The *Long and Winding Road* in the Search of Alternatives to Fetal Bovine Serum: Is there Light at the End of the Tunnel ?

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Alternatives to the Use of Fetal Bovine Serum: Human Platelet Lysates as a Serum Substitute in Cell Culture Media

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Short Communication

Preparation of Platelet Lysates for Mesenchymal Stem Cell Culture Media

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Plasma versus Serum

Proc. Natl. Acad. Sci. USA
Vol. 77, No. 5, pp. 2726–2730, May 1980
Cell Biology

Do plasma and serum have different abilities to promote cell growth?

(vascular smooth muscle/fibroblast growth factor/extracellular matrix)

D. GOSPODAROWICZ AND C. R. ILL

Cancer Research Institute and the Departments of Medicine and Ophthalmology, Medical Center, University of California, San Francisco, California 94143

Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5656–5660, September 1981
Cell Biology

Mitogenic factors present in serum but not in plasma

(chicken heart mesenchymal cells/Rous sarcoma virus/3T3 cells/platelet-derived growth factor/thrombin)

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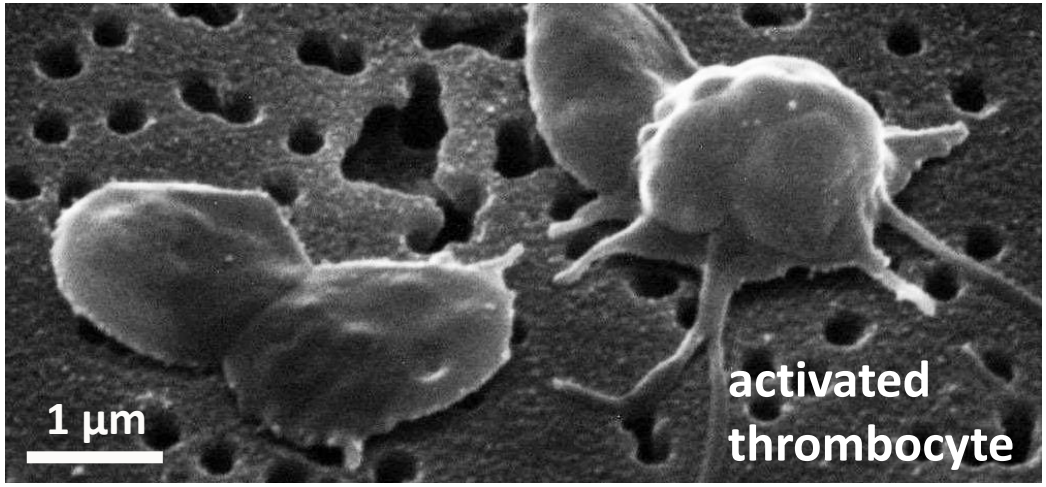
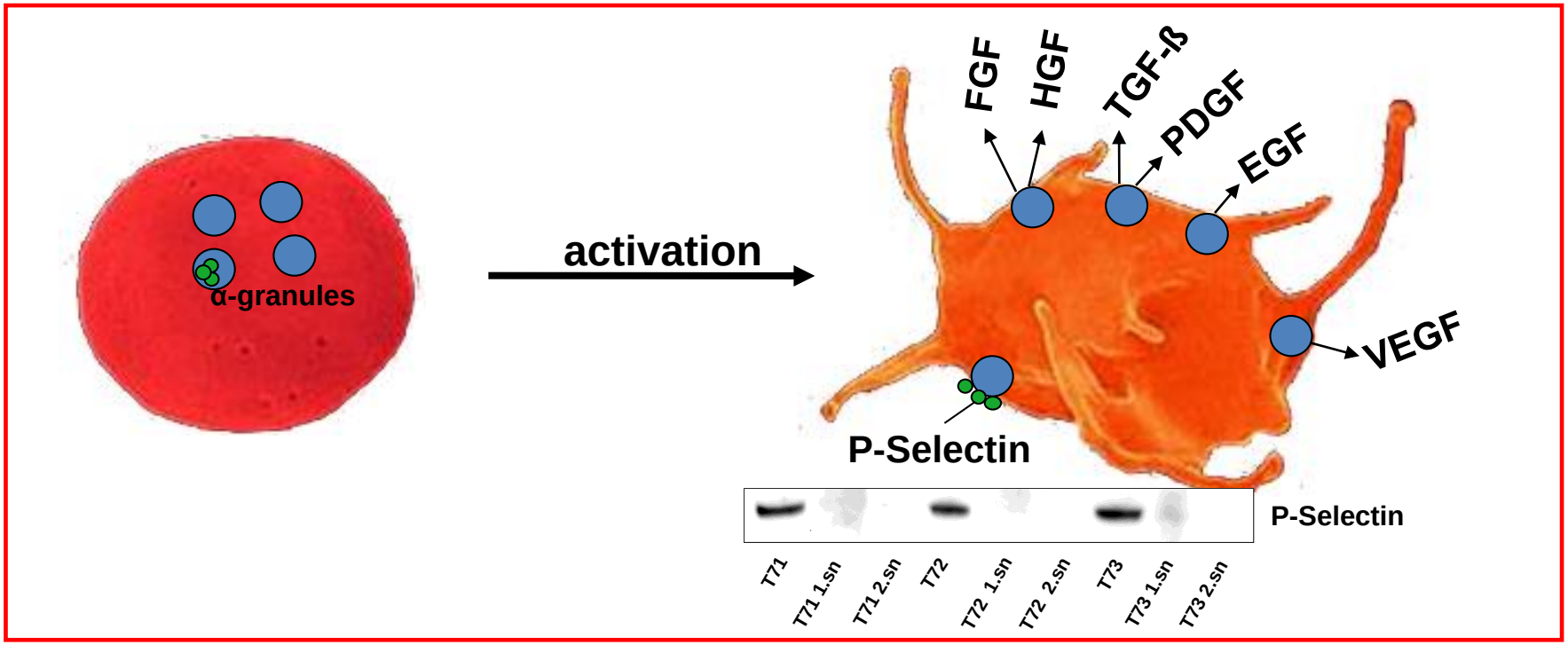




Rauch et al., 2011

Role of Serum in Cell Culture Media

Serum provides

> growth factors and hormones
> binding- and transport proteins
> attachment- and spreading factors
> additional amino acids, vitamins and trace elements
> fatty acids and lipids

> protease-inhibitors
> increased buffering capacity
> 'detoxification‘ (due to binding and inactivation)
> increased viscosity
> reduction of shear stress
> (colloid)osmotic pressure (serum-free vs. protein-free)

Gstraunthaler und Lindl, 2013

Disadvantages in the Use of Serum in Culture Media

- an ill-defined medium supplement
- lot-to-lot variability, quantitative and qualitative batch variations, geographical and seasonal differences (lack of consistency and standardization)
- may contain endotoxins and haemoglobin
- mycoplasma, viral contaminants, prion proteins

- may be unable to support growth of specific cell types or to prevent fibroblast overgrowth in primary cultures
- no *physiological* environment for cells
- **ethical concerns** about FBS harvest from bovine fetuses
- global demand vs. commercial supply (availability, costs)

Gstraunthaler und Lindl, 2013

Arguments for Serum-free Cell Culture

> **cell biological aspects:**
- chemically defined and controlled culture conditions *in vitro*
- reduced variability in qualitative and quantitative culture medium composition
- reduced risks of microbial contamination **(biosafety)**
- advantages in the isolation of cell culture products (down-stream processing)

> **physiological arguments**

> **ethical concerns (in terms of 3*R*):**
- to ***reduce*** or to completely avoid (***replace***) the suffering of fetuses and animals

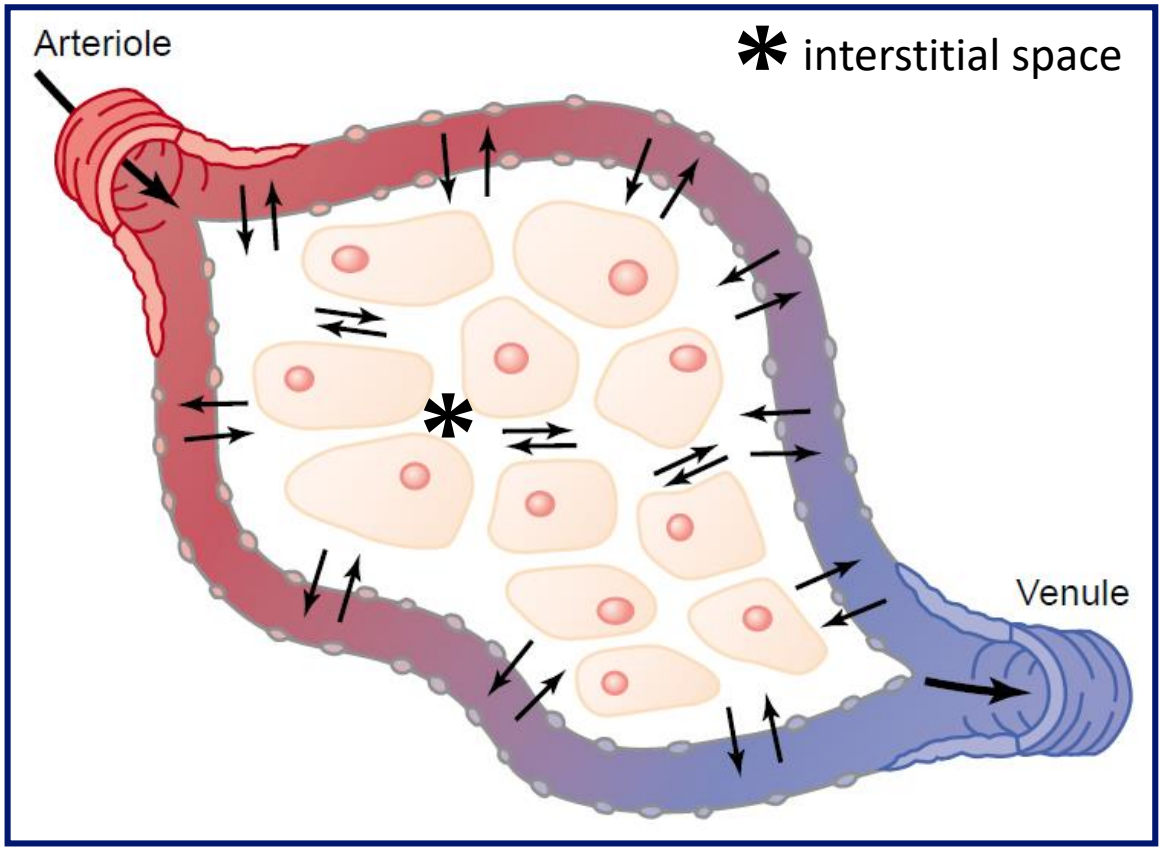
> **independence of commercial supply** (availability, costs)

Gstraunthaler und Lindl, 2013

Benefits of Serum-free Cell Culture

> physiological aspects:

serum proteins are **no** physiological environment for somatic cells



Benefits of Serum-free Cell Culture

> independence of commercial supply:

TECHNOLOGY

CELL CULTURE

Fetal Bovine Serum running short

Dr. Peter Häusl, Life Science Advisers/Raw Material Sourcing, Germany

Stocks of fetal bovine serum (FBS) – an essential active substance in cell culture – are low. New EU directives on meat imports, the weak US dollar and cultivation of energy crops have changed the topography of the market for the substance. The rise in price can be traced back to Brazil, the major supplier of FBS for medical research in Europe.

WELLINGTON: New Zealand Monday (May 28, 2018) ordered the culling of 126,000 cattle in an attempt to eradicate the painful Mycoplasma bovis disease, which causes udder infections, pneumonia and arthritis.

New Zealand to cull 150,000 cows in desperate measure to stop spread of deadly bacteria

WELLINGTON (Reuters) - New Zealand, the world's biggest dairy exporter, will spend more than NZ$880 million ($610 million) in a bid to eradicate the Mycoplasma bovis cattle disease, Prime Minister Jacinda Ardern said on Monday.

About 126,000 cows are expected to be culled, mainly over the next two years, as government and industry work to depopulate all infected farms, the government said in a statement.

The disease, which is common in many countries, was first detected in New Zealand at a farm in the South Island last July and some 37 properties have now tested positive for the illness.

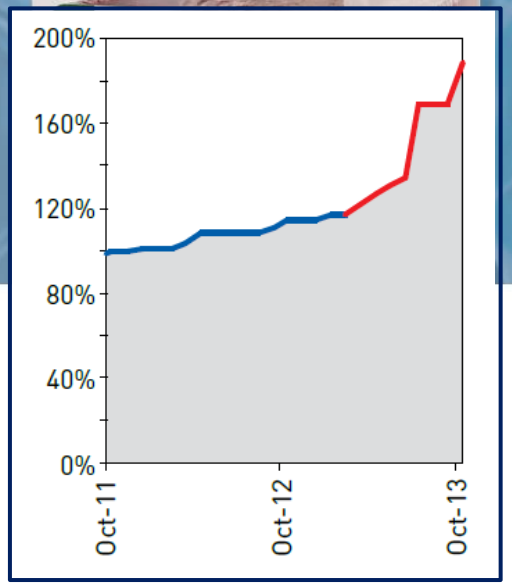
Benefits of Serum-free Cell Culture

> independence of commercial supply:

gibco

The key supply drivers causing the constant fluctuations in serum supply are:

Weather patterns	Droughts, floods, growing conditions
Beef demand	Determinant of harvesting rates
Dairy market	Dairy product prices
Feed costs	Escalating costs increase harvesting
Cattle cycle	Liquidation or rebuilding of herds



2017 FBS market update

Fetal or *Fatal* Bovine Serum

> **Quality, purity and safety of FBS:**

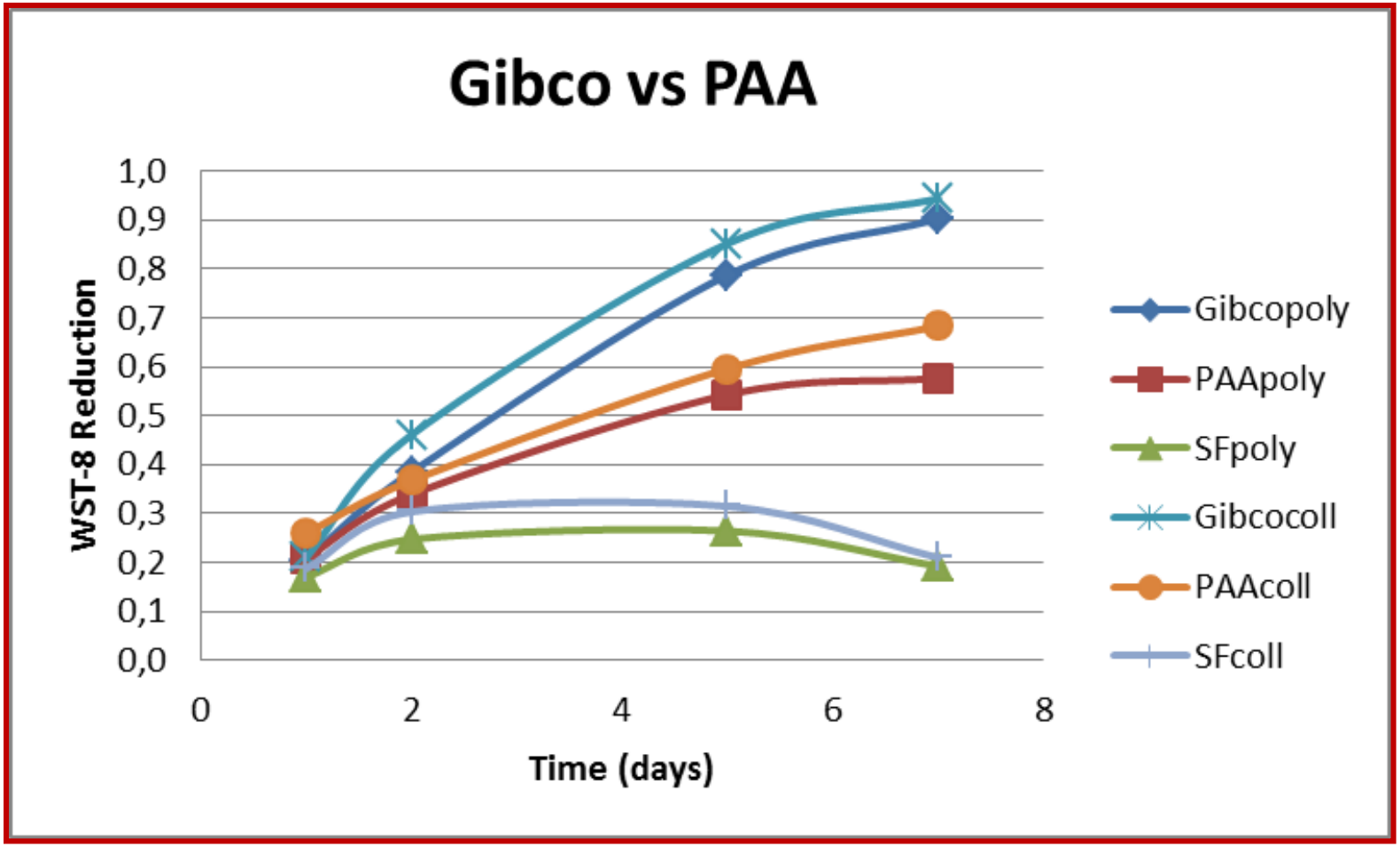
March 2008 – March 2013:

FBS blended with bovine serum albumin (BSA), water and/or cell growth promoting additives

U.S. Food and Drug Administration
Protecting and Promoting *Your* Health

143 batches, approx. 280.000 litres

Comparison FBS (Gibco) vs. FBS (PAA)



ATLA 42, 207–209, 2014

ATLA Alternatives to Laboratory Animals

Comment

A Severe Case of Fraudulent Blending of Fetal Bovine Serum Strengthens the Case for Serum-free Cell and Tissue Culture Applications

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Traceability of FBS (and raw serum) by stable isotope analysis

The application of stable isotope analysis in forensic investigations can distinguish materials that are chemically identical, however, from different geographic regions:

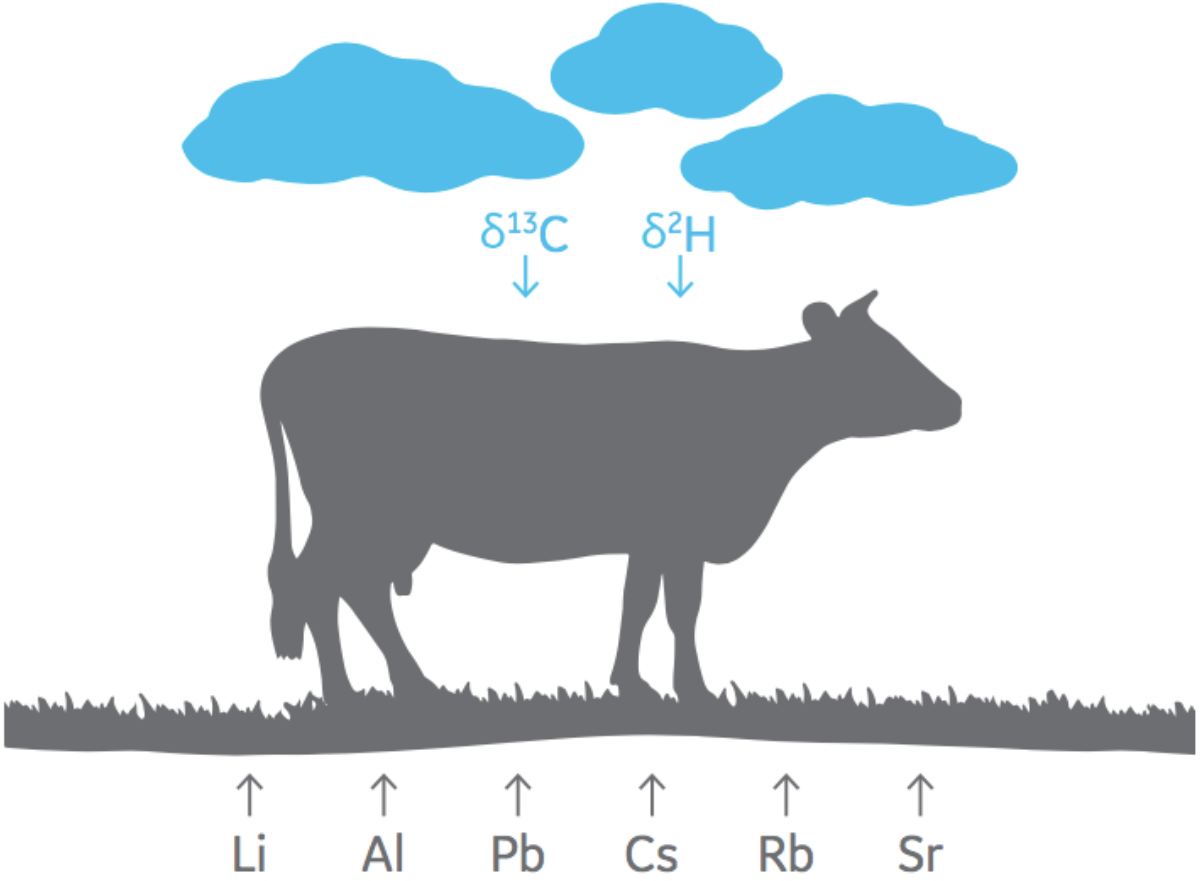
$^{18}O/^{16}O$ $^{13}C/^{12}C$ $^{34}S/^{32}S$ ^{14}C

$^{2}H/^{1}H$ $^{15}N/^{14}N$ $^{87}Sr/^{86}Sr$

INTERNATIONAL SERUM INDUSTRY ASSOCIATION

IsoForensics geo-location services

Animal tissues assimilate isotopes from dietary and drinking water inputs



IsoForensics
geo-location services

Methods for Reducing Fetal Bovine Serum Requirement

- Optimization of existing medium formulations
- Reduced serum media
- Alternative sera: newborn sera, calf sera, adult (donor) sera
- Chemically defined, serum-free culture media
- Alternative mammalian serum substitution

(Jayme D.W. et al., *Nature 334*: 547, 1988)

- **Search / browse for serum-free media or media formulations in literature or databases / databanks**

Calf/bovine sera according to the age (state of development) of the animals

- fetal calf/bovine serum (FCS/FBS)
- neonatal calf serum
- newborn calf serum (< 3 weeks)
- calf serum (< 12 months)
- adult bovine serum (> 12 months and older)
- donor bovine serum (from controlled prime cattle, 1-3 yrs)

Methods to *reduce* or *replace* Fetal Bovine Serum

Search for serum alternatives or serum-free media in literature or in databases:

Dr Hadwen Trust
Replacing Animals in Medical Research

Animal Free Research UK

ANIMAL FREE RESEARCH UK

https://www.animalfreeresearchuk.org/serum-free-media

FCS-free database

Utrecht University

https://fcs-free.org

Alternatives to Fetal Bovine Serum for Mammalian Cell Culture

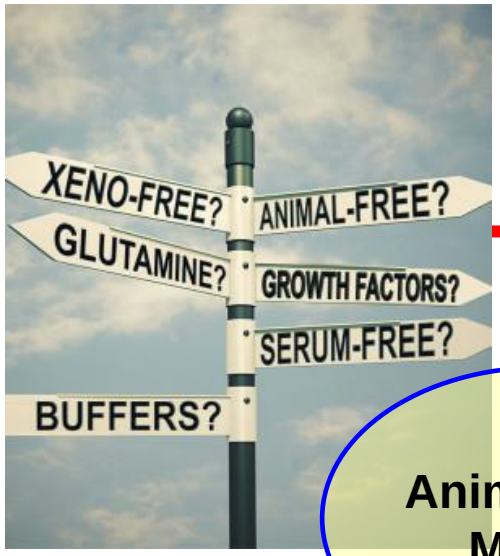
- **Serum alternatives:**

  > commercial alternative products (isolated from serum, process of isolation and production ?, composition ?, standardized, but still undefined)

  > biological alternatives: e.g. tissue extracts
  (pituitary extract, ***platelet lysates***)
  bovine (milk) colostrum
  milk proteins
  plant extracts („vegetal serum“)

  > chemically defined, serum-free cell culture

Evolution of Mammalian Cell Culture Media



Animal-derived Component-free

Defined Formulation

Basal Media + Serum

Serum-free Media

Animal-free Media

Protein-free Media

Chemically Defined Media

Chemically Defined Animal-free Media

(Hodge, 2005)

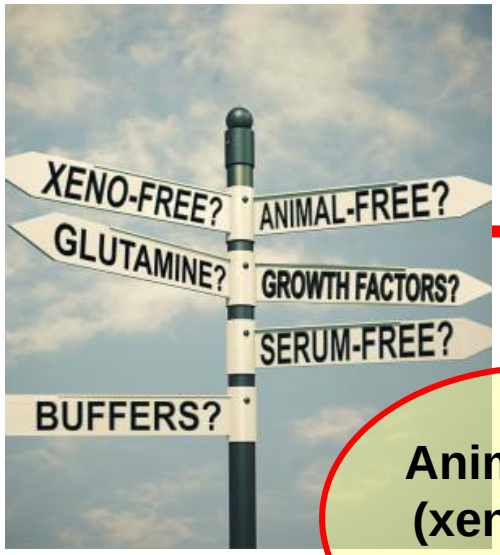
Culture Media (Definitions)

- ***Basal Medium + Serum (FBS)***
- ***Serum-Free Media***
- ***Protein-Free Media***
- ***Animal-Derived Component-Free Media***
- ***Chemically Defined Media***

Culture Media (Definitions)

- ***Basal Medium + Serum (FBS)***
- ***Serum-Free Media***
- ***Protein-Free Media***
- ***Animal-Derived Component-Free Media***
- ***Chemically Defined Media***

Evolution of Mammalian Cell Culture Media



Animal-derived Component-free

Defined Formulation

Basal Media + Serum

Serum-free Media

Animal-free (xeno-free) Media

Protein-free Media

Chemically Defined Media

Chemically Defined Animal-free Media

(Hodge, 2005)

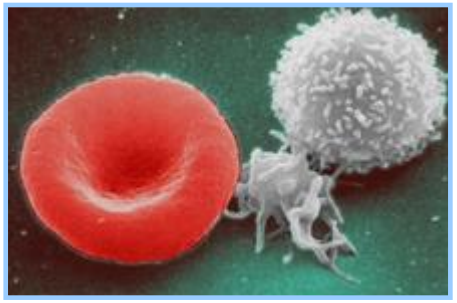
Contents of Thrombocyte Granules

Electron-dense Granules

- ADP
- Ca^{2+}
- Serotonin

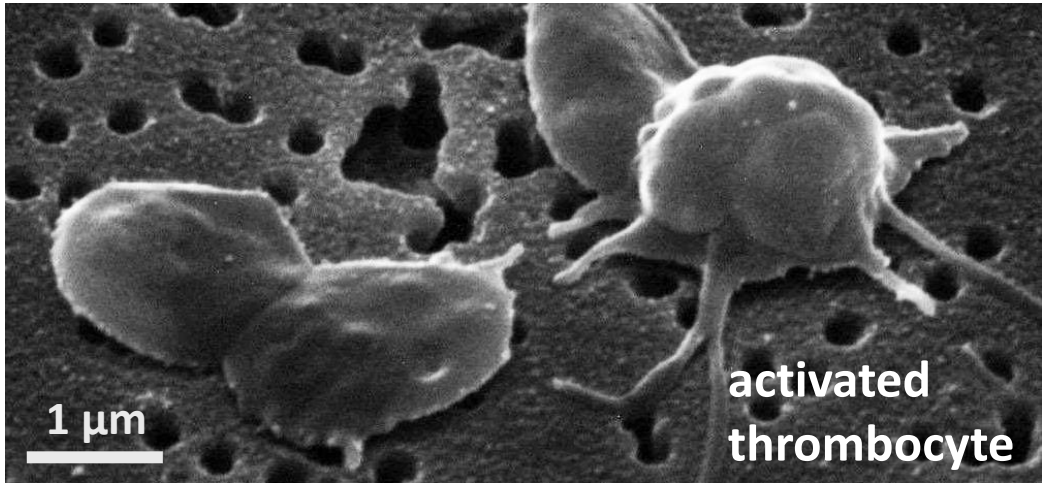
Lysosomes

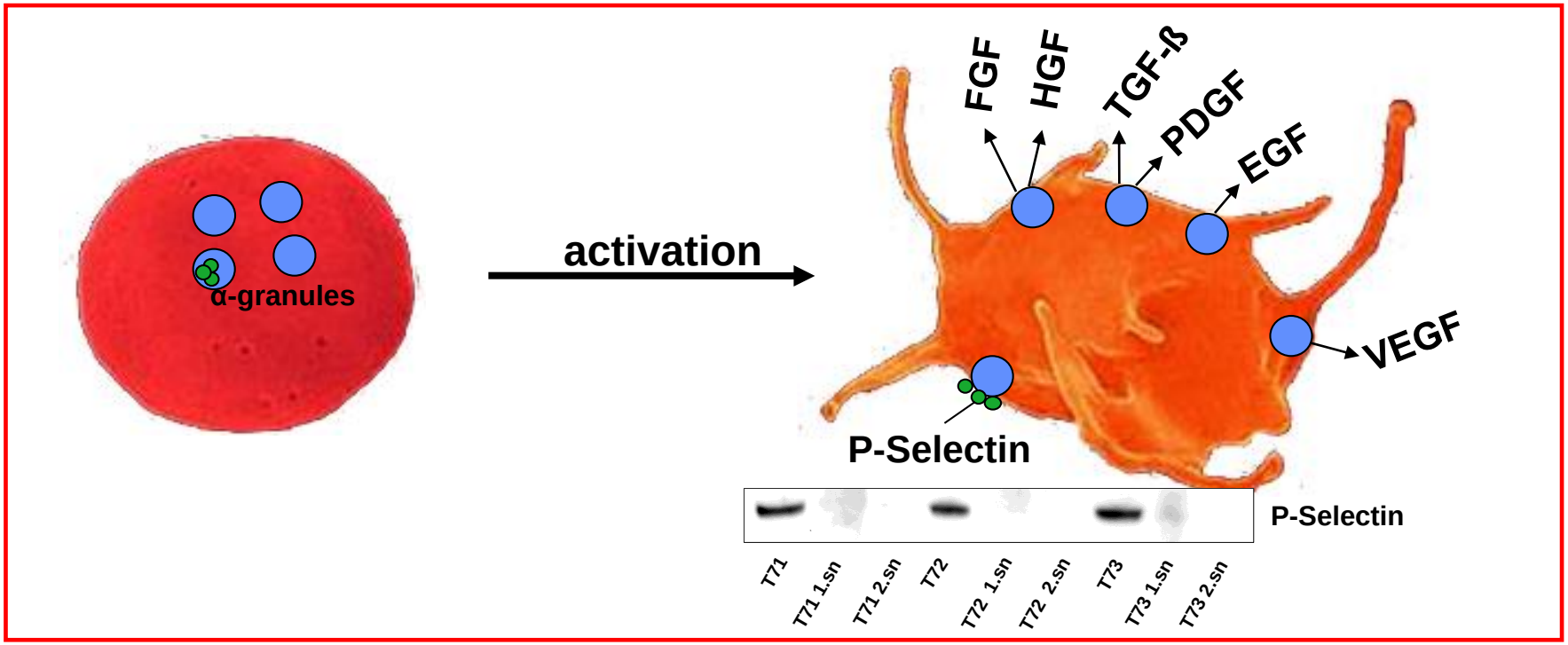
- Acid Hydrolases



α-Granules

- Fibrinogen
- Coagulation Factors V + VIII
- Fibronectin
- von-Willebrand-Factor, vWF
- Thrombospondin
- β-Thromboglobulin
- Platelet Factor 4
- Platelet-derived Growth Factor, PDGF
- Transforming Growth Factor-β, TGF-β
- Epidermal Growth Factor, EGF
- Fibroblast Growth Factor, FGF
- Vascular Endothelial Growth Factor, VEGF





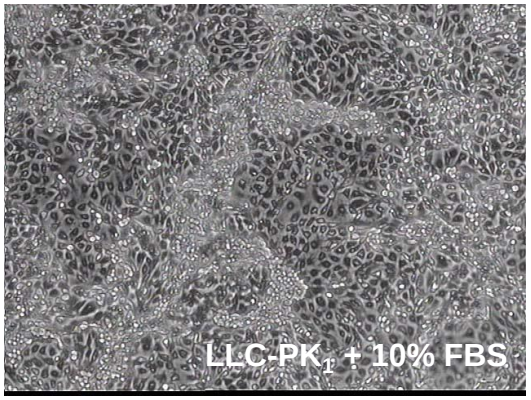
Rauch et al., 2011

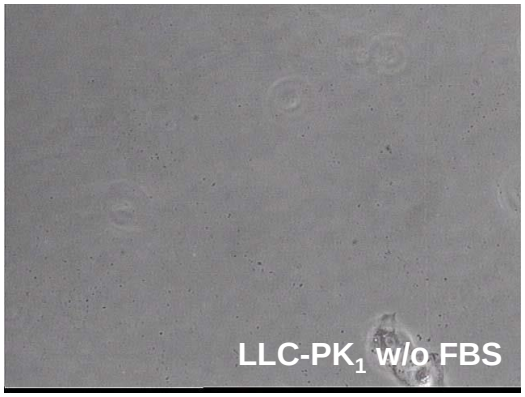
Growth Factor Contents in Platelet Lysates

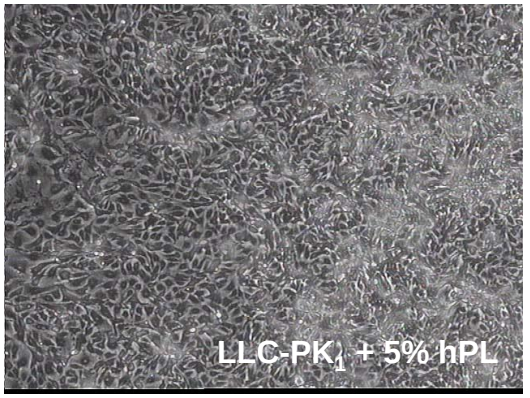
	Growth Factors [ng/ml]							Protein [mg/ml]
Lysates Samples	EGF	PDGF-AB	TGF-ß1	HGF	IGF-1	bFGF	VEGF	
# 56	11,63	57,28	1179,7	1,17	12,81	0,96	8,46	7,93
# 69	15,34	76,48	533,2	1,38	16,70	1,04	3,47	5,88
# 70	20,55	53,60	1462,6	1,46	8,90	1,05	10,63	9,03
# 71	13,45	58,26	503,7	2,48	16,13	0,90	19,65	7,10
# 72	16,69	46,34	1272,5	0,68	9,32	0,81	5,17	7,16
# 73	14,27	51,88	1084,3	1,22	16,66	0,98	19,99	6,94
# 108	13,44	89,26	518,1	1,65	25,89	1,33	1,27	9,34
Mean ± SD	15,05 ± 2,90	61,87 ± 15,31	963,3 ± 407,4	1,43 ± 0,55	15,20 ± 5,76	1,00 ± 0,17	9,81 ± 7,50	7,63 ± 1,22
Human Serum	1,52	4,64	46,87	0,80	75,96	0,0019	0,063	70,00
	0,02	5,20	33,85	0,62	83,98	0,018		70,00

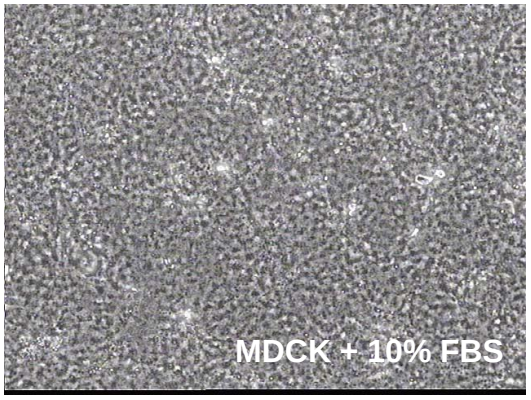
quality criteria: **high** contents of PDGF, VEGF, EGF, and TGF-β
low content of IGF-1
low protein content [< 10 mg/ml]

Rauch et al., 2011

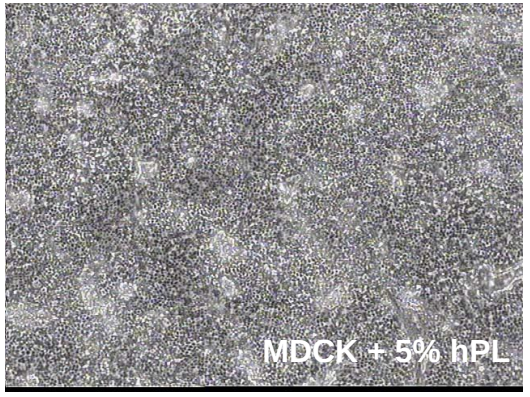

LLC-PK$_1$ + 10% FBS

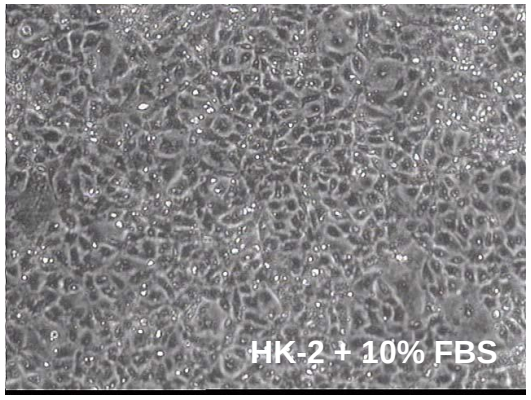

LLC-PK$_1$ w/o FBS

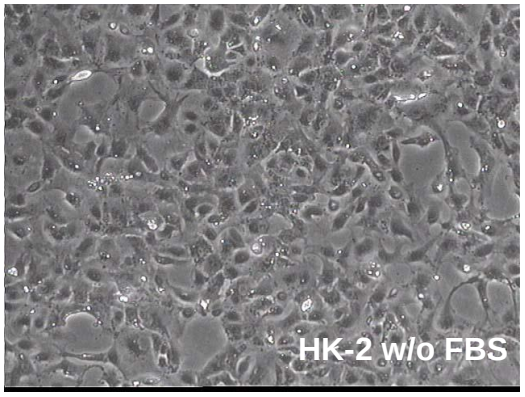

LLC-PK$_1$ + 5% hPL

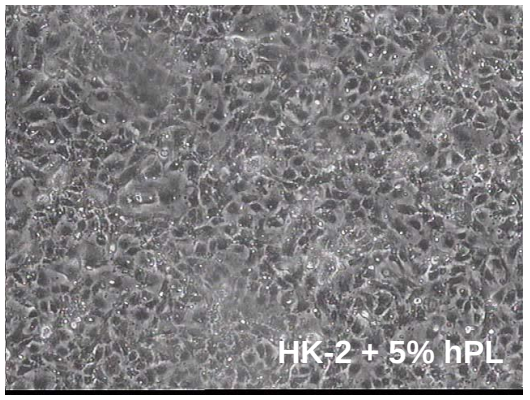

MDCK + 10% FBS

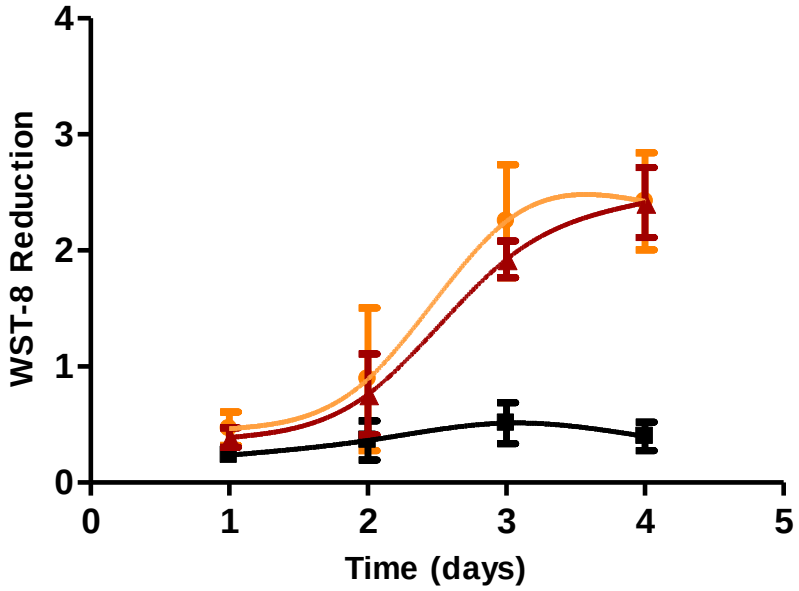

MDCK w/o FBS


MDCK + 5% hPL

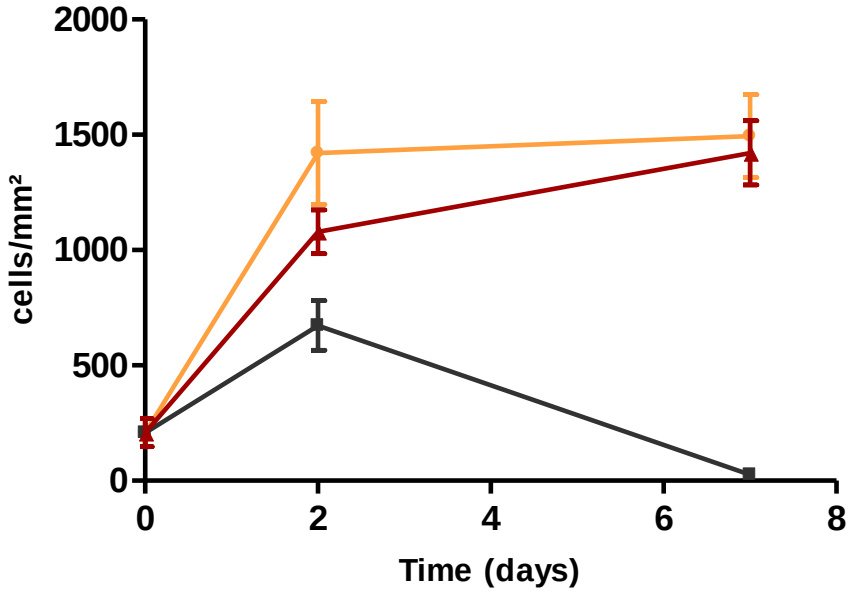

HK-2 + 10% FBS


HK-2 w/o FBS


HK-2 + 5% hPL

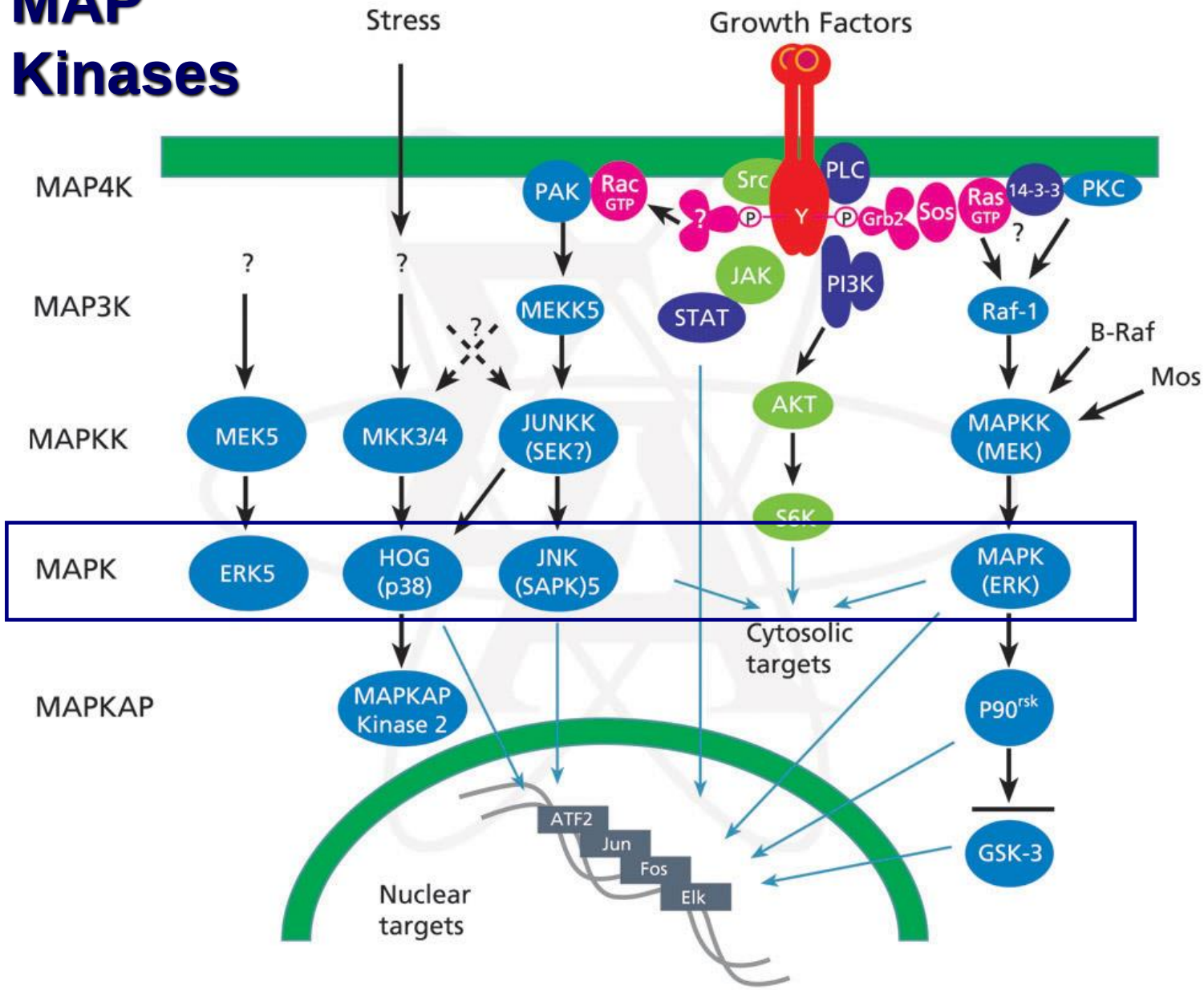


growth curve of MDCK cells

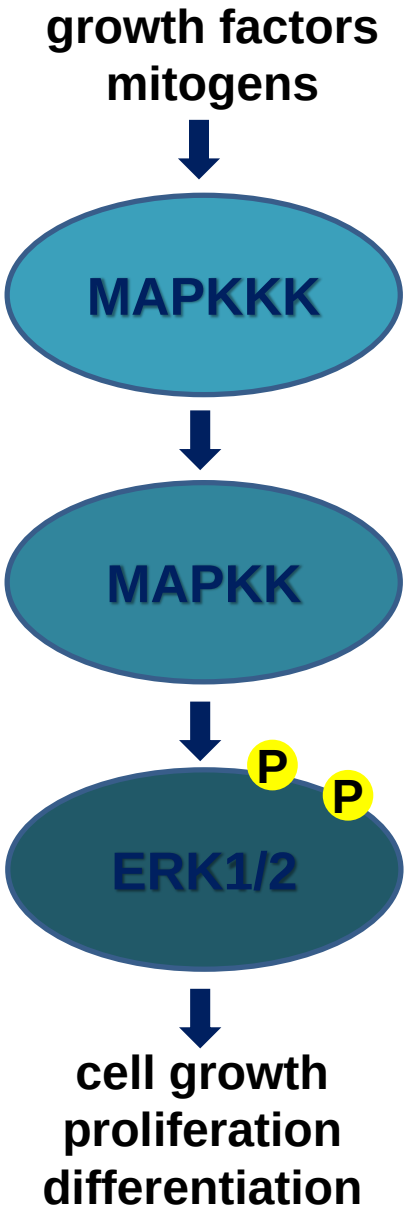


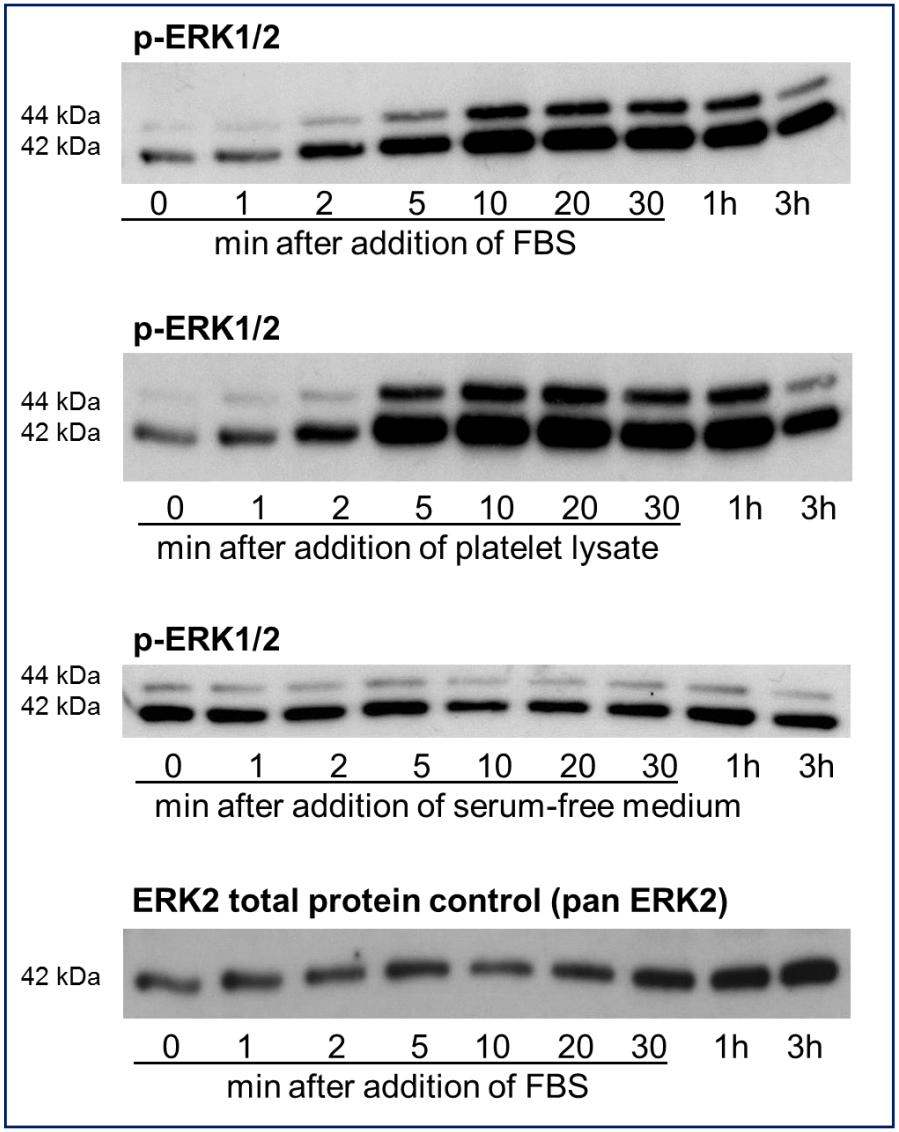
cell counts of LLC-PK_1 cells

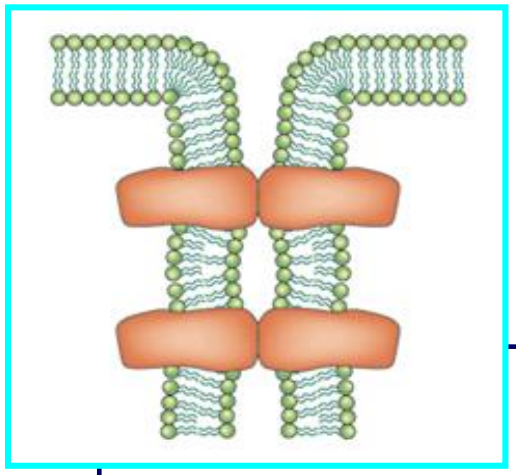
MAP Kinases


Stress
Growth Factors
MAP4K
MAP3K
MAPKK
MAPK
MAPKAP
PAK
Rac GTP
Src
PLC
Y
P
Grb2
Sos
Ras GTP
14-3-3
PKC
?
JAK
STAT
PI3K
MEKK5
Raf-1
B-Raf
Mos
AKT
MEK5
MKK3/4
JUNKK (SEK?)
MAPKK (MEK)
S6K
ERK5
HOG (p38)
JNK (SAPK)5
MAPK (ERK)
Cytosolic targets
MAPKAP Kinase 2
P90rsk
GSK-3
ATF2
Jun
Fos
Elk
Nuclear targets

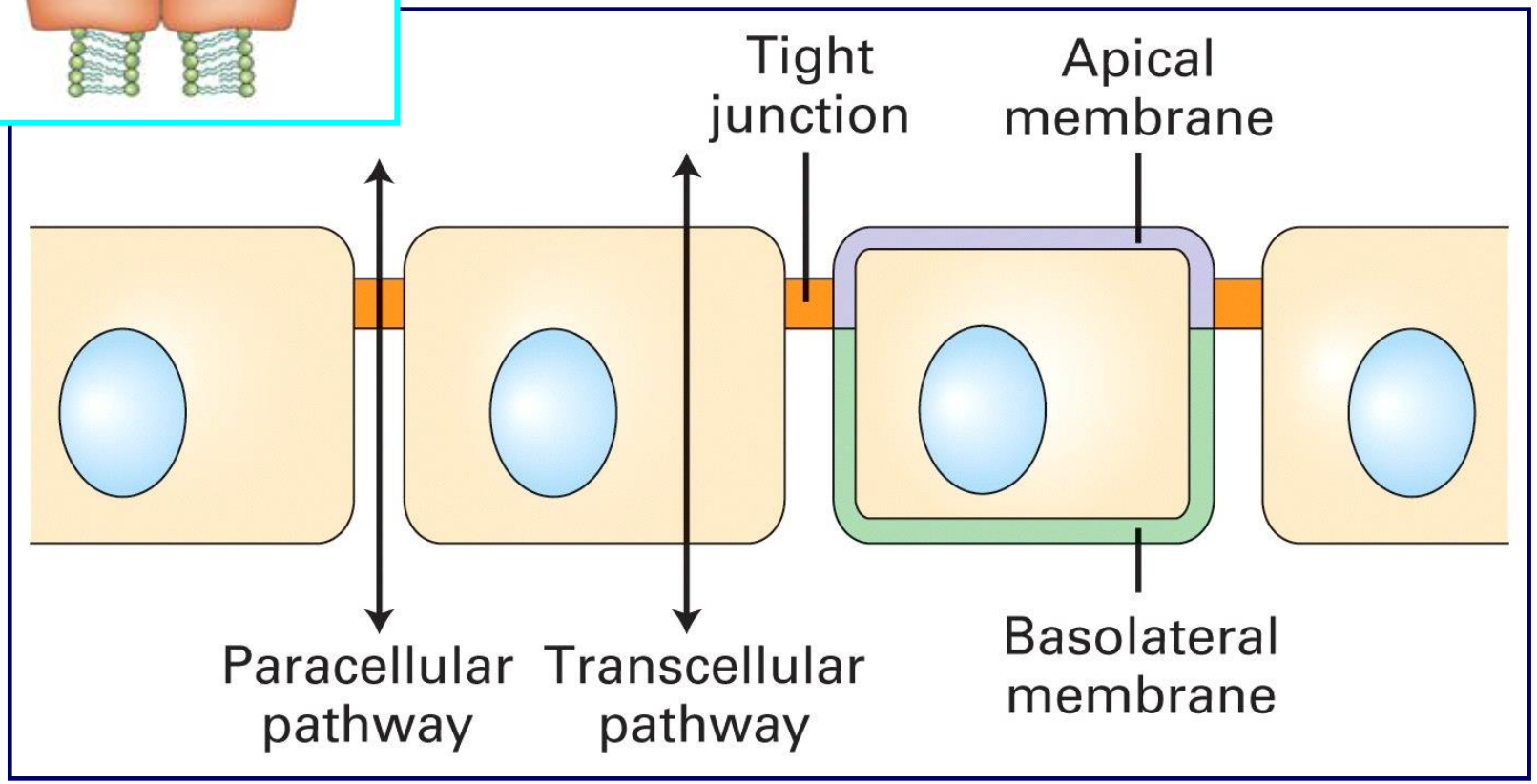
Activation of ERK1/2 MAP kinases



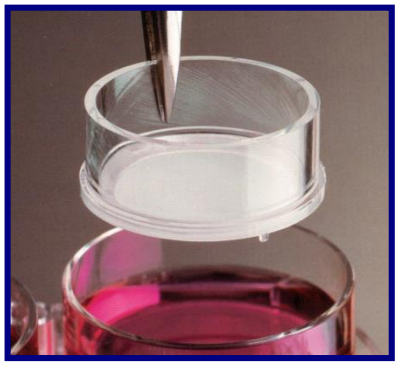


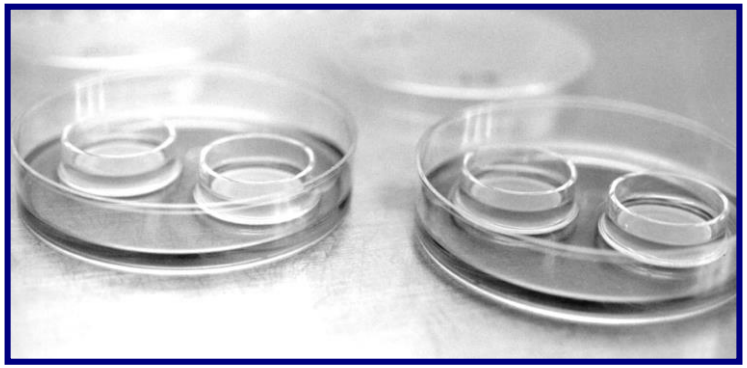


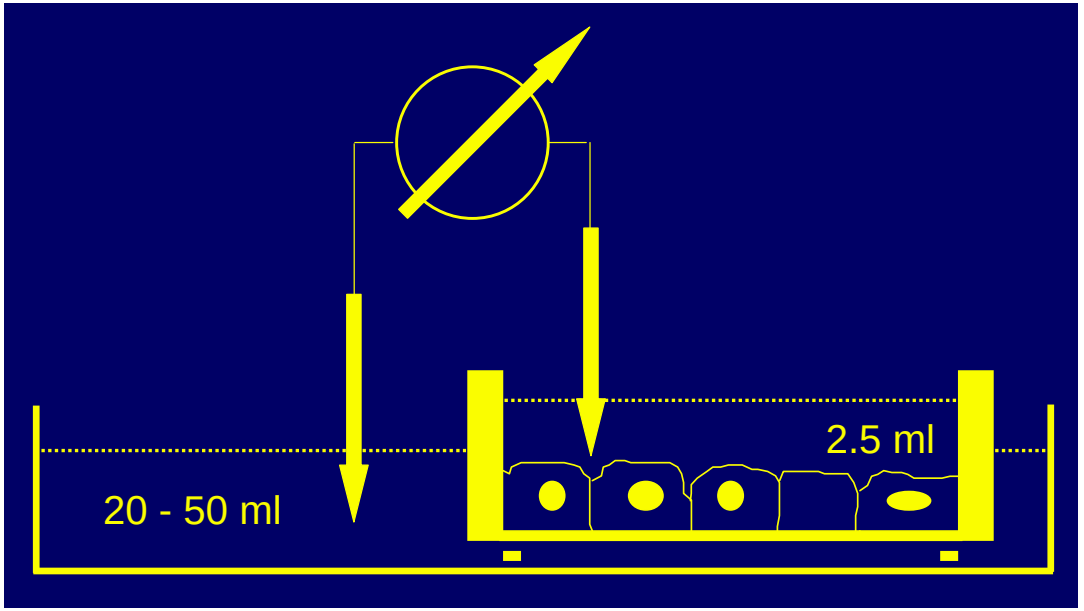
- **gate function**
- **fence function**

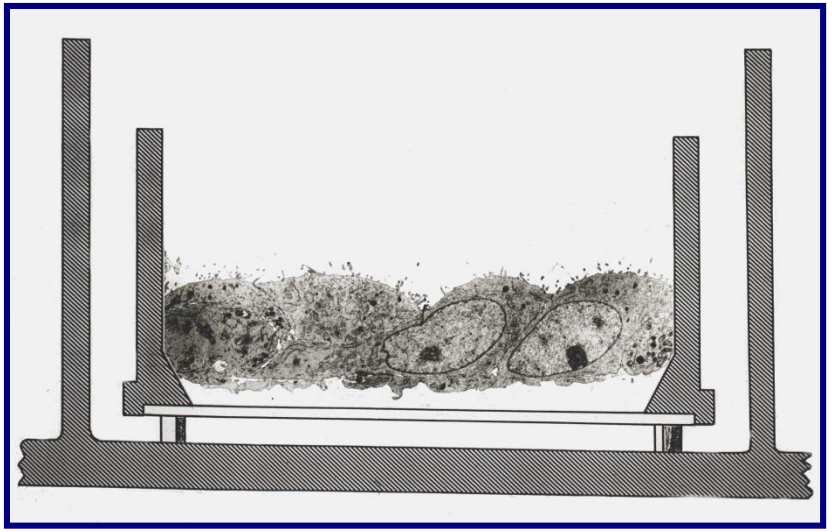


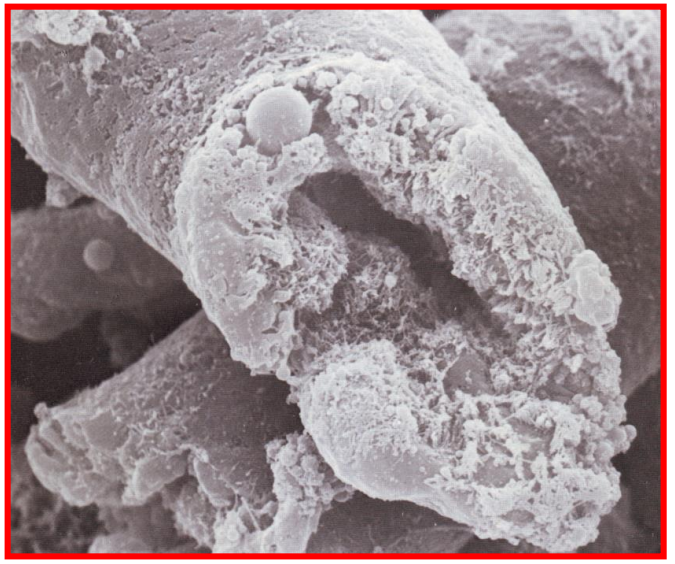
H. Lodish et al.: *Molecular Cell Biology*
5th Ed., Freeman, 2004



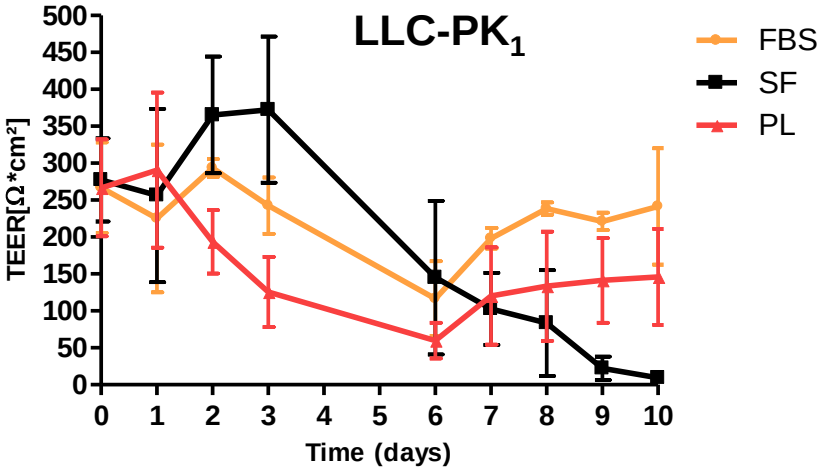


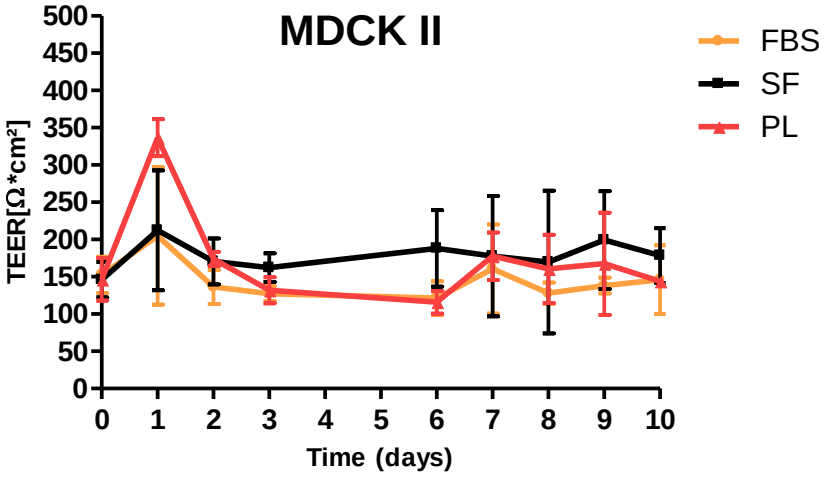


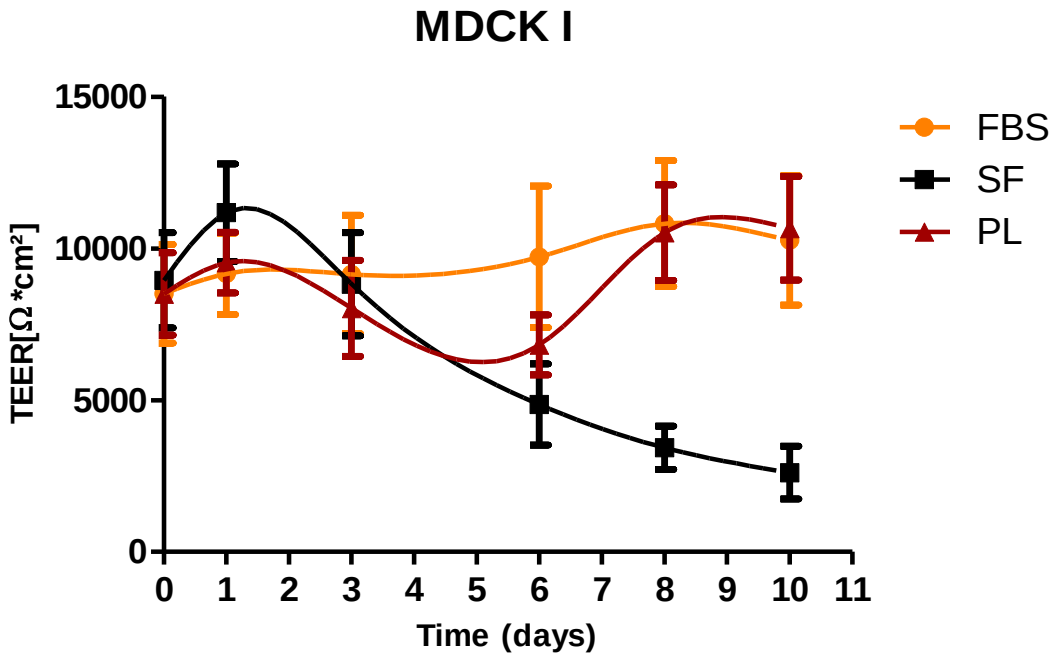




TEER in LLC-PK$_1$ and in low- and high-resistance MDCK epithelia







Summary and Conclusion (I)

Human donor platelets were activated by freeze-thawing and lysates prepared. The method of lysate preparation has been standardized.

Growth factors are released from platelet granules in sufficient amounts that can be quantified by ELISA.

Platelet lysates/releasates stimulate growth, proliferation and differentiation of a variety of human and animal cell lines.

The rates of growth and differentiation are comparable with FBS.

ERK1/2 MAP kinase cascade is specifically activated by platelet lysates.

The data show the high potential of platelet lysates as a valuable substitute for fetal bovine serum (FBS) in mammalian cell and tissue culture, tissue engineering and stem cell technology.

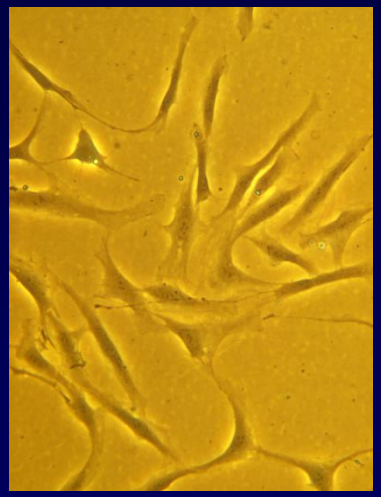
Adult mesenchymal stem cells

(adipose-derived stem cells, ADSC)

- Adherence to plastic culture surfaces
- Specific surface marker expression:
  positive for CD73, CD90 and CD105,
  negative for CD45
- Differentiation potential to differentiate into the adipogenic, chondrogenic and osteogenic lineage

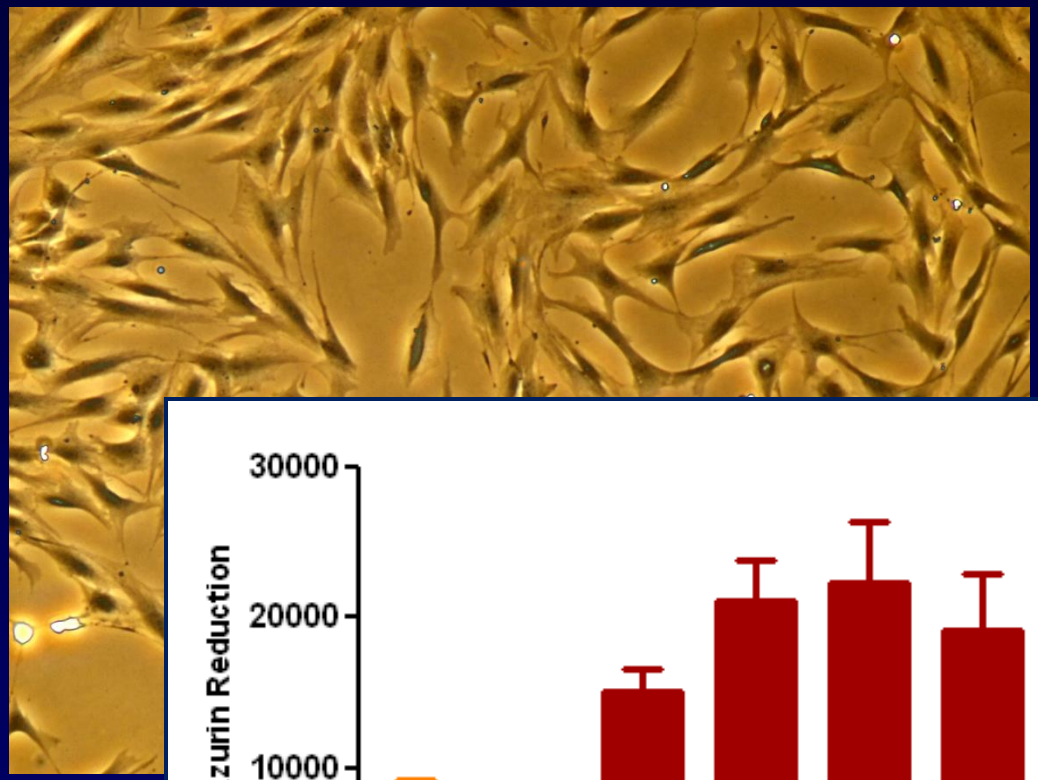
Dominici et al., 2006

Can stem cells be cultured in the presence of platelet lysates ?

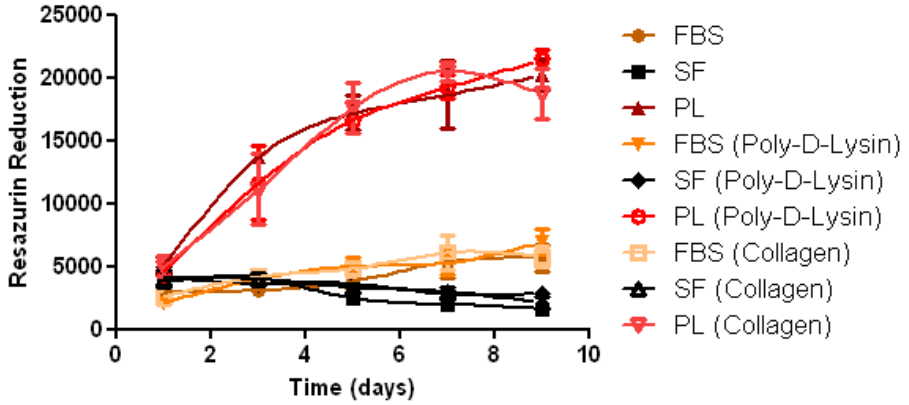


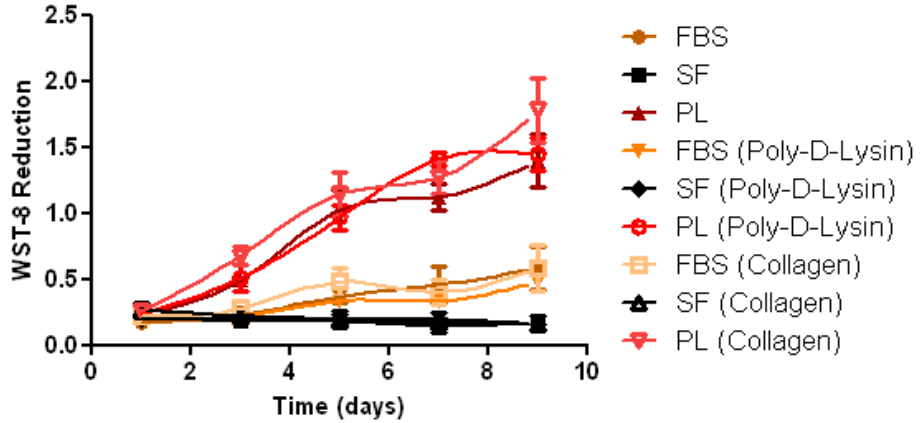
FBS

PL



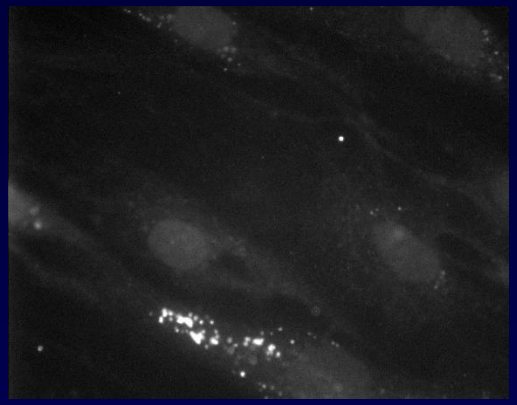
Adipose-derived Stem Cells (ADSC)



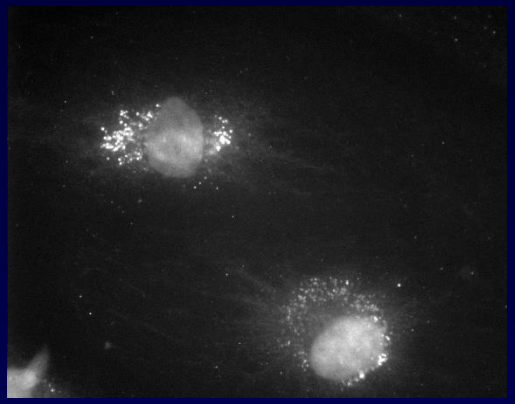


Can stem cells be kept in undifferentiated state ?

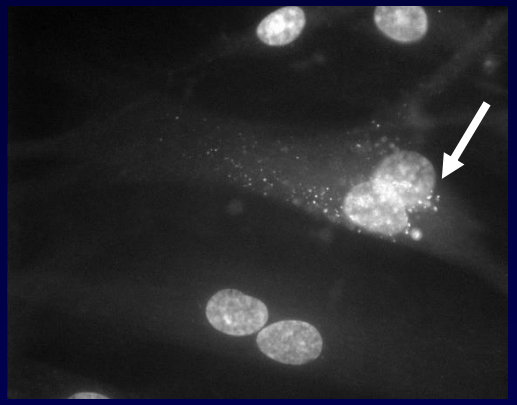
CD 73



10% FBS

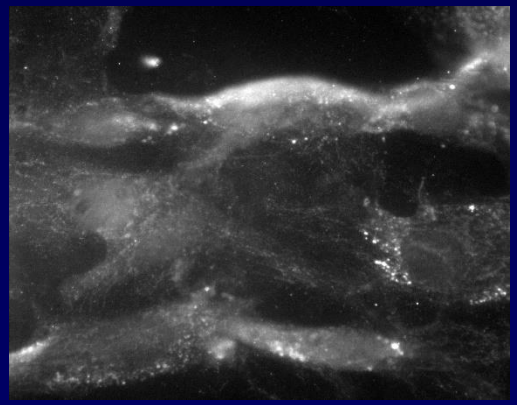


SF

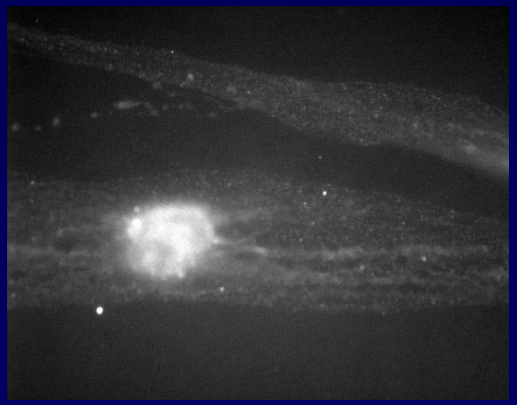


5% PL

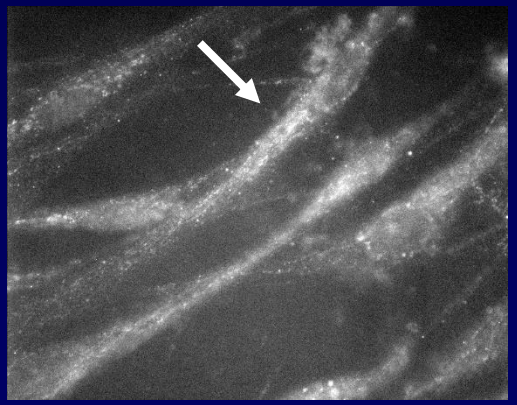
CD 90



10% FBS



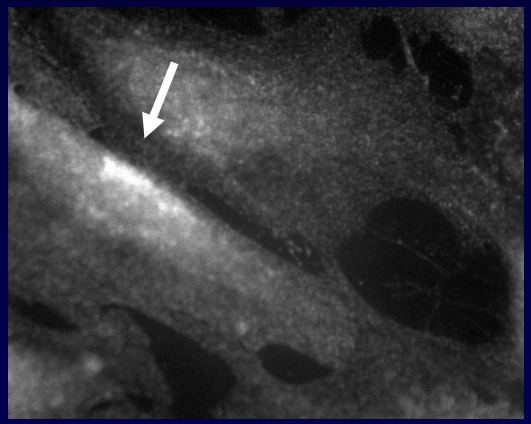
SF



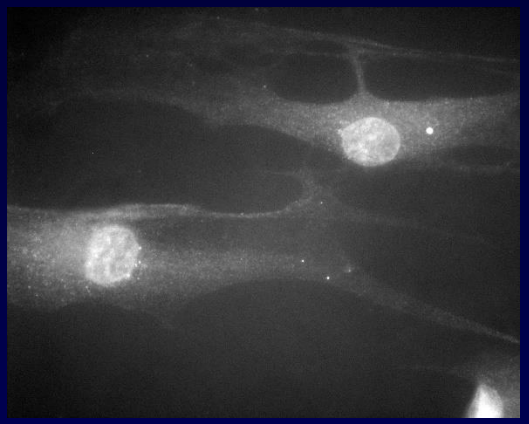
5% PL

Can stem cells be kept in undifferentiated state ?

CD 105



10% FBS



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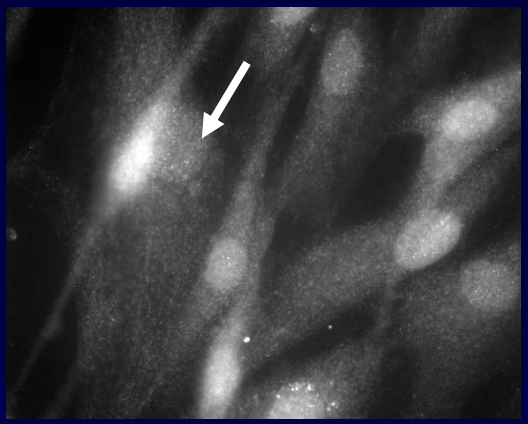
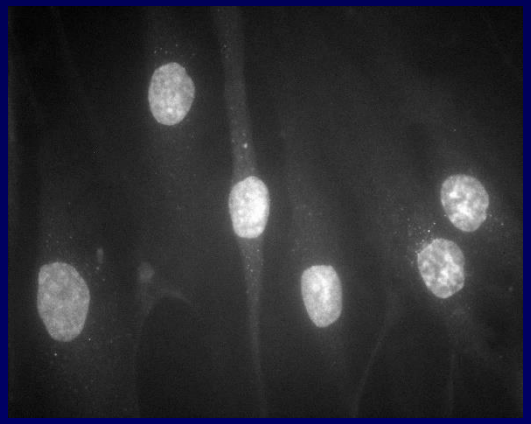
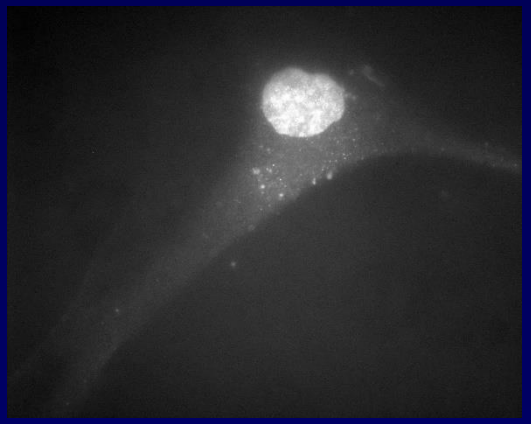


5% PL

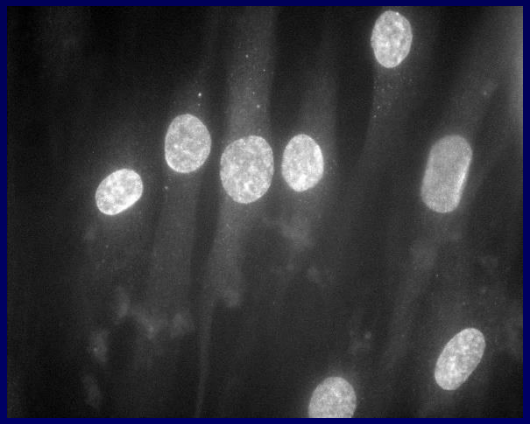
CD 45



10% FBS



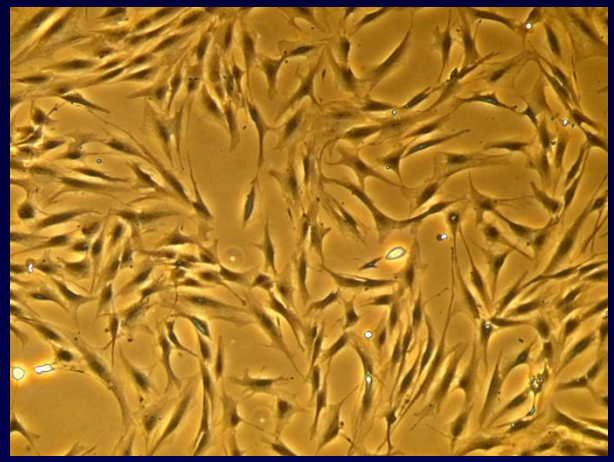
SF

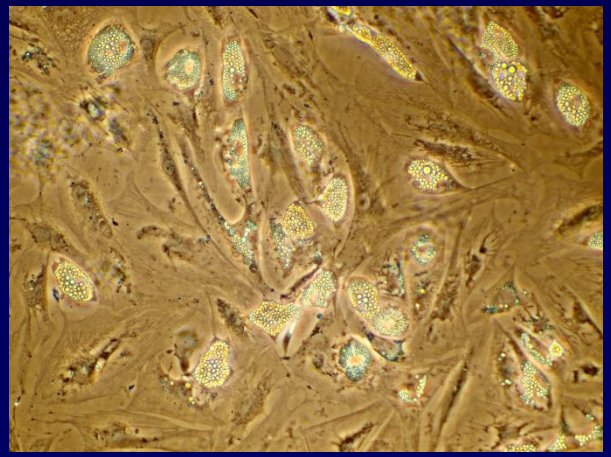


5% PL

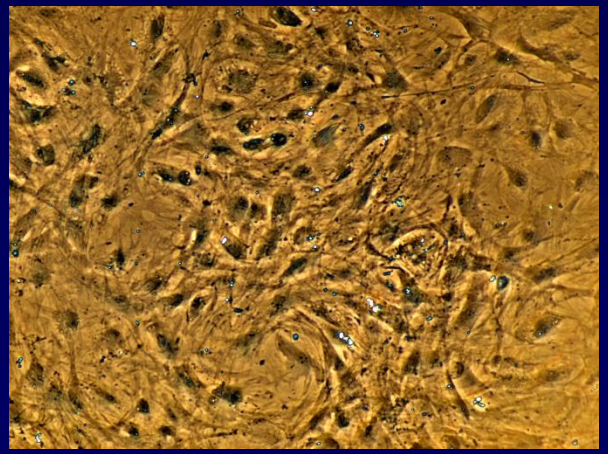
Can stem cells be triggered to differentiate into specific lineages ?

Differentiation potential of ADSC into specific lineages

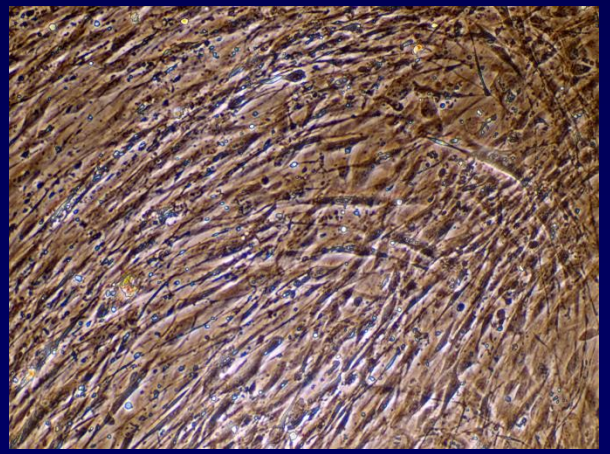




adipocytes



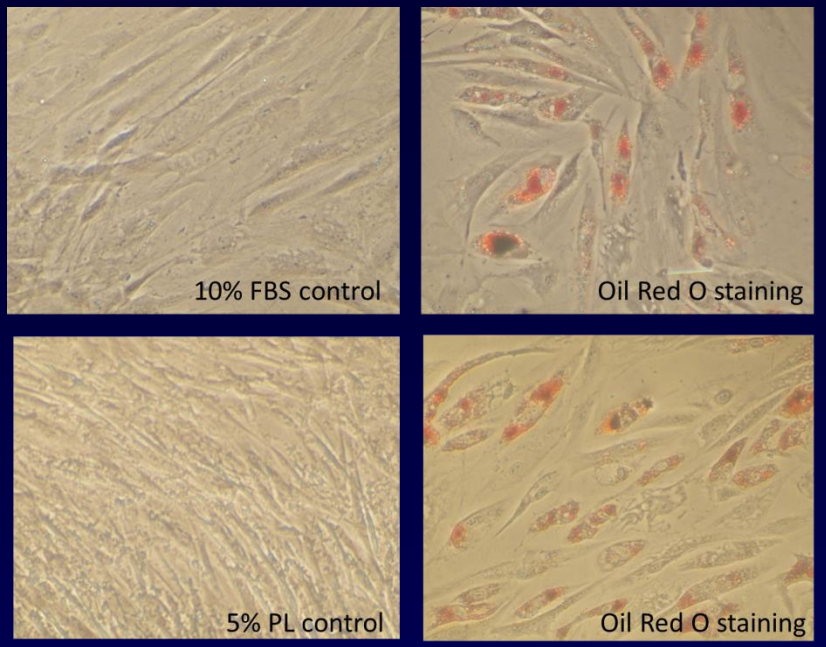
chondrocytes
(and aggregates)



osteoblasts

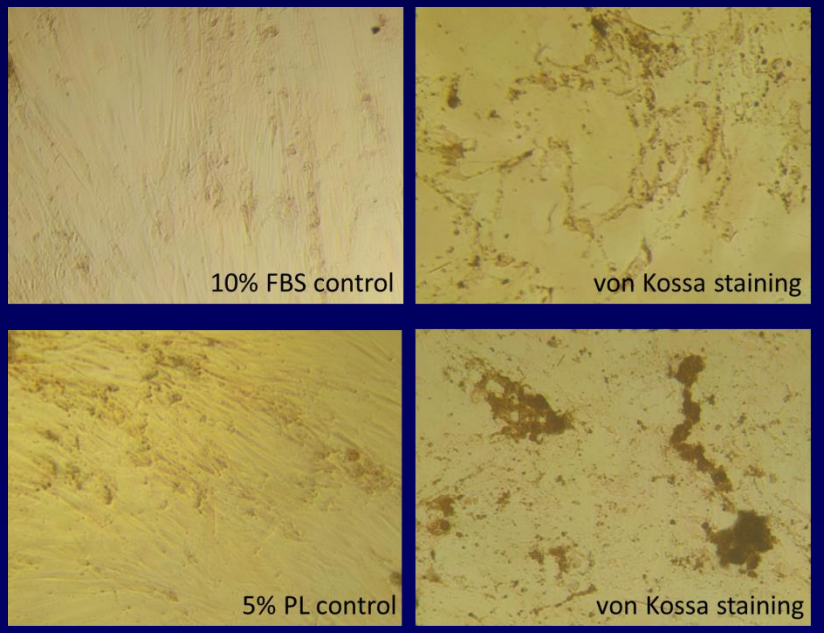
adipocyte differentiation

Oil Red O staining of
lipid droplets



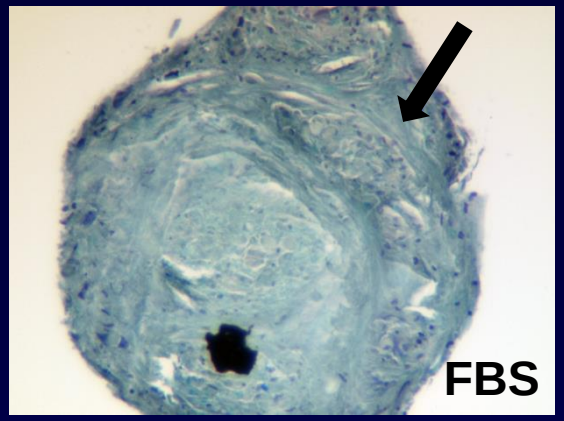
osteoblast differentiation

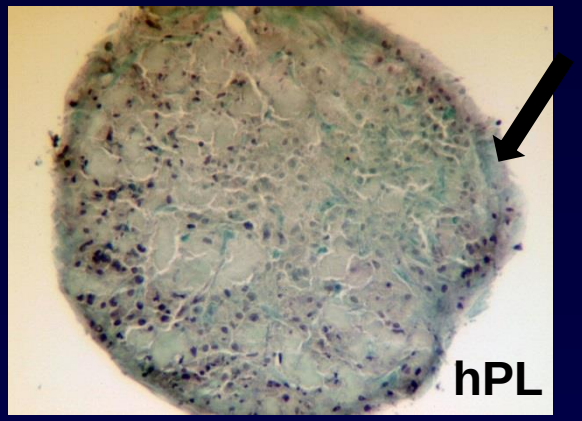
von Kossa staining of
calcium deposits



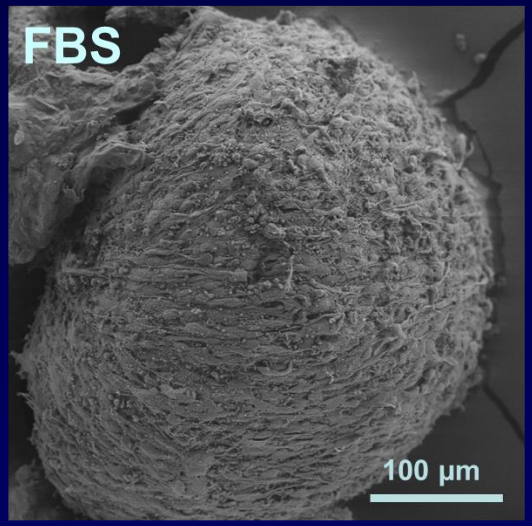
chondrogenic aggregates
semi-thin sections

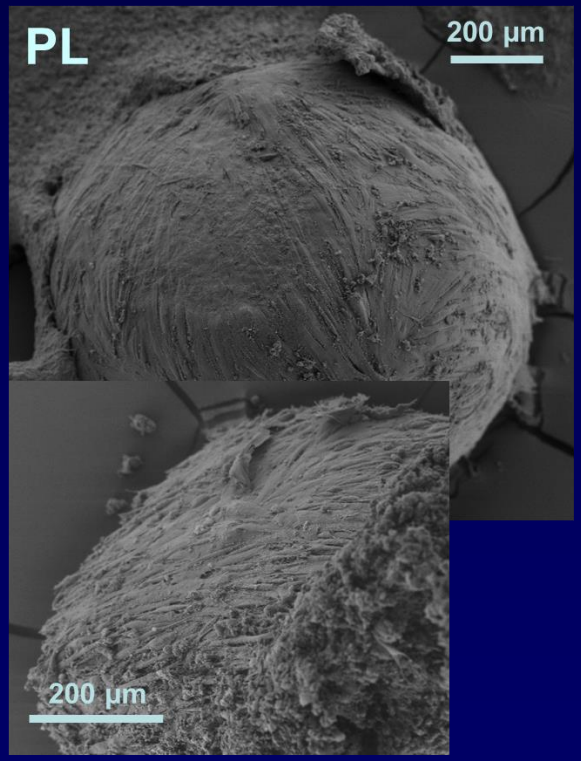
Toluidine Blue staining of proteoglycans





scanning electron micrographs





Summary and Conclusion (II)

Human platelet lysates have been tested as an alternative to FBS to ensure safe and animal-derived component-free cell culture conditions for human mesenchymal stem cells.

Cell morphology and proliferation assays confirmed the growth promoting effect of platelet lysates, comparable to high FBS.

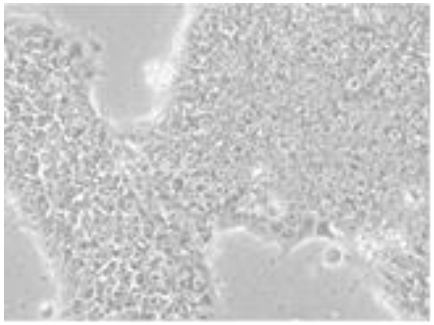
The cells also kept their undifferentiated state as proofed by the expression of the positive markers CD73, CD90, and CD105. Cells were negative for CD45.

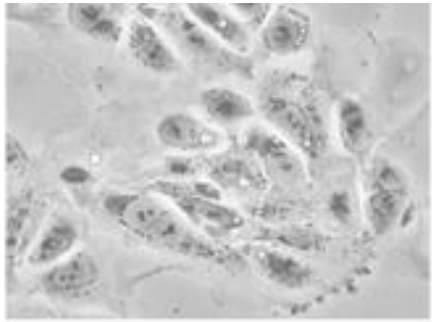
Furthermore, adipose-derived stem cells (ADSC) retained their full differentiation potential in platelet lysate-supplemented media.

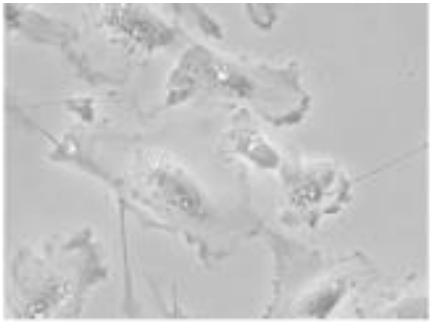
ADSC could be triggered to differentiate towards the adipogenic, chondrogenic, and osteogenic tissue lineage.

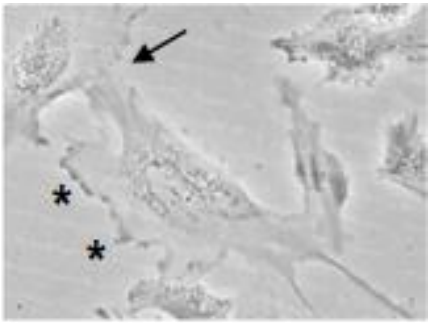
Differentiation of iPSC into Functional Podocytes

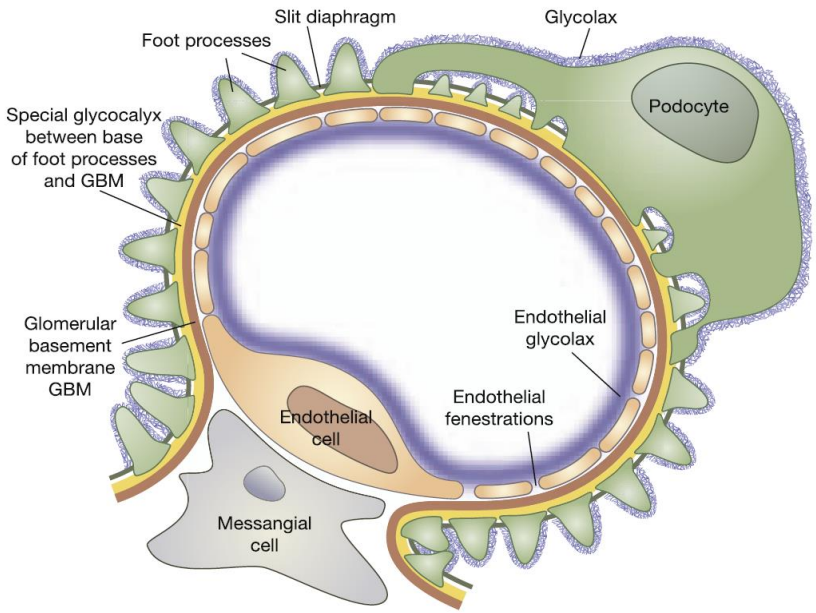
undifferentiated iPSC **day 1** **day 3** **day 10**

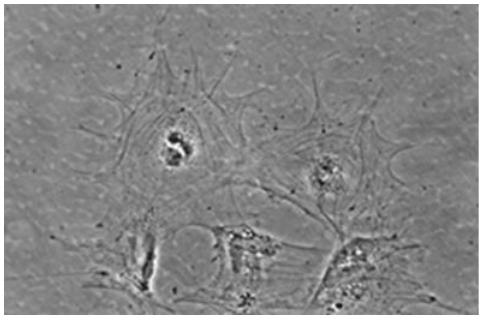


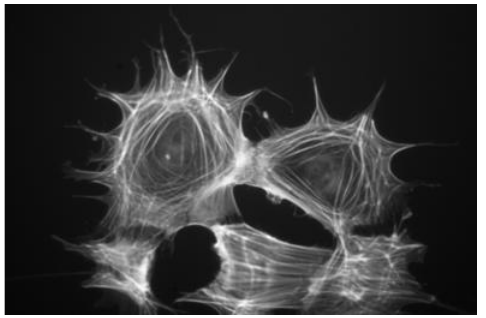


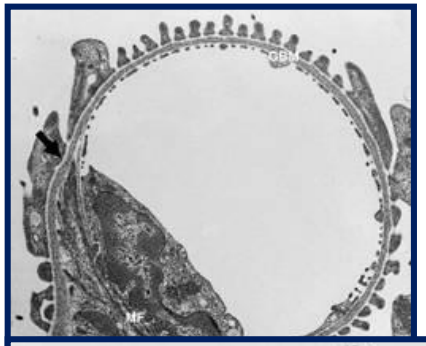


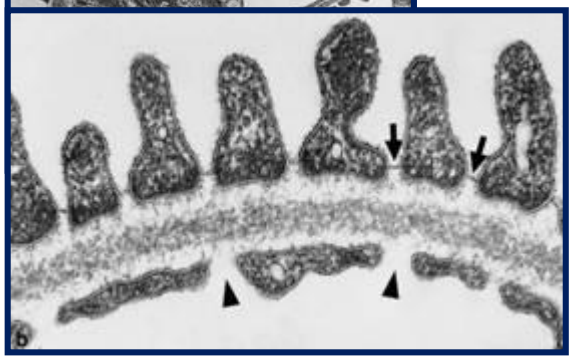


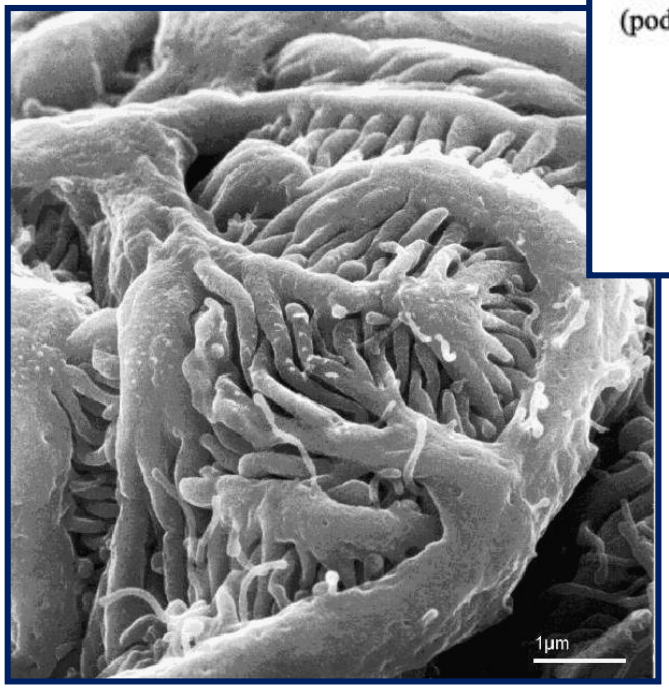


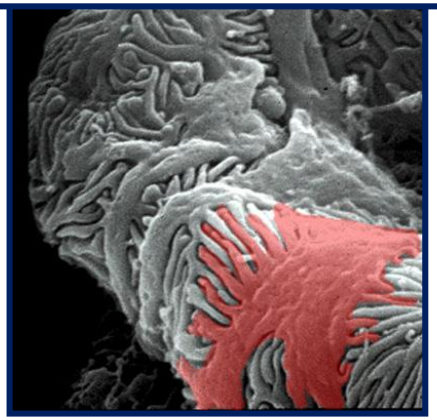


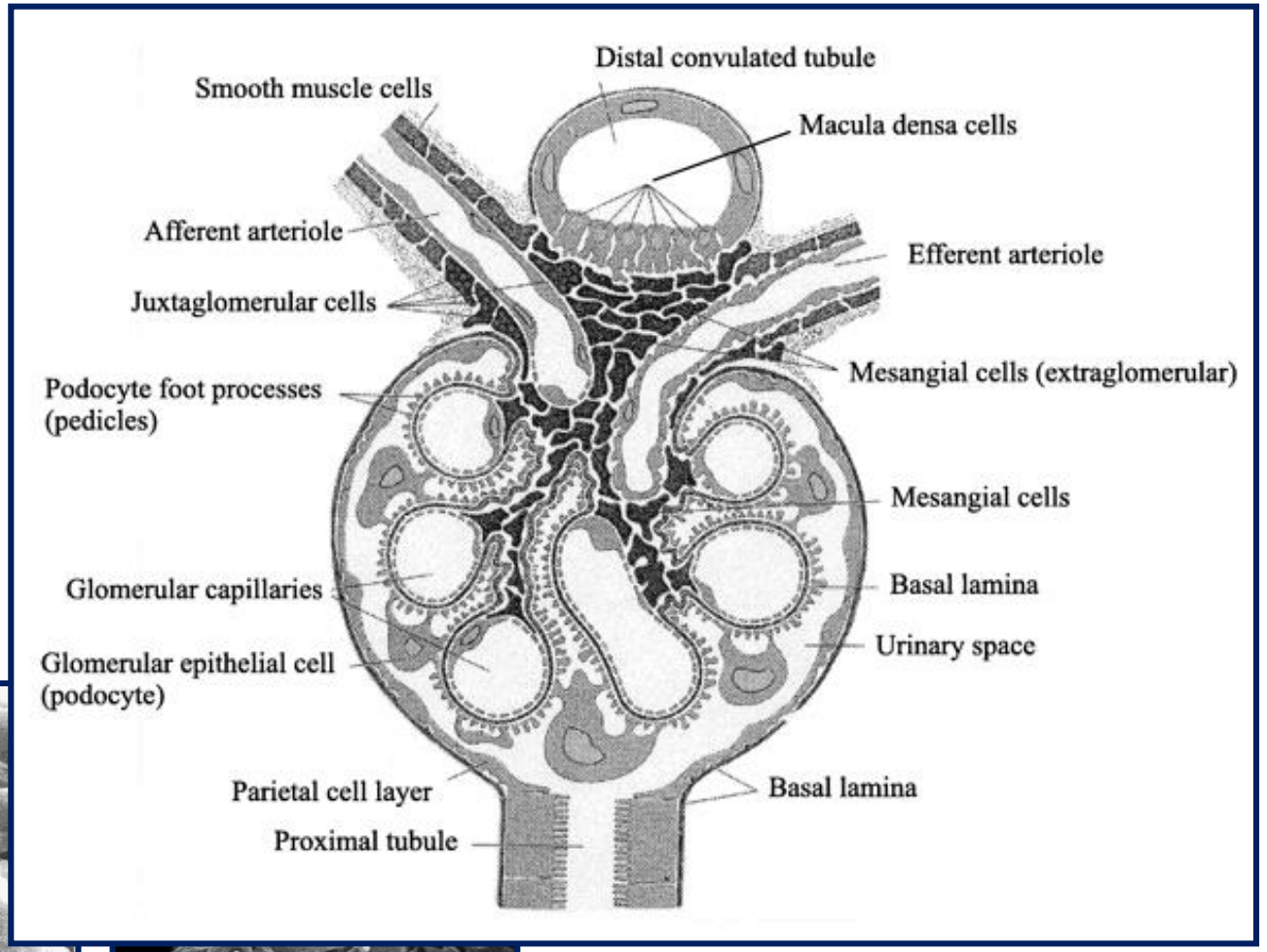




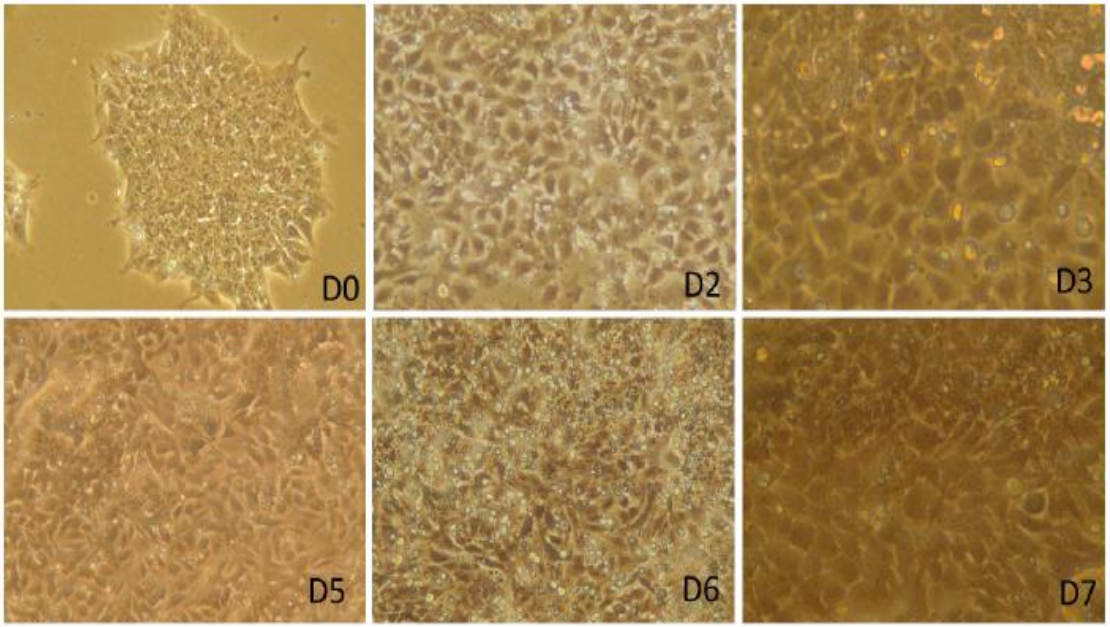


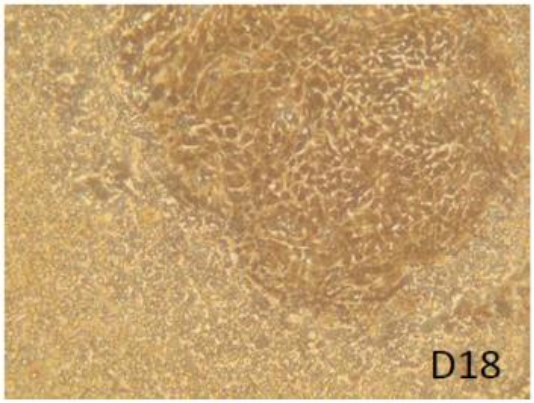


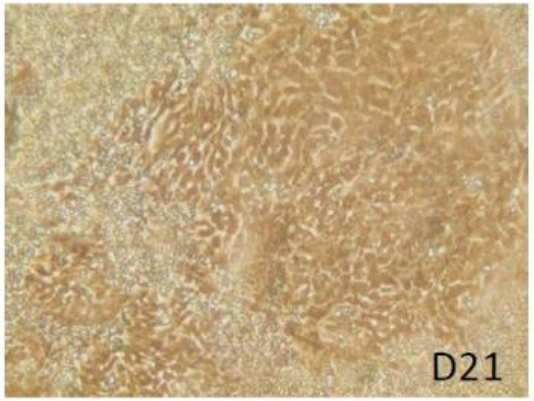


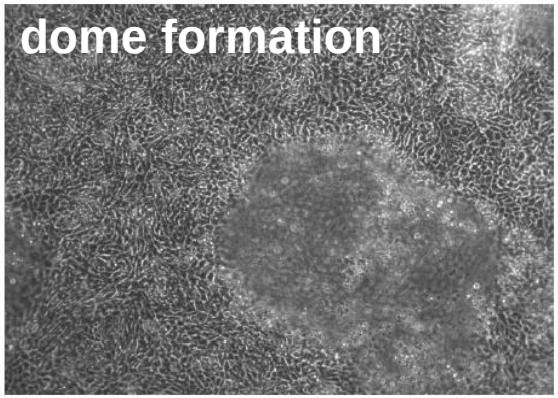


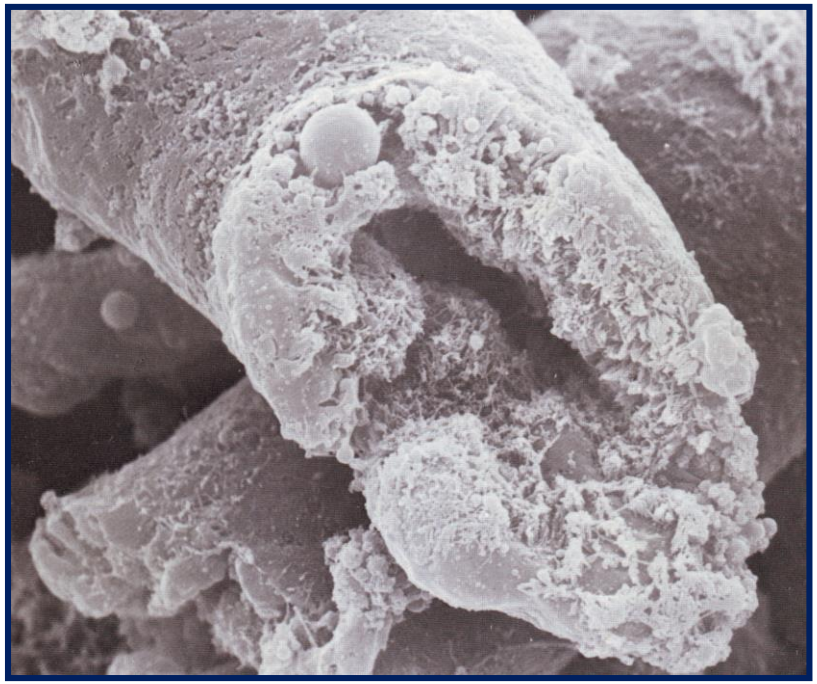
Differentiation of iPSC into Renal Proximal Tubular Cells

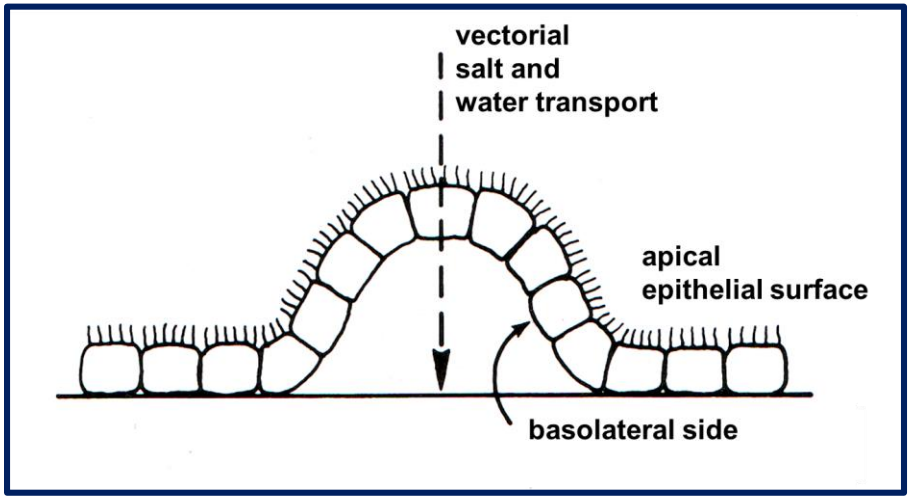


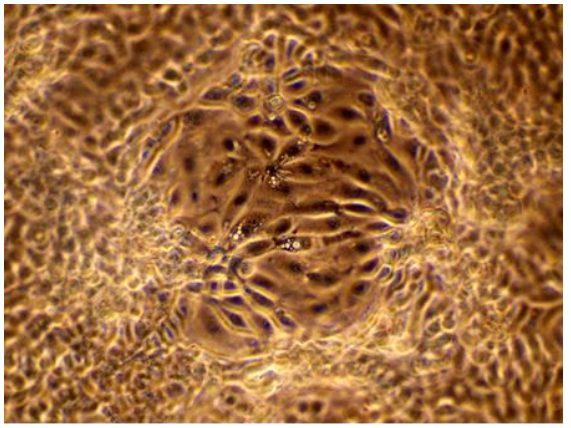


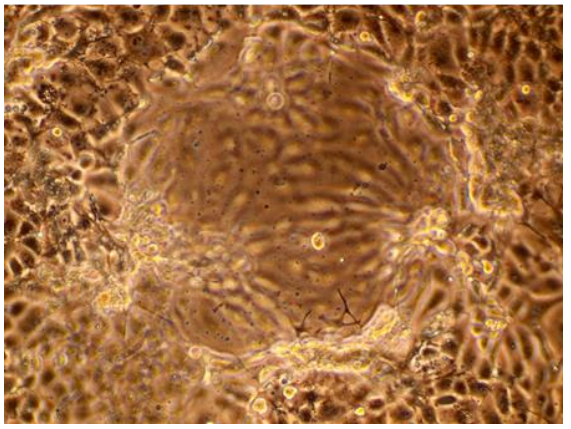


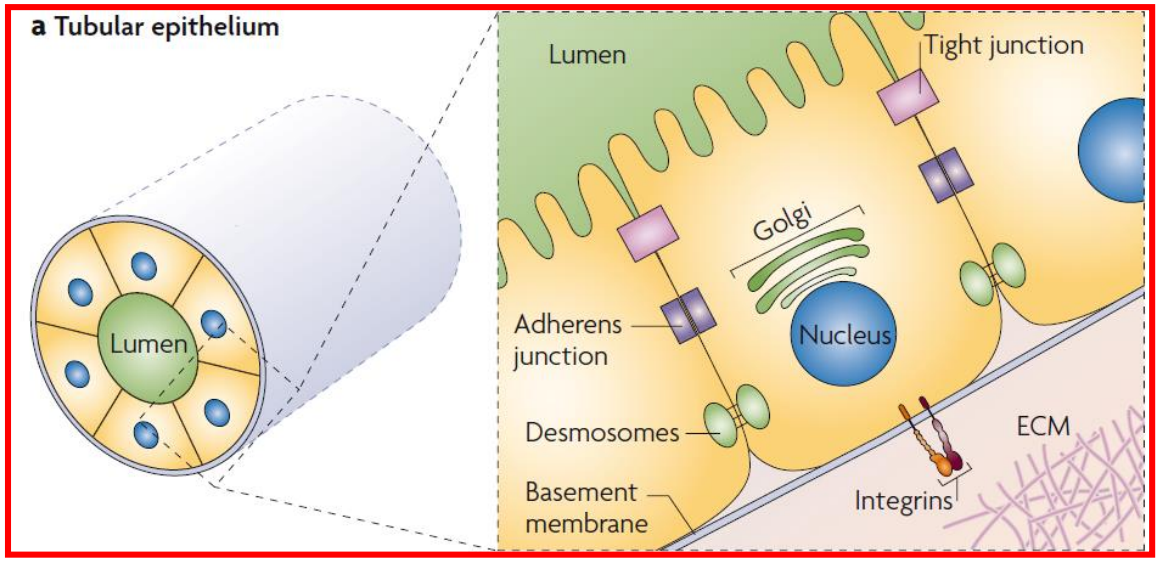


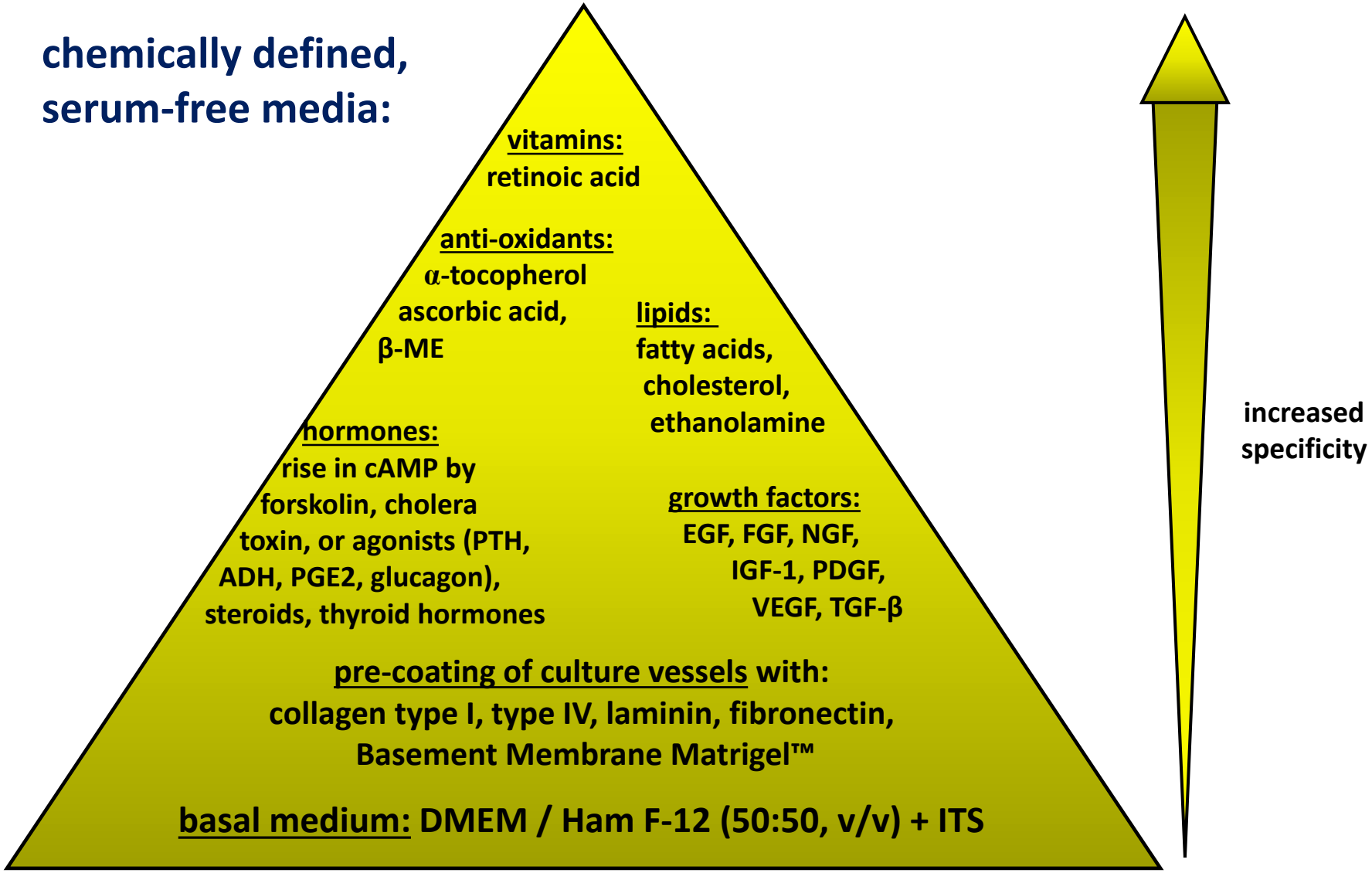












van der Valk et al., Toxicol. in Vitro 24: 1053-1063, 2010

ALTEX *35:* 99-118, 2018

Consensus Report

Fetal Bovine Serum (FBS): Past – Present – Future

Jan van der Valk[1], Karen Bieback[2], Christiane Buta[3], Brett Cochrane[4], Wilhelm G. Dirks[5], Jianan Fu[6], James J. Hickman[7], Christiane Hohensee[8], Roman Kolar[9], Manfred Liebsch[10], Francesca Pistollato[11], Markus Schulz[12], Daniel Thieme[13], Tilo Weber[9], Joachim Wiest[14], Stefan Winkler[15] and Gerhard Gstraunthaler[16]

Summary

The supplementation of culture medium with fetal bovine serum (FBS, also referred to as "fetal calf serum") is still common practice in cell culture applications. Due to a number of disadvantages in terms of quality and reproducibility of *in vitro* data, animal welfare concerns, and in light of recent cases of fraudulent marketing, the search for alternatives and the development of serum-free medium formulations has gained global attention. Here, we report on the 3rd Workshop on FBS, Serum Alternatives and Serum-free Media, where regulatory aspects, the serum dilemma, alternatives to FBS, case-studies of serum-free *in vitro* applications, and the establishment of serum-free databases were discussed.

The whole process of obtaining blood from a living calf fetus to using the FBS produced from it for scientific purposes is *de facto* not yet legally regulated despite the existing EU-Directive 2010/63/EU on the use of animals for scientific purposes. Together with the above-mentioned challenges, several strategies have been developed to reduce or replace FBS in cell culture media in terms of the 3Rs (Refinement, Reduction, Replacement). Most recently, releasates of activated human donor thrombocytes (human platelet lysates) have been shown to be one of the most promising serum alternatives when chemically-defined media are not yet an option. Additionally, new developments in cell-based assay techniques, advanced organ-on-chip and microphysiological systems are covered in this report. Chemically-defined serum-free media are shown to be the ultimate goal for the majority of culture systems, and examples are discussed.

ATLA 45, 329–332, 2017

ATLA Alternatives to Laboratory Animals

Comment

Fetal Bovine Serum (FBS) — A Pain in the Dish?

Jan van der Valk and Gerhard Gstraunthaler

The use of Fetal Bovine Serum in replacement alternative methods is associated with serious animal welfare concerns, as well as worrying reproducibility issues

Thank you !