Cattedra di
Endocrinologia

Modelli animali Cre-LoxP e sfingosina 1-fosfato

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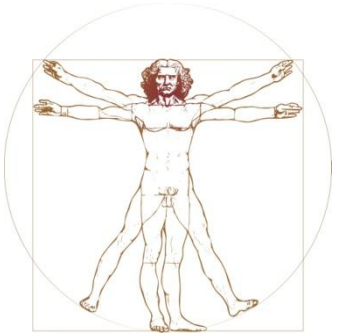
Di cosa parleremo…

- Il topo, questo sconosciuto..
- Progetto di ricerca
- Modelli condizionali
- Risultati preliminari
- Prospettive

Of mice and men…



Topo

- 2,5 Gb
- 19 coppie di autosomi + XY
- ≈ 25-30 x 10^3 geni
- ≈ 27-29 x 10^3 proteine

Uomo

- 2,9 Gb
- 22 coppie di autosomi + XY
- ≈ 30 x 10^3 geni
- ≈ 40 x 10^3 proteine

≈ 40% identità (allineamento sequenze)

≈ 99% geni murini hanno un corrispettivo umano (omologhi)

≈ 96% sintenia e 80% “best match” (ortologhi)

The mouse genome. *Nature* **420**, 520-562 (5 December 2002)

Circa il 95% degli animali utilizzati nella ricerca biomedica è rappresentato da roditori:

topi e ratti	90 %
criceti	2%
cavie	2%
altre specie	1%

ALTRE SPECIE UTILIZZATE NELLA SPERIMENTAZIONE

Mammiferi

- Primati non umani
  - *Macaca mulatta* (scimmia Rhesus)
  - *Macaca fascicularis* (Cynomolgus)
  - *Callithrix* (marmoset)
  - *Pan troglodytes* (scimpanzé)
- Cane *(Canis familiaris)*
- Gatto *(Felis catus)*
- Furetto (*Mustela putorius)*
- Maiale *(Sus scrofa)*
  - mini- e micro-pig
- Pecora *(Ovis aries)*
- Capra *(Capra hircus)*

PERCHE' I RODITORI?

- ✓ Facilità di mantenimento e manipolazione
- ✓ Elevata capacità riproduttiva
- ✓ Tempo di generazione e durata della vita relativamente brevi
- ✓ Disponibilità di dati di base sulle specie roditrici
- ✓ Disponibilità di numerosi ceppi e linee ben definiti
- ✓ Possibilità per alcune specie di adottare tecniche di produzione *germ-free* o *pathogen-free*
- ✓ Possibilità di generare numerosi modelli di malattia rilevanti per la ricerca biomedica

TECNICHE DI ALLEVAMENTO

- Linee INBRED

TECNICHE DI ALLEVAMENTO

Topo C57BL/6

➔ Linee INBRED

- Incroci tra fratello e sorella (o genitori e figli) per > 20 generazioni consecutive
- Identità genica pari a circa il 98%
- Caratteristiche di istocompatibilità
- Necessità di programmare e registrare gli incroci in modo rigoroso
- Necessità di pianificare un corretto programma di monitoraggio genico per il controllo delle caratteristiche della linea

TECNICHE DI ALLEVAMENTO

- ➔ Linee INBRED
- ➔ Linee OUTBRED

TECNICHE DI ALLEVAMENTO

➔ Linee INBRED

➔ Linee OUTBRED

Topo CD-1

- Accoppiamenti random
- Eliminare il più possibile ogni grado di parentela
- Animali con max variabilità genica osservabile in una popolazione
- Le diverse caratteristiche degli animali sono un ottimo modello per studi applicati ad animali eterogenei
- Progenie molto robusta e numerosa

DATI BIOLOGICI DI BASE DEL TOPO

Adult body weight	20-40gm
Life Span	1.5-3 years
Chromosome n. (2n)	40
Food consumption	15 g/100 g/day
Water consumption	15 ml/100 g/day
Breeding onset	50 days
Gestation Period	19-21 days
Weaning	21-28 days
Body Temperature	36.5-38.0 °C
Heart rate	300-800 bpm
Respiratory Rate	84-230 per minute
Blood volume	76-80 ml/kg → 20g ≈ 1,5 ml

WORKING ON MOUSE MODELS…..

Breeding:

1. Wild-type mice: **C57BL/6J** ***(Jax n°: 000664)***
2. "Athero-prone" model: **LDLR-/-** ***(Jax n°: 002207)***
3. Macrophage-specific *Cre* strain: **Lyz-*Cre*** ***(Jax n°: 004781)***
4. Endothelium-specific *Cre* strain: **Cad-*Cre*** ***(Jax n°: 006137)***

Conditional knock-in *LoxP* ("*floxed*") strains:

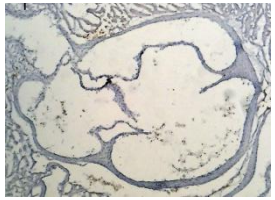
5. **Lox-STOP-Lox-$S1P_1R$**
6. **Lox-STOP-Lox-$S1P_3R$**

C57BL/6J background

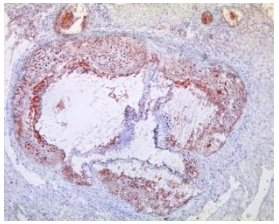
?

Hosted in a Specified Pathogen Free (SPF) animal facility (CSSI, *Biostab – Unimore*)

Perché C57BL/6J come background?



Suscettibilità all'aterosclerosi



- BALB/cJ, C3H/J, A/J, SWR/J, NZB/J < 129/J, AKR/J, DBA/2J, < C57L/J < **C57BL/6J**

  (Paigen B et al, Atherosclerosis. 1985 Oct;57(1):65-73.)

- C57BL/6J sviluppano aterosclerosi solo in seguito a somministrazione di diete "estreme" (1,25% cholesterol; 21% fat; 0,5% cholate) e per lungo tempo (> 16 weeks)

Modelli geneticamente modificati: **ApoE$^{-/-}$** e **LDL-R$^{-/-}$**

University of Modena and Reggio Emilia

FIRB-IDEAS Project RBID08777T

"Diagnostic and therapeutic potential of lysosphingolipids and their mimetics in the atherosclerosis "

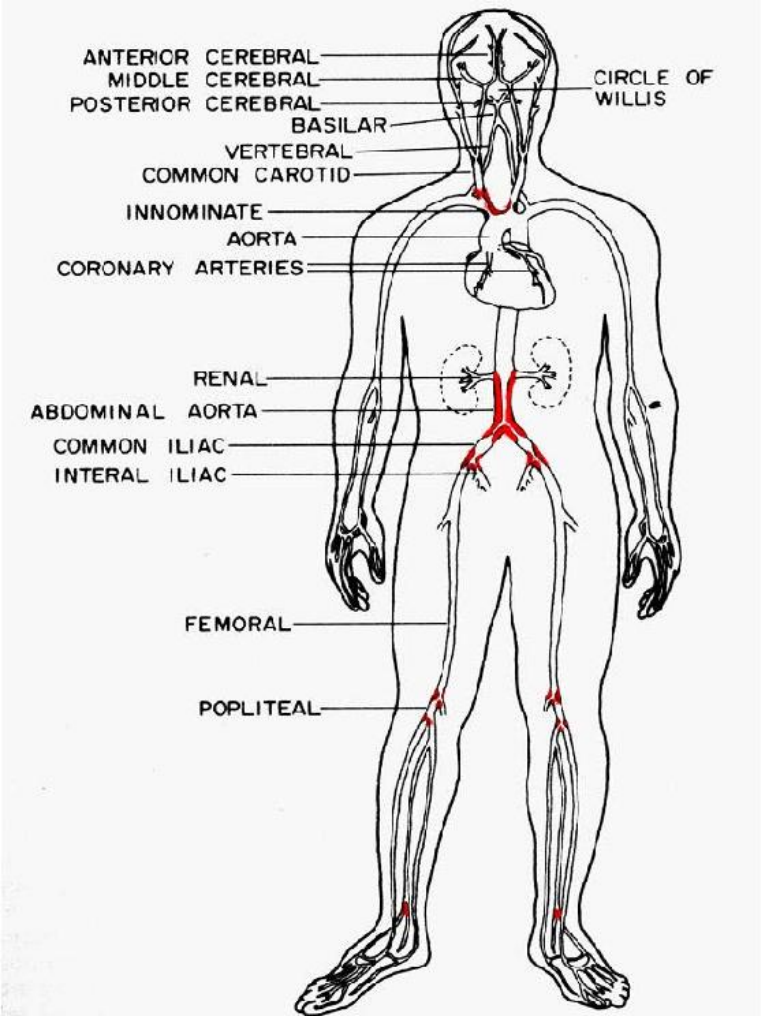
Prof.ssa Manuela Simoni

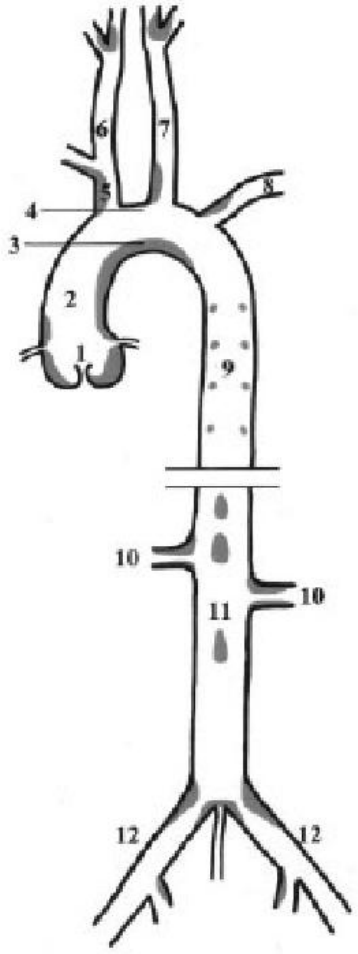
Università di Modena e Reggio Emilia

Prof. Jerzy-Roch Nofer

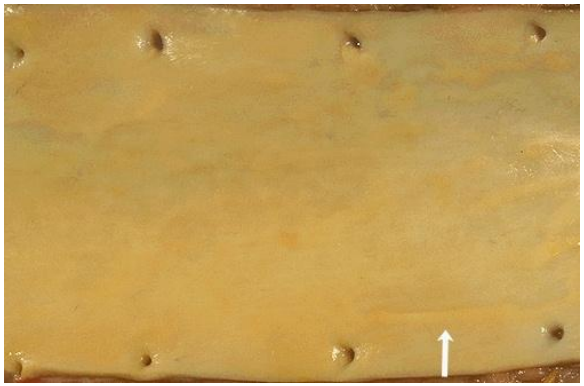
University Hospital of Münster (Germany)

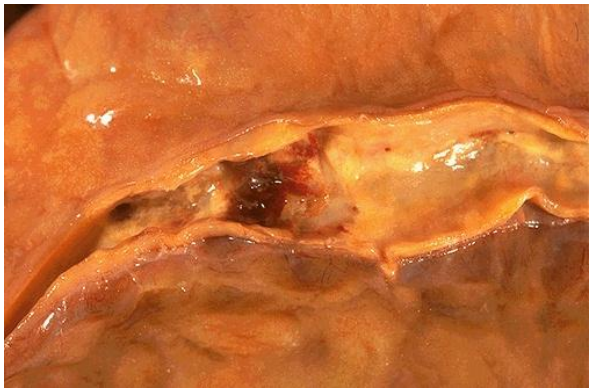
Aterosclerosi





Aterosclerosi





ENDOTHEHELIAL DYSFUNCTION

Initial lesion
- histologically "normal"
- macrophage infiltration
- isolated foam cells

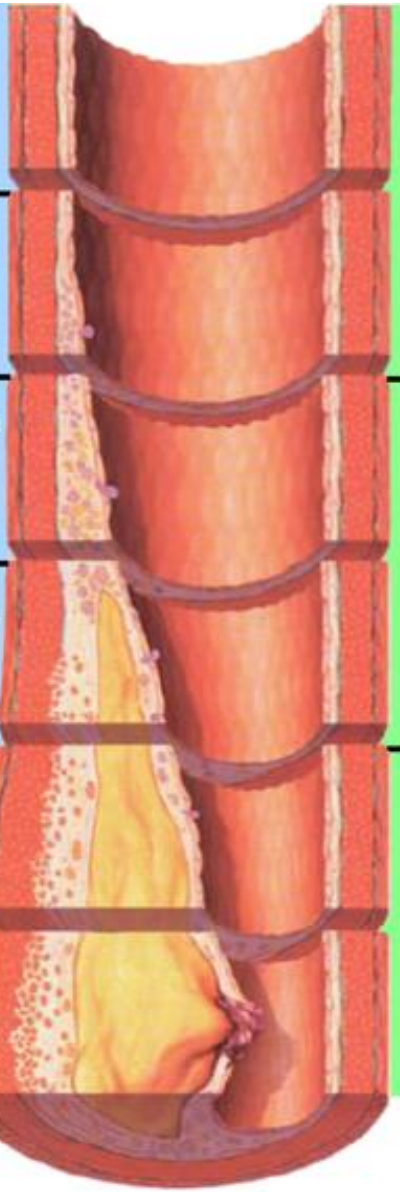
Fatty streak
mainly intracellular lipid accumulation

Intermediate lesion
- intracellular lipid accumulation
- small extracellular lipid pools

Atheroma
- intracellular lipid accumulation
- core of extracellular lipid

Fibroatheroma
- single or multiple lipid cores
- fibrotic/calcific layers

Complicated lesion
- surface defect
- hematoma-hemorrhage
- thrombosis



from first decade

from third decade

from fourth decade

growth mainly by lipid addition

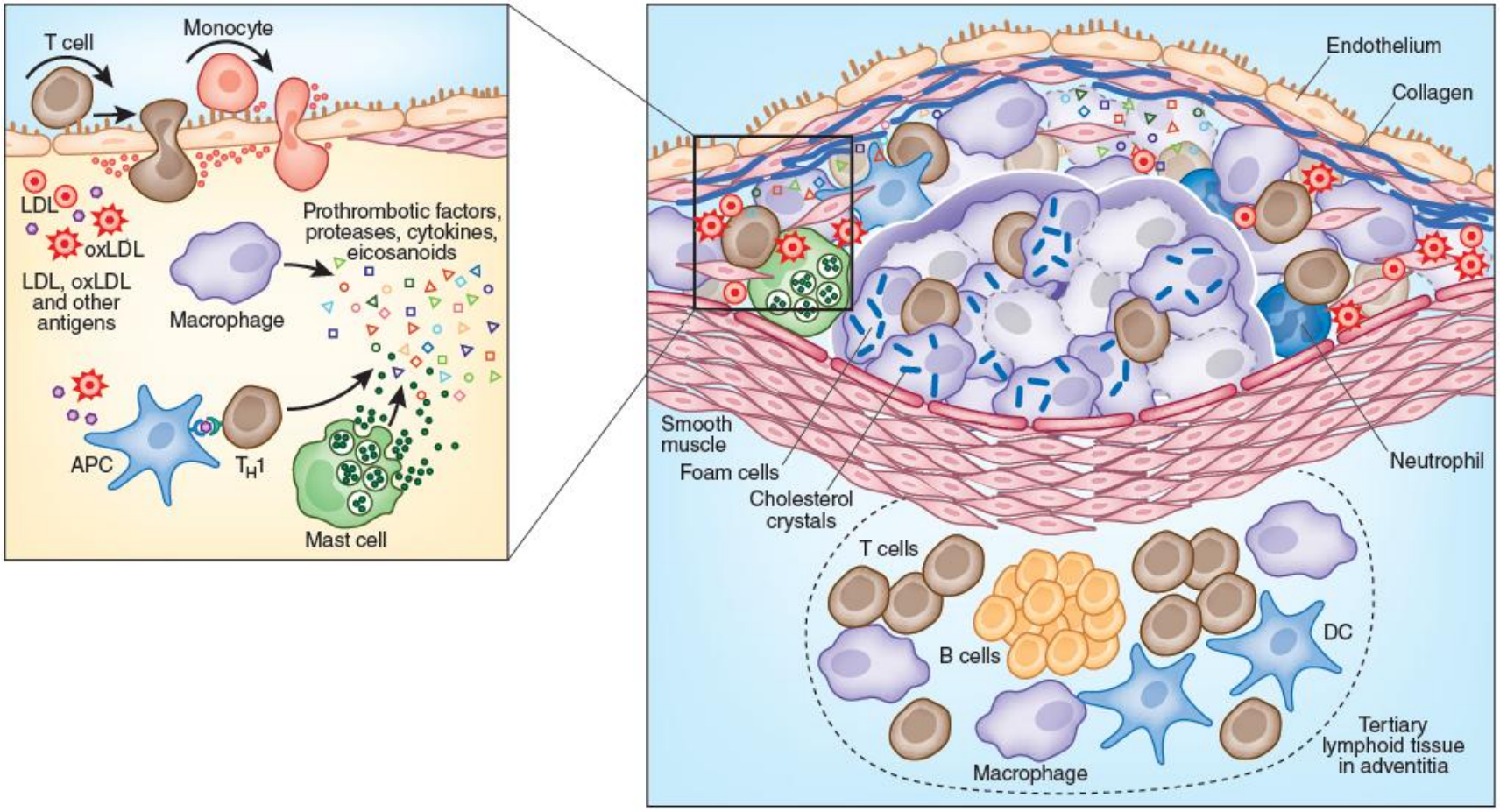
increased smooth muscle and collagen increase

thrombosis and/or hematoma

clinically silent

clinically silent or overt

Immune components of the atherosclerotic plaque

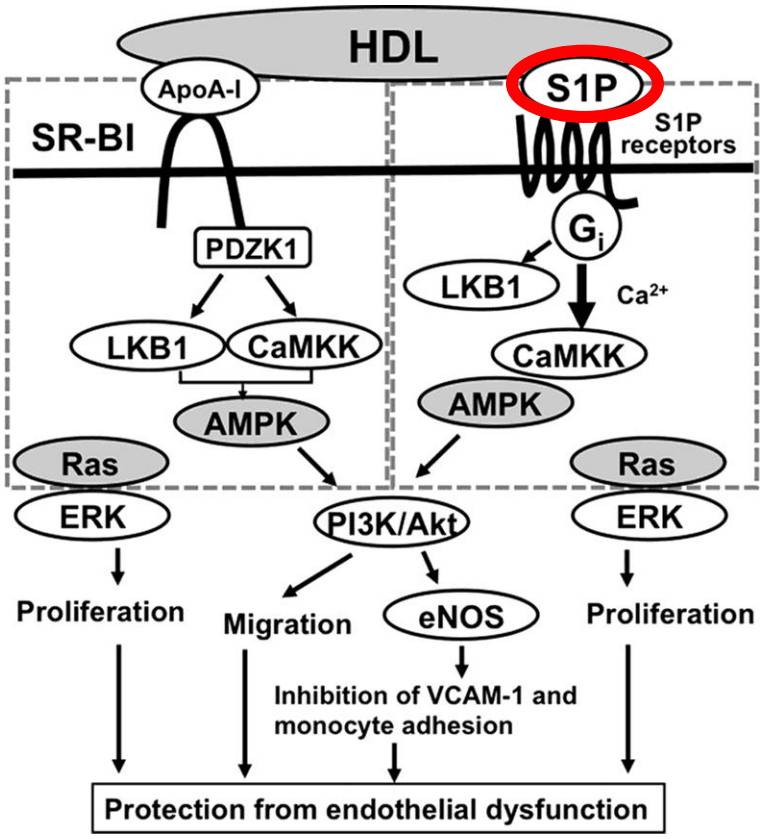


Meccanismi protettivi ed approcci terapeutici

Endogeni ⇨
- **High Density Lipoproteins (HDL)**
  - Trasporto inverso del colesterolo (RCT)
  - Proprietà antiinfiammatorie

Farmaci ⇨
- **Statine**
- …

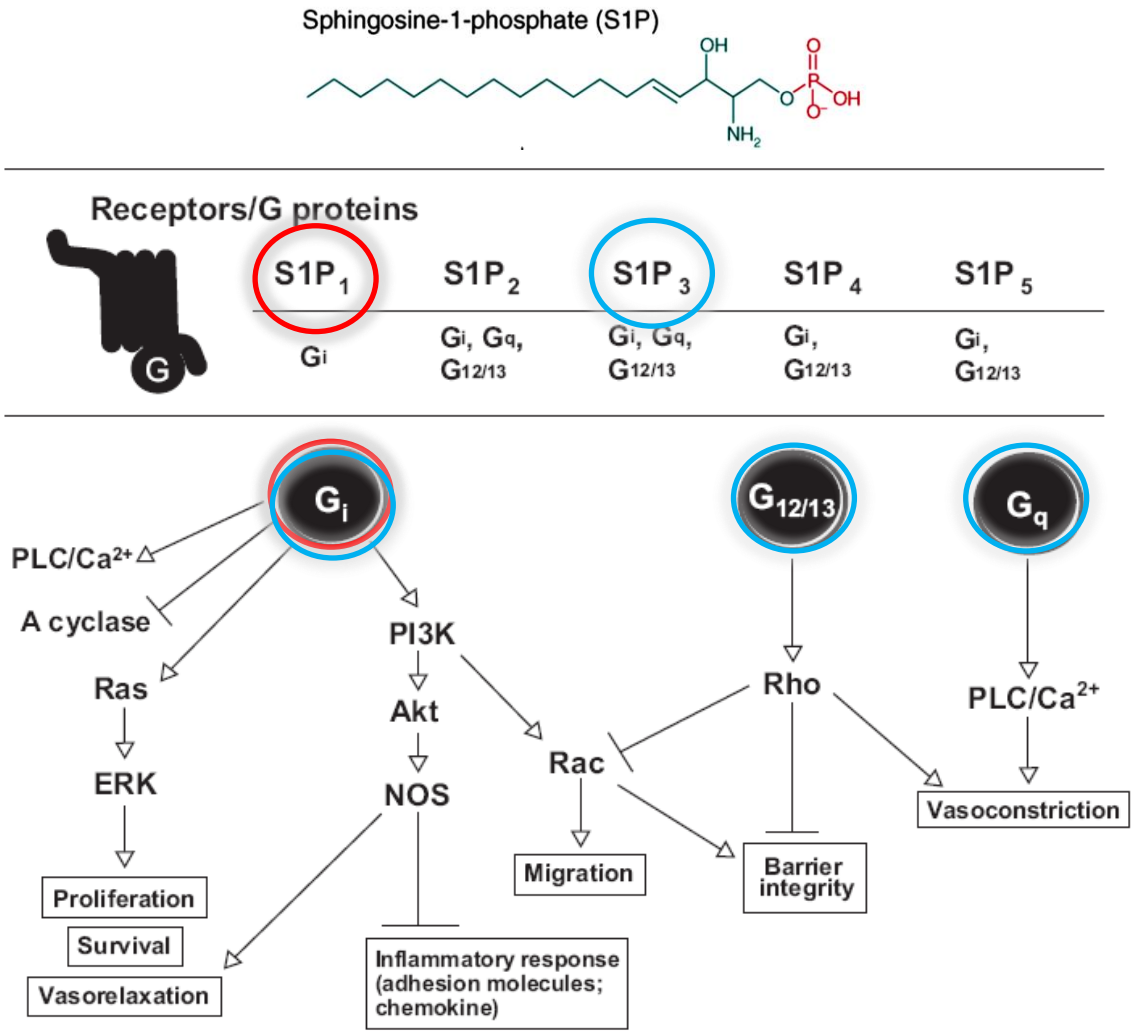
S1P mediated effects relevant to atheroprotection



- inhibition of endothelial apoptosis
- downregulation of the expression of adhesins (VCAM-1, ICAM-1)
- inhibition of monocyte adhesion
- stimulation of NO and PGI2 production → vasorelaxation
- inhibition of NADPH oxidase and ROS production
- secretion of proinflammatory cytokines and chemokines by activated SMCs.

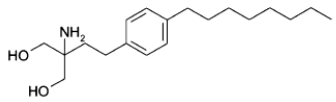
Kimura T et al. J. Biol. Chem. 2010;285:4387-4397

S1P and S1P-receptors (S1PRs)



$S1P_{1\text{-}2\text{-}3}$: lymphocytes, monocytes/macrophages, EC, VSMC

Immunosuppressive effects through S1PRs stimulation



FTY 720

(not selective $S1P_{1\text{-}3\text{-}4\text{-}5}$ agonist)

New drug for the multiple sclerosis therapy (Gilenya®)

Okajima F. *et al*. Endocr. J. 2009

Main topics of the project:

1. SISTEMIC INTERFERENCE WITH S1P SIGNAL TRANSDUCTION THROUGH

PHARMACOLOGICAL INTERVENTION

LDL-R -/- mice

2. PRODUCTION OF **CONDITIONAL AND TISSUE-SPECIFIC EXPRESSING MOUSE MODELS**

Strain Name: B6.129S7-*Ldlr*^{tm1Her}/J

Stock Number: 002207 Place order

Availability: Level 2

Mice homozygous for the *Ldlr*^{tm1Her} mutation have an elevated serum cholesterol level of 200-400 mg/dl and they have very high levels (>2,000 mg/dl) when fed a high fat diet. Normal serum cholesterol in the mouse is 80-100 mg/dl.

Strain Information

View larger image

Type Congenic; Mutant Strain; Targeted Mutation;
Additional information on Genetically Engineered and Mutant Mice.
Visit our online *Nomenclature tutorial*.
Additional information on Congenic nomenclature.

Mating System Homozygote x Homozygote (Female x Male) 01-MAR-06

Breeding Considerations This strain is a good breeder.

Species laboratory mouse

Background Strain C57BL/6J

Donor Strain 129S7 via AB1 ES cell line (+*Hprt-bm2*)

Generation N13F14 (17-SEP-12)
Generation Definitions

Donating Investigator IMR Colony, The Jackson Laboratory

Appearance
black
Related Genotype: *a/a*

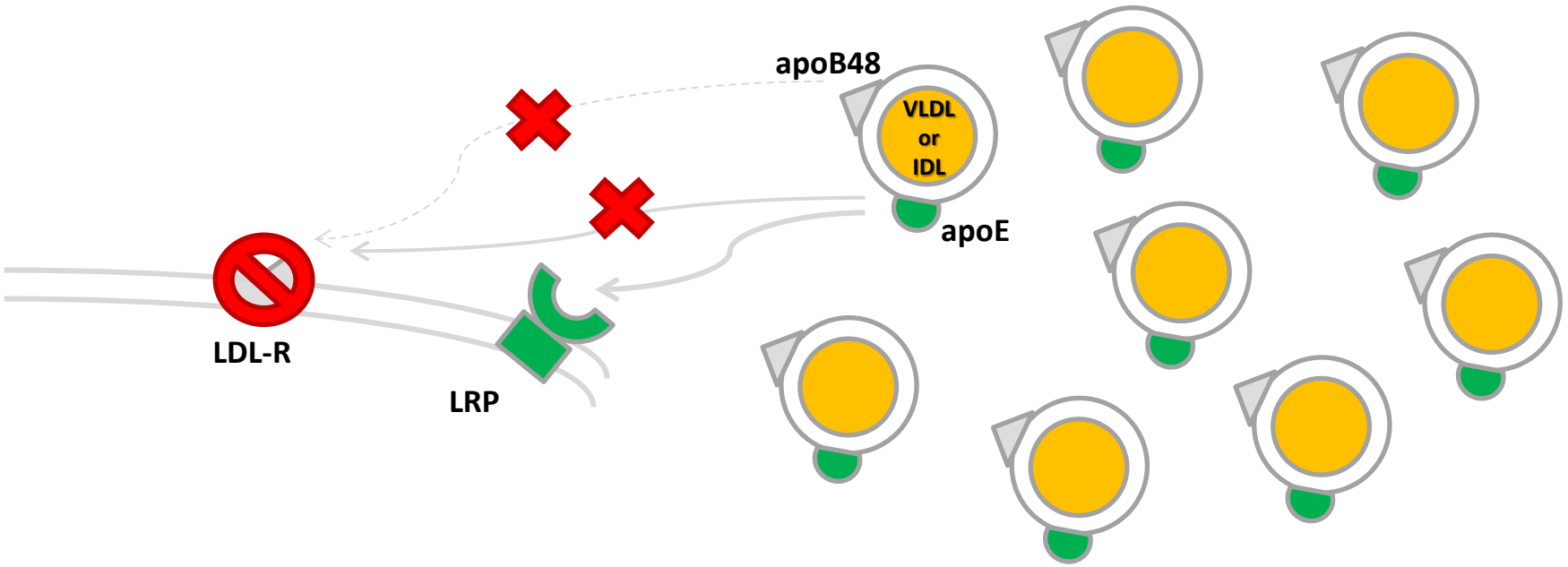
Description
Mice homozygous for the *Ldlr*^{tm1Her} mutation have an elevated serum cholesterol level of 200-400 mg/dl and they have very high levels (>2,000 mg/dl) when fed a high fat diet. Normal serum cholesterol in the mouse is 80-100 mg/dl.

Development
The *Ldlr*^{tm1Her} mutant strain was developed in the laboratory of Dr. Robert Hammer and Dr. Joachim Herz at the Howard Hughes Medical Institute Research Laboratories, University of Texas Southwestern Medical Center at Dallas. The 129-derived AB1 ES cell line was used. The strain has been backcrossed to C57BL/6J mice for 10 generations (N10).

Control Information

Control
000664 C57BL/6J

Atherosclerotic phenotype induction in LDL-R$^{-/-}$ mice

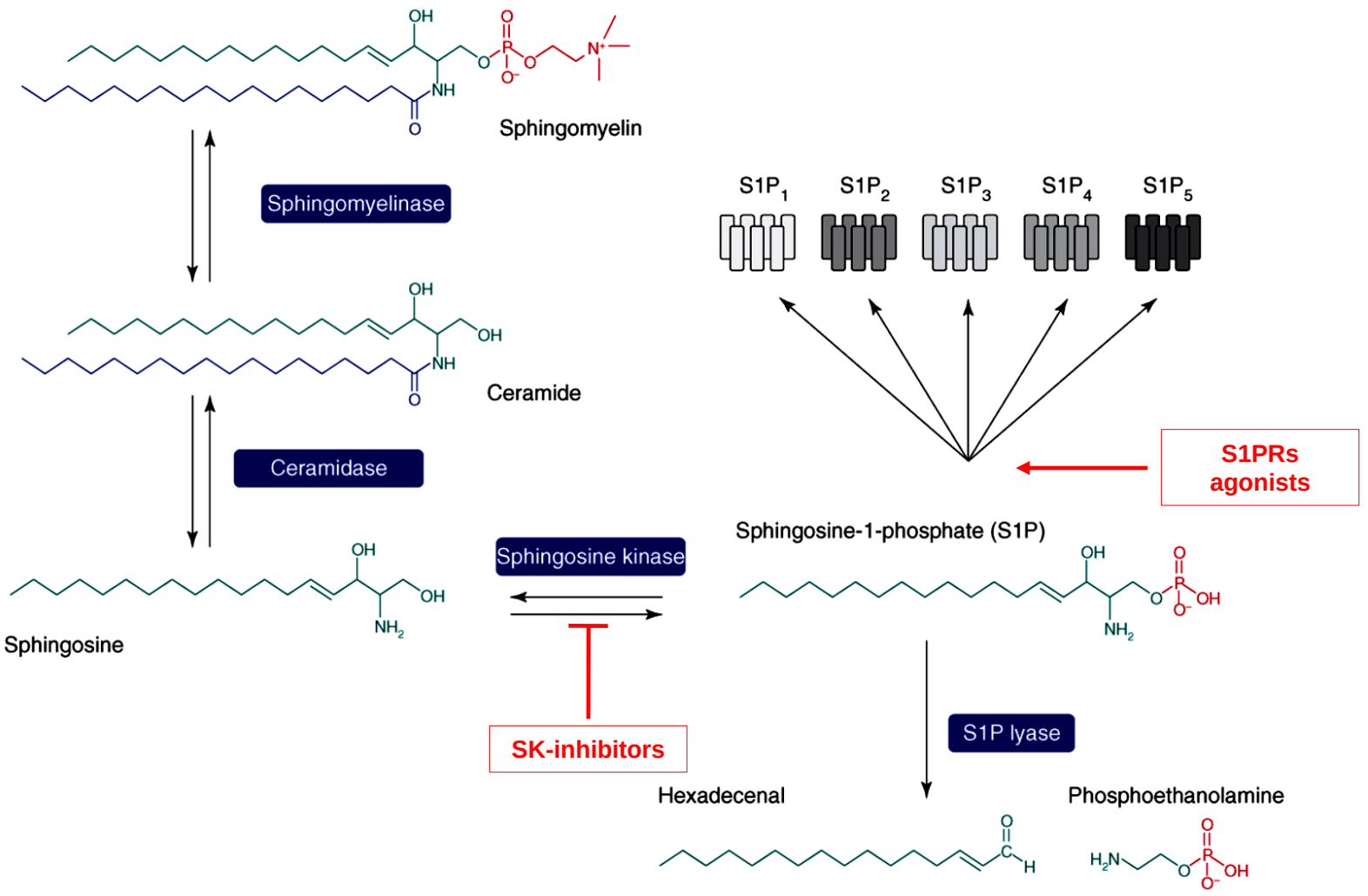


In **LDL-R$^{-/-}$**, plasma VLDL and IDL can be cleared only by LRP

1) on <u>standard chow</u> diet
   → moderate increase in VLDL/IDL plasma concentration (≈200 mg/dL)
   → no spontaneous or very small atherosclerotic lesions

2) on <u>Western type</u> diet
   → very high plasma VLDL/IDL concentrations (≈700-2000 mg/dL)
   → spread and large atherosclerotic lesions

1. SISTEMIC INTERFERENCE WITH S1P SIGNAL TRANSDUCTION THROUGH PHARMACOLOGICAL INTERVENTION



Sphingosine kinase inhibition exerts both pro- and anti-atherogenic effects in low-density lipoprotein receptor-deficient (LDL-R$^{-/-}$) mice

Francesco Poti[1]; Martine Bot[2]; Sara Costa[1]; Valeria Bergonzini[1]; Lynn Maines[3]; Georg Varga[4]; Hendrik Freise[5]; Horst Robenek[6]; Manuela Simoni[1*]; Jerzy-Roch Nofer[1,7*]

[1]Department of Medicine, Endocrinology, Metabolism and Geriatrics, University of Modena and Reggio Emilia, Modena, Italy; [2]Division of Biopharmaceutics, Gorlaeus Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands; [3]Apogee Biotechnology Corporation, Hershey, Pennsylvania, USA; [4]Institute of Immunology, University of Münster, Münster, Germany; [5]Department of Anaesthesiology and Intensive Medicine, University Hospital Münster, Münster, Germany; [6]Leibniz-Institute for Arteriosclerosis Research, University of Münster, Münster, Germany; [7]Center for Laboratory Medicine, University Hospital Münster, Münster, Germany

Thromb Haemost 2012; 107: 552–561

Effect of sphingosine 1-phosphate (S1P) receptor agonists FTY720 and CYM5442 on atherosclerosis development in LDL receptor deficient (LDL-R$^{-/-}$) mice

Francesco Poti [a], Sara Costa [a], Valeria Bergonzini [a], Margherita Galletti [a], Elisa Pignatti [a], Christian Weber [b], Manuela Simoni [a,1], Jerzy-Roch Nofer [a,c,*,1]

[a] *Department of Medicine, Endocrinology, Metabolism and Geriatrics, University of Modena and Reggio Emilia, Modena, Italy*
[b] *Institute for Cardiovascular Prevention, Chair in Vascular Medicine, Ludwig-Maximilian-University of München, München, Germany*
[c] *Center for Laboratory Medicine, University Hospital Münster, Münster, Germany*

Vascular Pharmacology 57 (2012) 56–64

KRP-203, Sphingosine 1-Phosphate Receptor Type 1 Agonist, Ameliorates Atherosclerosis in LDL-R $^{-/-}$ Mice

Francesco Potì, Fabio Gualtieri, Sandro Sacchi, Gabriele Weißen-Plenz, Georg Varga, Martin Brodde, Christian Weber, Manuela Simoni and Jerzy-Roch Nofer

Arterioscler Thromb Vasc Biol. 2013;33:1505-1512;

Arteriosclerosis, Thrombosis, and Vascular Biology
Journal of the American Heart Association

American Heart Association®

S1PRs agonists and atherosclerosis: state of the art

FTY 720 ($S1P_{1\text{-}3\text{-}4\text{-}5}$ not selective agonist)

↓↓ **atherosclerosis** in murine models of atherosclerosis
under conditions of severe hypercholesterolemia

1) LDL-R KO upon 1,25% chol diet, Nofer JR, et al. *Circulation. 2007 Jan 30;115(4):501-8.*

2) ApoE KO upon 0,15% chol diet, Keul P, et al. *Arterioscler Thromb Vasc Biol. 2007 Mar;27(3):607-13*.

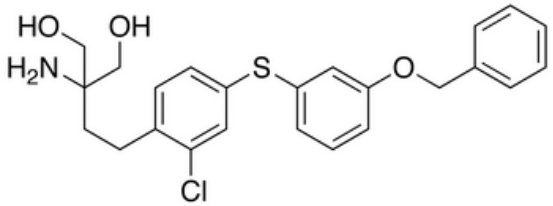
FTY 720 ($S1P_{1\text{-}3\text{-}4\text{-}5}$ not selective agonist)

NO effects on atherosclerosis in murine models of atherosclerosis
under conditions of moderate hypercholesterolemia

3) ApoE KO upon normal chow diet, Klingenberg R, et al. *Arterioscler Thromb Vasc Biol. 2007 Nov;27(11): 2392-9*.

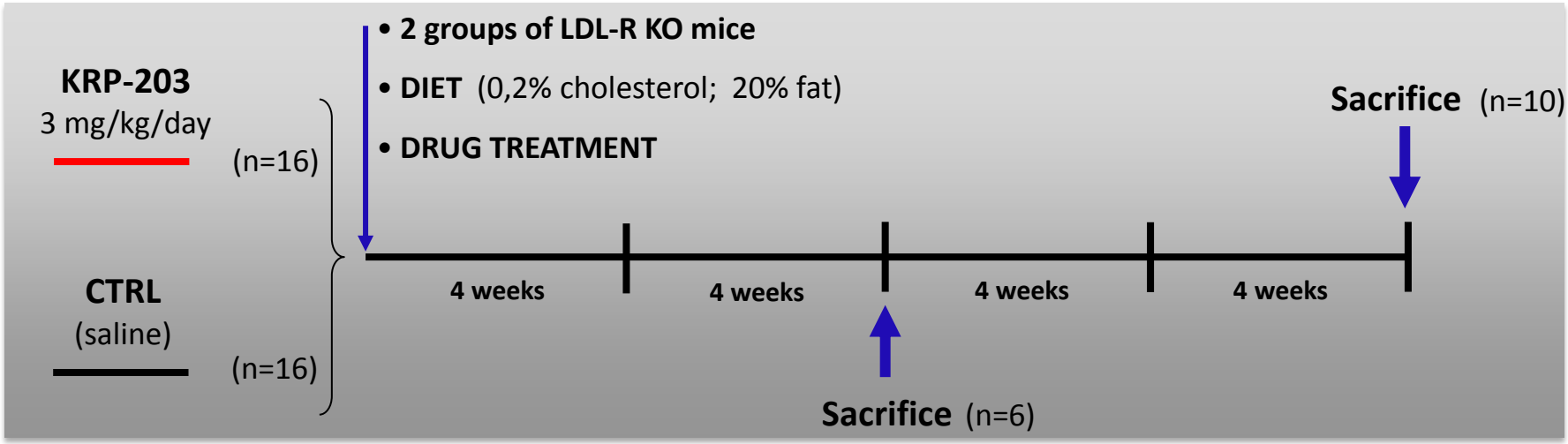
4) LDL-R KO upon 0,25% chol diet, Potì F, et al. *Vascular Pharmacology. 2012 Aug 19;57(1):56-64.*

AIM: to evaluate the effects of **selective S1P1 receptor agonist** in a murine model of atherosclerosis upon moderate dietary cholesterol challenge

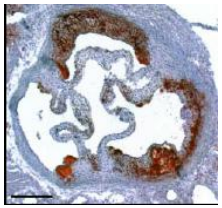


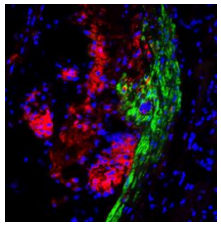
KRP-203

(selective $S1P_1$ agonist)



Experimental procedures





- **Evaluation of atherosclerosis development**:

  isolation, fixation, processing and Oil Red O lipid staining of the aorta;

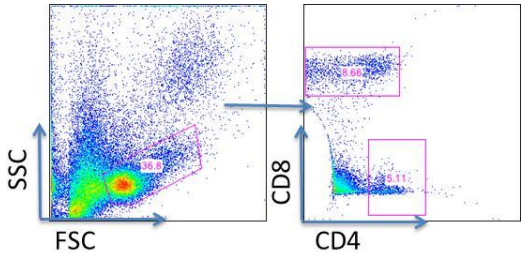
  cryosectioning of the heart at the aortic sinus level;

- **Histology and Immunohistochemistry of atherosclerotic lesions**

  lipid staining and presence of macrophages, lymphocytes, smooth muscle cells;

- **Analysis of splenocytes**:

  evaluation of proliferation; analysis through flow cytometry; cytokine secretion;

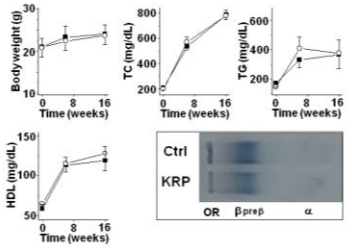


- **Analysis of lymph nodes**:

  characterization through flow cytometry;

- **Analysis of resident peritoneal macrophages**:

  characterization through flow cytometry; cytokine secretion; signal transduction pathways;

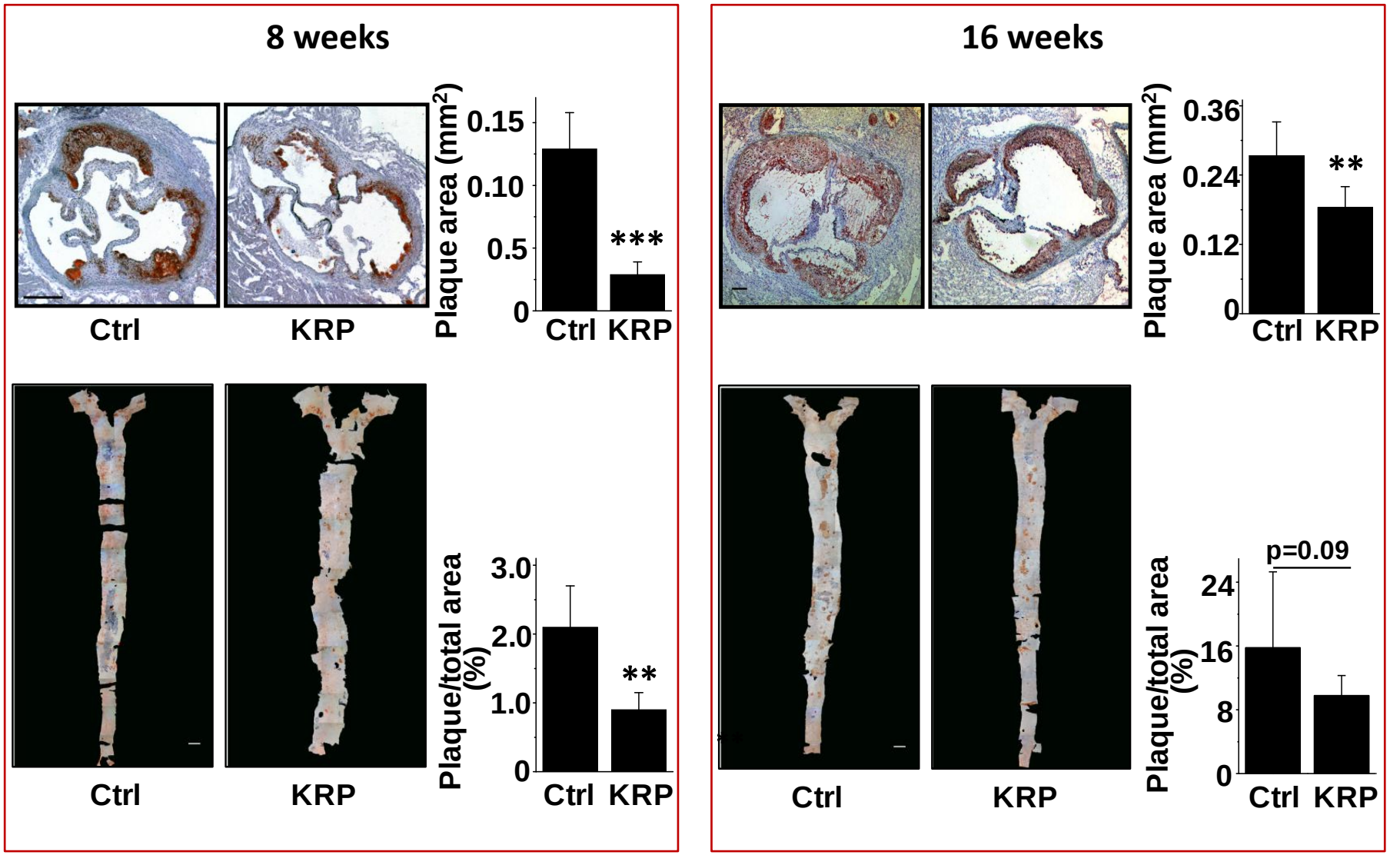


- **Blood analysis**:

  evaluation of plasma lipids and glucose; measurement of circulating cytokines;

  characterization through flow cytometry;

Effect of KRP-203 on atherosclerosis development



Potì F et al. *Arterioscler Thromb Vasc Biol* 2013;33:1505-1512

Main topics of the project:

1. SISTEMIC INTERFERENCE WITH S1P SIGNAL TRANSDUCTION THROUGH

PHARMACOLOGICAL INTERVENTION

2. PRODUCTION OF **CONDITIONAL AND TISSUE-SPECIFIC EXPRESSING MOUSE MODELS**

2. Generation of conditional S1PR1 and S1PR3 Knock-in mouse lines

Aim:

to evaluate

a) the effects of **endogenous S1P** on the distribution and quality of atherosclerotic lesions

b) the **specific contribution of one or more S1PRs** to the potential atheroprotective effect of S1P

c) the specific contribution of S1P signal transduction in **selected cells**

2. Generation of conditional S1PR1 and S1PR3 Knock-in mouse lines

Esigenza: modello sperimentale

con segnale endogeno S1P-S1PRs amplificato

(tessuto specifico)

Topo transgenico che sovraesprima il recettore d'interesse

in modo tessuto specifico → "**knock-in**" condizionale (**Cre-LoxP**)

Outsourcing: gen O way

PRODUCTION OF TRANSGENIC MOUSE MODELS…..

Cre recombinase

Cre

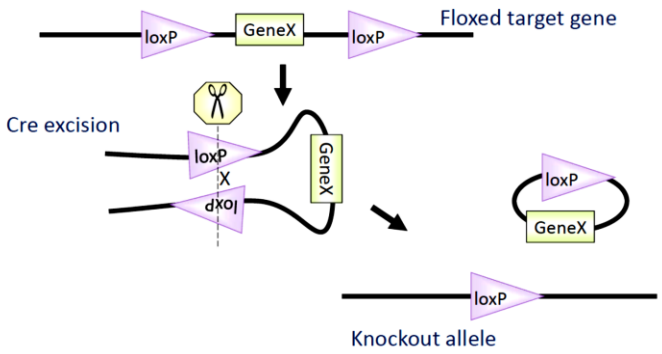
- Site-specific enzyme, catalyzes recombination between two *loxP* sites

loxP site

- 34 base pair DNA sequence
- Location and orientation determines recombination result:
  - Deletion
  - Inversion
  - Translocation

Abundant possibilities for genome manipulation!

Cre - lox Deletion



Reviewed in Nagy A. 2000. *Genesis* 26(2):99-109. PMID: 10686599

Cre-Lox technology allows conditional knock-in

LoxP-STOP-*Lox*P-**S1P1** o **-S1P3**

ATG

LoxP

LoxP

3'-Short Homology Arm

pA

gene

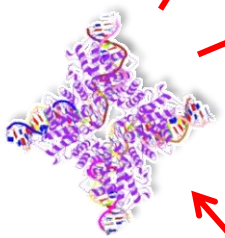
STOP

Neo

pCAG

5'-Long Homology Arm

S1P1 or S1P3



Lyz-*Cre*

3'-end

Cre

Tissue specific promoter

5'-end

Targeting vector construction

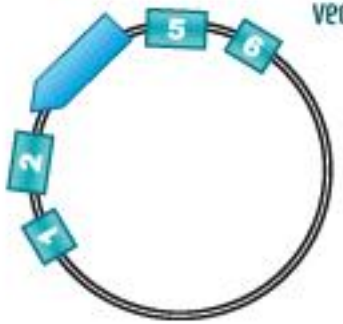
GenBank number

Model design: Bio-informatics analysis, bibliography search, project feasibility assessment

Gene targeting strategy

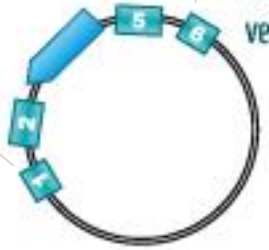
Targeting vector construction

Targeting vector



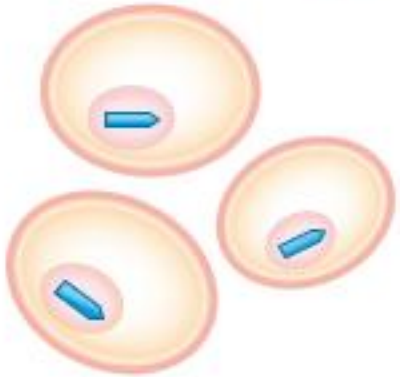
Homologous recombination into ES cells

Targeting vector

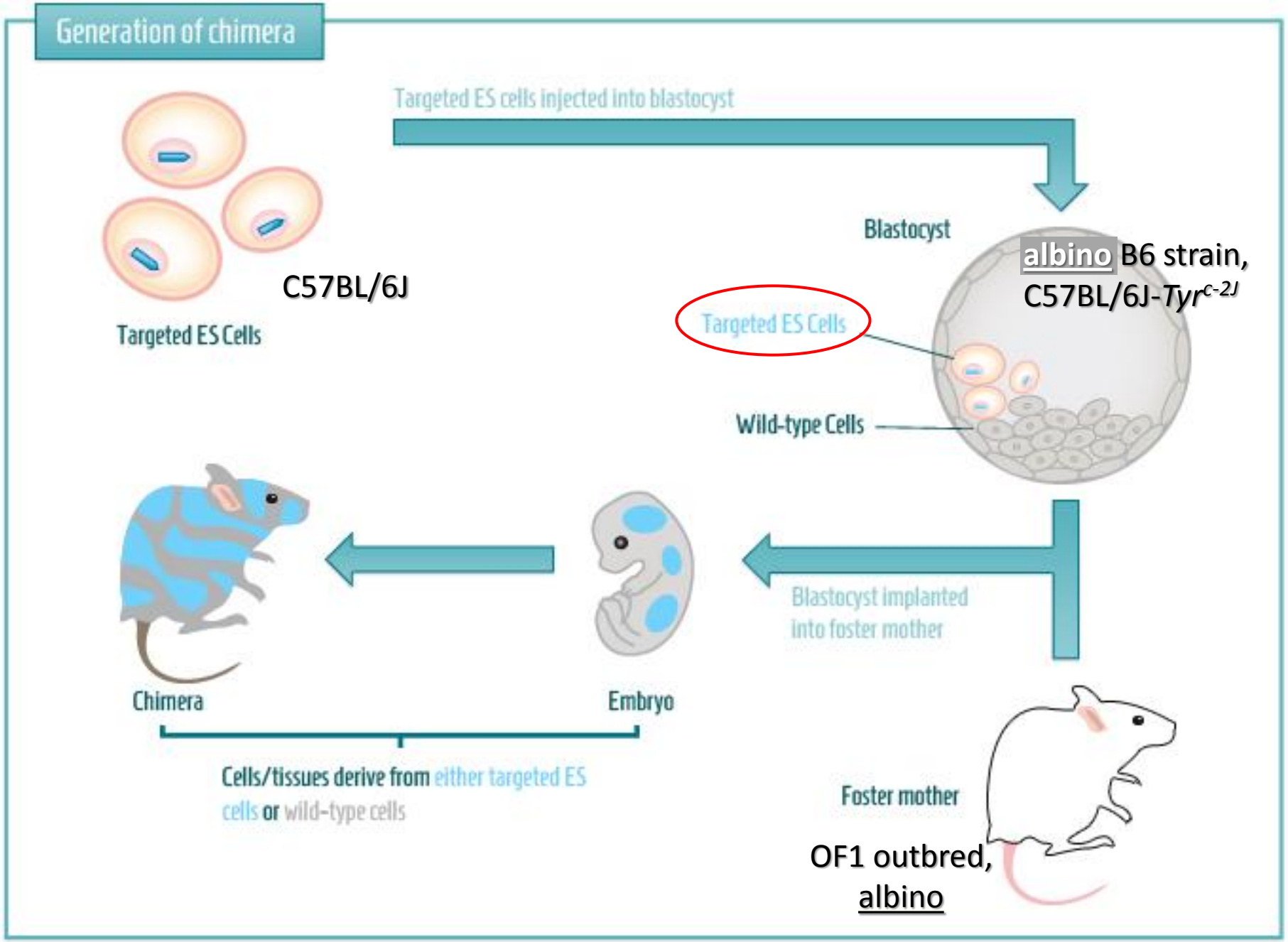


ES from C57BL/6J

Electroporation

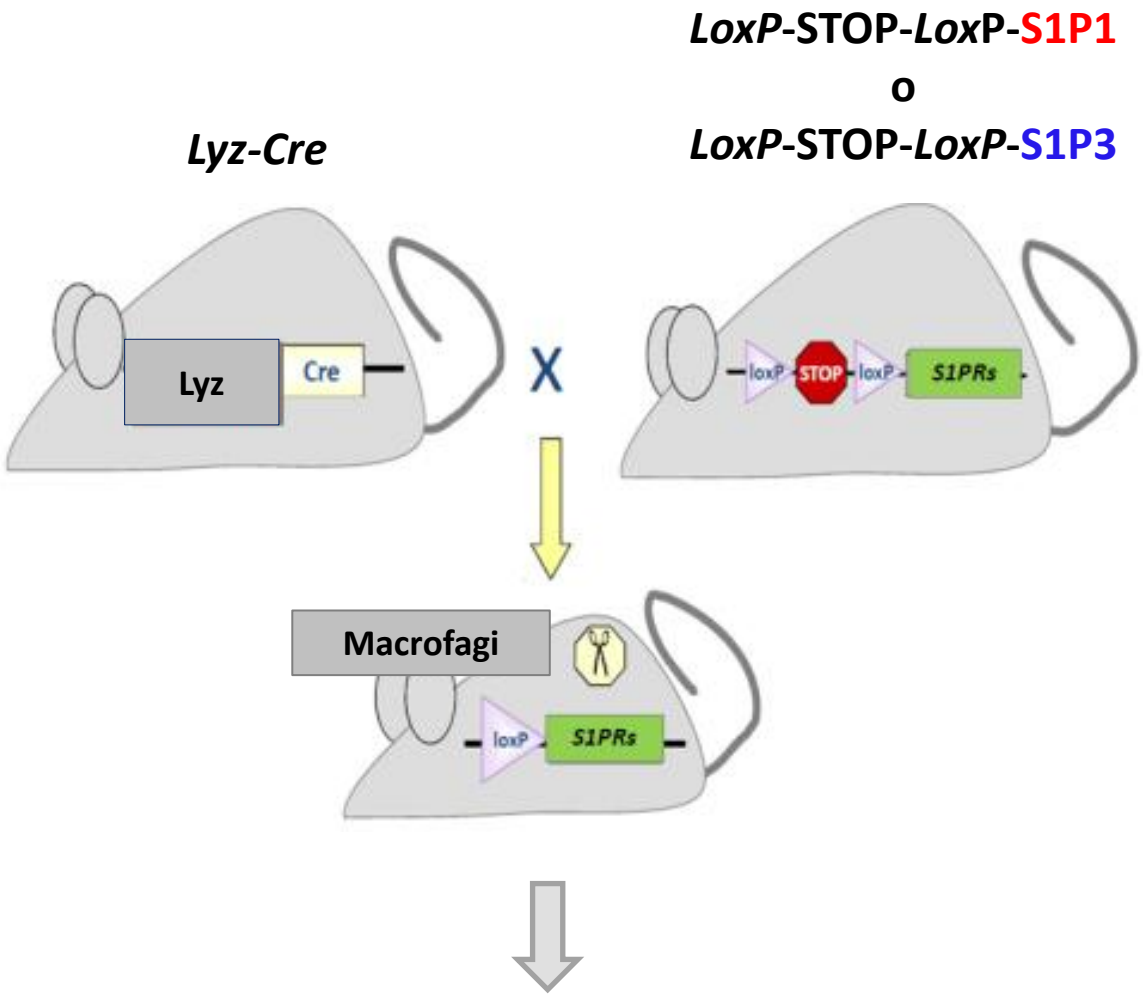


ES cells genotyping and characterization of targeted ES Cell


Generation of chimera
Targeted ES cells injected into blastocyst
C57BL/6J
Targeted ES Cells
Blastocyst
albino B6 strain,
C57BL/6J-Tyr c-2J
Targeted ES Cells
Wild-type Cells
Blastocyst implanted
into foster mother
Foster mother
OF1 outbred,
albino
Chimera
Embryo
Cells/tissues derive from either targeted ES
cells or wild-type cells

Germline transmission

Overview…


LoxP-STOP-*Lox*P-S1P1
o
LoxP-STOP-*LoxP*-S1P3
Lyz-Cre
Lyz
Cre
X
loxP
STOP
loxP
S1PRs
Macrofagi
loxP
S1PRs
Topi sovraesprimenti S1P1 o S1P3
nei **monociti/macrofagi**

Acknowledgements

Prof. Jerzy-Roch Nofer

University Hospital of Münster (Germany)

Prof.ssa Manuela Simoni

Dott.ssa Elisa Pignatti

Dott.ssa Laura Riccetti

Eleonora Daini

Dott. Lorenzo Canti

Dott. Daniel Cavalli

Dott.ssa Eleonora Forghieri

Dott. Sandro Sacchi

Dott. Fabio Gualtieri

Dott.ssa Elisa Resca