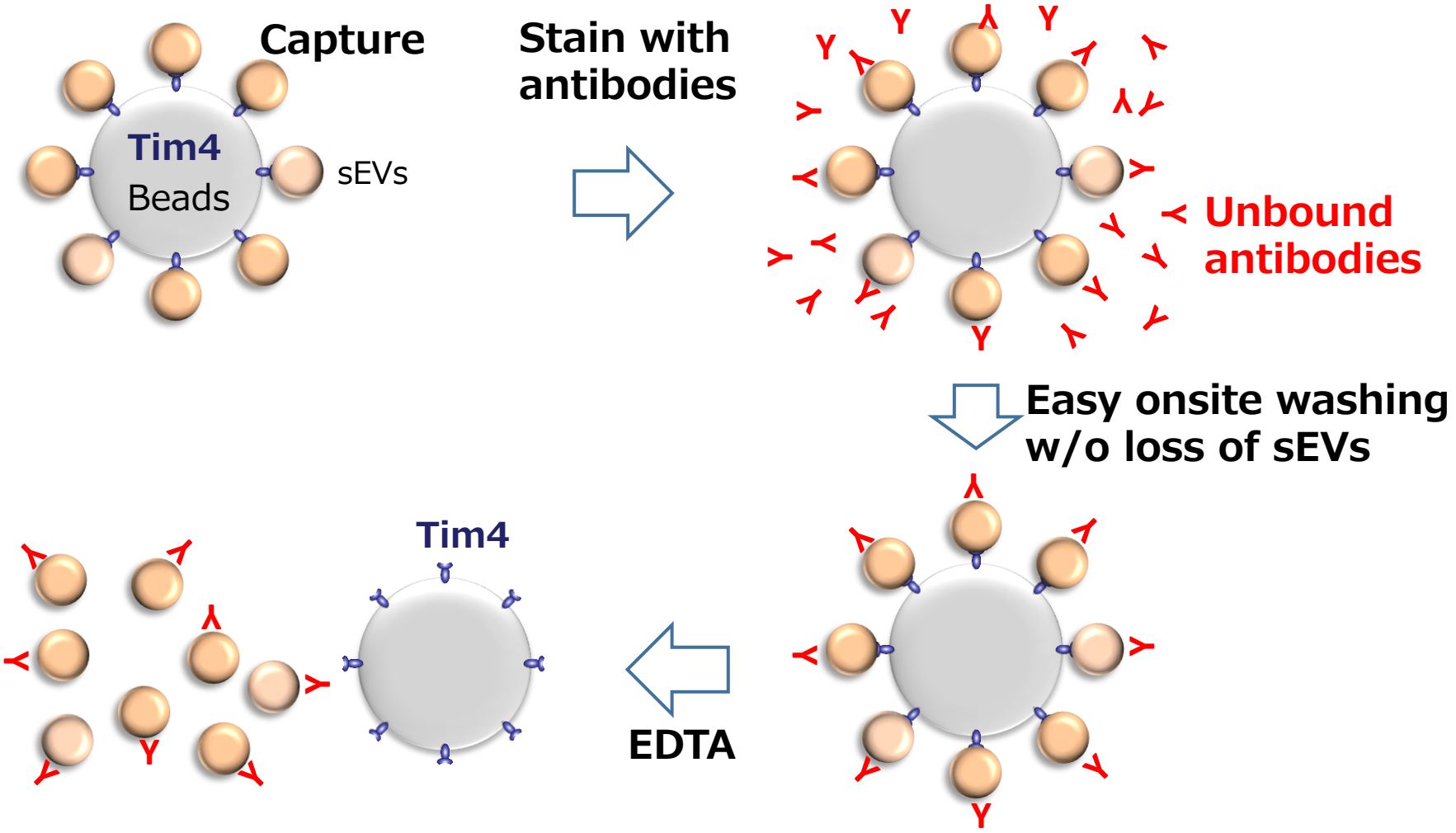
Simultaneous detection of multiple antigens

High-sensitive qualitative analysis

Direct detection without purification

With easy operation and high reproducibility

Sample preparation for flow cytometry using Tim4



Single particle analyses of sEVs using Tim4-beads

NanoFCM

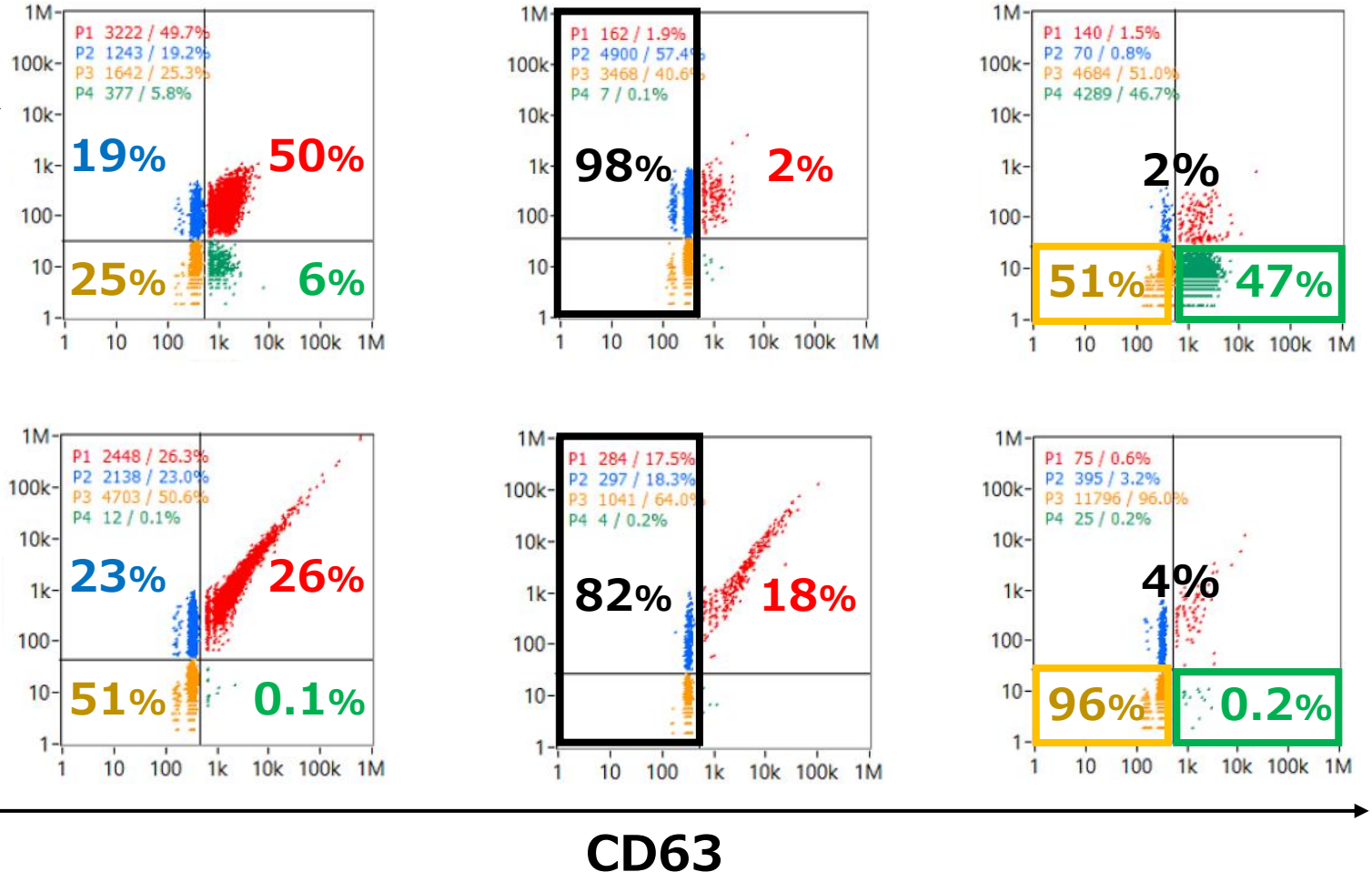
WT-sEV | **CD63KO-sEV** | **CD9KO-sEV**

Tim4 beads

UC

CD9

CD63



Tim4 method is more suitable for sEV sample preparation for FCM analyses

Acknowledgements