CNRS UMR 5164

Hypoxia contributes to quiescence and chemoresistance of Leukemia Initiating Cells in B Acute Lymphocytic Leukemia

Arnaud Villacreces

Supervisor : Pr V. Praloran

10th July 2014

B-ALL : Generalities

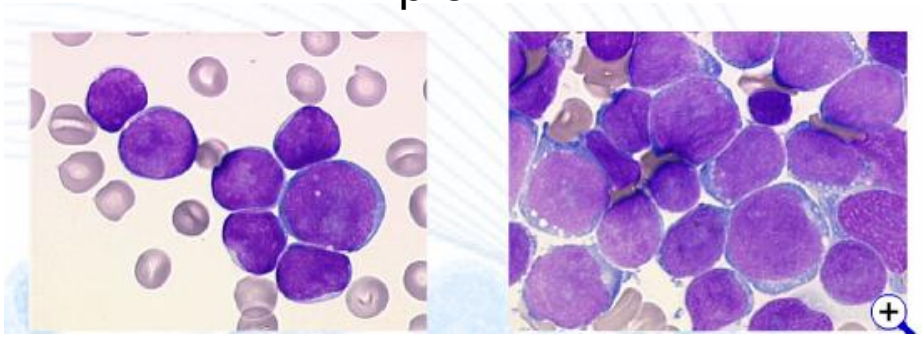
Definition :

Haematopoietic malignancy,

Affecting B lymphoid lineages

Affecting haematopoiesis (thrombopenia, anemia, neutropenia)

pro B ALL



Blood Bone marrow

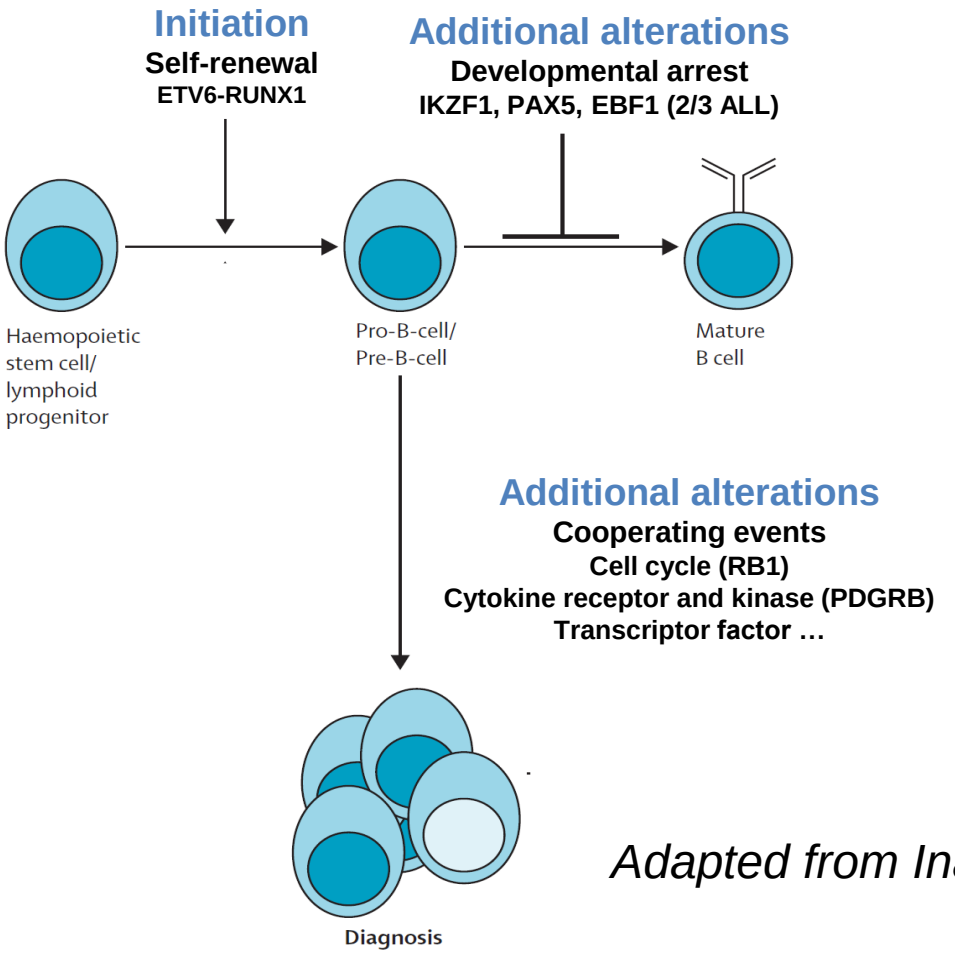
Epidemiology :

1.25 / 100 000

Children: 2 to 10 years (20% of cancers)

Adult: ≥ 50 years (< 1% of cancers)

B-ALL: leukaemogenesis



Adapted from Inaba et al., The Lancet, 2013

Systematic research of recurrent genetic abnormalities
→ Classification and stratification

ALL: Molecular abnormalities, frequencies and prognosis

LAL	Anomalie	Incidence	Pronostic
B+T	Hyperdiploïdie	< 10 % chez l'adulte/30-35 % en pédiatrie	Favorable
	Formes hypodiploïdes, haploïdes et quasi triploïdes	< 5 % chez l'adulte	Défavorable
	Réarrangements de MLL	80 % chez les < 1 an, exceptionnel au-delà de cet âge chez l'enfant, 10 % chez l'adulte	
	Caryotypes complexes (> 5 anomalies)	< 5 % chez l'adulte	
B	t(9;22)(q34;q11)/*BCR-ABL1*	30-40 % chez l'adulte/< 5 % chez l'enfant	Défavorable
	TCF3-PBX1 et *TCF3-HLF*	4 % des LAL de l'enfant et jeune adulte	
	(iAMP21)	Enfants et adolescents principalement	
	Mutations/délétions du gène *IKZF1*	~ 3/4 LAL Ph+, 1/3 LAL non Ph+/15 % des LAL B de l'enfant	
	LAL BCR-ABL1 *like*	15 % des LAL B de l'enfant, et vraisemblablement une proportion significative chez les 15-25 ans	
	Réarrangements de *CRLF2*	50 % des LAL associées au syndrome de Down et jusqu'à 50 % des LAL BCR-ABL1 *like*	
	Mutation de *PAX5*	Précoce dans la leucémogenèse B environ 30 % des LAL B	?
	t(12;21)(p12;q22)/ETV6-RUNX1	1-3 % chez l'adulte/25 % chez l'enfant	Favorable
	Délétion intragénique de *ERG*	-/3 %	

Adapted from Lara et Gauthier., Hématologie, 2014

Prognosis stratification criteria

❖**Clinical characteristics**

- ➢Age (Tolerance – different genetic abnormalities)
- ➢Innate genetic background (Trisomy)
- ➢Signs of central nervous system invasion
- ➢Signs of testis invasion

❖**Biological characteristics**

- ➢Leucocyte enumeration (Hyperleucocytosis > 30 000 / mm^3)
- ➢Extended B-cell phenotyping : ($CD10^-$)
- ➢Genetics abnormalities (BCR-ABL)

❖**Evolution Characteristics : Response to treatment**

- ➢Initial corticoresistance
- ➢Chemoresistance **(MRD level)**

ALL Treatment : Polychemotherapy

Long (≈3years) in 3 phases preceded by 1 week of corticotherapy

Induction : ≈ 1 month

- Anthracyclines (Daunorubicine)
- Corticoïds (MéthylPrednisolone)
- Vincristine
- L-Asparaginase
- Alkylating agents

Allograft
Adult

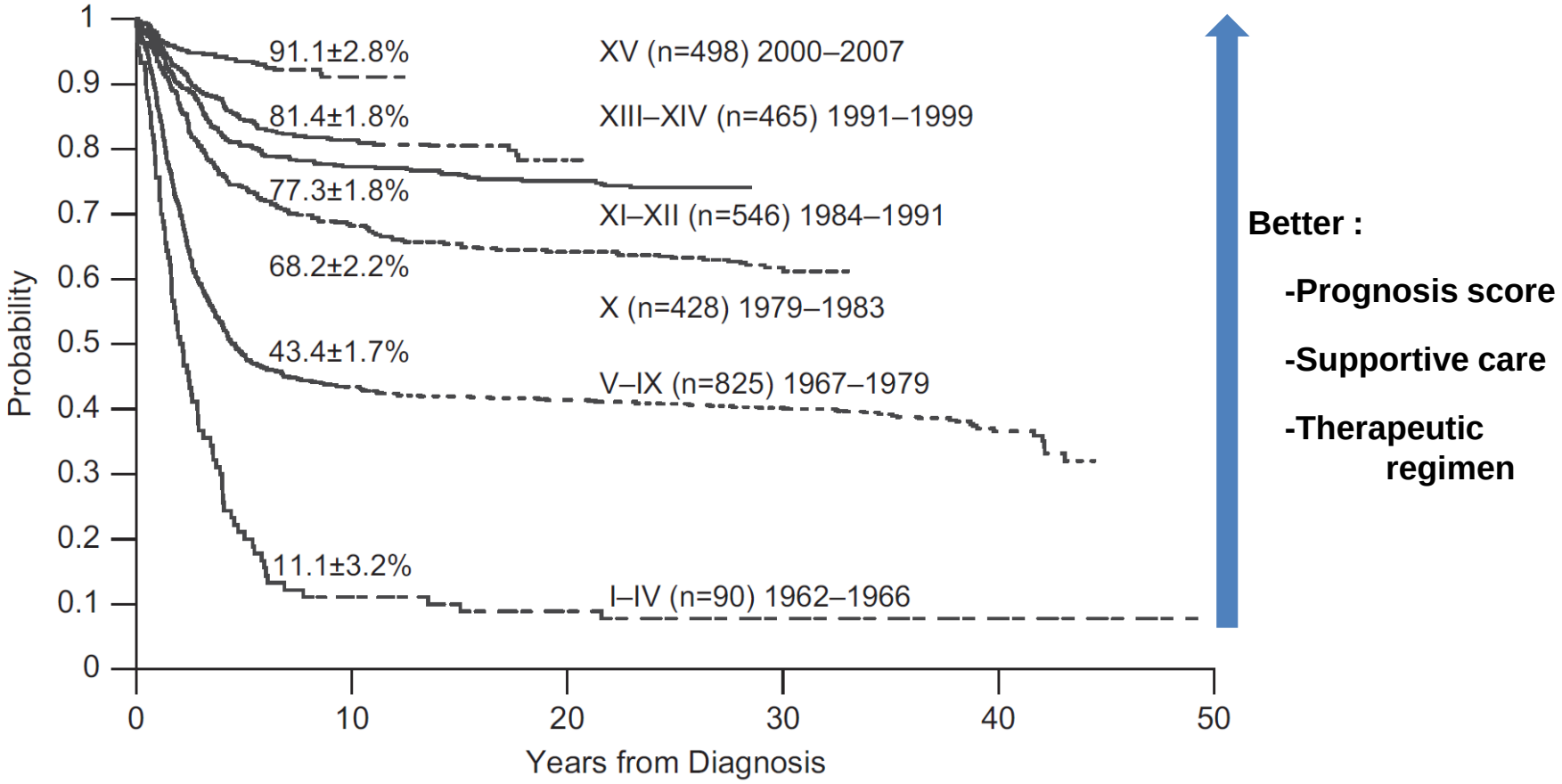
Consolidation : ≈ 7 months

- Methotrexate
- Cytarabine (Ara C)
- Cyclophosphamide
- Other induction therapy

Maintenance : ≈ 2-3 years

- 6-mercaptopurine
- Methotrexate
- Vincristine
- Corticoïds

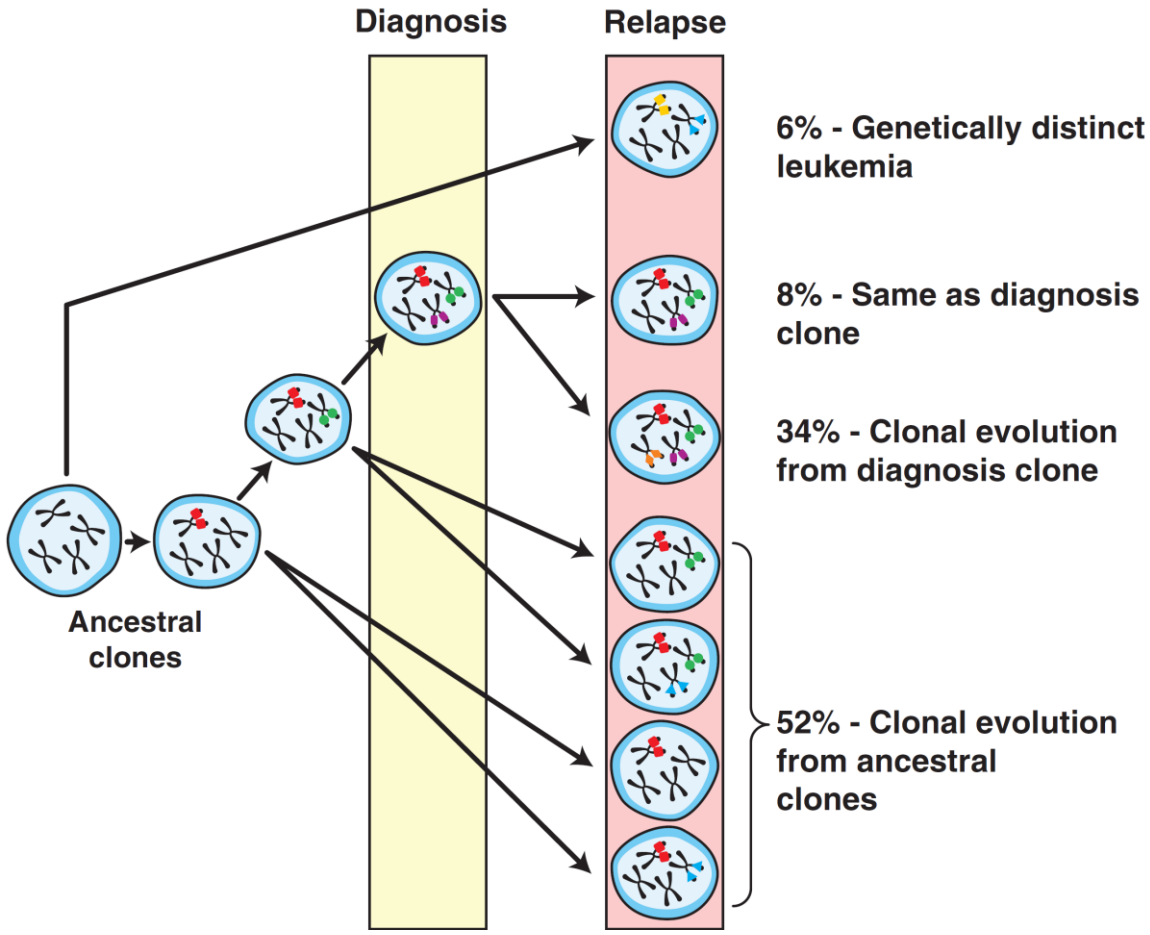
Results



Kaplan-Meier analysis of survival for 2852 children enrolled in 15 consecutive studies from 1962 to 2007

Adapted from Pui and Evans, Semin Hematol., 2013

Clonal relationship of diagnosis and relapse samples in ALL

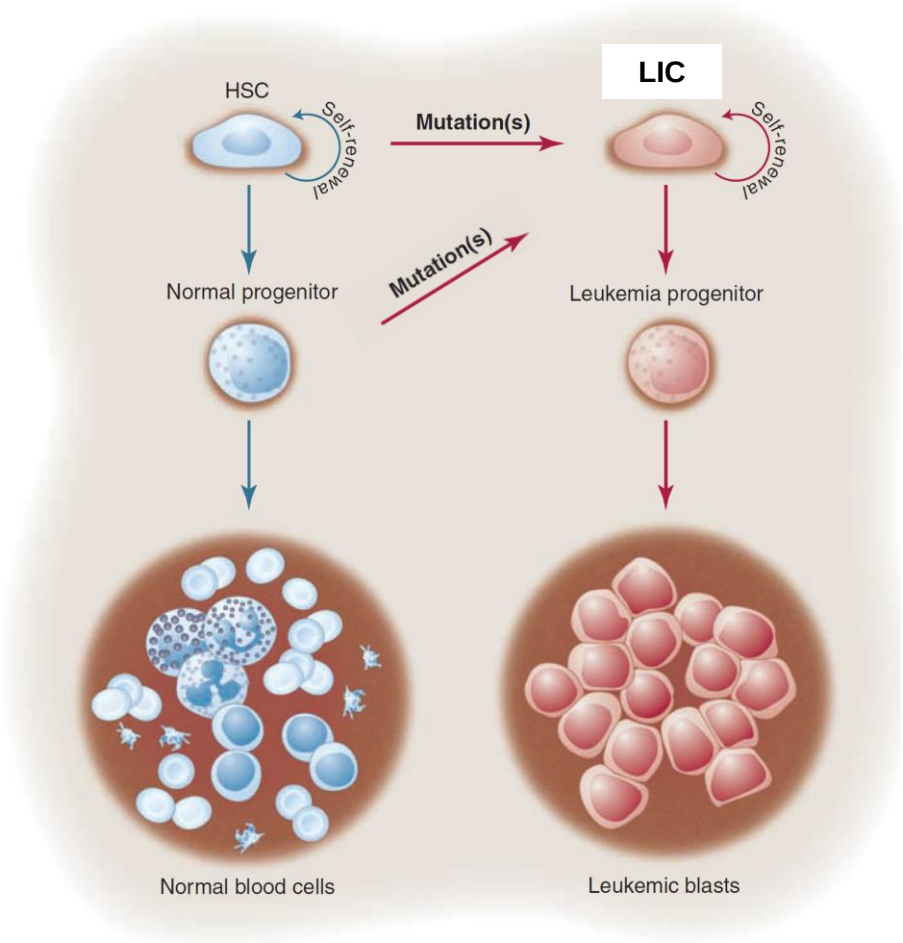


61 paediatric patients

Mullighan et al., Science, 2008

Relapses : Leukaemia Initiating Cells persistence

Leukemia initiating cells (LIC)



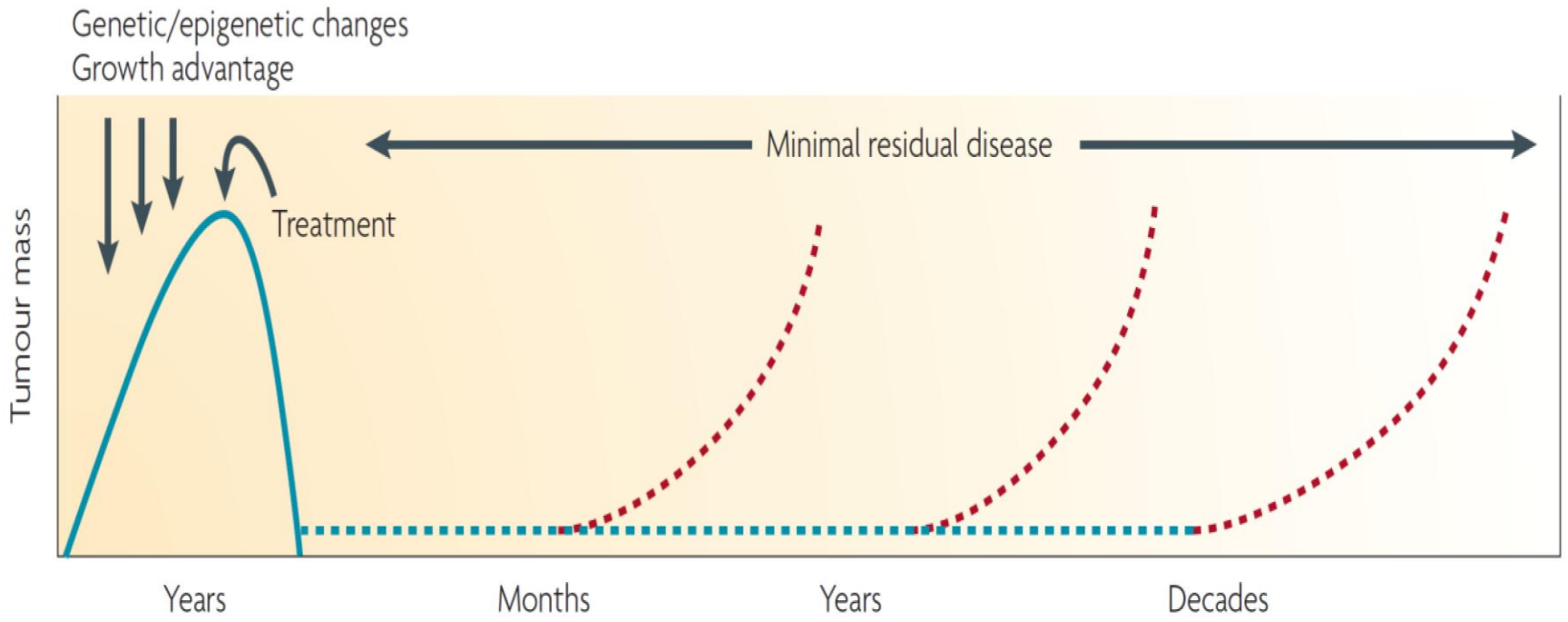
Leukaemic transformation model
Adapted of Rosen and Jordan, Science, 2009

❖Malignant cells that initiate leukaemia

❖**That kept/reacquire HSC properties**

- ✓ Self-renewal
- ✓ Differentiation (partial – tumoral heterogenity)
- ✓ Homing
- ✓ **Protection mechanisms**

Relapses are due to residual LIC



Aguirre-Ghiso, Nat Rev Cancer., 2007

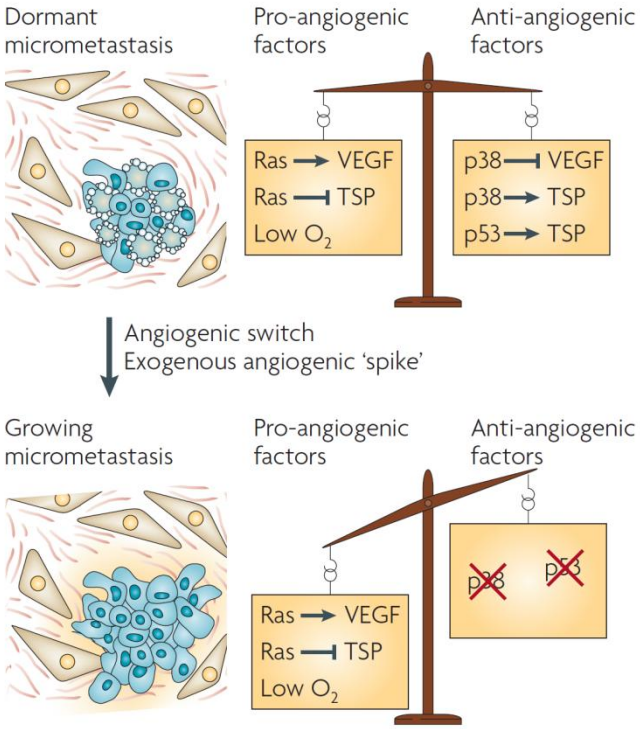
Relapses : Leukaemia Initiating Cells in dormancy state

Dormancy: the three possible mechanisms

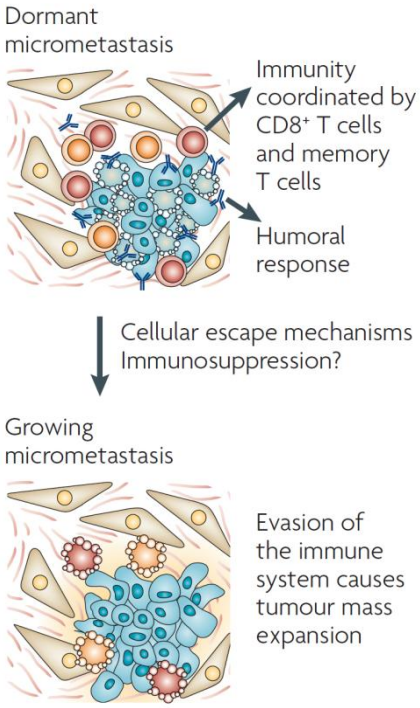
Tumor mass dormancy

% Proliferation = % Apoptosis

Angiogenic dormancy

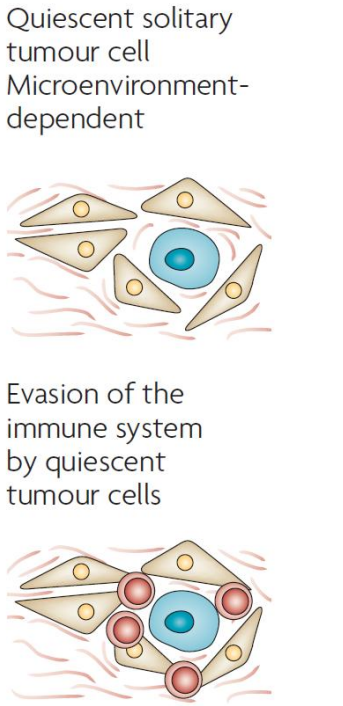


Immunosurveillance



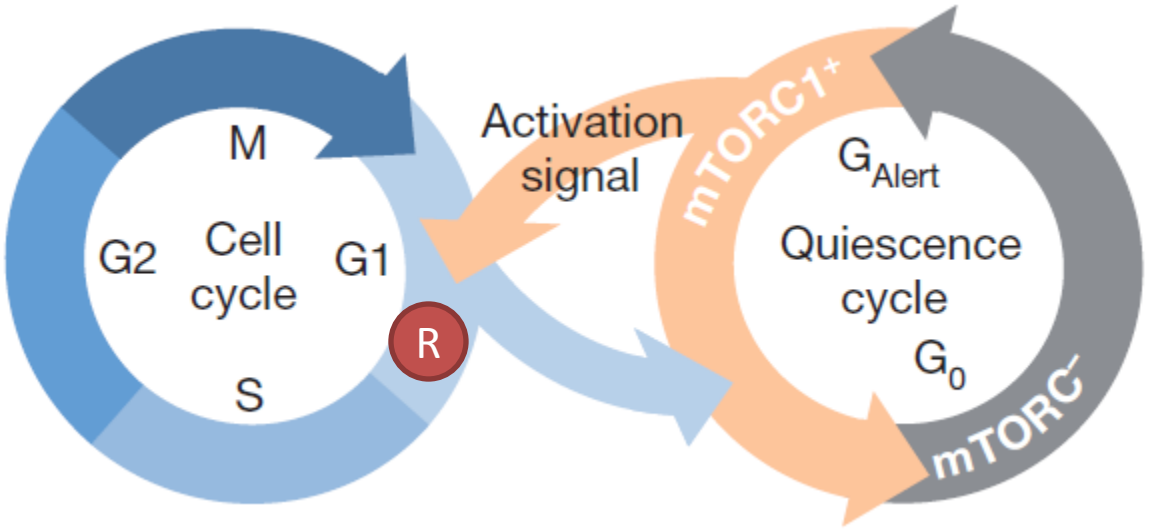
Cellular dormancy

Cellular dormancy



Adapted from Aguirre-Ghiso, Nat Rev Cancer., 2007

Cellular dormancy = quiescence



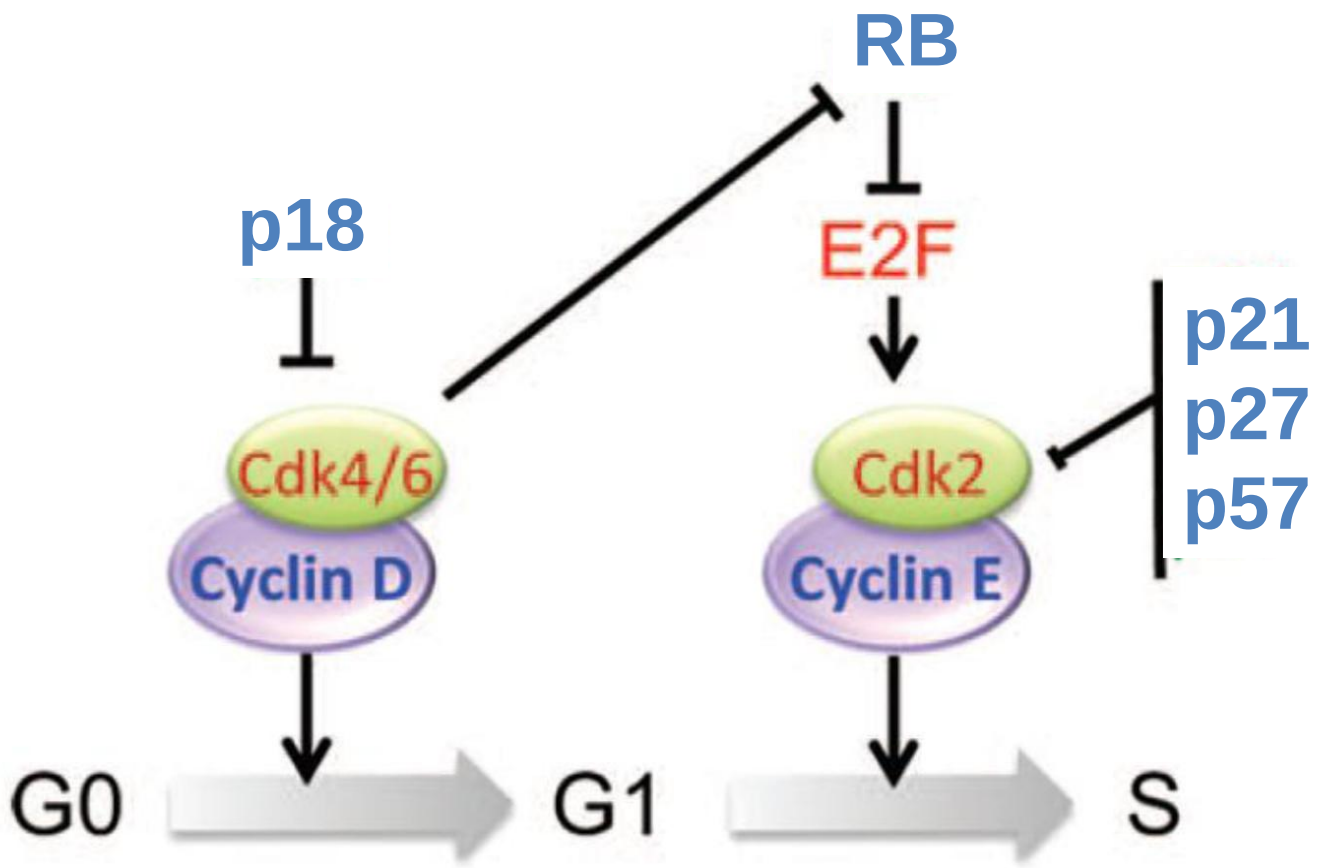
Reversibility of the G0 state of the cell cycle
Adapted from Rodgers et al., Nature, 2014

- ✓Cell cycle arrest with low metabolic rate
- ✓Quiescence = Reversibility ≠ senescence
- ✓Poorly characterized (rare)
- ✓Crucial to maintaining the HSC pool (preservation of key functions)
- ✓**Protection mechanism of the HSC**

Identification of quiescent cells

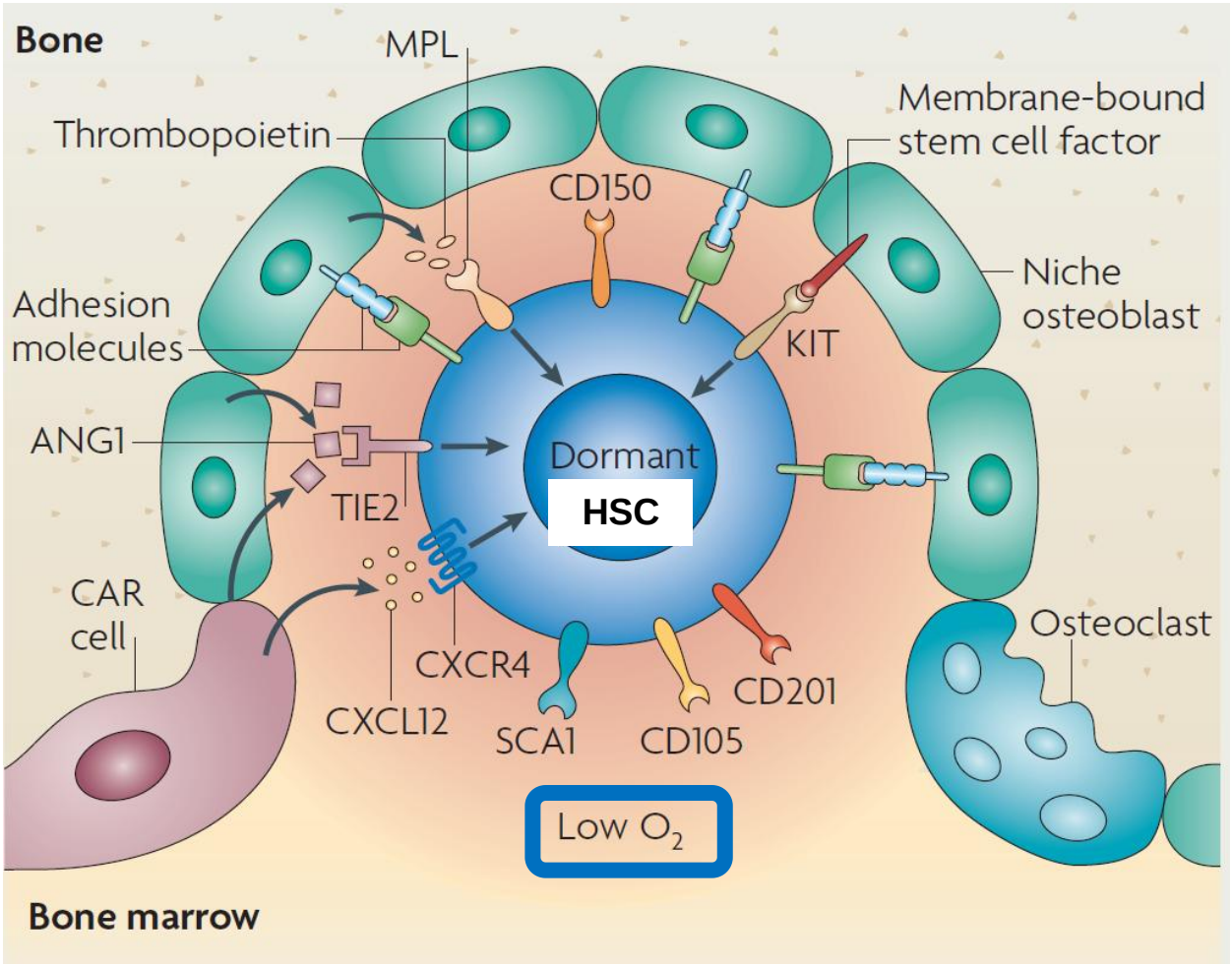
Markers	Effects	Properties
EdU	No S phase incorporation	Nucleic acid analogue
DiD	Retention	Membrane Lipophilic marker
Ki67	Absence	Proliferation marker
Pyronine Y	Low	RNA marker
Rhodamine 123	Low	Mitochondrial activity marker

Control of stem cell quiescence



Regulation of murine HSC quiescence exit.
Adapted from Yamada et al., Cell Cycle, 2013

BM micro-environment modulates quiescence : HSC

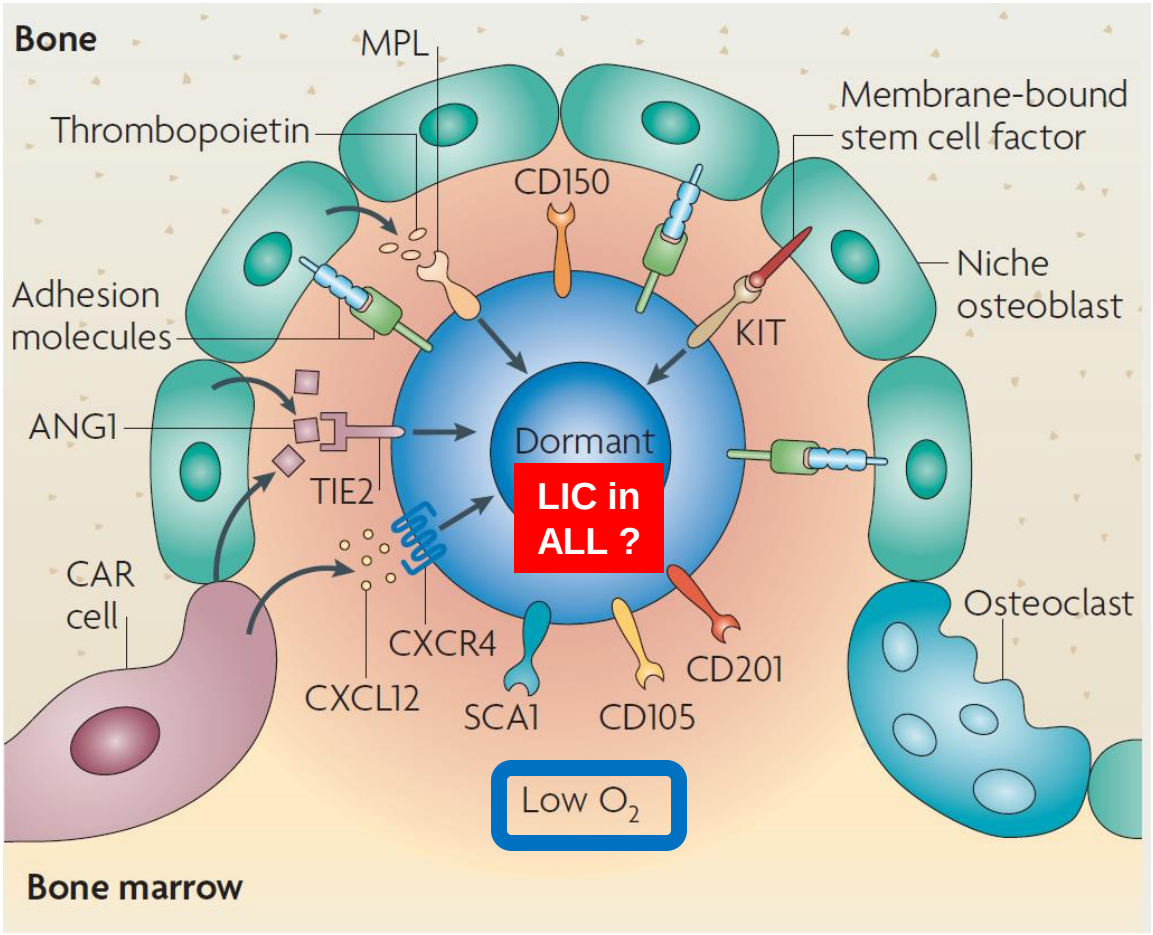


HSC localisation in the bone marrow

adapted from Trumpp et al., Nat Rev Immunol., 2010

The role of hypoxia in the maintenance of hematopoietic stem cells.
Cipolleschi MG et al, Blood. 1993

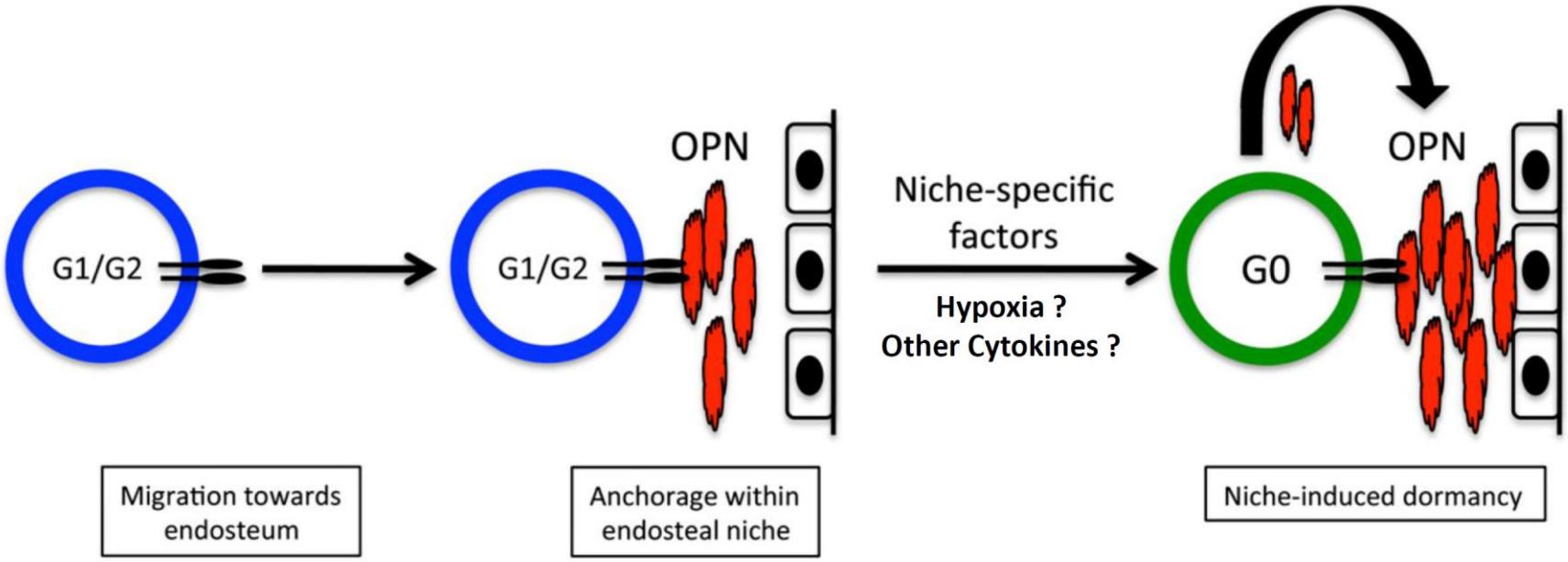
BM micro-environment modulates quiescence : LIC hypothesis



LIC localisation in the bone marrow niche ?

adapted from Trumpp et al., Nat Rev Immunol., 2010

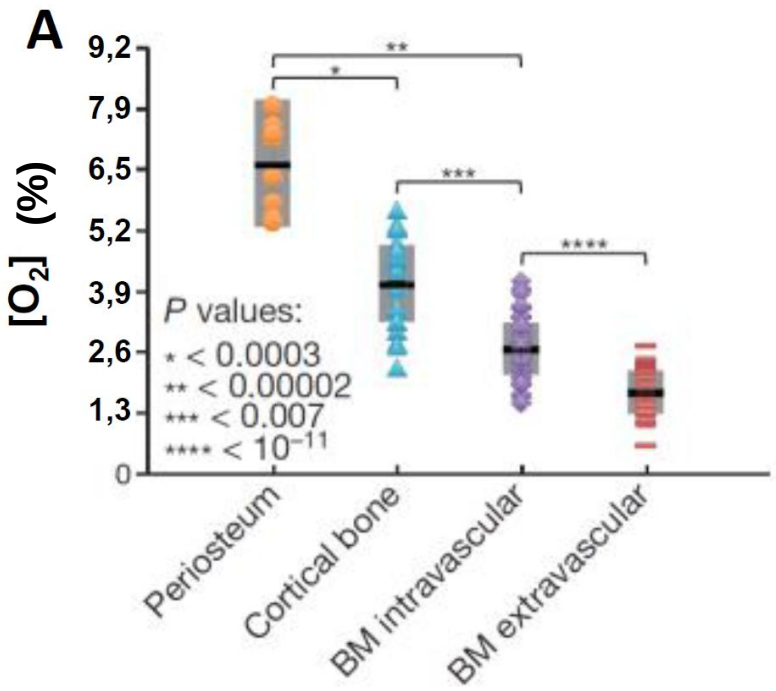
Role of the BM micro-environment in quiescence : What is known about ALL



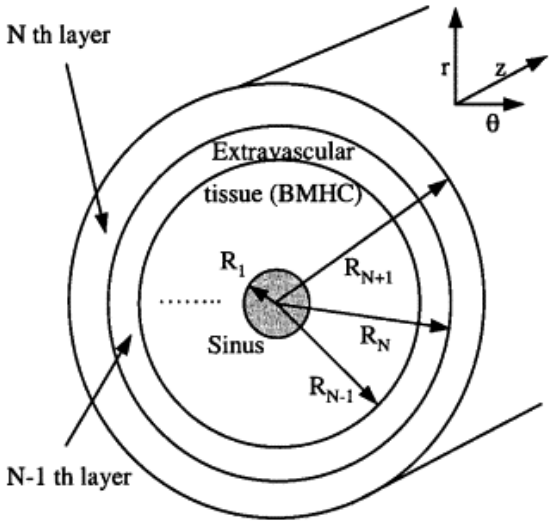
Model for osteopontin-induced dormancy of ALL at the endosteum

Adapted from Boyerinas et al., Blood, 2013

A BM environmental factor : O_2
Why hypoxia ?

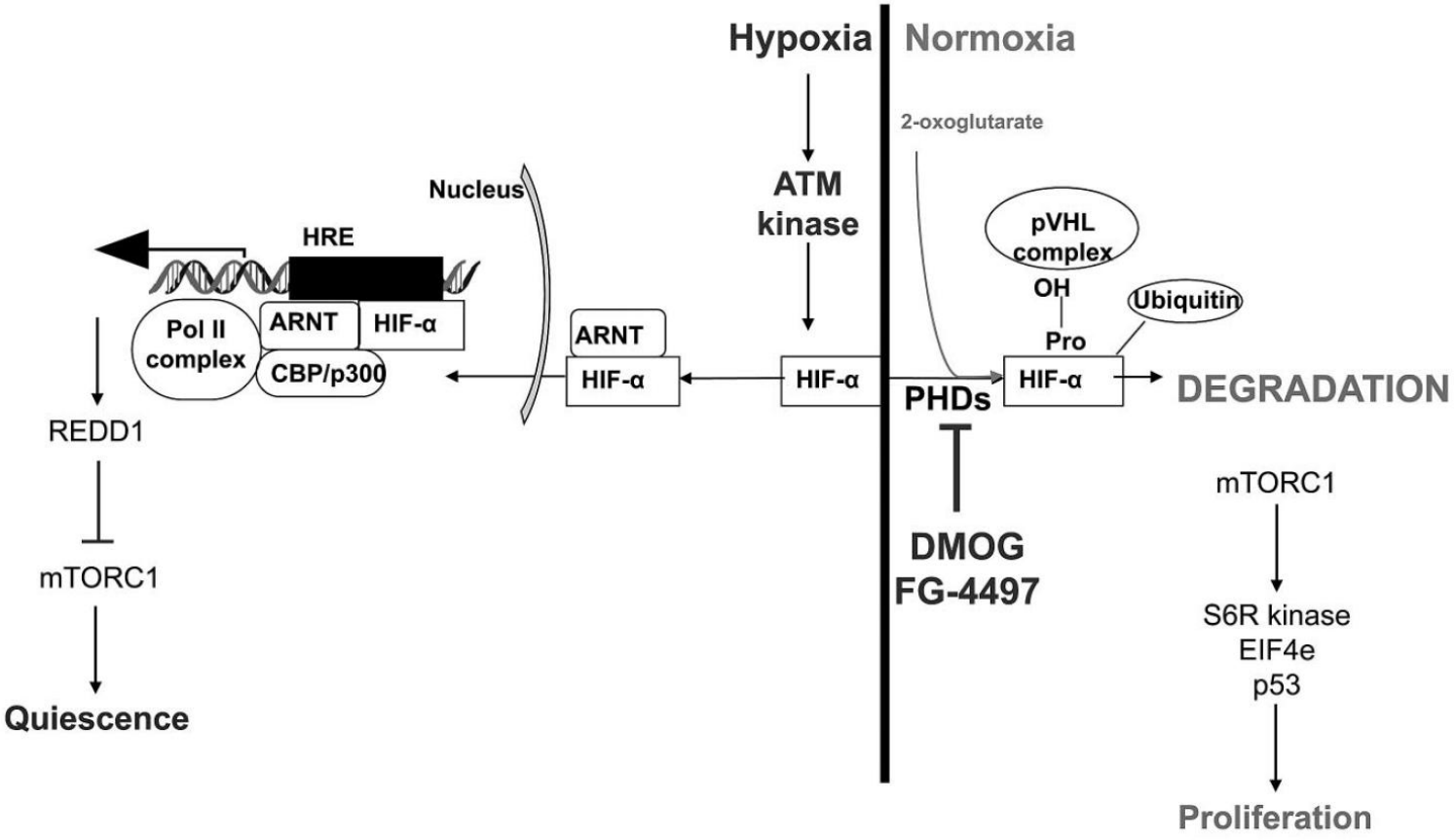


Oxygenation of murine calvaria.
Spencer et al., Nature, 2014



Human bone marrow $[O_2] \approx 0\%$ after 10 cellular layers
Chow et al, Biophysical Journal, 2001

A BM environmental factor : O_2
HIF-1α and quiescence

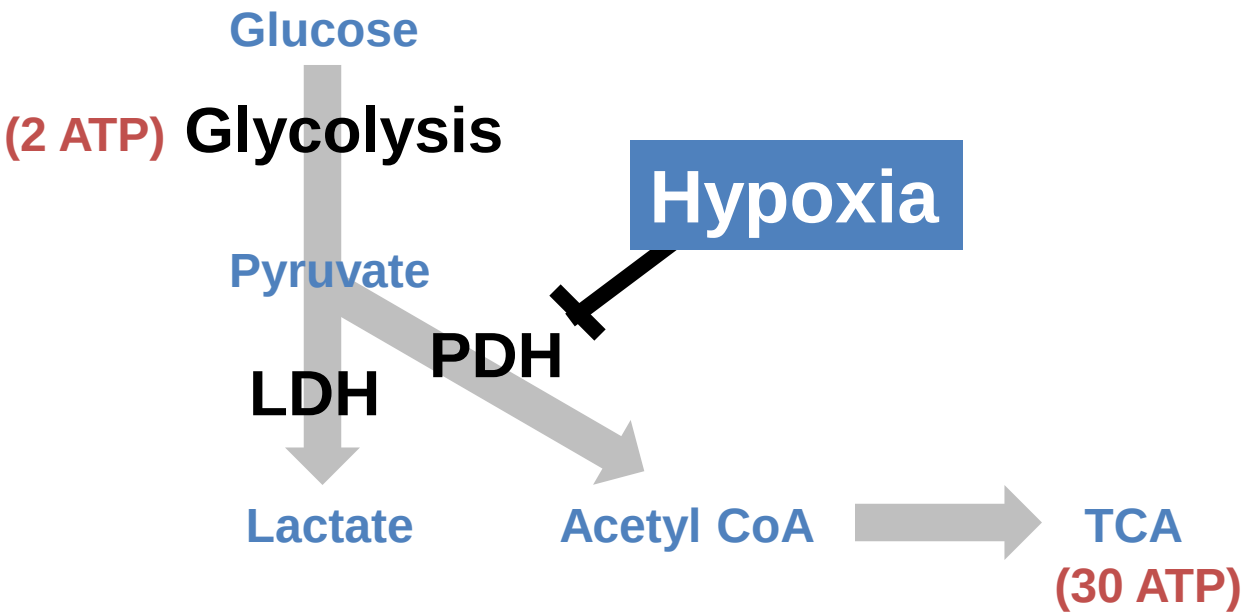


Regulation of quiescence through HIF1α in normoxia and hypoxia

Forristal et al., Blood, 2013

A BM environmental factor : O_2
Metabolism and quiescence

Role in energy metabolism



Effect of hypoxia on the energetic metabolism
adapted from Takubo et al., Cell Stem Cell, 2013

Objectives of the present study

Development of an *in vitro/in vivo* model for exploring the relationships between hypoxia and ALL LIC

Role of low [O_2] on self-renewal and quiescence of ALL cells

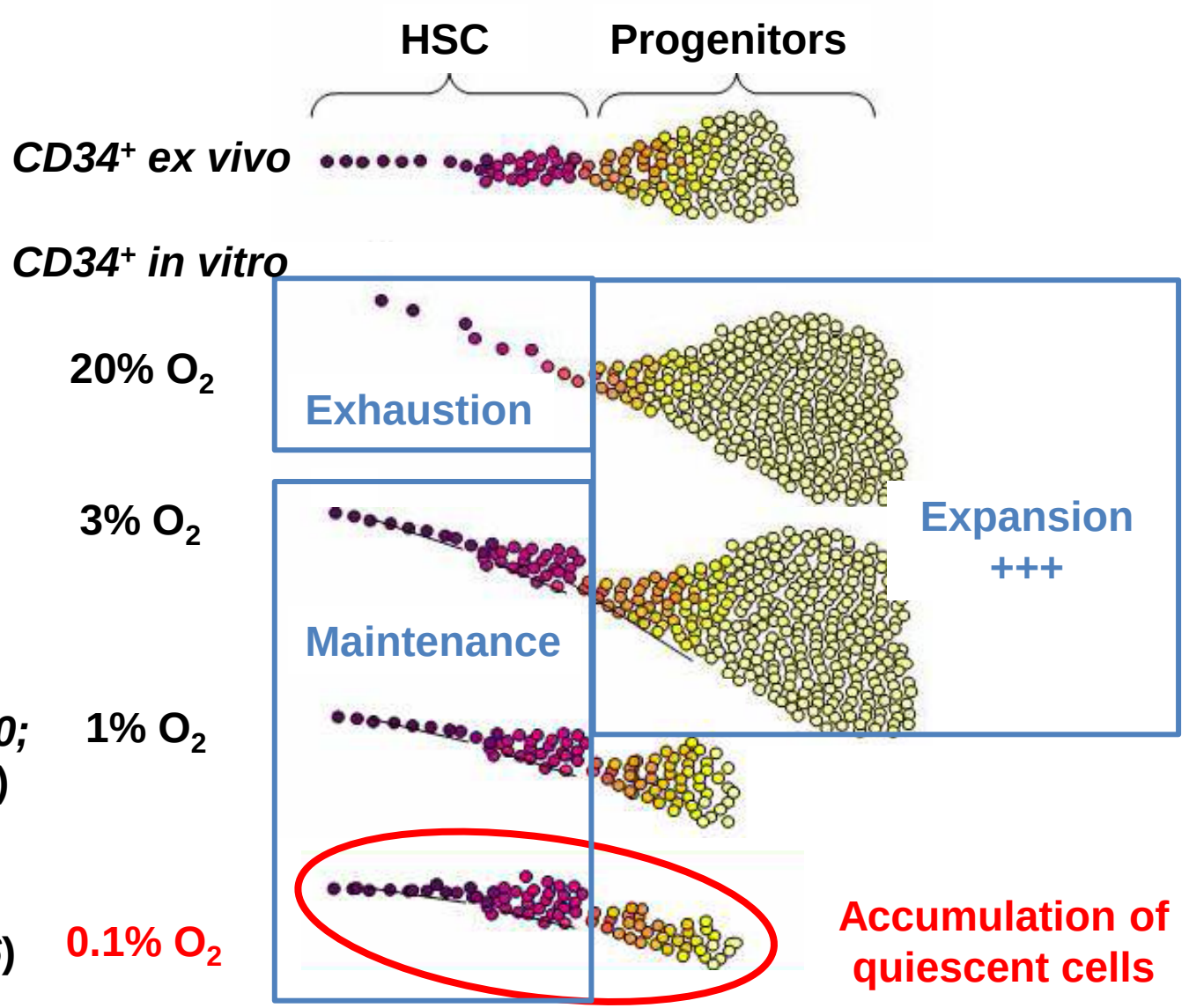
Role of quiescence in chemoresistance

Potential Clinical benefit

To explore mechanisms of persistence of residual LIC in ALL

Potential role in relapse

A BM environmental factor : O_2
What do we know ? Previous results



Material

Human cell line : NALM6 (Pre-B)

✓**Origin : 19 year-old man in relapse; peripheral blood (1976)**

✓**Immunophenotype : $CD3^-$, $HLA\text{-}DR^+$, $CD10^+$, $CD19^+$, $HLA\text{-}DR^+$, $CD34^-$, $cyCD79a^+$, $CD37^-$, $CD80^-$, $CD138^+$, sm/cy IgG^-, cy IgM^+, sm IgM^-**

✓**Genetic : closed to diploid caryotype**

46 (43-47)<2n>XY

t(5 ; 12) (q33.2 ; p13.2) (ETV6 – PDGFRB)

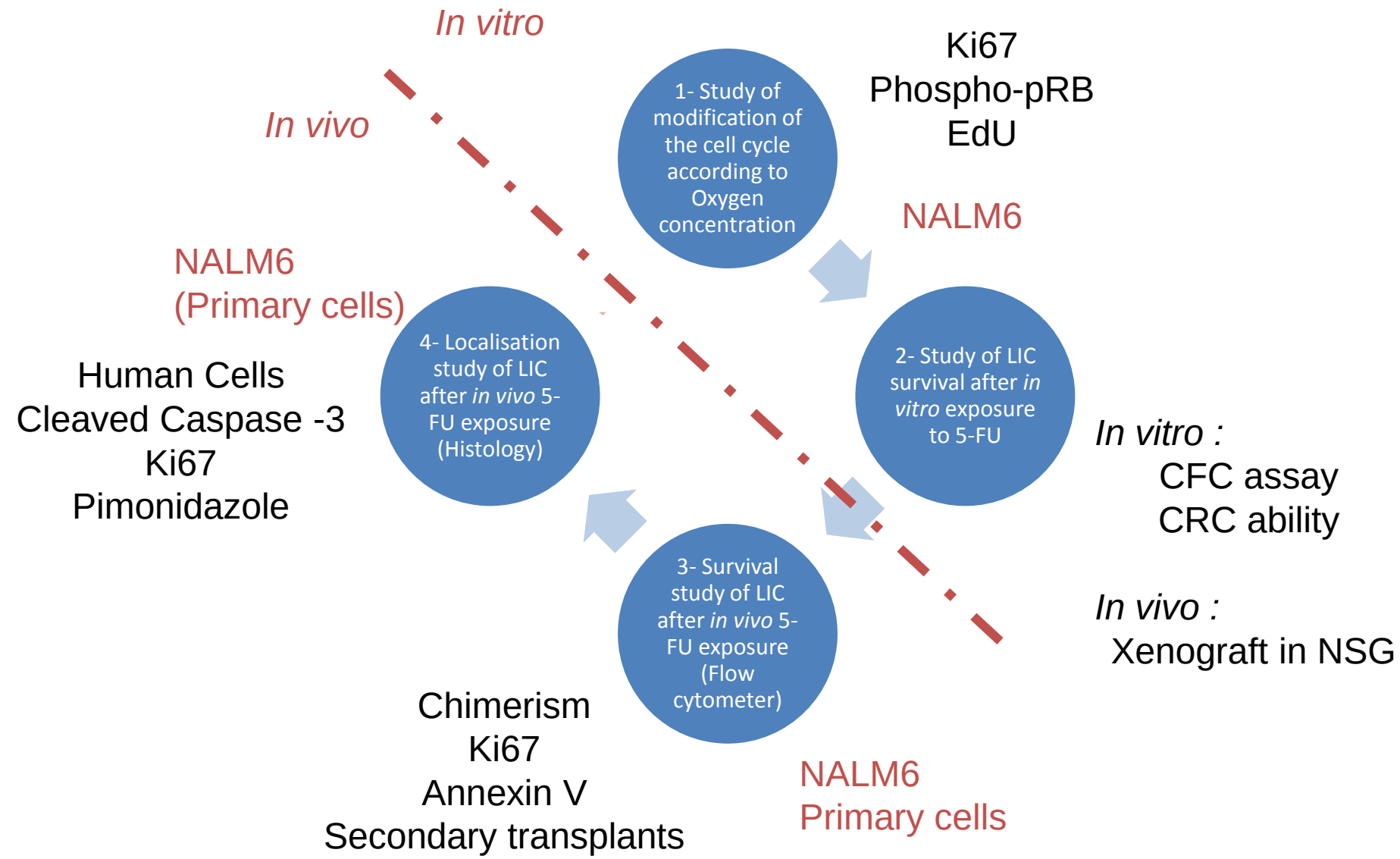
Primary cells from patients :

✓ **Samples heterogeneity (Dif genetic Abnormalities…)**

✓***In vitro* culture difficulties**

✓**Transplant difficulties**

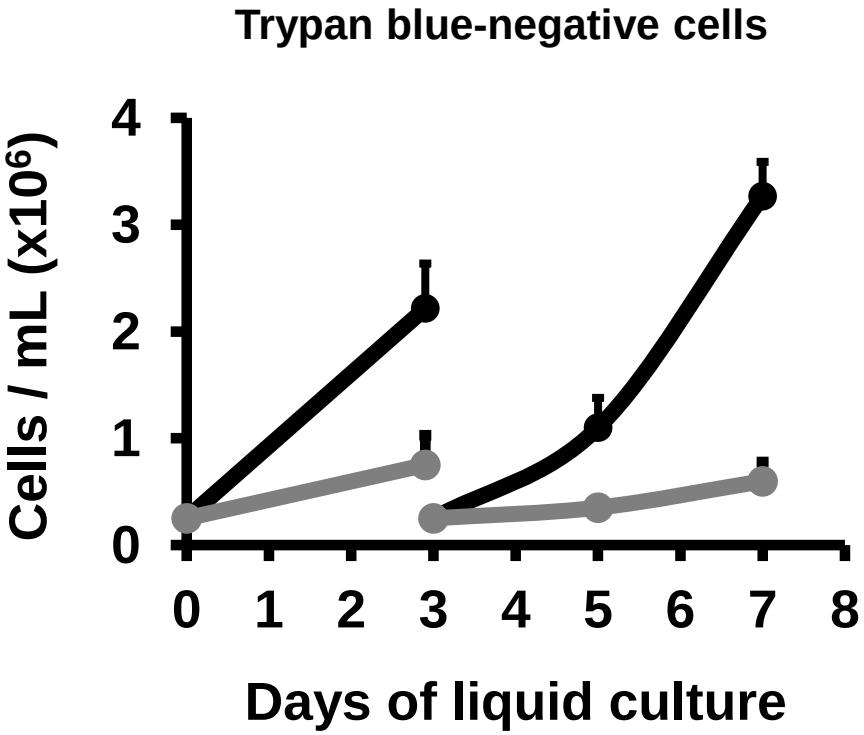
Methods

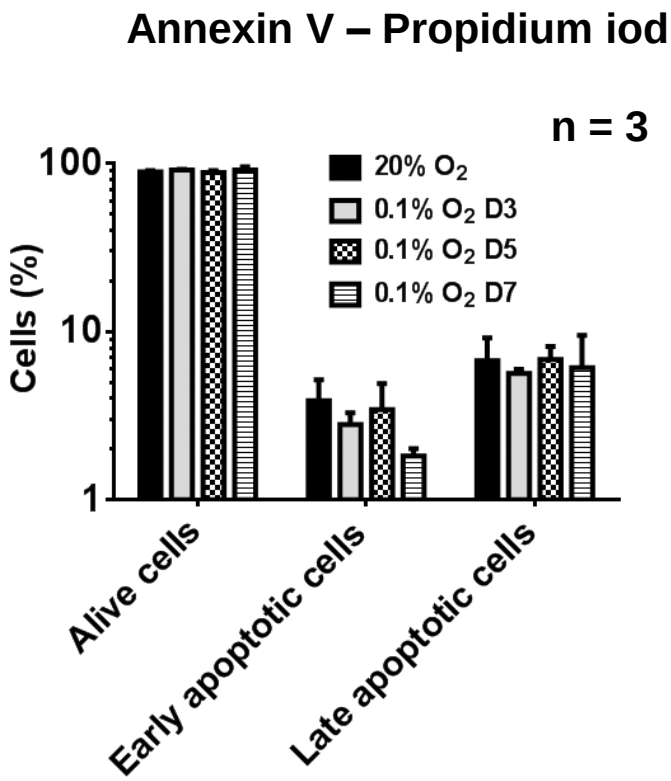

In vitro
In vivo
1- Study of modification of the cell cycle according to Oxygen concentration
Ki67
Phospho-pRB
EdU
NALM6
2- Study of LIC survival after *in vitro* exposure to 5-FU
In vitro :
CFC assay
CRC ability
In vivo :
Xenograft in NSG
NALM6
Primary cells
3- Survival study of LIC after *in vivo* 5-FU exposure (Flow cytometer)
Chimerism
Ki67
Annexin V
Secondary transplants
4- Localisation study of LIC after *in vivo* 5-FU exposure (Histology)
NALM6
(Primary cells)
Human Cells
Cleaved Caspase -3
Ki67
Pimonidazole

1- Study of modification of the cell cycle according to oxygen concentration

NALM6 : *In vitro* part 1

Culture at 0.1% O_2 slows down NALM6 proliferation





Conclusion : NALM6 cells survive at 0.1% (low mortality) low proliferation

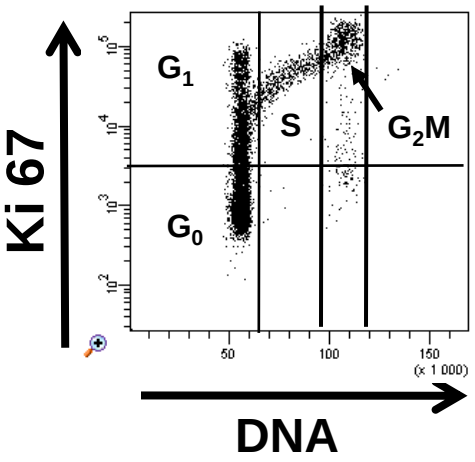
Which is their cell cycle status ?

Culture at 0.1% O_2 induces cell cycle arrest in G_0

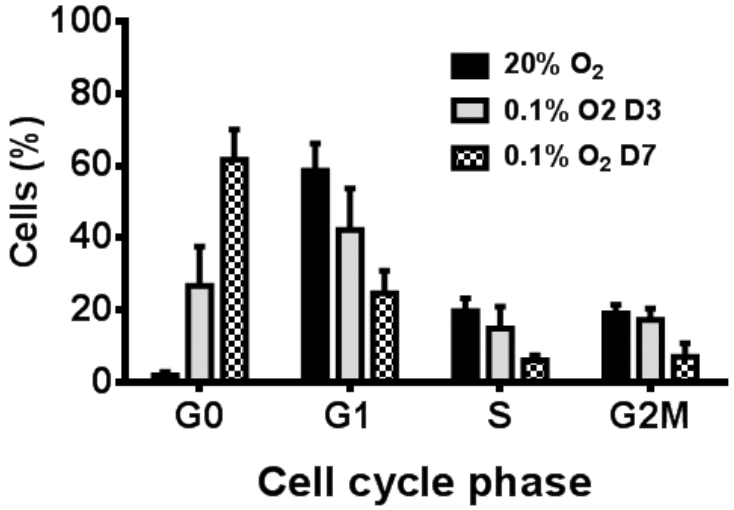
Ki67 :

- **Nuclear protein associated to hétérochromatin**
- **Role ??**

  DNA organisation ? rRNA synthesis ?
- **Required for cell proliferation**



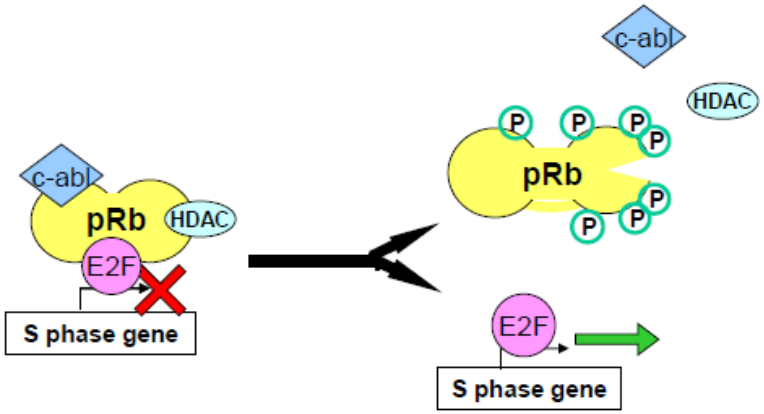
Cell cycle distribution during culture
(n = 5)



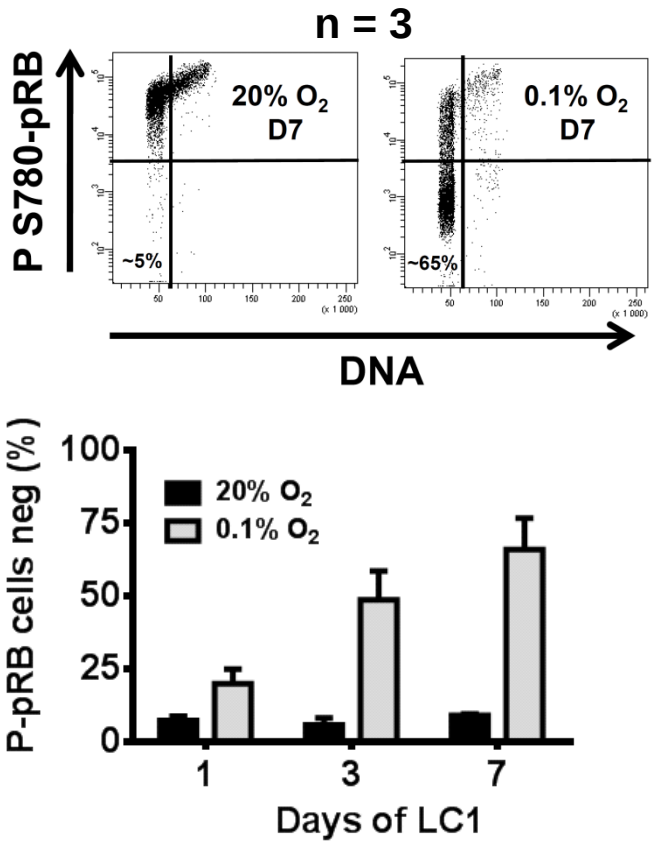
Conclusion : NALM6 cells become quiescent in severe hypoxia

Molecular actors ?

Culture at 0.1% O_2 modifies the phosphorylation of pRB



From Guitart, University of Bordeaux, Thesis n°1672



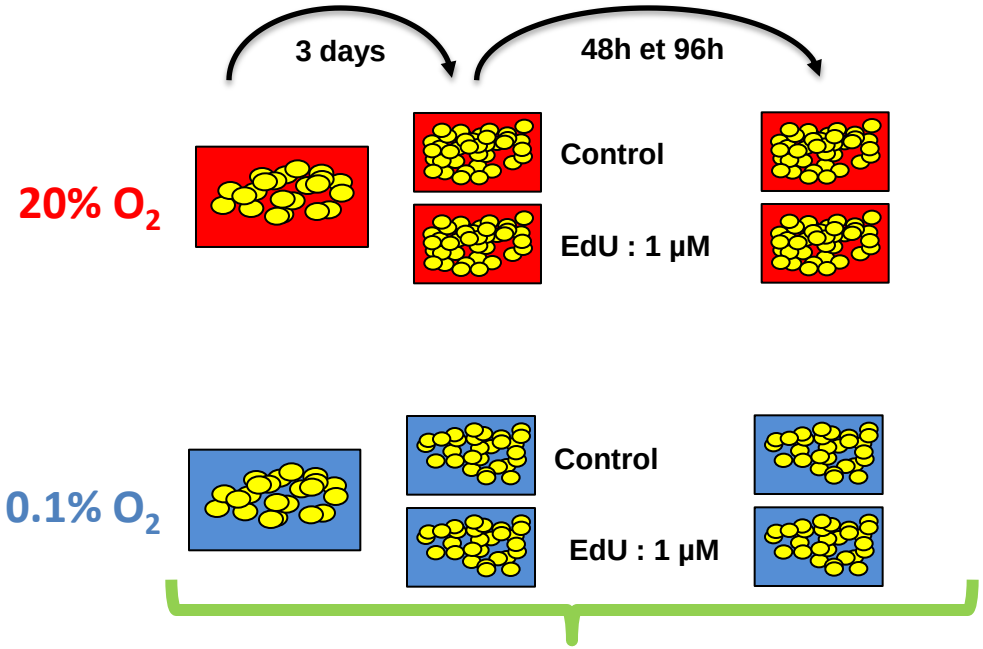
Conclusion : quiescent NALM6 cells express dephosphorylated pRb

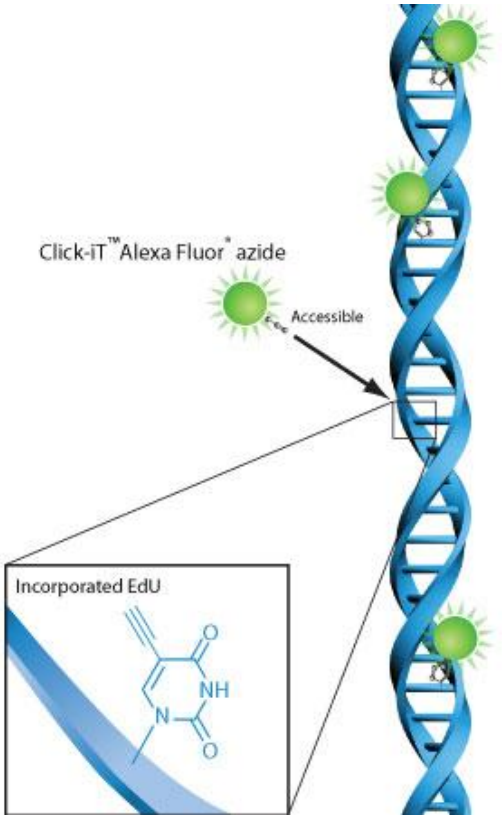
Do these cells remain undivided from day 3 to day 7 ? EdU assays

EdU Protocol

EdU (analog of thymidine) :
-Incorporated in DNA during S phase

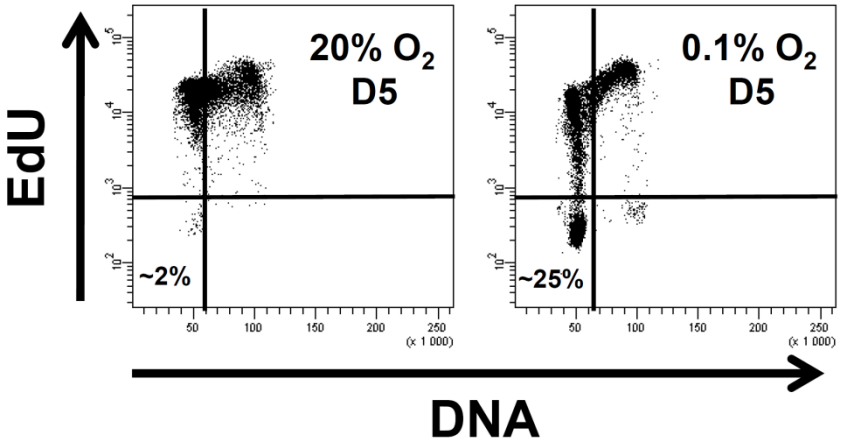
Incorporated EdU is detected using AlexaFluor488 azide

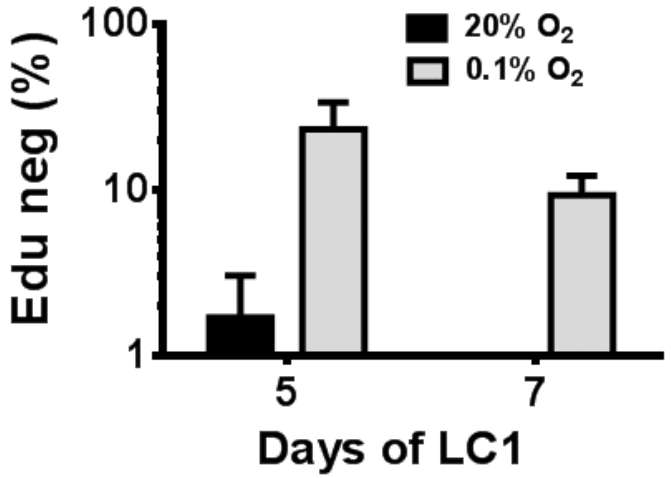




Culture at 0.1% O_2 induces G_0/G_1 cell cycle arrest of a subset of NALM6 cells

Cell cycle kinetics (EdU incorporation) during culture (n = 3)

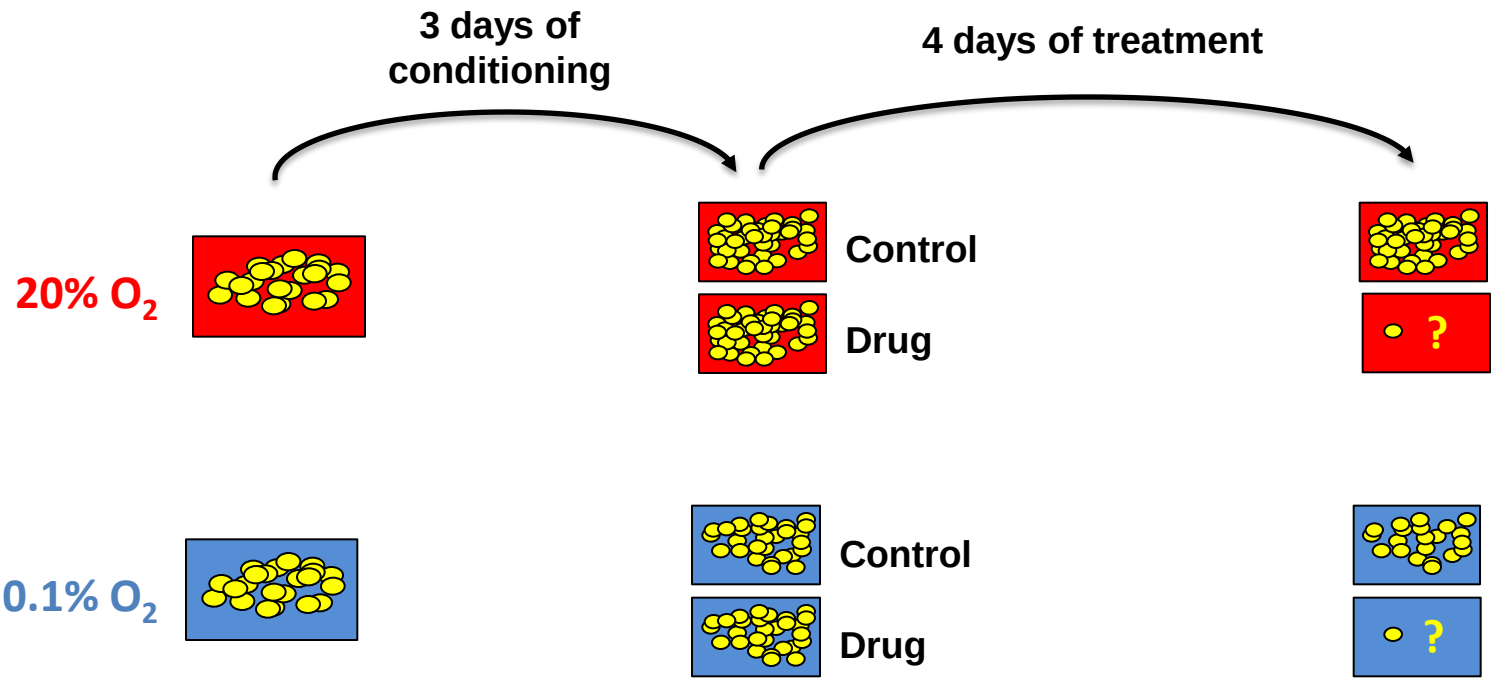




Conclusion :
13% of NALM-6 cells did not enter in S phase after 4 days of exposure to hypoxia

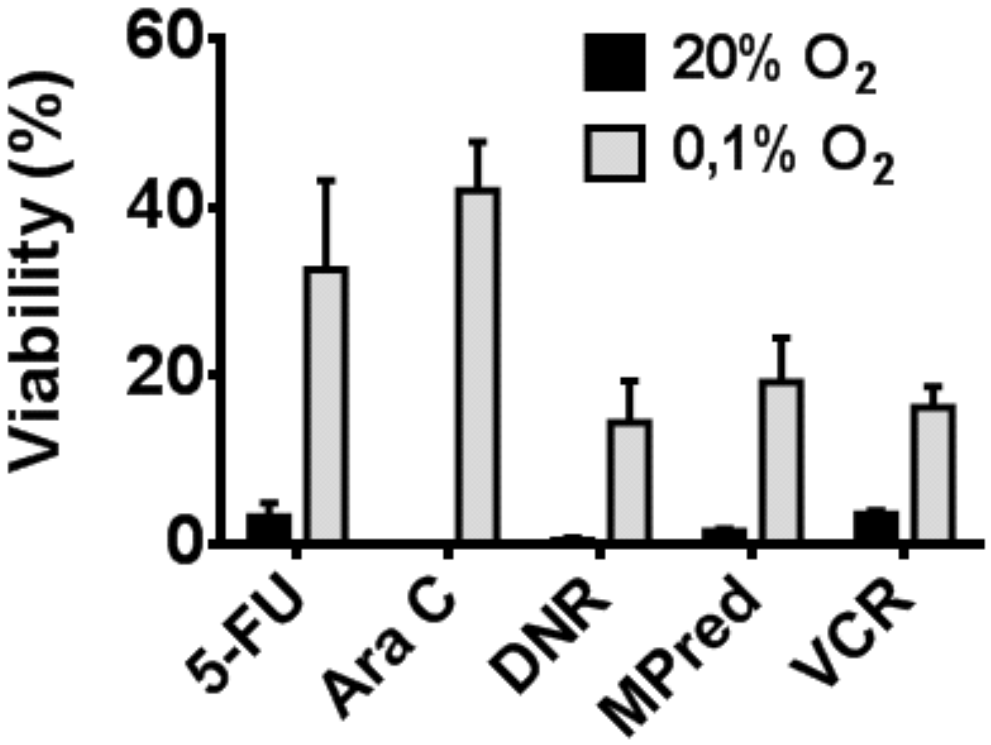
Is this arrest related to chemotherapeutic agents resistance ?

Protocol of NALM6 Primary culture



Culture at 0.1% O_2 contributes to NALM6 chemoresistance

Chemoresistance during culture (n = 3)



At 1 and 3%, the results are similar to those at 20%

Do these residual viable cells behave as LIC ?

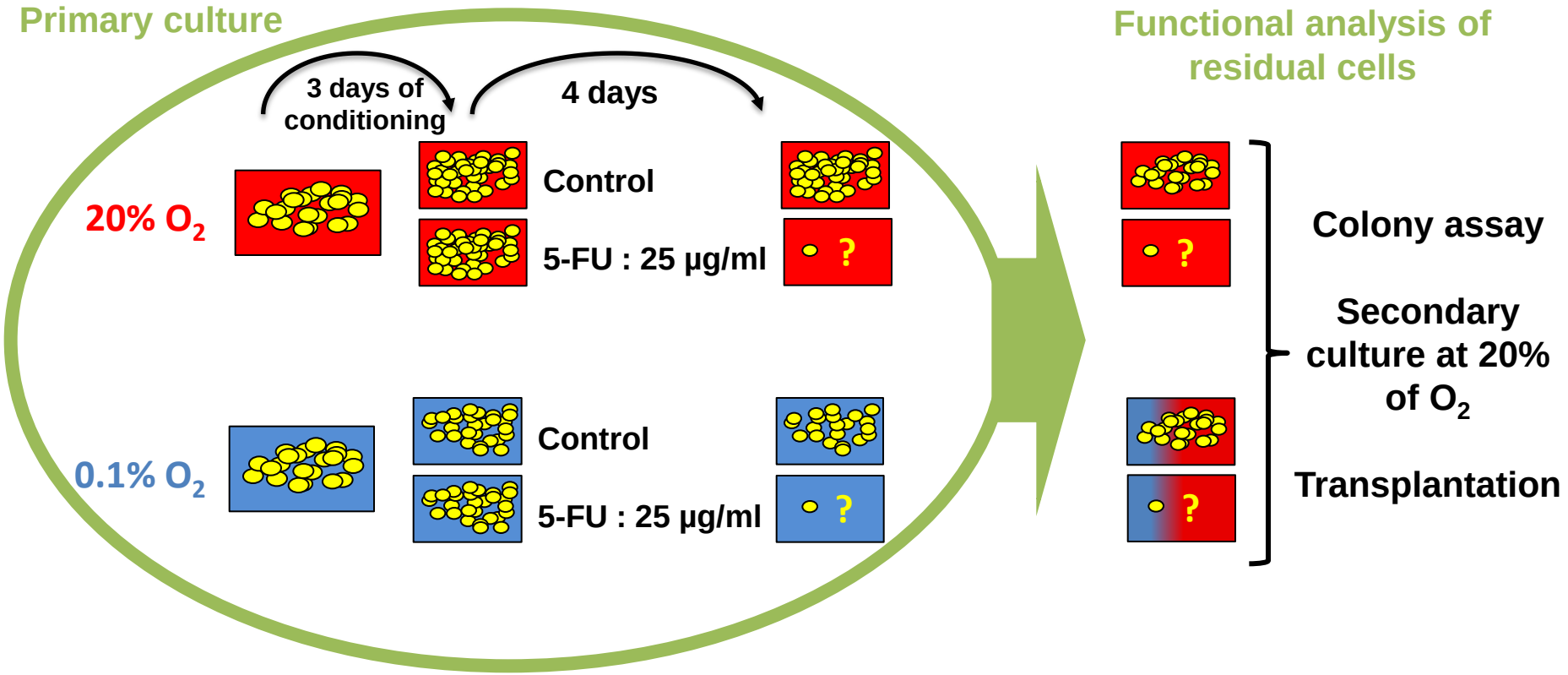
2- Study of LIC survival after *in vitro* exposure to 5-FU

NALM6 : *In vitro* part 2

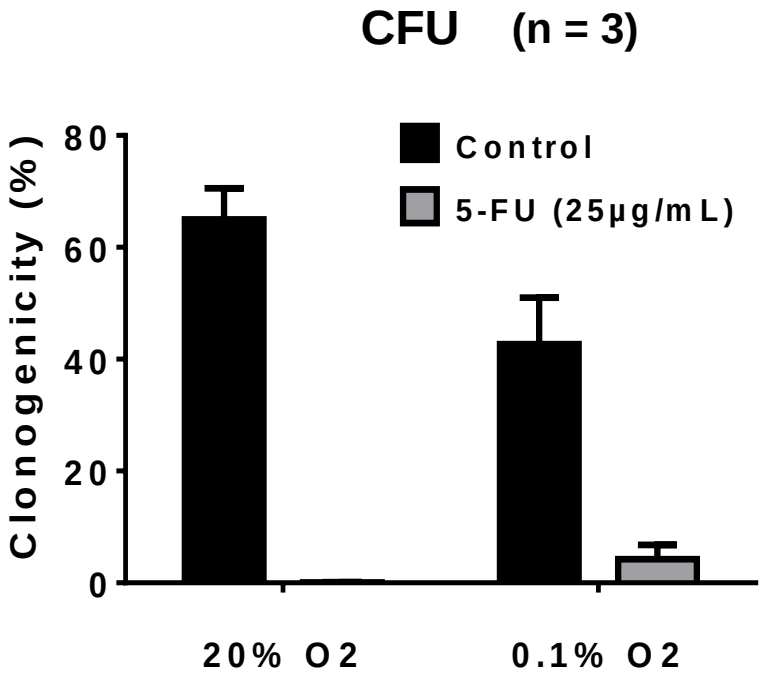
Protocol of primary and secondary culture

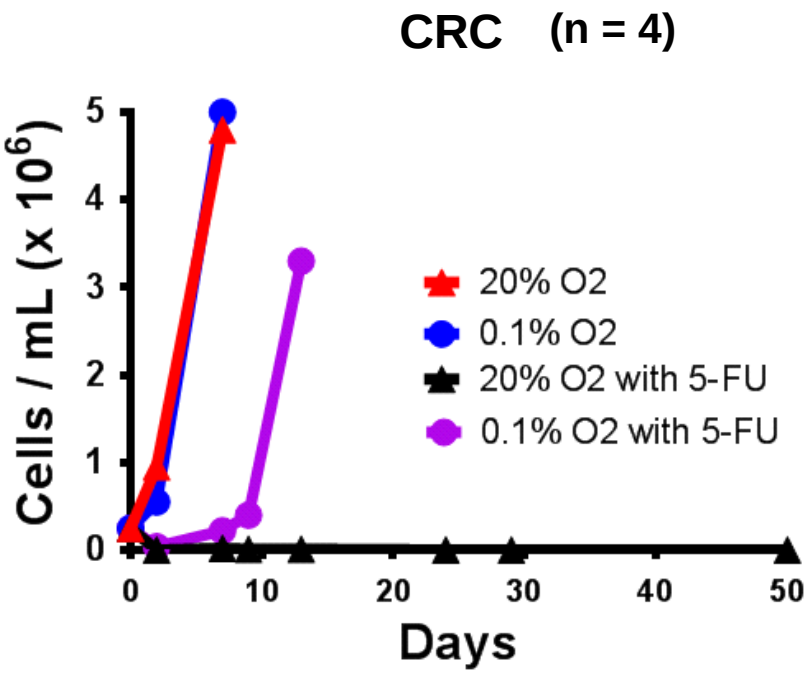
5-FU (5-Fluoro-Uracil) : target S phase

***In vitro* assay to detect :**
-clonogenic cells
-LIC, by repopulating liquid culture



5-FU resistant cells in Hypoxia contain CFU and CRC populations

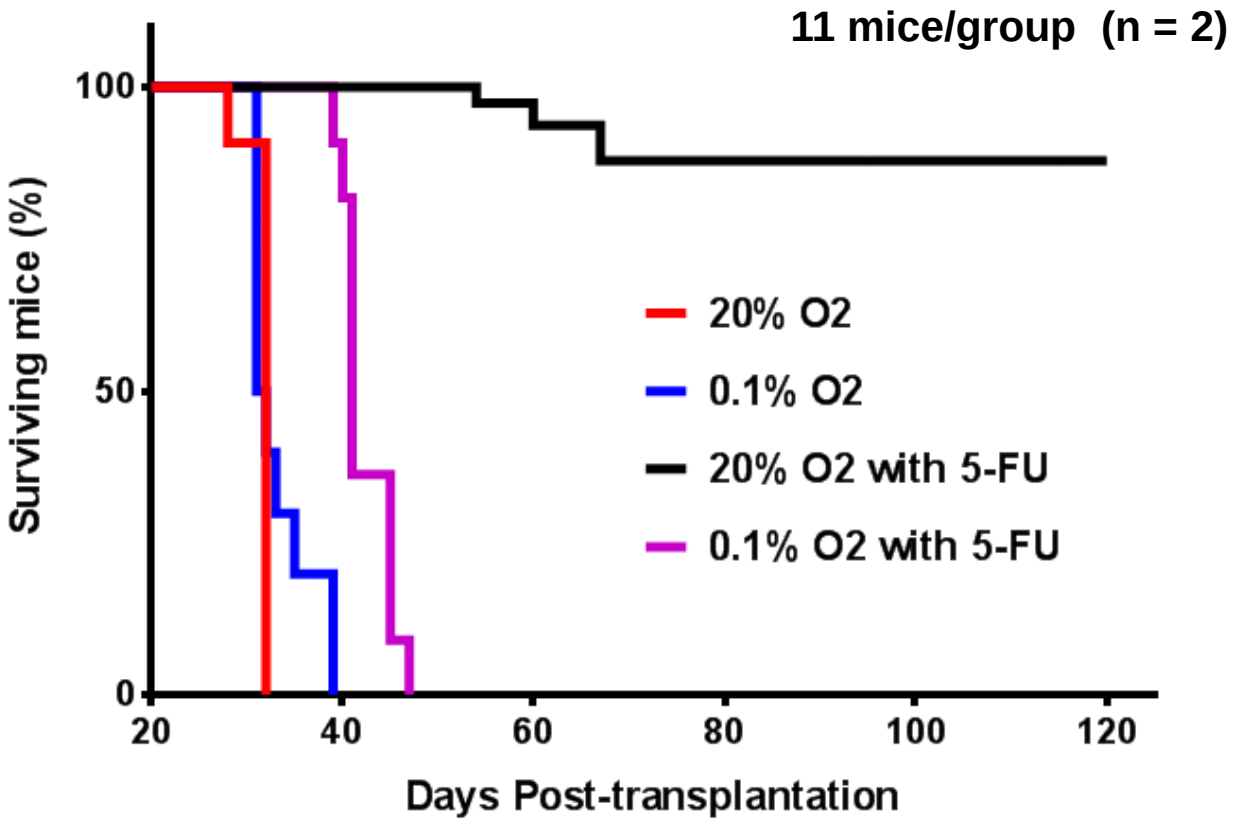




Conclusion :
NALM6 is heterogeneous
After 7 days in hypoxia, CFU-L and CRC are maintained
Hypoxia protects CFU-L and CRC from 4 days of 5-FU exposure

What would happen *in vivo?*

5-FU resistant cells in Hypoxia contain LIC

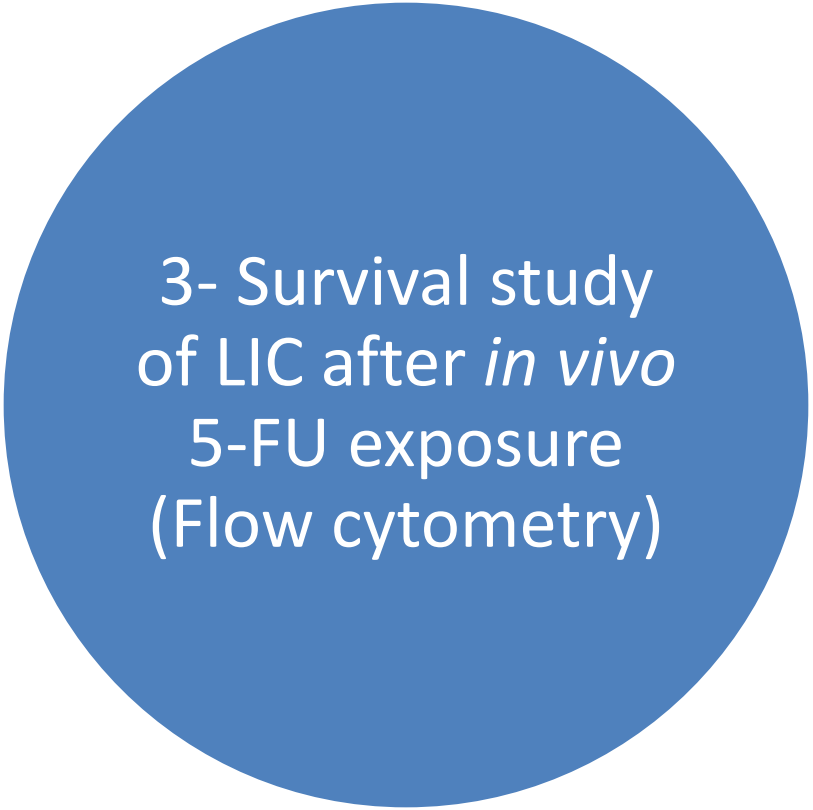


Conclusions :
Control conditions : no significant difference
5-FU conditions :
At 20%, all LIC are killed by 5-FU
Primary culture at 0.1% maintains LIC

In vitro results: Conclusion

NALM6 culture at 0.1% O_2 leads to:

- **Proliferation slowing down without mortality increase**
- **G0 arrest of a limited proportion of cells (75% are still cycling after 3 days)**
- **Resistance to several drugs used in ALL**
- **Resistance to 5-FU of rare quiescent LIC able to**
  - **Repopulate secondary liquid cultures *in vitro***
  - **Engraft leukemia into mice**

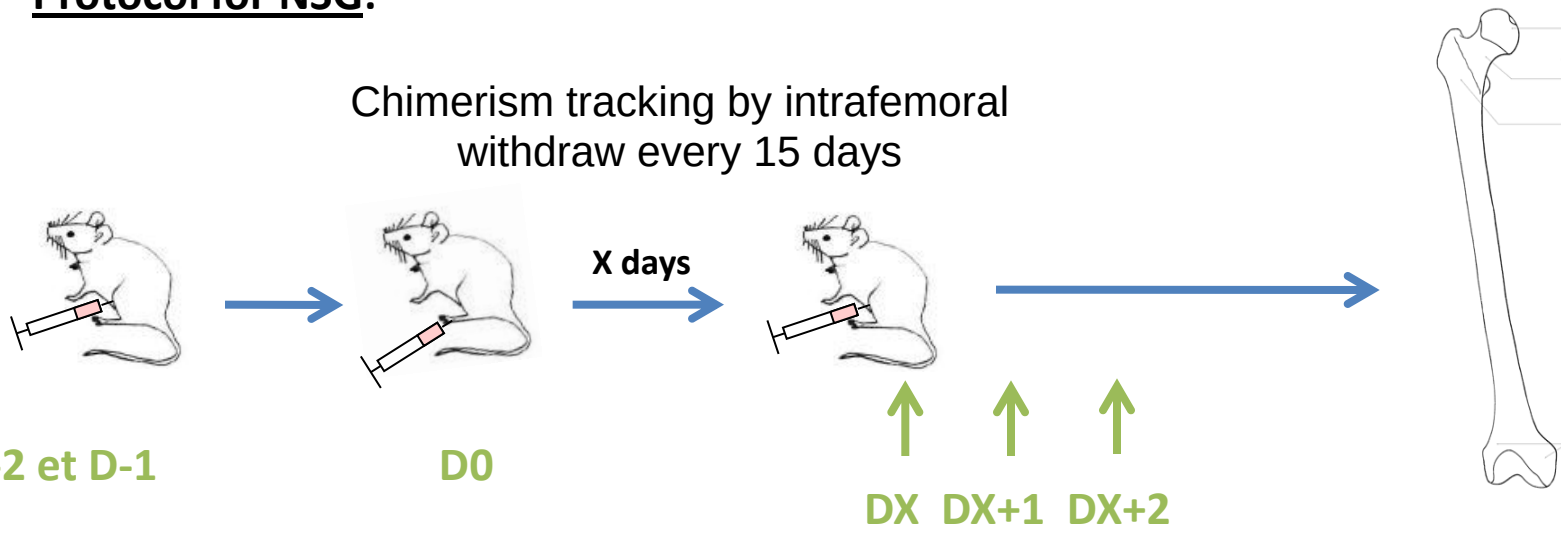


NALM6 : *In vivo* assay 1
Patient : *In vivo* assay (Experimental bias)

Flow cytometry analysis of *in vivo* chemoresistant cells

Protocol for NSG:

Chimerism tracking by intrafemoral withdraw every 15 days



Busulfan conditioning (20mg/kg)

Transplantation of 250 000 primary cells (IF) or 10 000 NALM6 cells (IV)

Chimerism > 50% : Intra-peritoneal 5-FU treatment (150mg/kg)

Sacrifice DX+3
Cell analysis
-Bone marrow

in vivo experiments

➢**Residual human cells FACS analysis (NALM6 and primary cells from patients)**

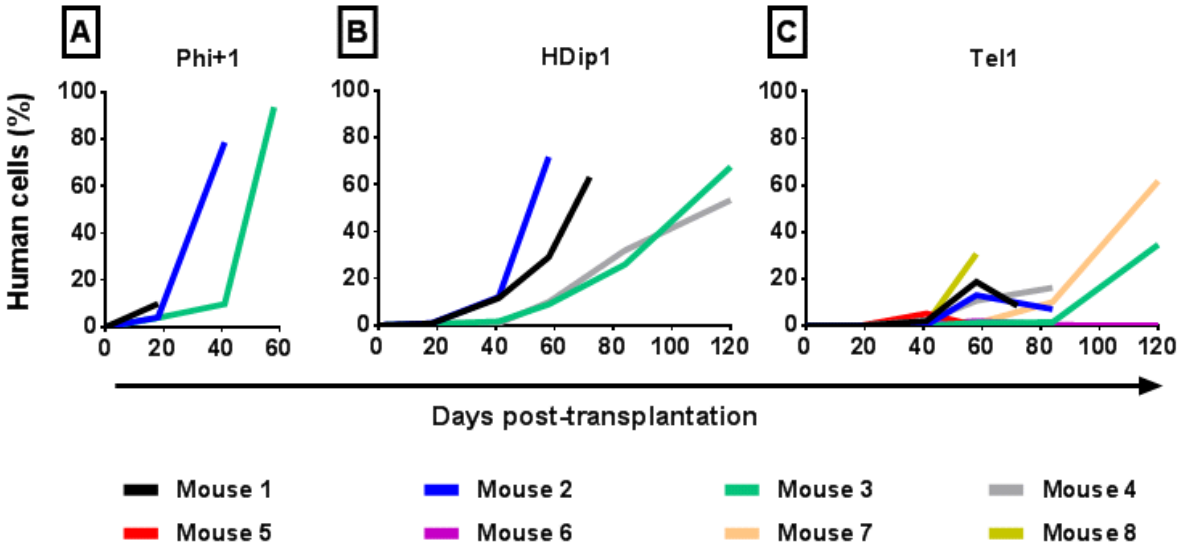
- **phenotype :**
  - **Human : HLA-DR ; hCD45 ;**
  - **Leukaemic B : hCD10 ; hCD19 ;**
  - **Stemness : hCD34 ;**

- **Cell cycle : Ki67**

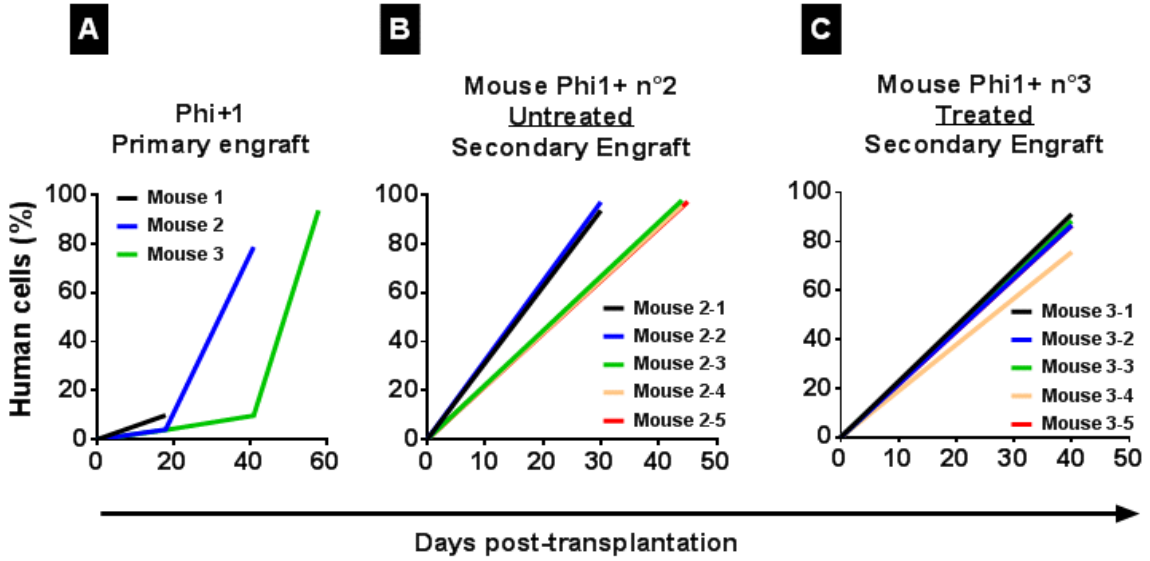
- **Secondary transplant.**

Kinetics of primary cell engraftment in femoral BM

Profiles of 3 tested samples



Secondary transplants of Phi+ patient cells



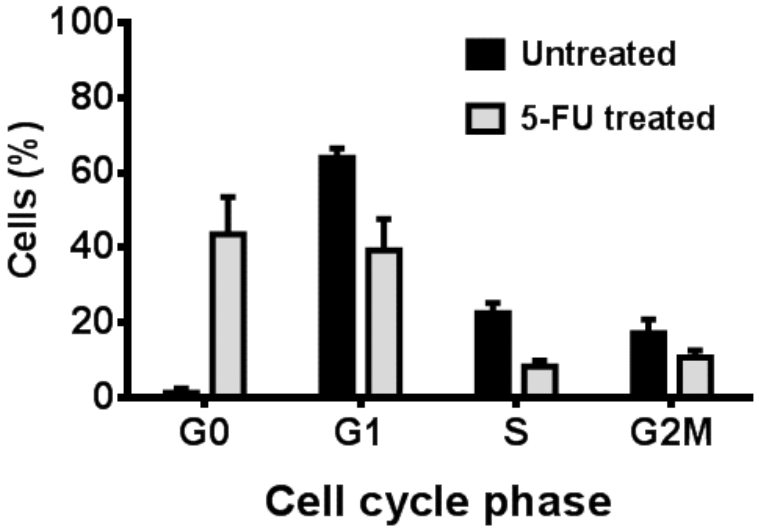
Serial transplant is lethal

BM residual human cells are mainly alive and quiescent

Proportion of Ki67 negative human cells in femur

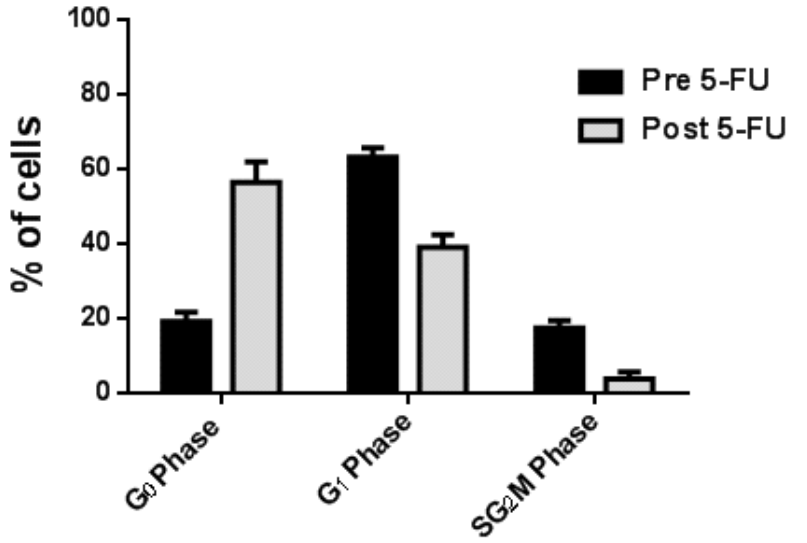
NALM6

(n = 2; 8 mice)



Patients (Secondary recipients)

(n = 1; 3 mice)



In vivo chemoresistant cells: major results of flow cytometry

➢ **NALM6 : 5-FU treatment of xenografted mice increases the percentage of quiescent cells among residual BM cells.**

➢ **Patients : Intrafemoral engraftment of human primary ALL cells in NSG mice**

- **Engraftment kinetics vary from patient to patient**
- **Serial transplantation (IV) evidences the presence of LIC whose phenotypic profile did not vary**
- **Secondary transplantation improves the mouse to mouse reproducibility kinetics of engraftment, arguing for the selection of LIC adapted to xenotransplantation**
- **Post 5-FU residual cells contain the quiescent LIC**

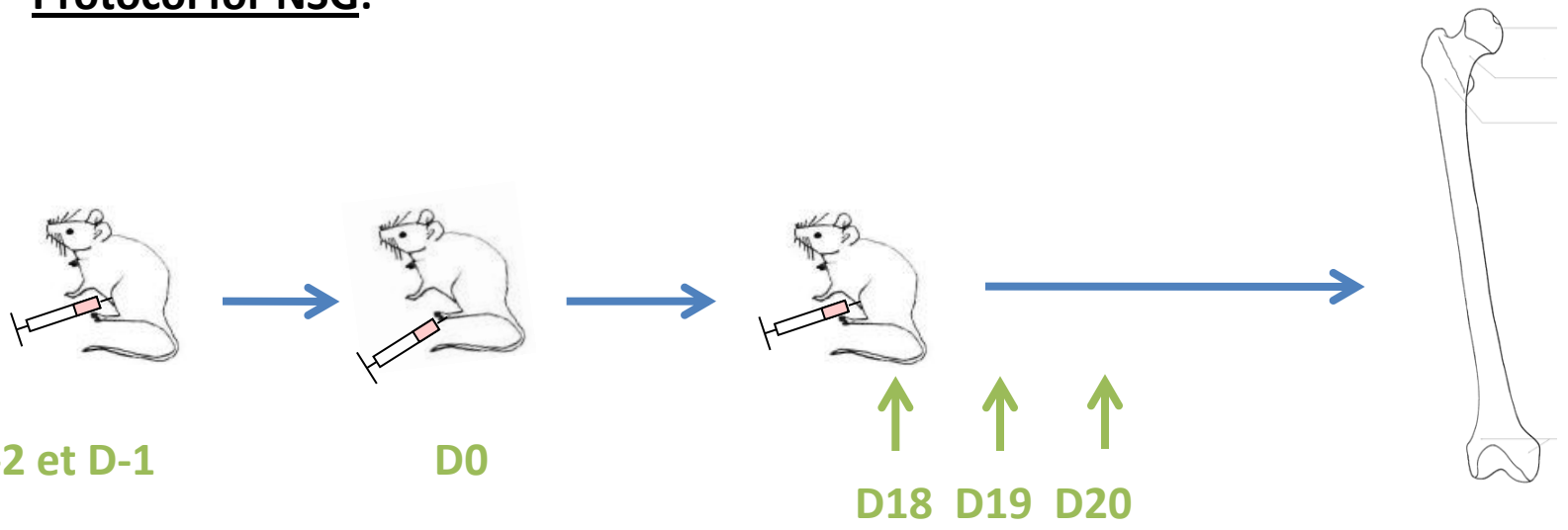
Where are the residual quescient LIC localized in BM ?

- **BM Histological sections (femurs, dorsal and caudal vertebrae)**
  - **Localisation of LIC before and after 5-FU treatment,**
  - **oxygenation and perfusion rates**

4- Localisation study of LIC after *in vivo* 5-FU exposure

Histological examination of engrafted cells

Protocol for NSG:



Busulfan conditioning (20mg/kg)

Transplantation of 10 000 NALM6 cells (IV)

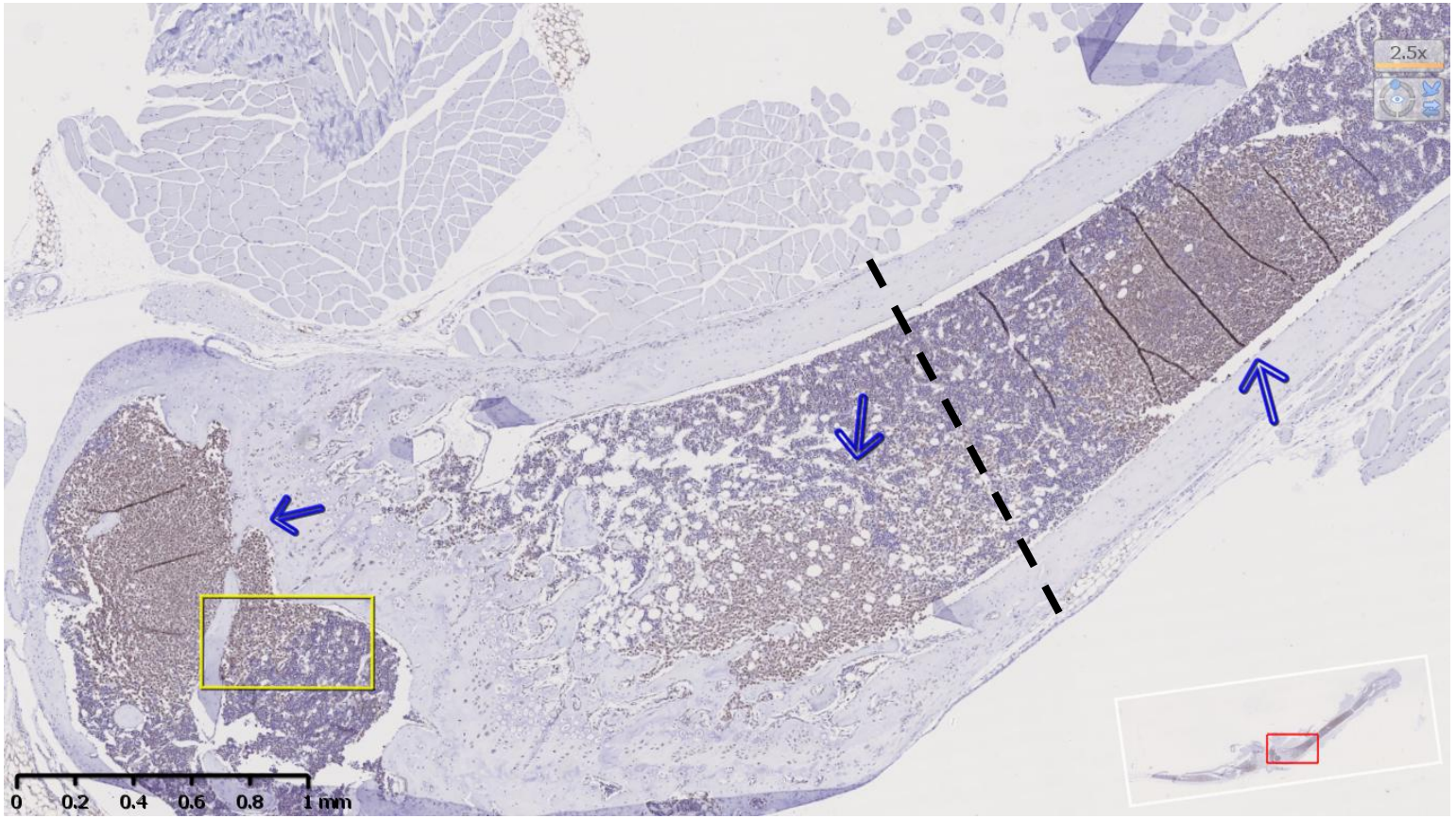
Intra-peritoneal 5-FU treatment (150mg/kg)

Sacrifice D21 Histological analysis of BM

LPRO
Laboratoire Physiopathologie de la Résorption Osseuse
INSERM - UN UMR 957

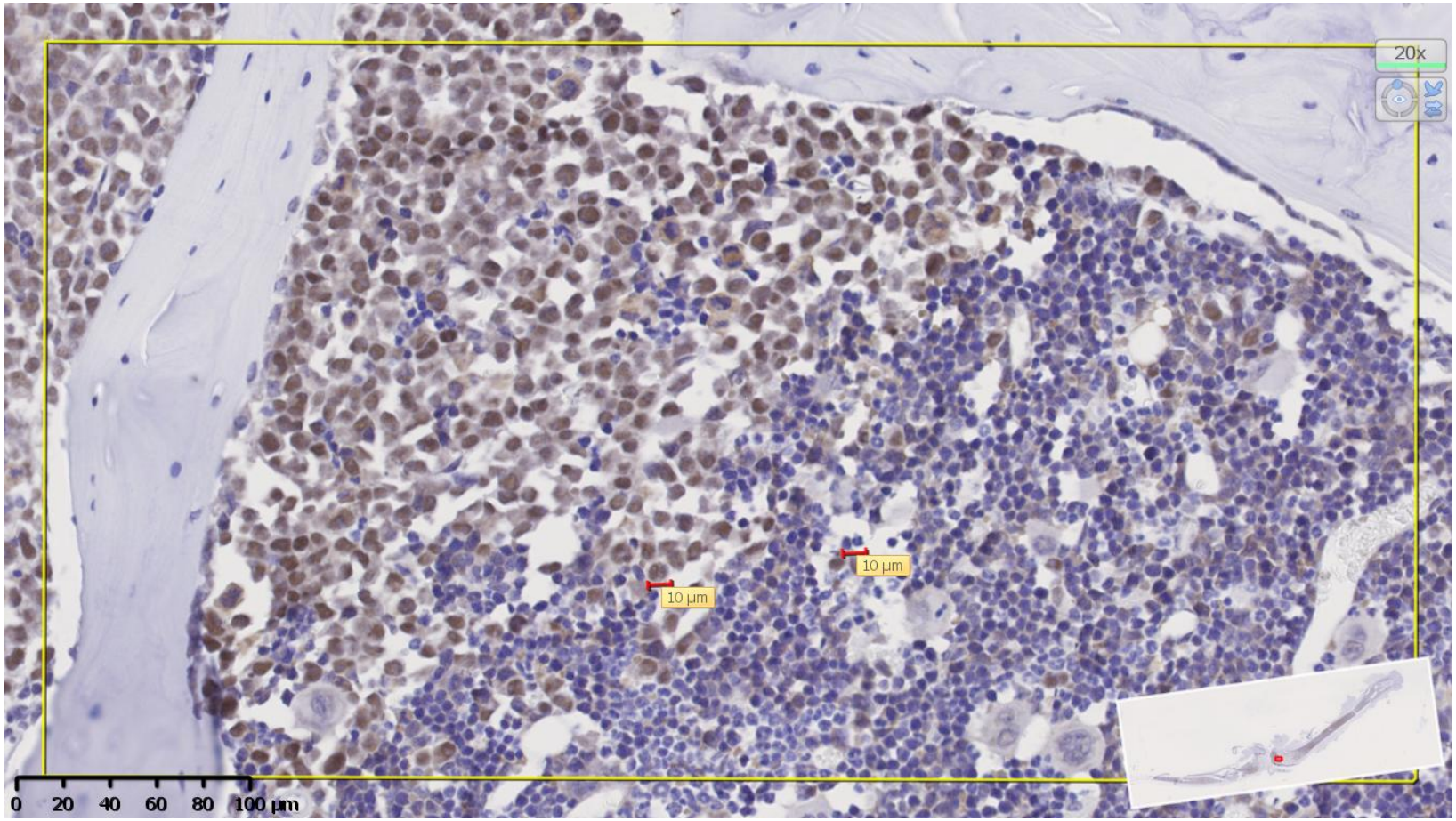
At day 21 after transplantation, NALM6 cells aggregates are dispersed in mouse TBA and LBA

ALU sequences labelling; one representative femoral section (n = 3 mice)



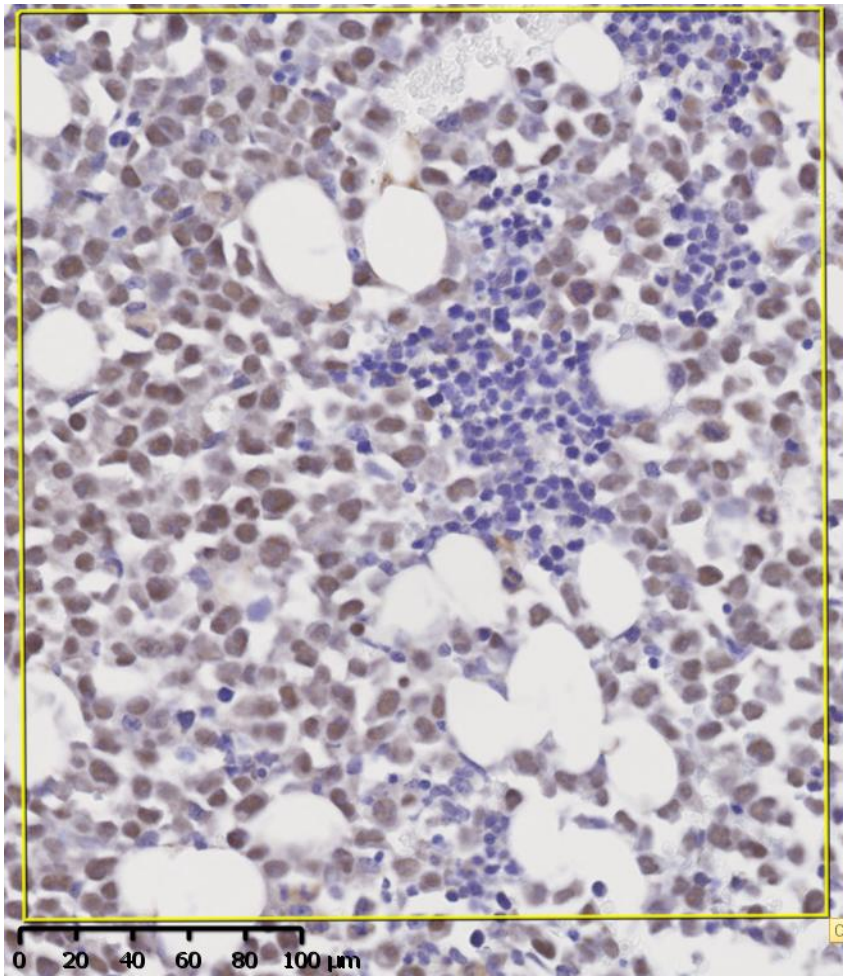
At day 21 after transplantation, NALM6 cells aggregates are dispersed in mouse TBA and LBA

x200 ; ALU labeling; one representative section (n = 2 souris)

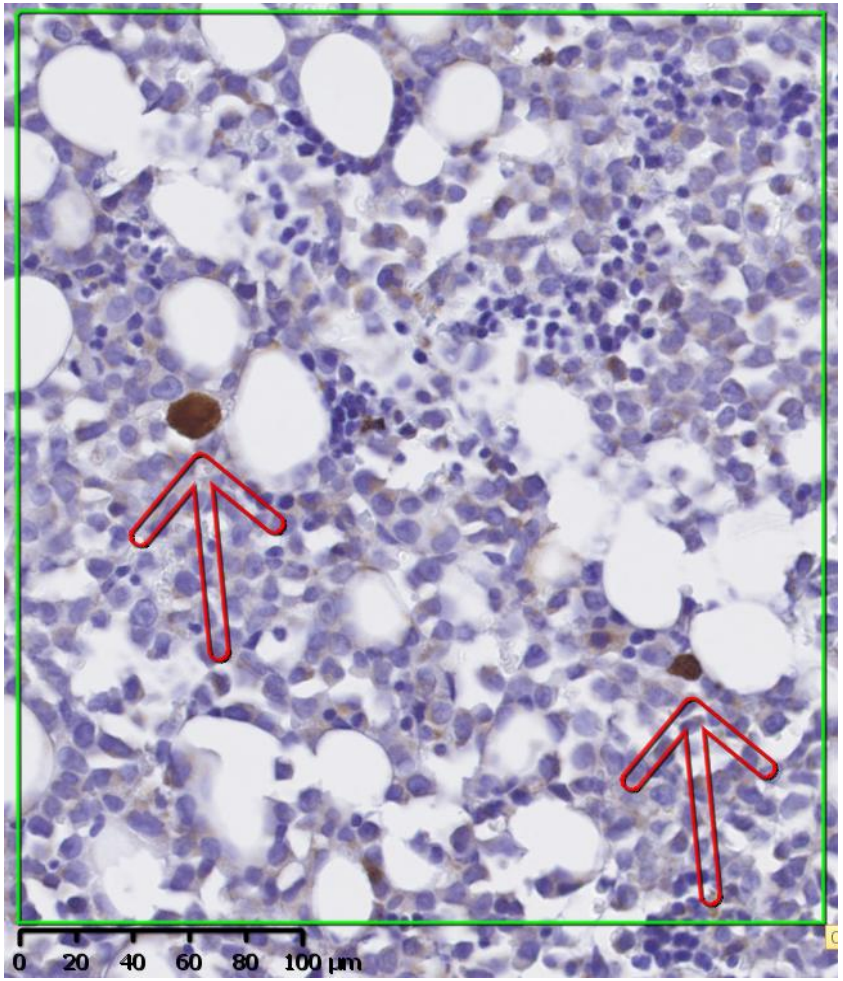


At day 21 after transplantation, apoptotic NALM6 cells are very rare in the BM

x200 ; cleaved Caspase 3 labelling; one representative section (n = 2 mice)



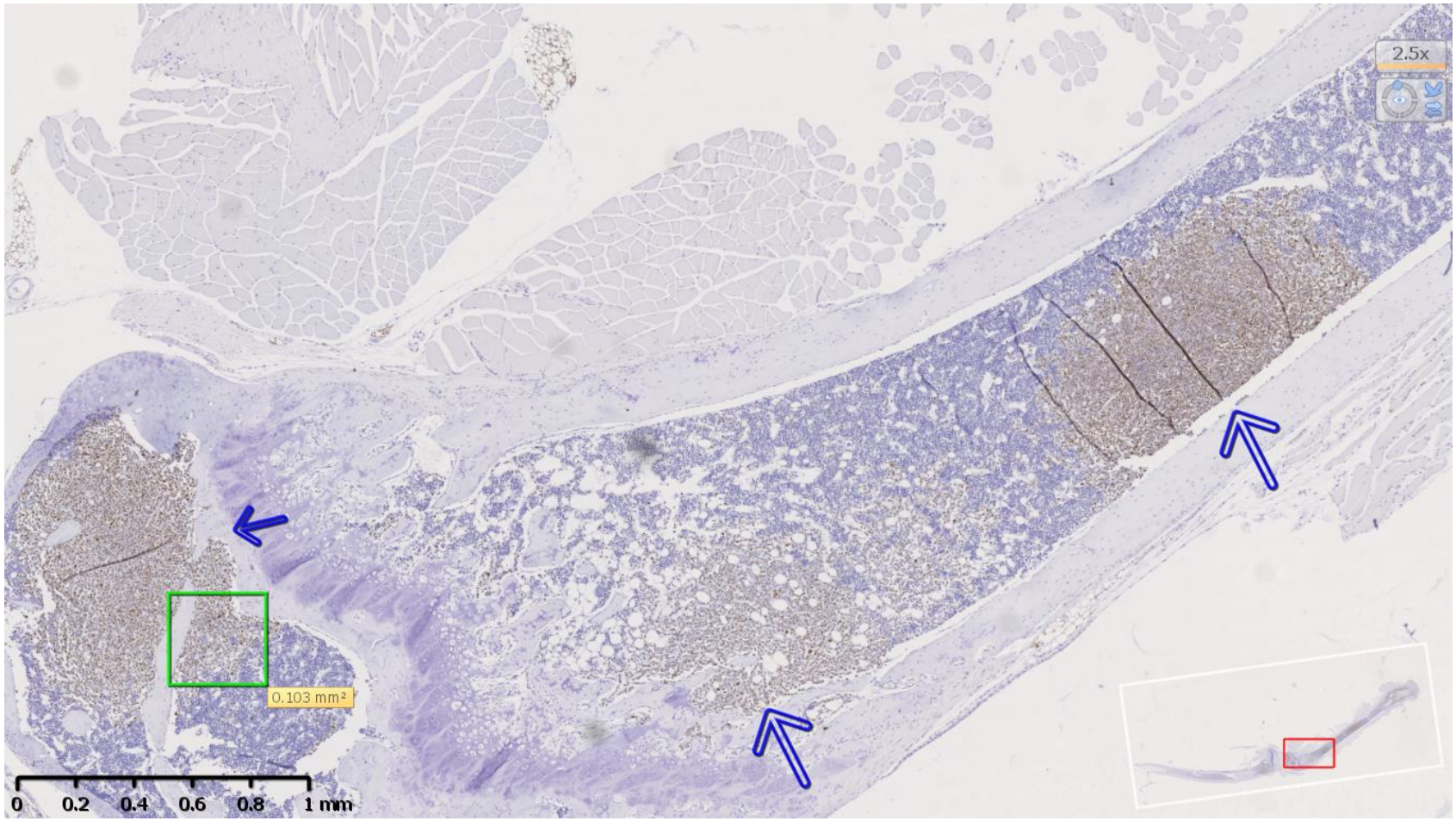
Human cells



Apoptotic cells

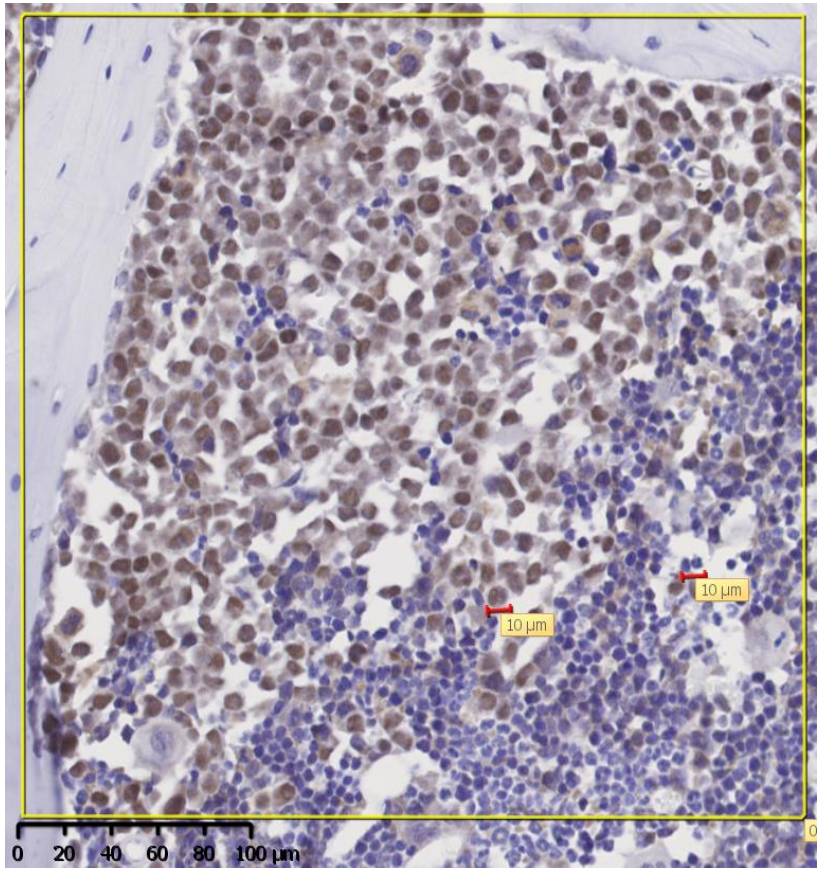
At day 21 after transplantation, quiescent NALM6 cells are very rare in the BM

Ki67 labelling; one representative section (n = 2 mice)

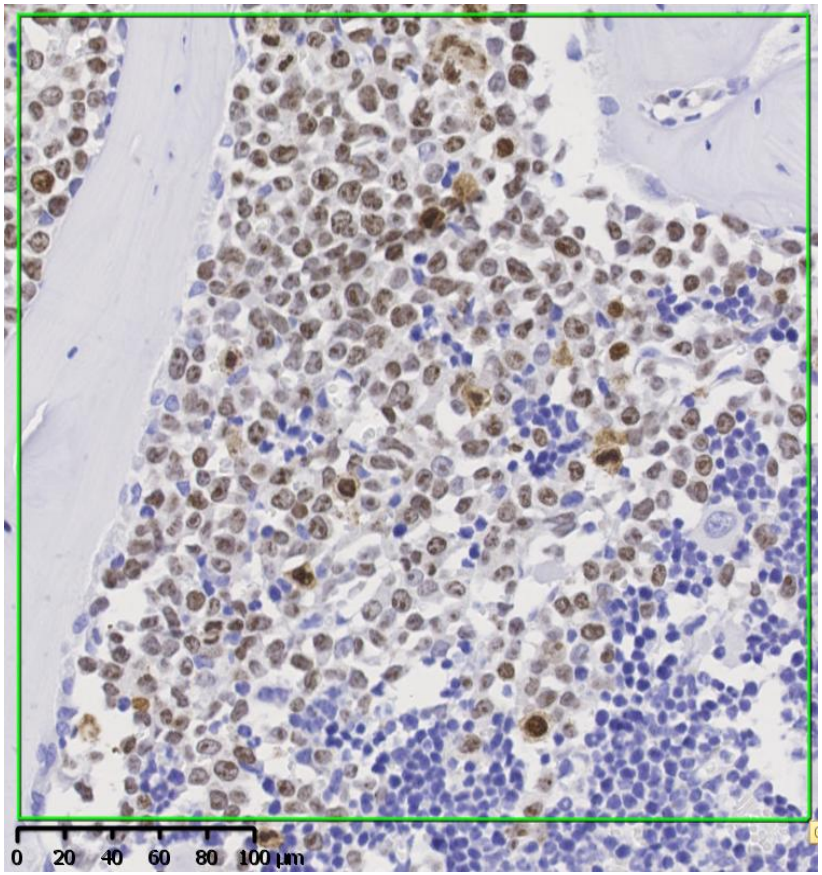


At day 21 after transplantation, quiescent NALM6 cells are very rare in the BM

Ki67 labelling; one representative section (n = 2 mice)



Human cells



Proliferating cells

Conclusion

- Leukemic aggregates dispersed in intact residual murine hematopoietic areas suggest a clonal NALM6 BM seeding and development (to be confirmed)

- Apoptotic/ dead cells are very rare

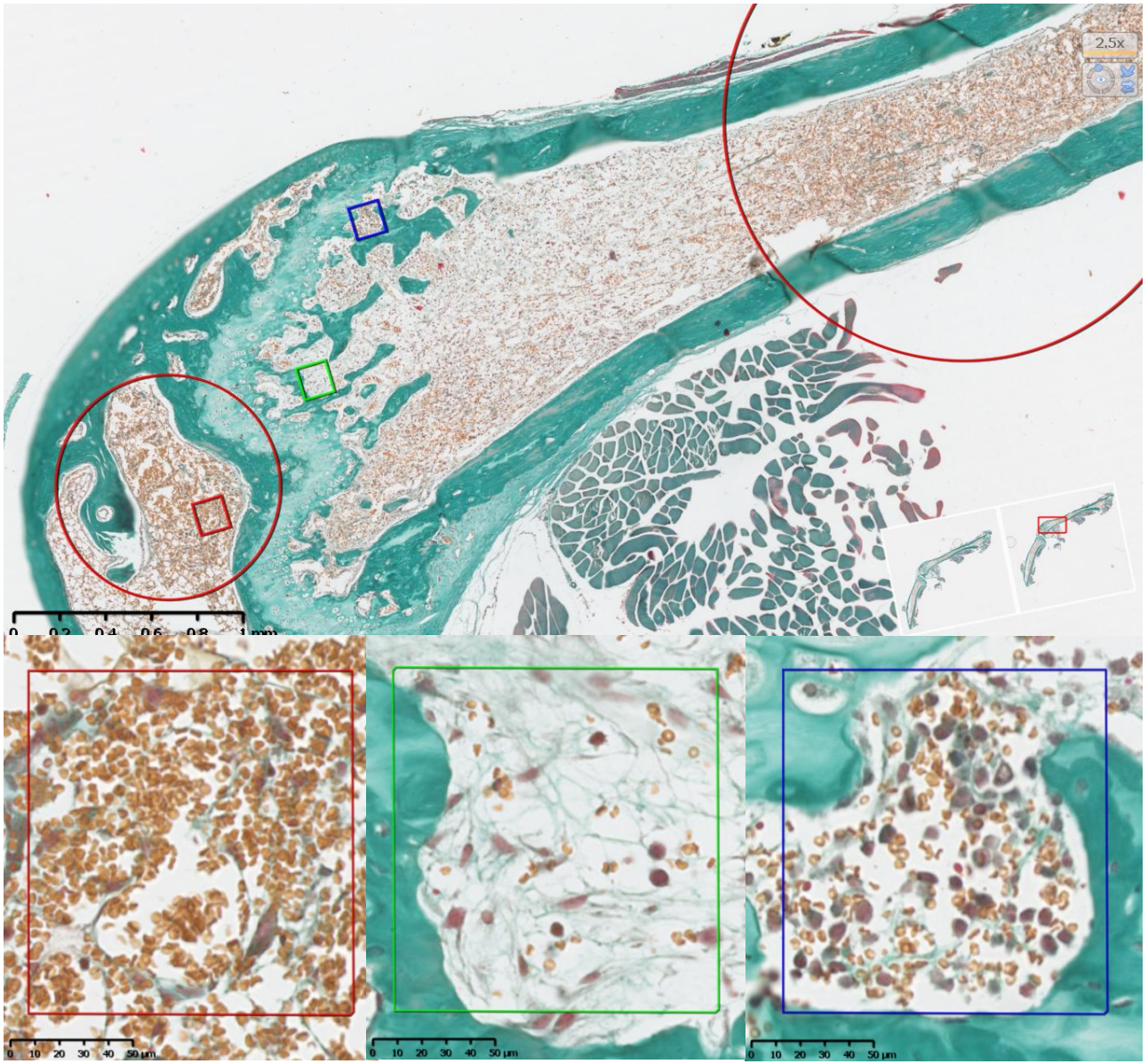
- Most cells are proliferating

- Are these cell aggregates hypoxic ? *Benito et al, PLoS One. 2*011

❑ Identifying specific BM homing/seeding sites (niches?) will require analysis of earlier engraftment time points and use of other methods.

5-FU treatment induces mice BM cytopenia

Trichrome de Masson ; representative section (n = 3 mice)



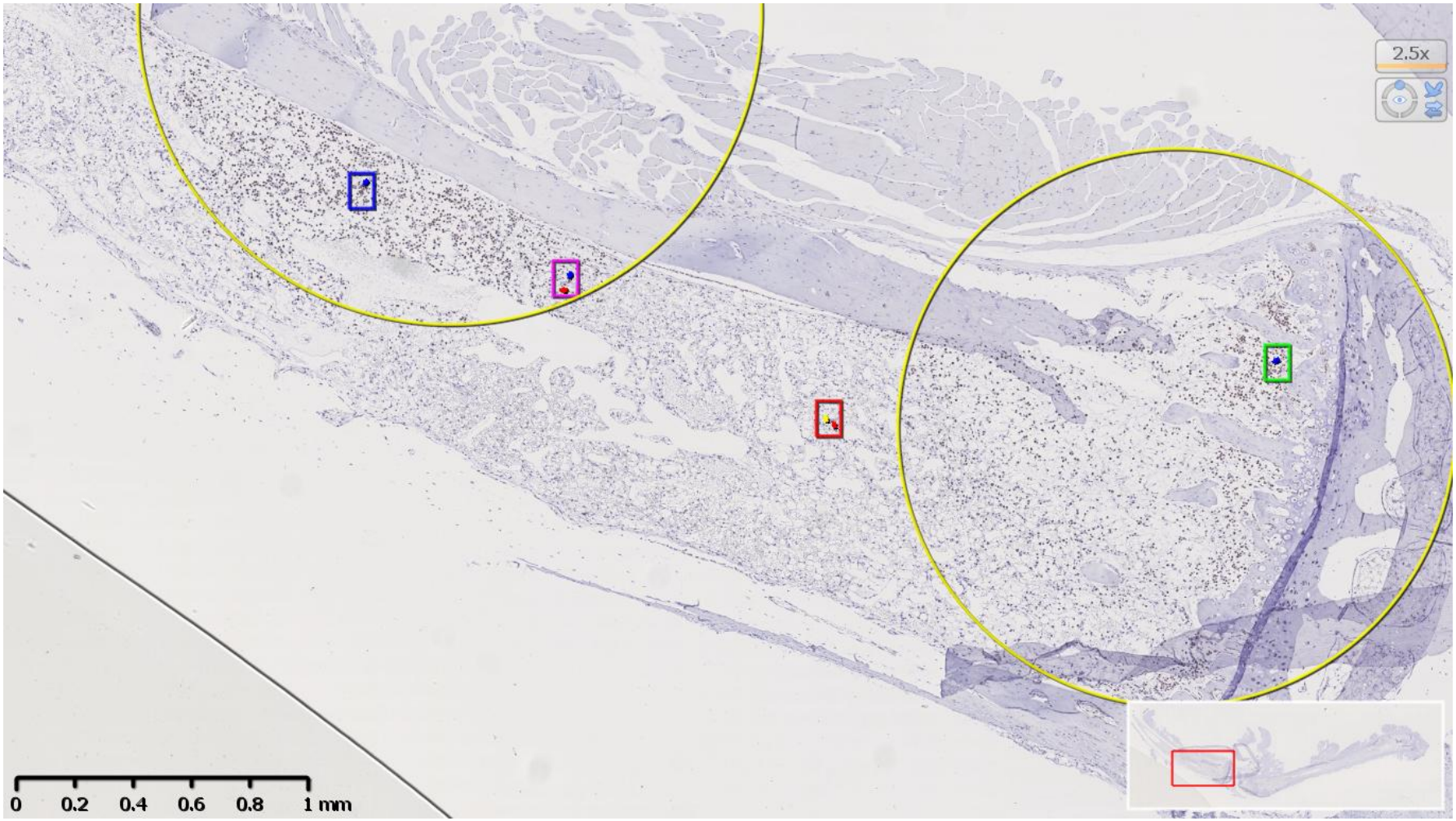
Hemorragic Suffusion

Residual hematopoiesis

Cytopenia

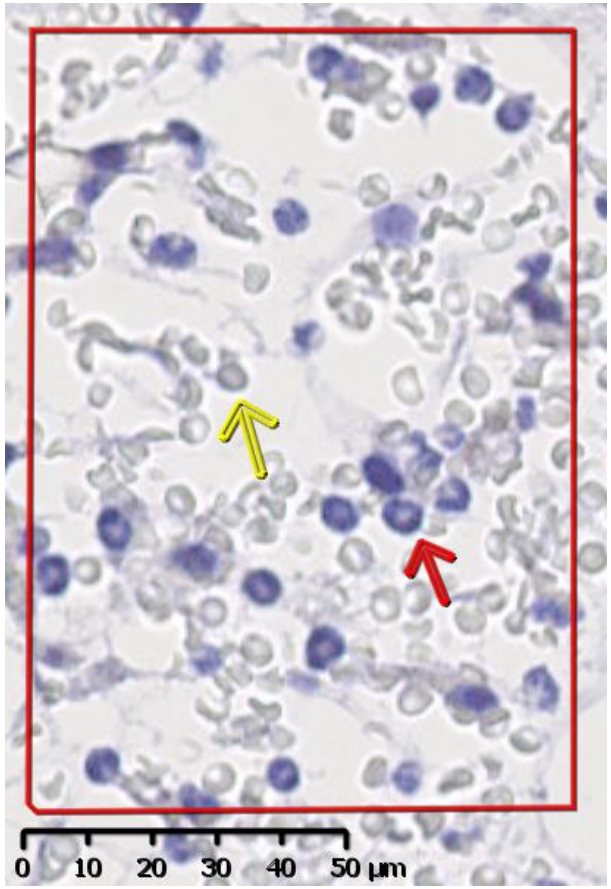
5-FU treatment induces human and murine cells lysis

ALU labelling ; one representative section (n = 3 mice)

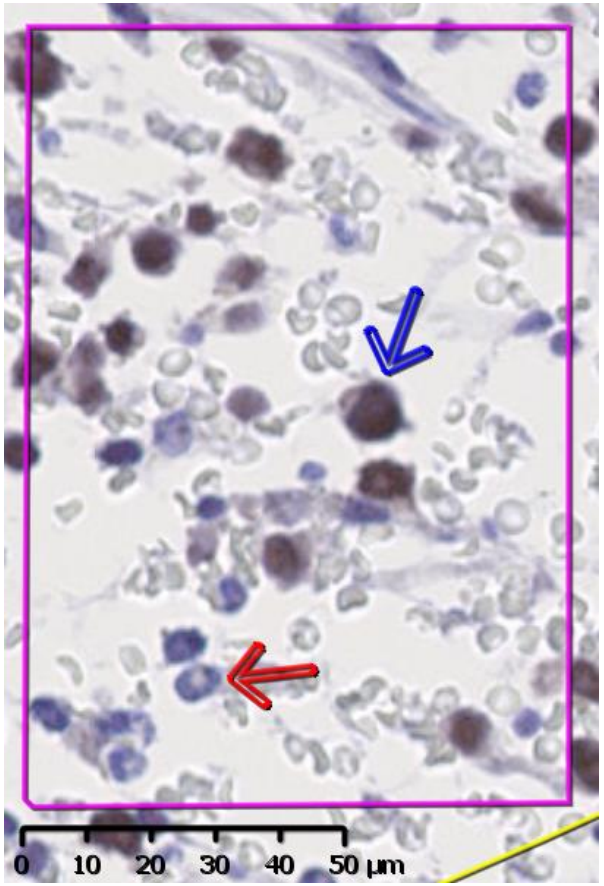


21 days after transplantation, NALM6 cells aggregates are detected in murine femoral BM

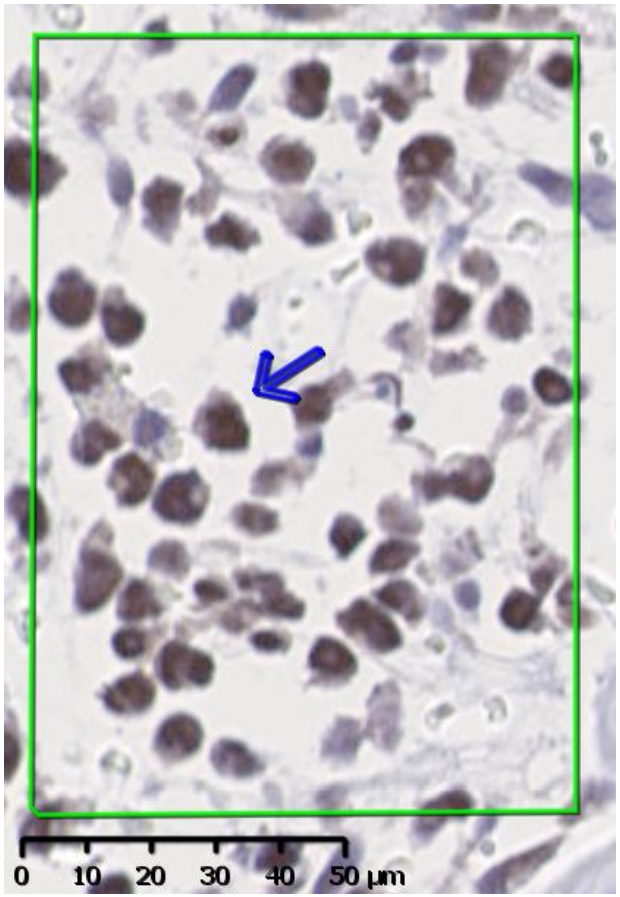
ALU labelling ; one representative section (n = 3 mice)



Murine cells area



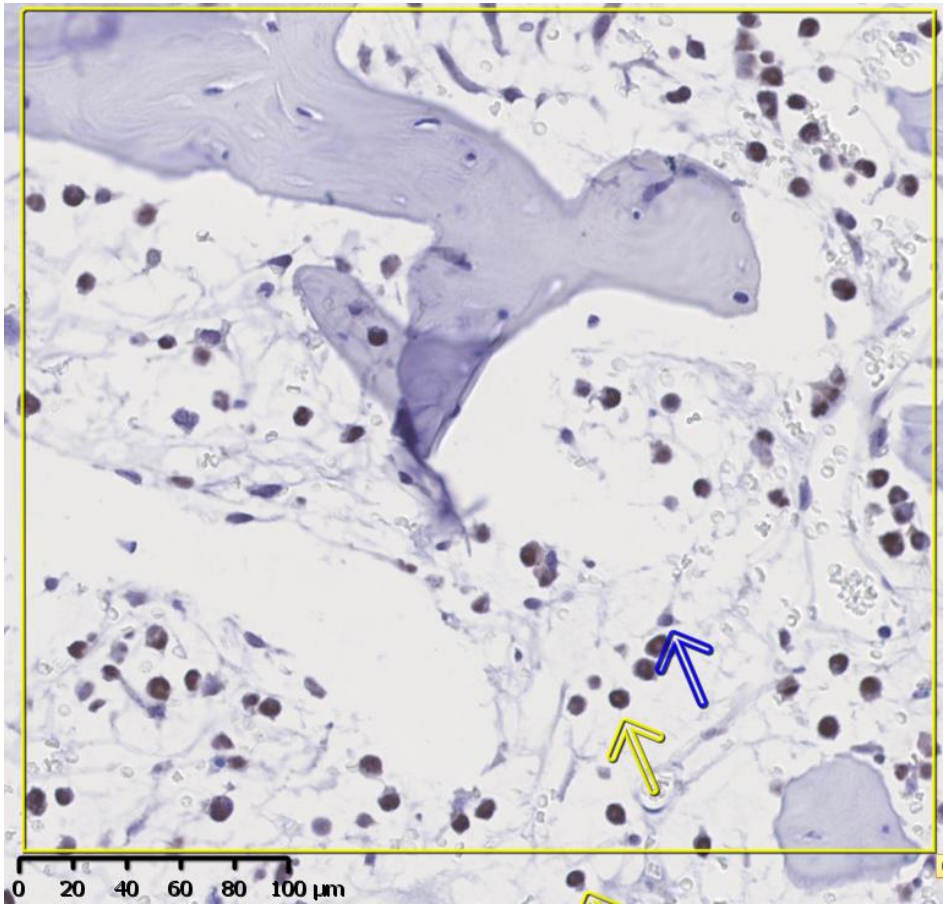
Murine/human mixed area



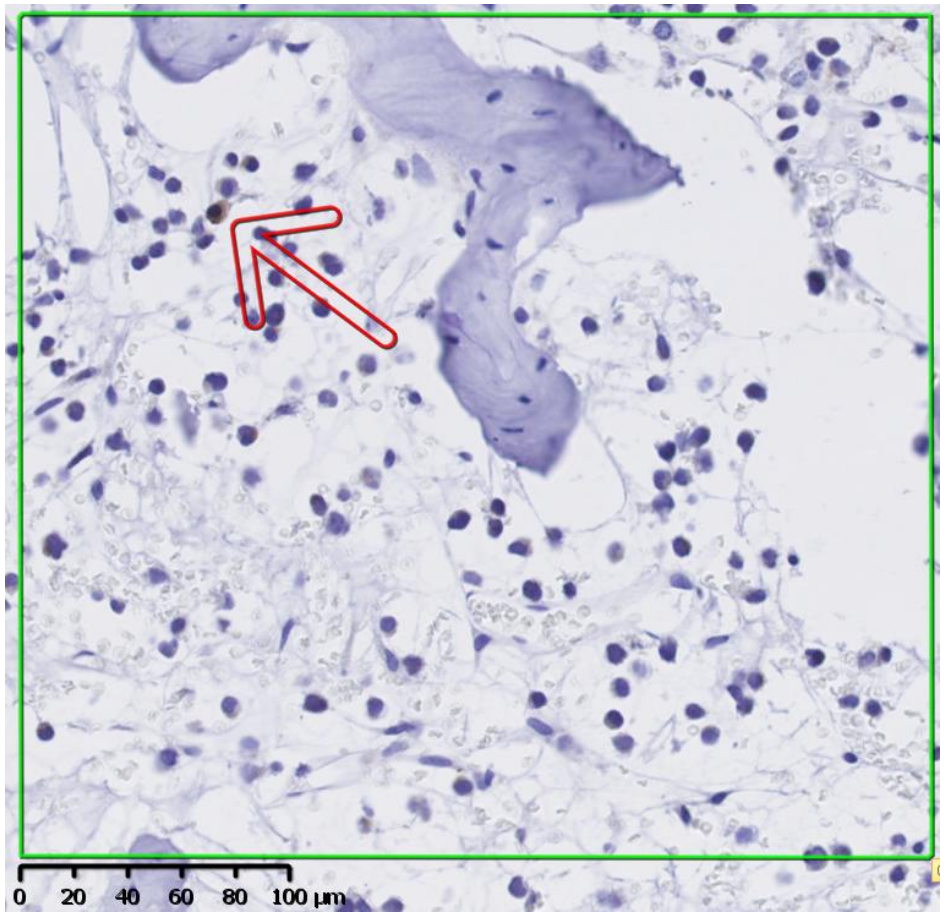
Human cells area

Most 5-FU residual cells are alive

Cleaved Caspase 3 labelling ; one representative section (n = 3 mice)



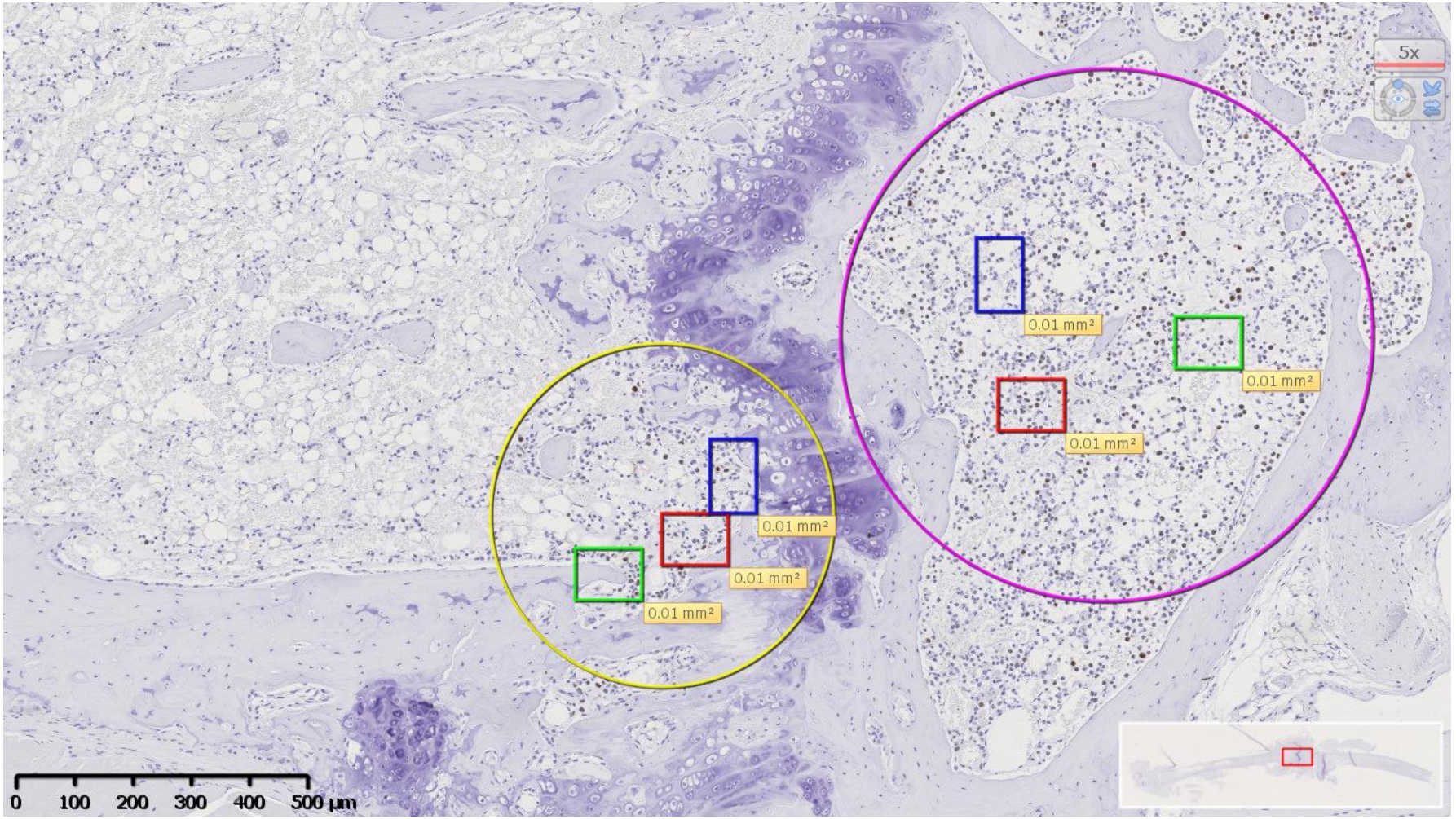
Human cells



Apoptotic cells

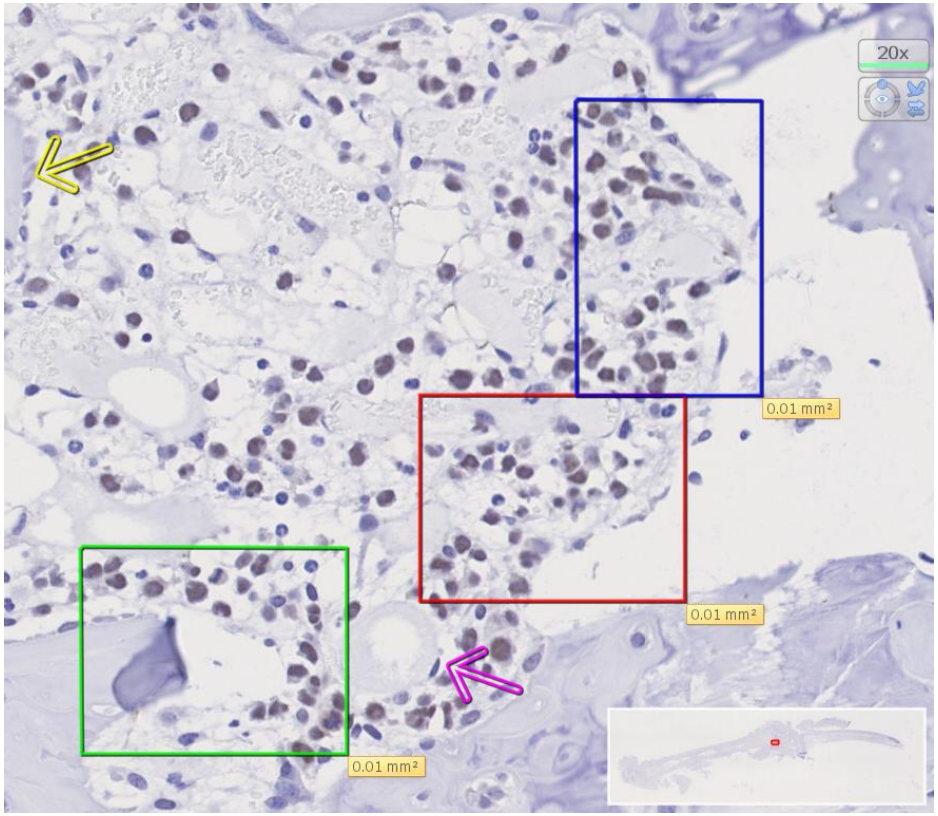
Post 5-FU residual NALM6 cells are mostly quiescent

Ki67 labelling ; one representative section (n = 3 mice)

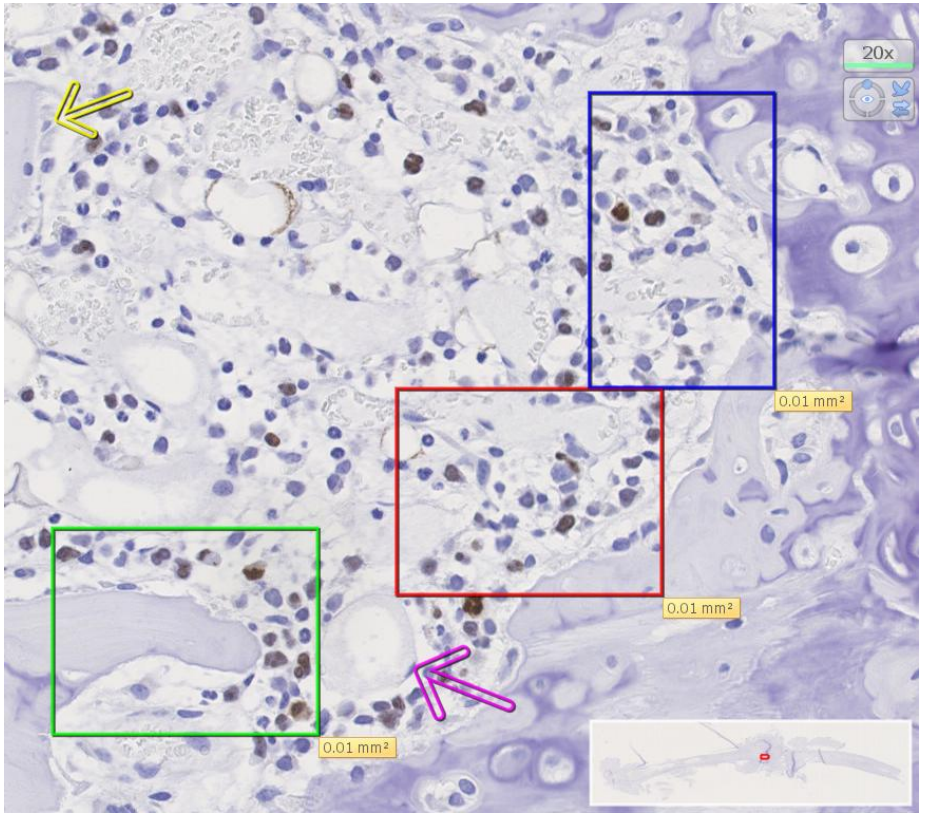


Post 5-FU residual NALM6 cells are mostly quiescent

Ki67 labelling ; one representative section (n = 3 mice)



80% of cells are human



70% of human cells are quiescent

Conclusion

- Heterogeneous BM cytopenia: are some areas (metaphyse) better protected ?

- Rapid elimination of apoptotic cells

- A large percentage of 5-FU resistant NALM6 cells are quiescent

- No preferential endosteal localisation of quiescent leukemic cells

Perspectives (1)
Pathophysiological studies

❖Our model will allow to continue *in vitro* and *in vivo* investigations on aspect of ALL biology:

- *Existence of preferential Metabolic pathways*
- *Existence of preferential « niches » harboring quiescent and resistant LIC*
- *Mechanisms of quiescence and their relationships with LIC resistant to therapy*
- *Relationships between LIC quiescence and vascularisation, perfusion and innervation of their « niches ».*

Perspectives (2)
Clinical translation

- *New parameters involved in individual prognosis evaluation*

- *innovative therapeutic approaches taking into account the results of pathophysiological studies*

Thank you for your attention

Acknowledgments :

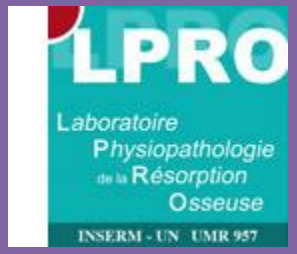
UMR CNRS 5164
Jean-François Moreau

Equipe Cellules Souches
Hélène Boeuf

Groupe Hémato :
Vincent Praloran

Grégory Harvet
Philippe Brunet de la Grange
Yann Peytour
Savitha Varatharajan
Zoran Ivanovic
EFS

U957 : Dominique Heyman



Céline Charrier
Rachel Lanel
Jérôme Amiaud
Fréderic Blanchard

A2 : Benoit Rousseau

CRB : Melyssa Reault
Eric Lippert

HL : Marie Jeanneteau
Kelly Airiau

Cytometry :
Vincent Pitard