Clinical Cancer Research

Effective activity of cytokine-induced killer cells against autologous metastatic melanoma including cells with stemness features

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Presented by

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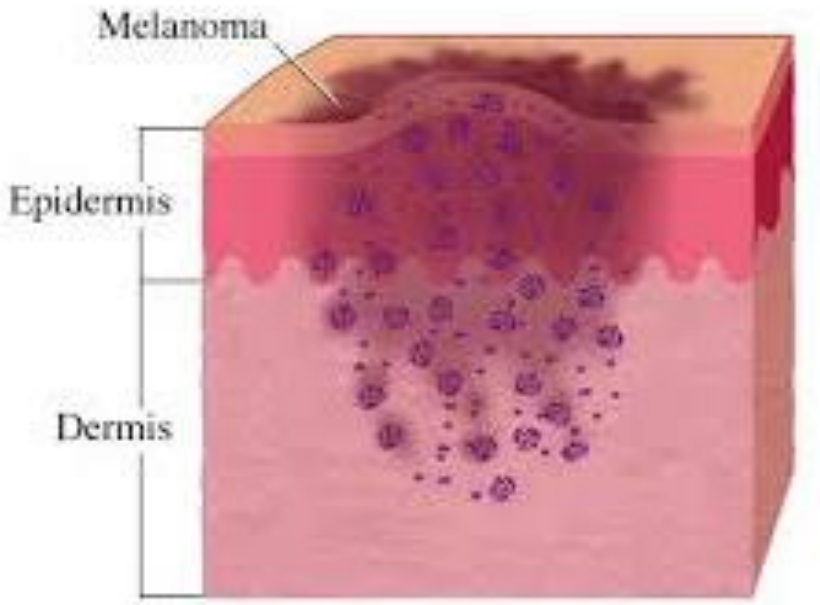
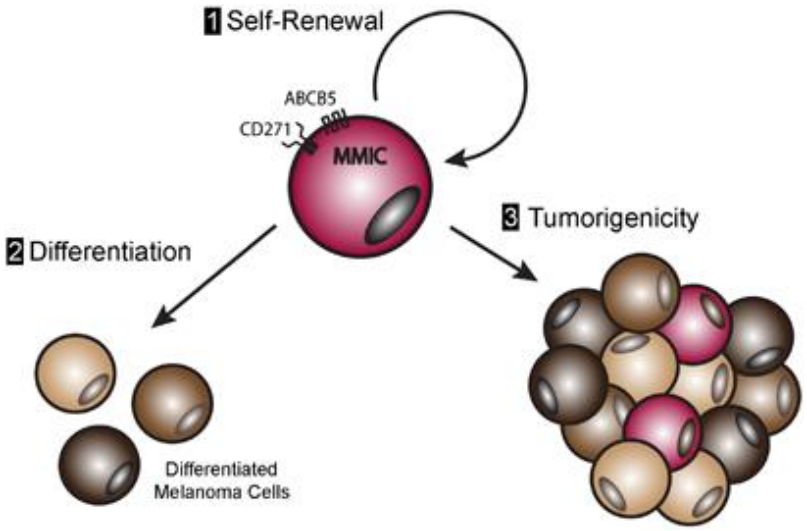
Melanoma



- Malignant tumor of the pigment-producing melanocytes
- Surgical resection
- Conventional therapies is less effective
- Highly metastatic cancer
  - **Melanoma cancer stem cells (mCSC)**

Melanoma cancer stem cells (mCSC)



- Tumor cell subpopulations
- Stemness features
  - Self-renew
  - Differentiation
- Heterogenous lineages of cancer cells
- Drug resistance and disease relapse

- New target for therapeutic approaches
- **Adoptive immunotherapy**
  - **Cytokine-induced killer (CIK) cells**

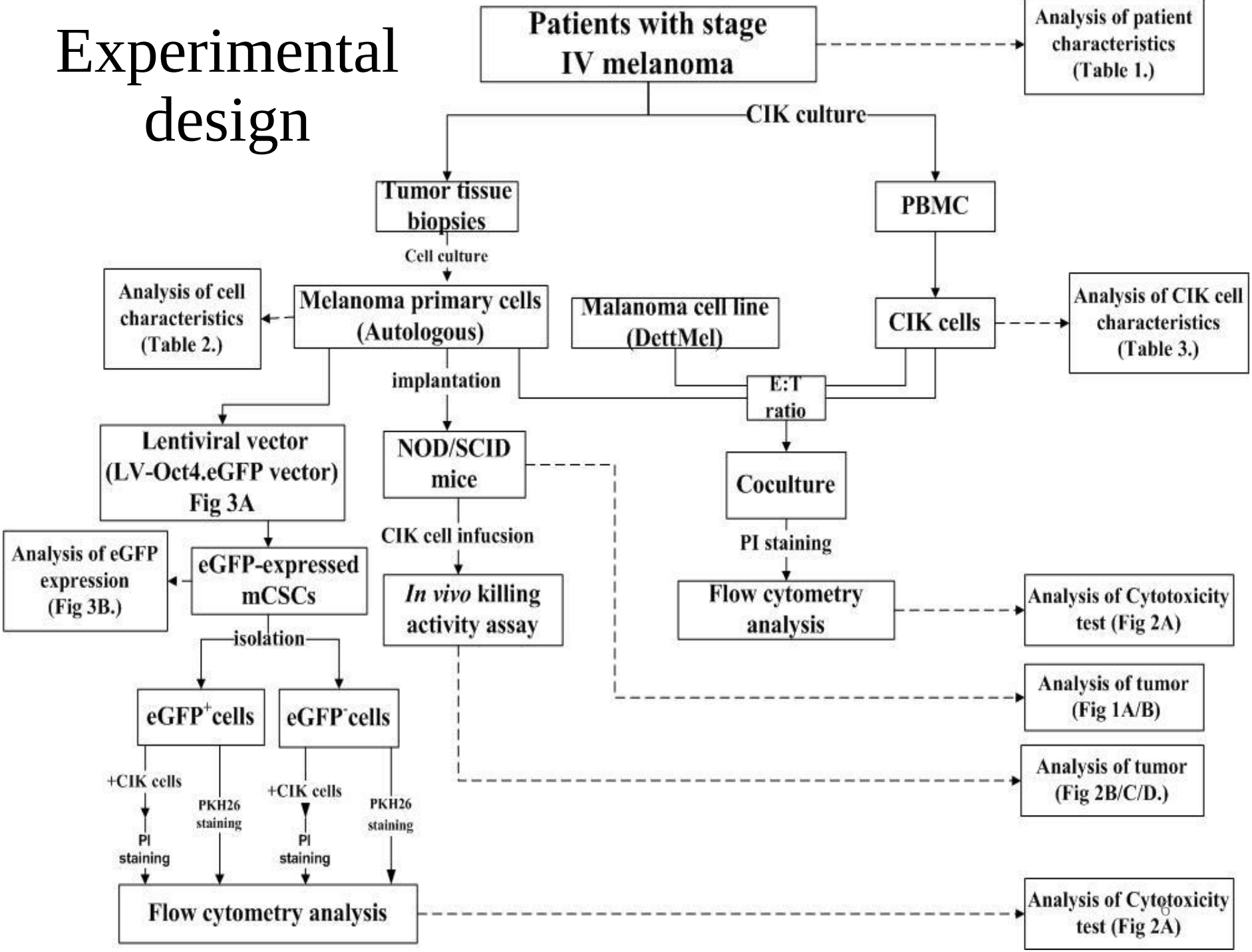
CIK cells

- *Ex vivo* expanded T-natural killer (NK) lymphocytes
- Generation of CIK cells
  - Peripheral blood mononuclear cells (PBMC)
  - IFN-γ, OKT-3, IL-2
- Elimination of tumor cells
  - Non-HLA restriction process
  - NKG2D signaling
    - NKG2D receptor
    - Ligands of NKG2D receptor (stress-inducible molecules)
      - MHC class I-related chain A and B (MIC A/B)
      - UL-16-binding proteins (ULBPs)

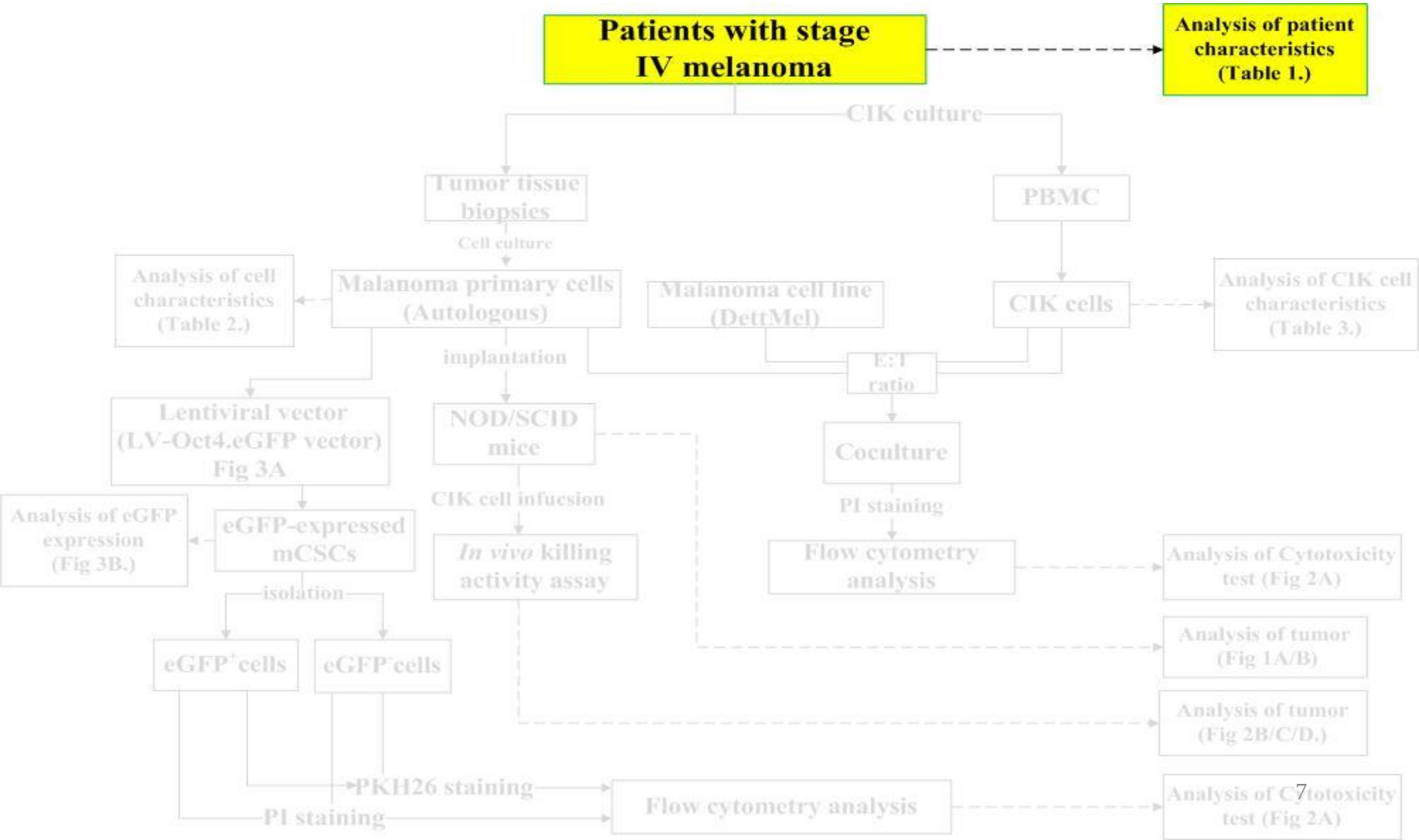
Objective

To investigate the unknown tumor-killing activity of cytokine-induced killer (CIK) cells against autologous metastatic melanoma and the elusive subset of putative cancer stem cells (mCSC)

Experimental design



Establishment and characterization of autologous melanoma primary cell cultures



Characterization of melanoma patients

Table 1. Main characteristics of melanoma patients and corresponding samples

Subject number	Age/sex	Status	Lesion site[a]	Primary cell culture[b]	CIK cell expansion[c]	Autologous cytotoxicity assay[d]
mMel1	67/M	PD	SC	Y[e]	115	Y
mMel2	64/F	PD	SC	Y[e,f]	133	Y
mMel3	69/F	PD	SC	Y[e,f]	419	Y
mMel4	54/M	PD	LN	Y[e]	314	Y
mMel5	67/M	PD	LN	Y[e,f]	1870	Y
mMel6	78/M	PD	LN	Y[e,f]	109	Y
mMel7	79/F	PD	LN	Y	125	Y
mMel8	87/F	PD	SC	Y	1387	Y
mMel9	74/F	PD	LN	Y	49	Y
mMel10	67/M	PD	LN	Y	195	Y

Abbreviations: Y, yes; mMel, metastatic melanoma.

[a]Biopsy was conducted at different metastatic sites including subcutaneous (SC) or lymph node (LN) sites.

[b]*In vitro* primary cell cultures derived from tumor biopsies.

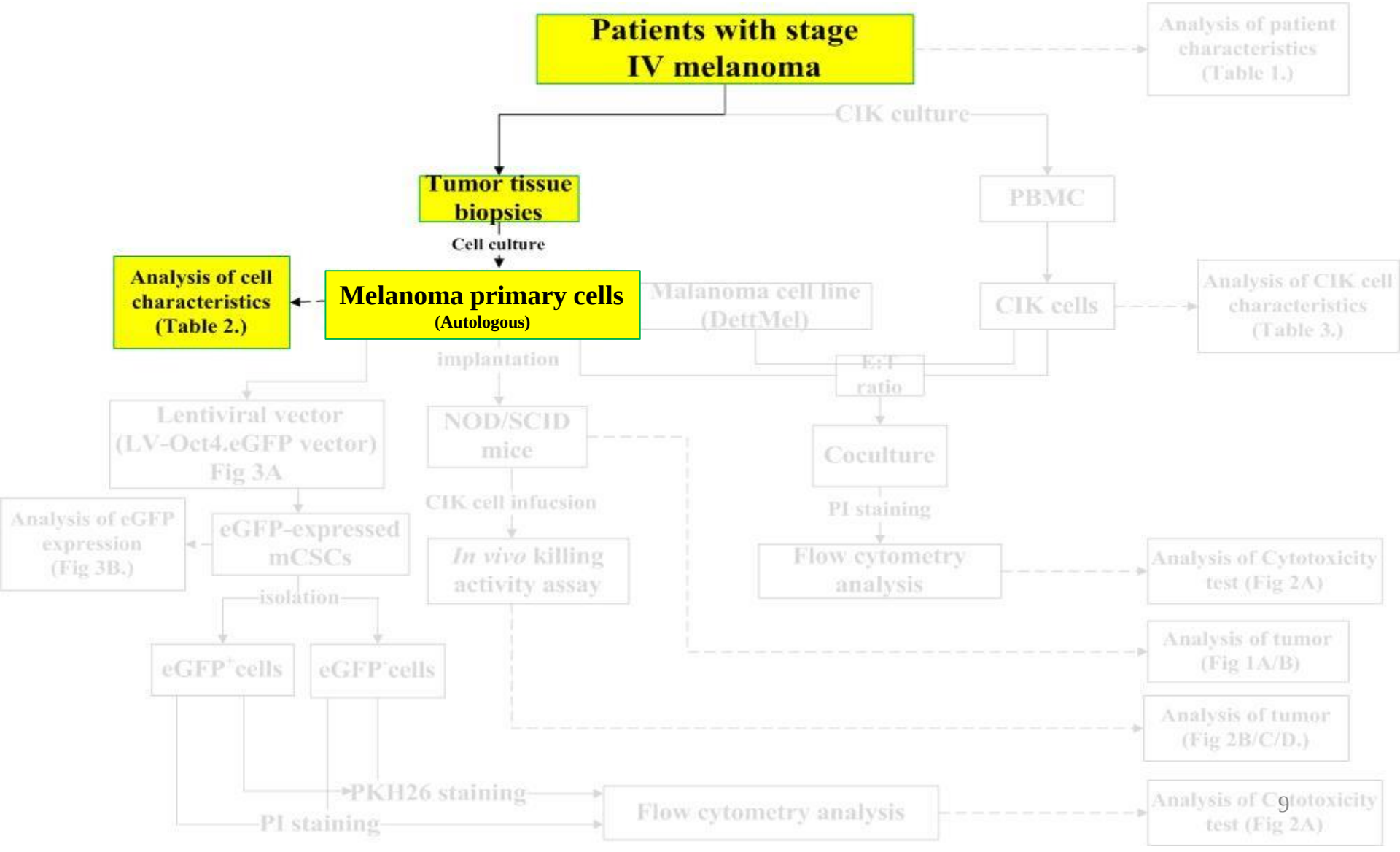
[c]$CD3^+/CD56^+$ cell number-fold increase after 3 weeks of expansion.

[d]Cytotoxicity assay with CIK cells versus autologous tumor target cells.

[e]mMel samples assessed for tumorigenic capacity *in vivo* in NOD/SCID mice. The same mMel samples were transduced with LV-Oct4-eGFP to visualize mCSC and subsequently were sorted on the basis of eGFP expression.

[f]mMel samples used for cytotoxicity assay with CIK cells versus LV-Oct4-eGFP–transduced target cells sorted on the basis of eGFP expression.

Establishment of autologous melanoma primary cell cultures



Main melanoma markers

- Stem cell-like markers
  - CD271 or Nerve growth factor receptor (NGFR)
  - CD133
  - CD117
  - CD34
  - Oct4
  - ATP-binding cassette G2 (ABCG2)
  - Microphthalmia associated transcription factor (Mitf)
  - Aldehyde dehydrogenase (ALDH)

Main melanoma markers

- Ligands for NKG2D receptor
  - MHC class I-related chain A and B (MIC A/B)
  - UL-16-binding proteins (ULBPs)
- Marker-associated tumor cells
  - Melanoma-associated chondroitin sulfate proteoglycan (MCSP)
  - Vascular endothelial growth factor receptor1 (VEGFR1)

Characterization of autologous melanoma primary cells

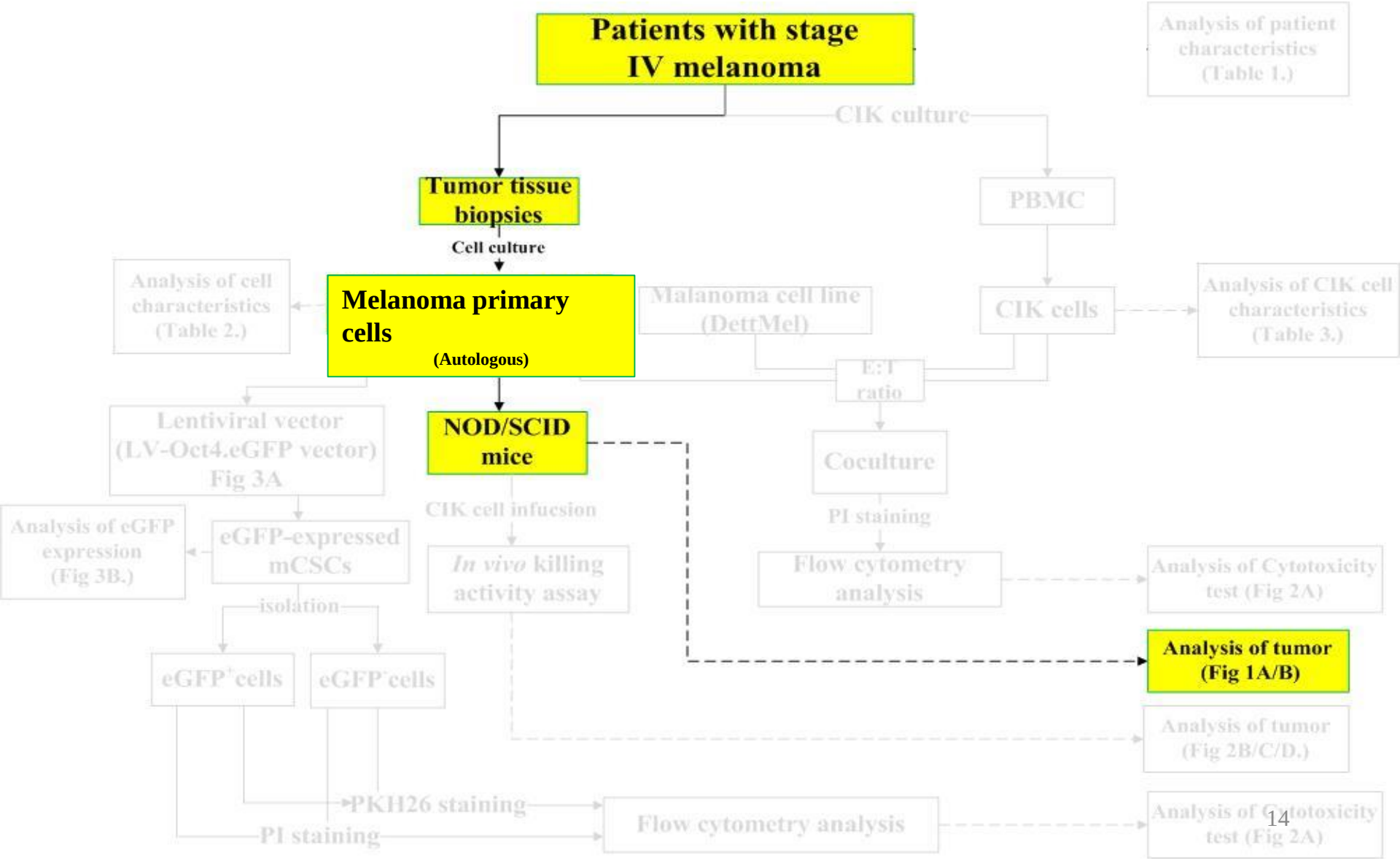
Table 2. Phenotype characterization of primary melanoma cell cultures

Subject number	% MIC A/B	% ULBP1	% ULBP2	% ULBP3	% NGFR	% MCSP	% Oct4	% ALDH bright	% ABCG2	% Mitf-	% VEGFR1 bright	% CD34	% CD117	% HLA-ABC
mMel1	33.7	0	62.8	0	74.2	64.7	15.5	8.3	11.8	16.8	19.3	7.6	neg	99.9
mMel2	15.2	1.6	40.7	1.9	58.3	71.4	11.5	8.7	12.4	16.9	10.0	18.0	neg	99.5
mMel3	90.1	1.6	97.9	0	93.0	81.5	8.3	28.9	12.2	14	12.5	25.1	neg	99.6
mMel4	64.9	0	60.6	7.4	19.9	95.0	10.1	8.2	1.3	10.7	10.6	6.3	neg	99.2
mMel5	7.3	1.1	40.3	1.3	82.4	88.9	10.7	11.6	7.3	6.8	12.3	12.3	neg	99.7
mMel6	32.4	0	55.0	0	50.8	95.1	7.3	2.8	11.2	6.5	10.7	6.4	88.8	99.1
mMel7	20.5	0	64.5	0	39.1	78	10.7	8.9	14.1	9.8	5.0	3.1	30.1	99.4
mMel8	53.5	0	52.7	0	28.9	74.2	11.4	3.3	17.5	15	4.4	3.1	neg	99.6
mMel9	7.4	0	65.5	0	82.3	99.7	8.3	6.0	10.2	3.4	2.7	2.1	neg	neg
mMel10	14.2	1.17	62.1	16.6	33.6	73.6	15.2	9.9	13	8.3	5.6	7.0	70.3	neg

Discussion

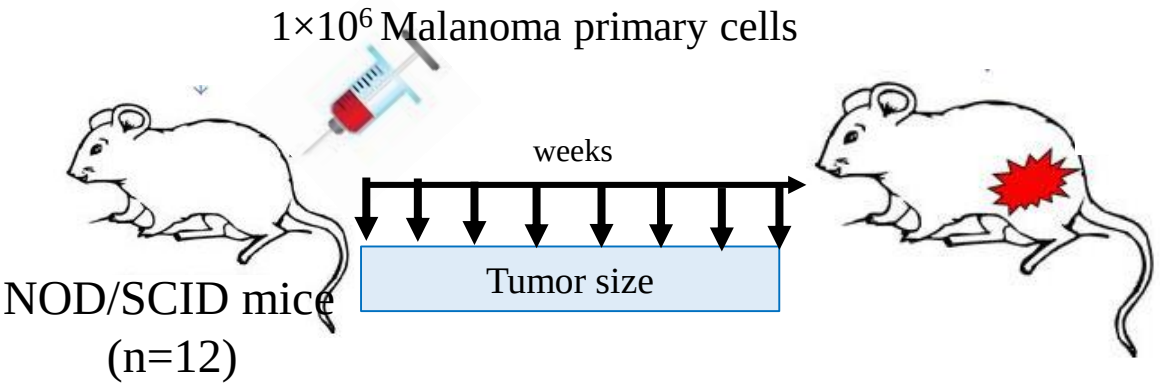
- Melanoma primary cells retained original tumor characteristics and displayed great immunophenotypic heterogeneity among samples.
- Most differentiation antigens detected on metastatic melanoma cells showed variable levels of expression.

Melanoma primary cell implantation



Melanoma primary cell implantation

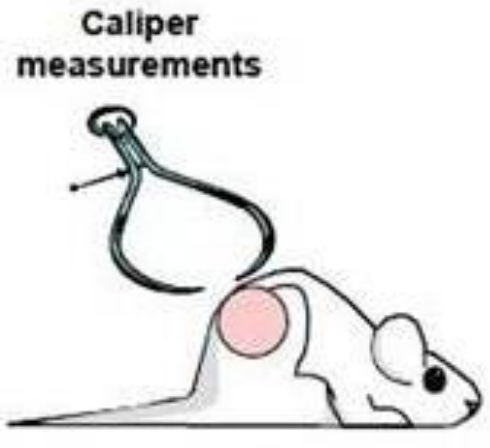
To show tumorigenicity of primary metastatic melanoma cells *in vivo*



- Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice
  - 4-week-old female mice
- Melanoma primary cells from 6 different patients

Melanoma primary cell implantation

To show tumorigenicity of primary metastatic melanoma cells *in vivo*



$$\text{Volume} = 4/3 \times \pi \times (l/2)^2 \times (L/2)$$

L = Length
l = Width

- Caliper
- Tumor volume calculation
- Euthanazia
  - Reached 2 cm in diameter
- Histopathology
  - H&E staining

In vivo melanoma primary cell growth

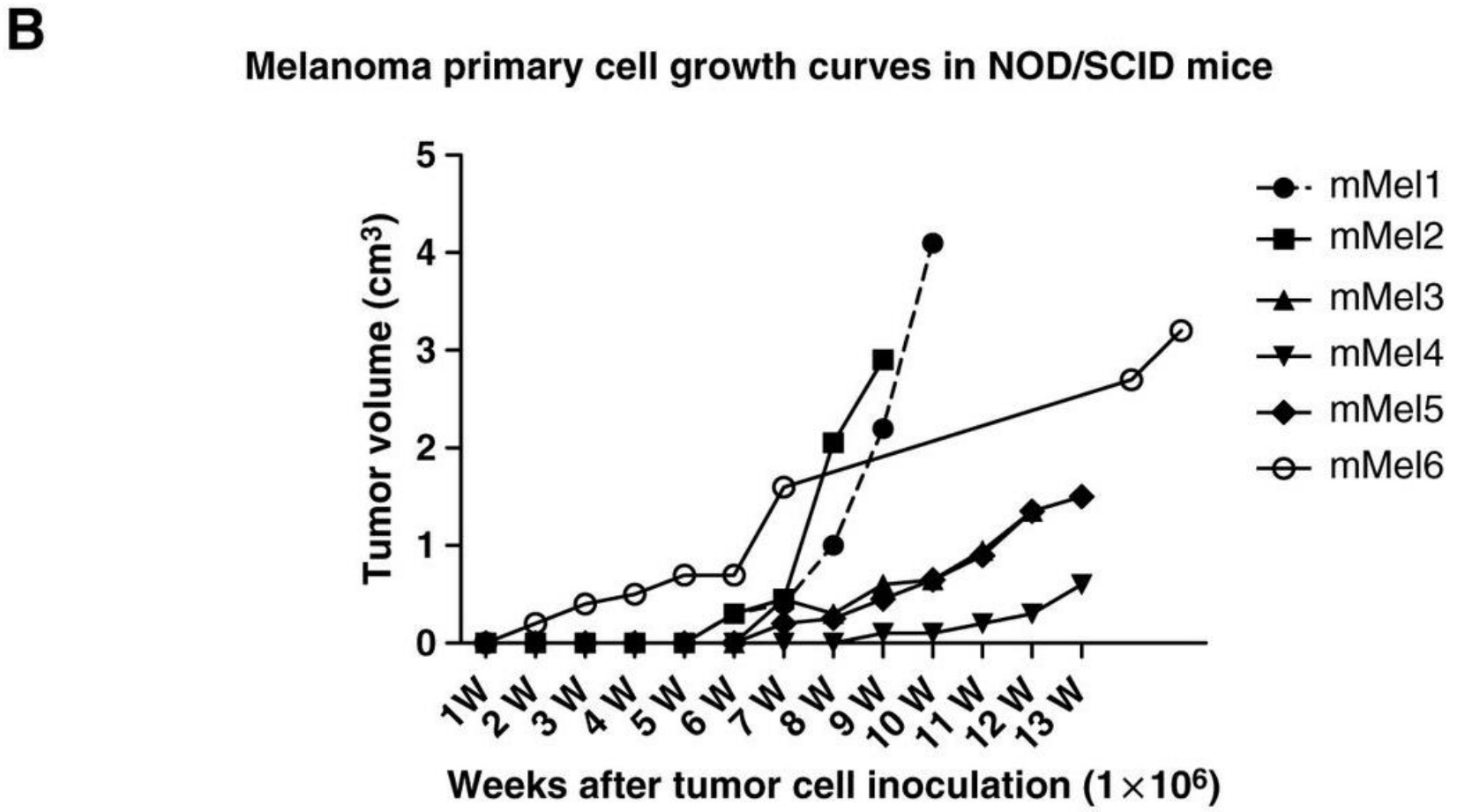


Figure 1B

Tumor growth curves of the melanoma cell lines transplanted into the NOD/SCID mice

Tumor cell morphologic feature
(Pathology evaluation)

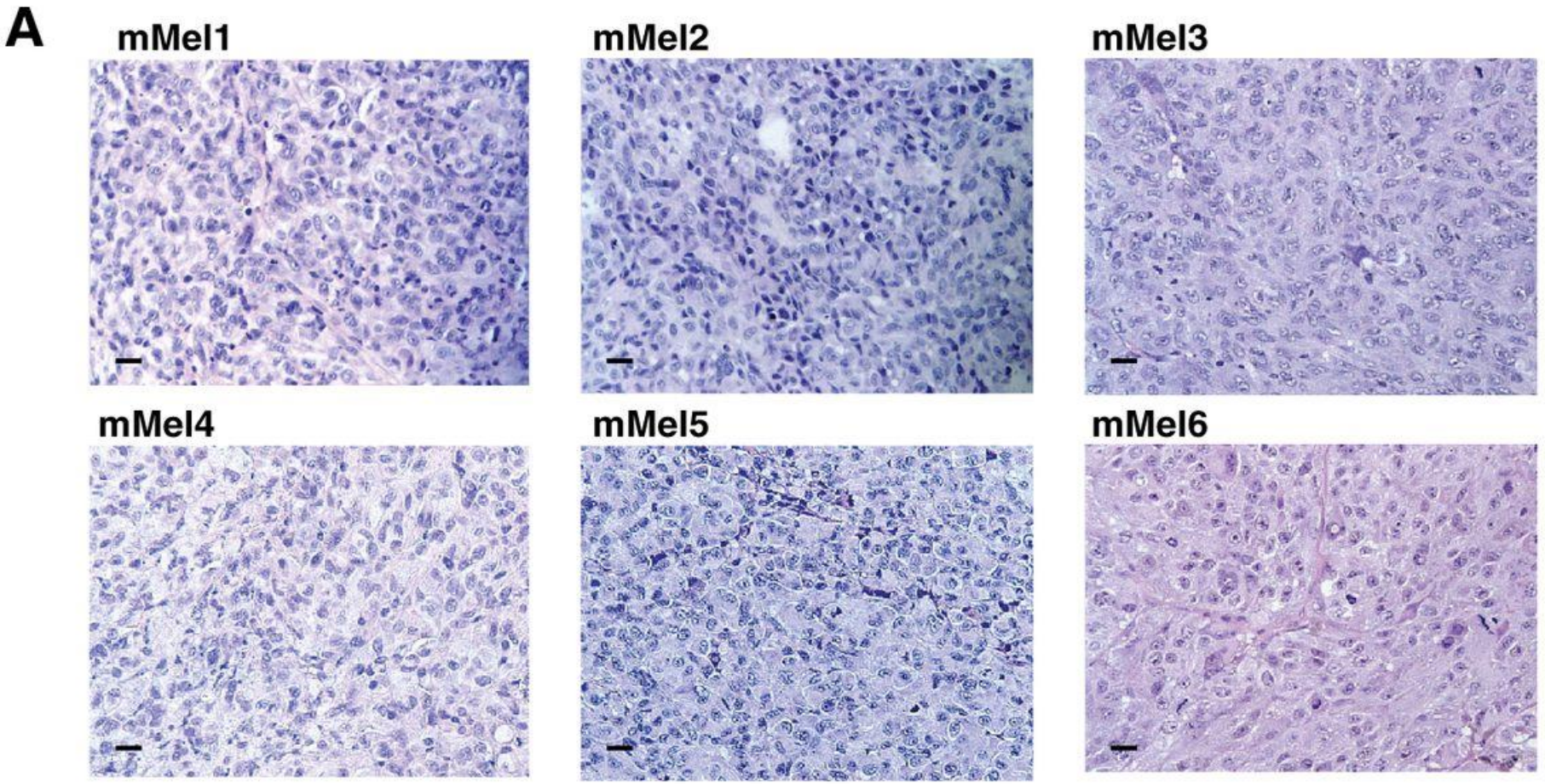
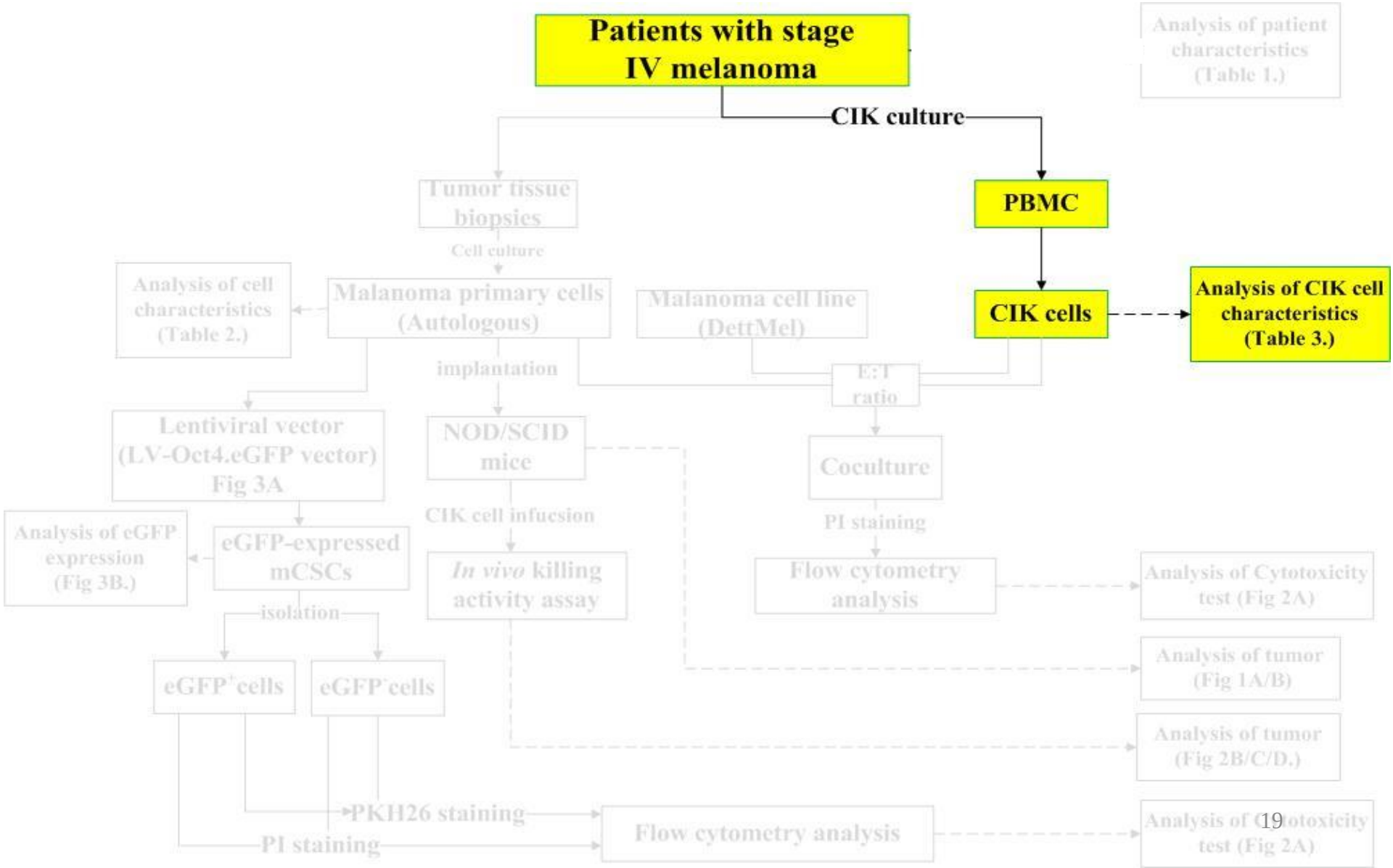
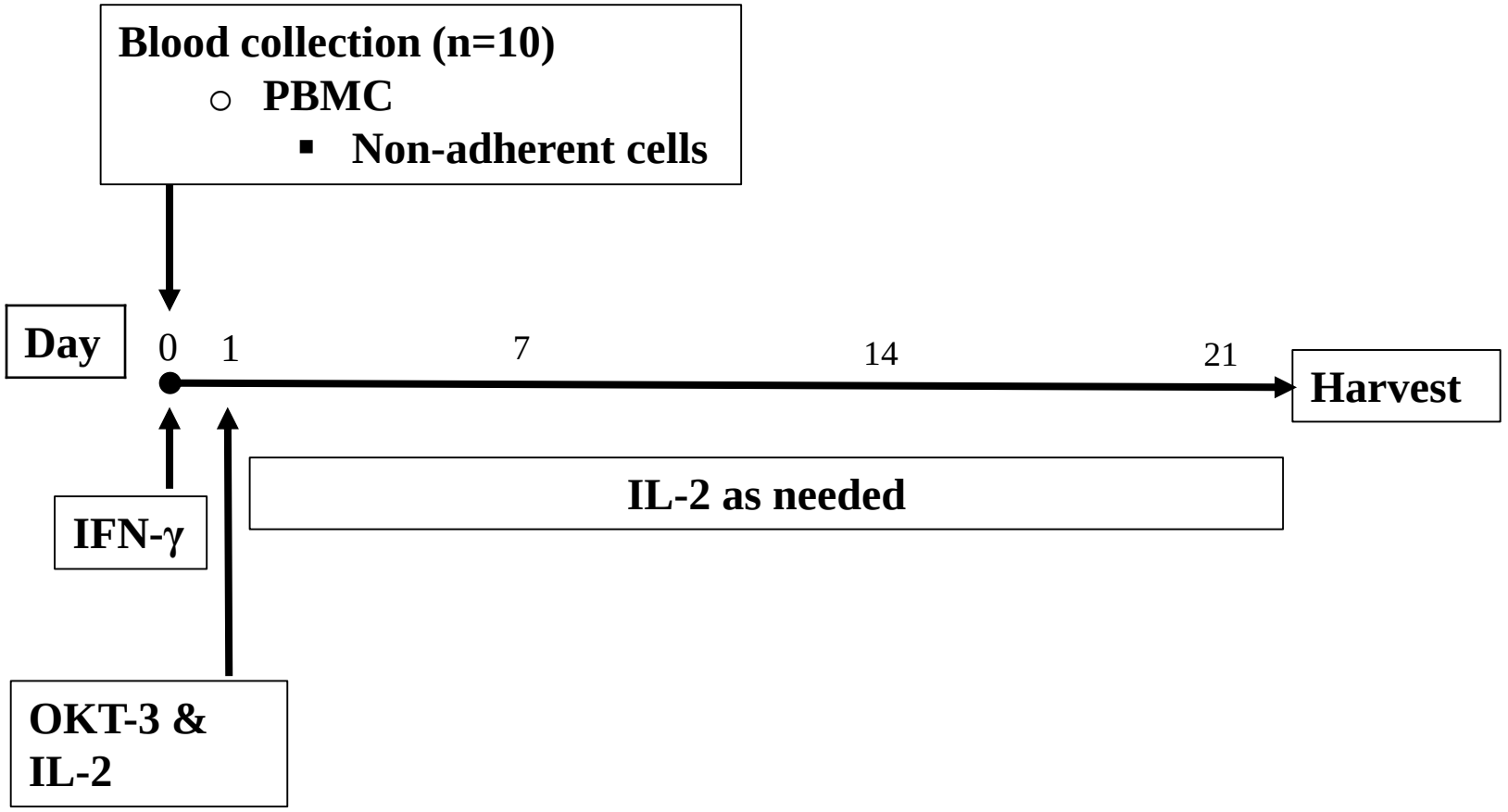


Figure 1A. Representative H&E-stained paraffin-embedded sections from tumor generated after subcutaneous injection in NOD/SCID mice. Tumor xenograft morphology was consistent with the original human tumor as confirmed by a pathology review.

Expansion and phenotype of CIK cells



Expansion of CIK cells



Phenotype of CIK cells

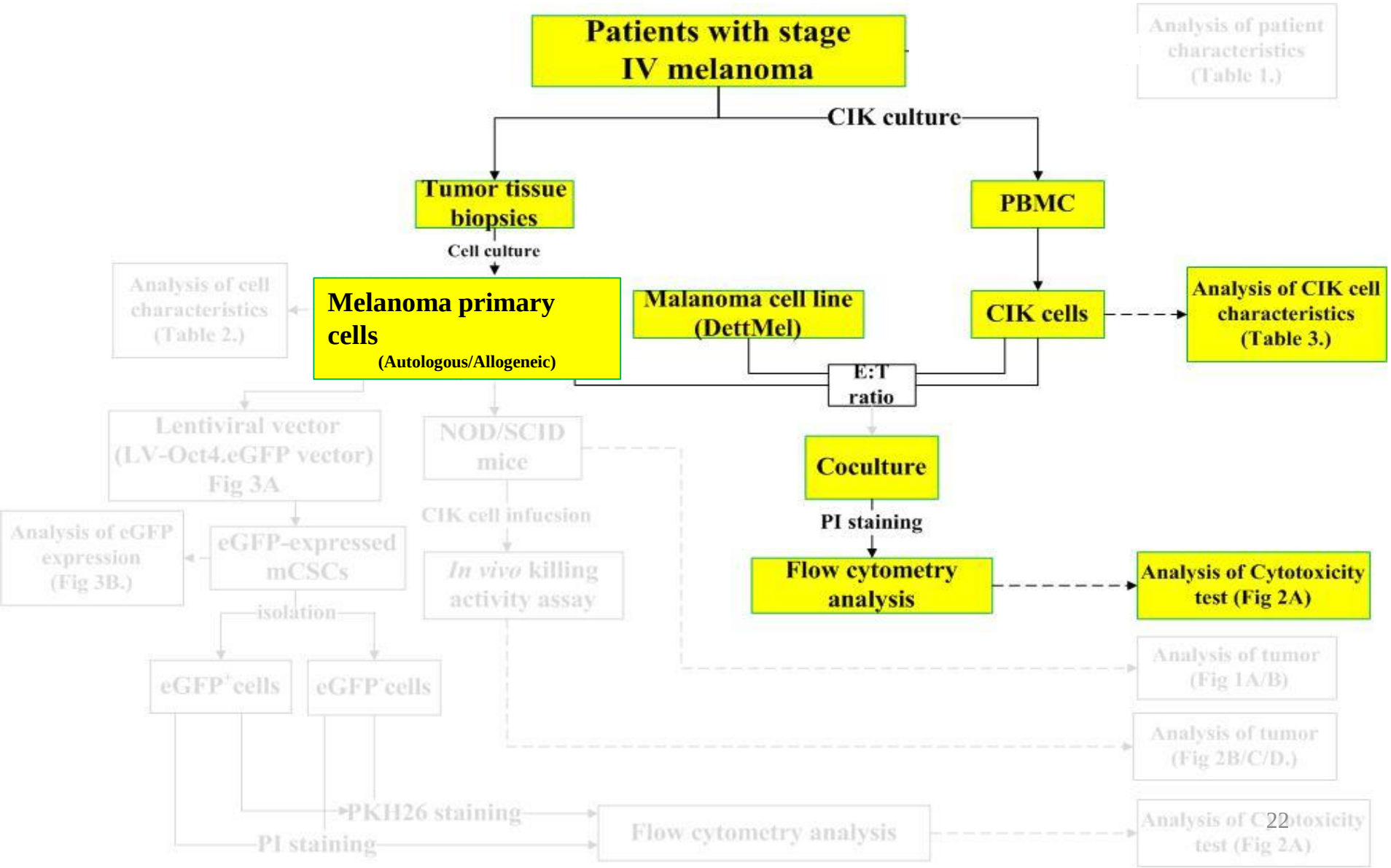
Table 3. Expansion rate and phenotype characterization of CIK cells

Subject number	FI cells[a]	% iCD3[b]	% fCD3[c]	FI[d] CD3	% iCD3/CD56[b]	% fCD3/CD56[c]	FI[d] CD3/CD56	% iCD3/CD8[b]	% fCD3/CD8[c]	% iCD3/NKG2D[b]	% fCD3/NKG2D[c]
mMel1	11	69	99	16	6	60	115	25	91	10	93
mMel2	21	74	99	28	4	25	133	20	69	10	74
mMel3	41	60	99	67	4	41	419	15	73	17	85
mMel4	55	93	99	58	4	23	314	20	82	20	57
mMel5	117	73	99	159	5	80	1870	11	76	14	91
mMel6	11	50	94	20	5	50	109	17	80	11	91
mMel7	22	68	100	32	10	58	125	37	69	37	79
mMel8	27	49	95	53	1	51	1387	4	59	16	83
mMel9	14	76	99	18	14	48	49	59	80	50	93
mMel10	13	57	98	22	3	45	195	15	79	14	79

[a]Fold increase [FI = (cell number $_{T = week\ 3}$)/(cell number $_{T = 0}$)] of total cell number after 3 weeks of expansion.
[b]Percentage of cells expressing different surface antigens at the basal time (T = 0).
[c] Percentage of cells expressing different surface antigens after 3-week expansion.
[d]Fold increase [FI = (cell number $_{T = week\ 3}$)/(cell number $_{T = 0}$)] of absolute cell number after 3 weeks of expansion calculated for every subpopulation of cells expressing different surface antigens.

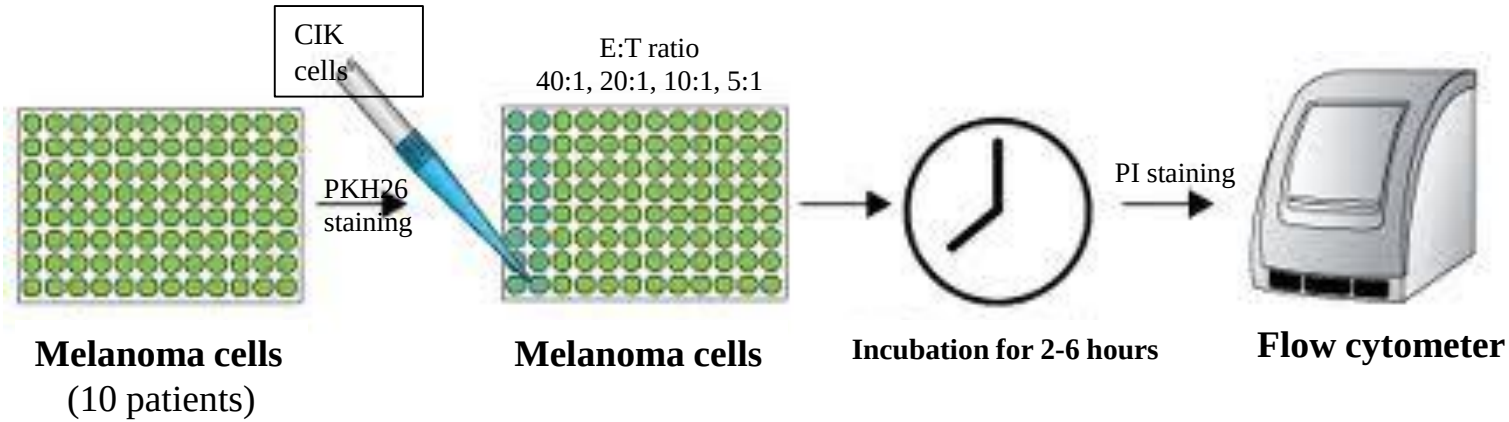
- CIK cells from patients with metastatic melanoma were expanded at clinically relevant levels.

In vitro killing activity of CIK cells against metastatic melanoma cells



Killing activity of CIK cells against melanoma cells

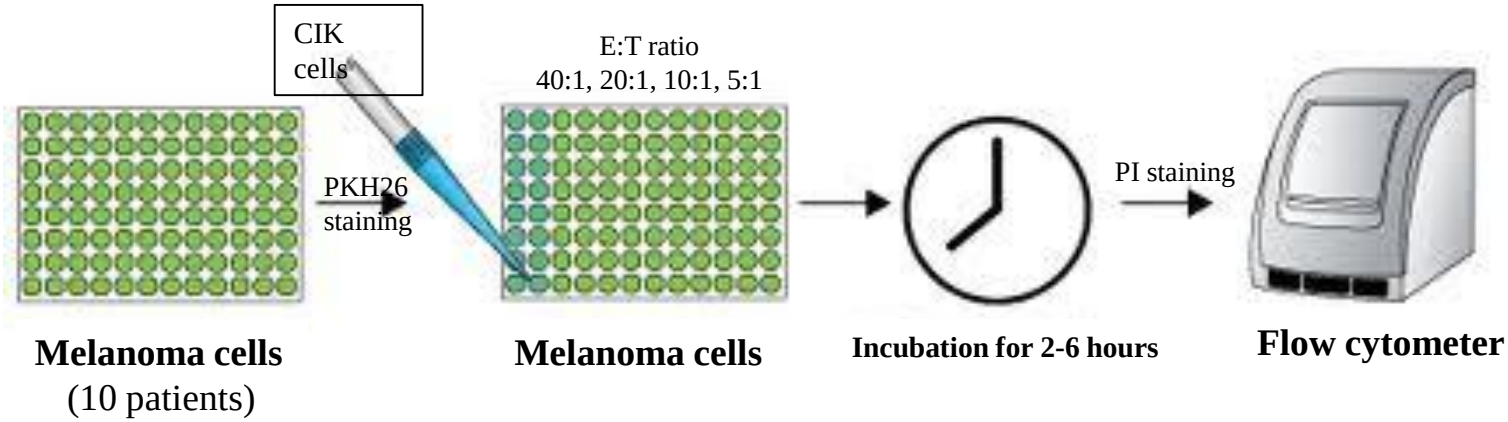
To evaluated the ability of CIK cells to kill *in vitro* a melanoma cells



- Target cells
  - Melanoma cell line (DettMel)
  - Autologous melanoma primary cells
  - Allogeneic melanoma primary cells

Killing activity of CIK cells against melanoma cells

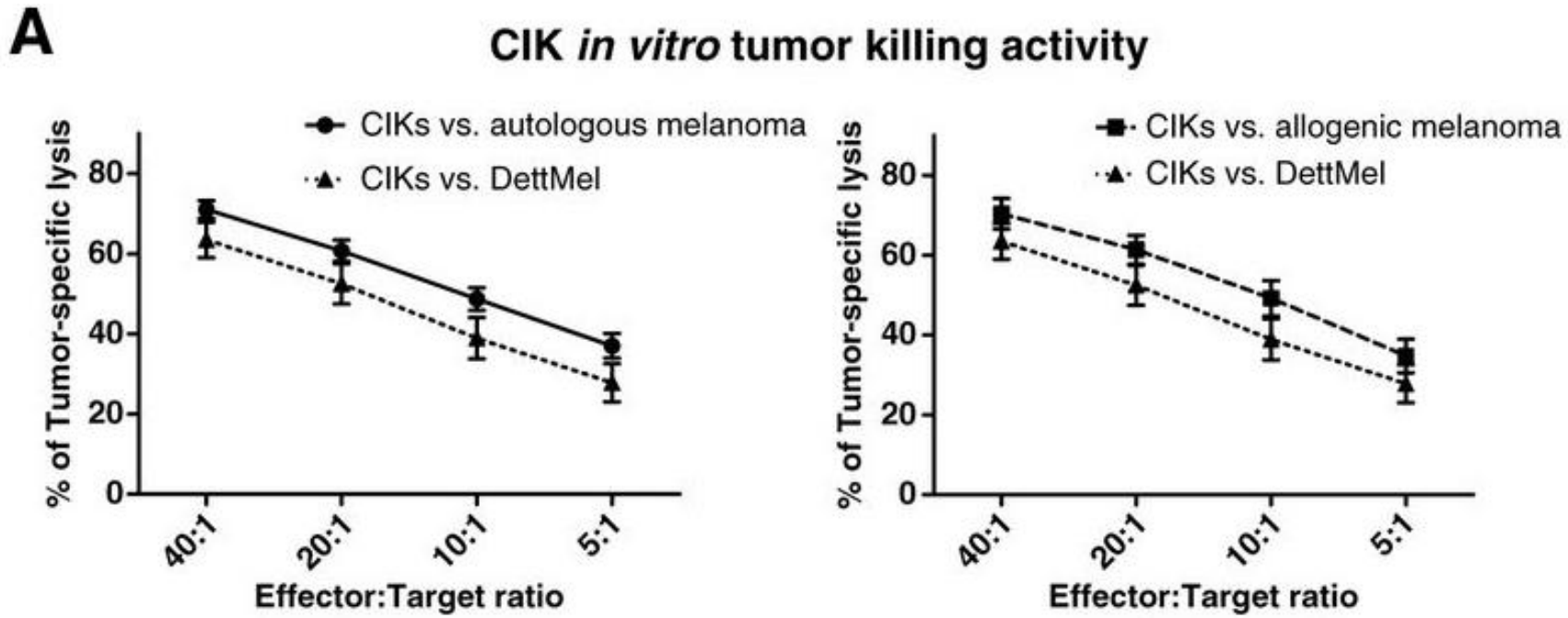
To evaluated the ability of CIK cells to kill *in vitro* a melanoma cells



- Perentage of tumor-specific lysis formula

$$\% = \frac{(\text{experimental-spontaneous mortality}) \times 100}{100\text{-spontaneous mortality}}$$

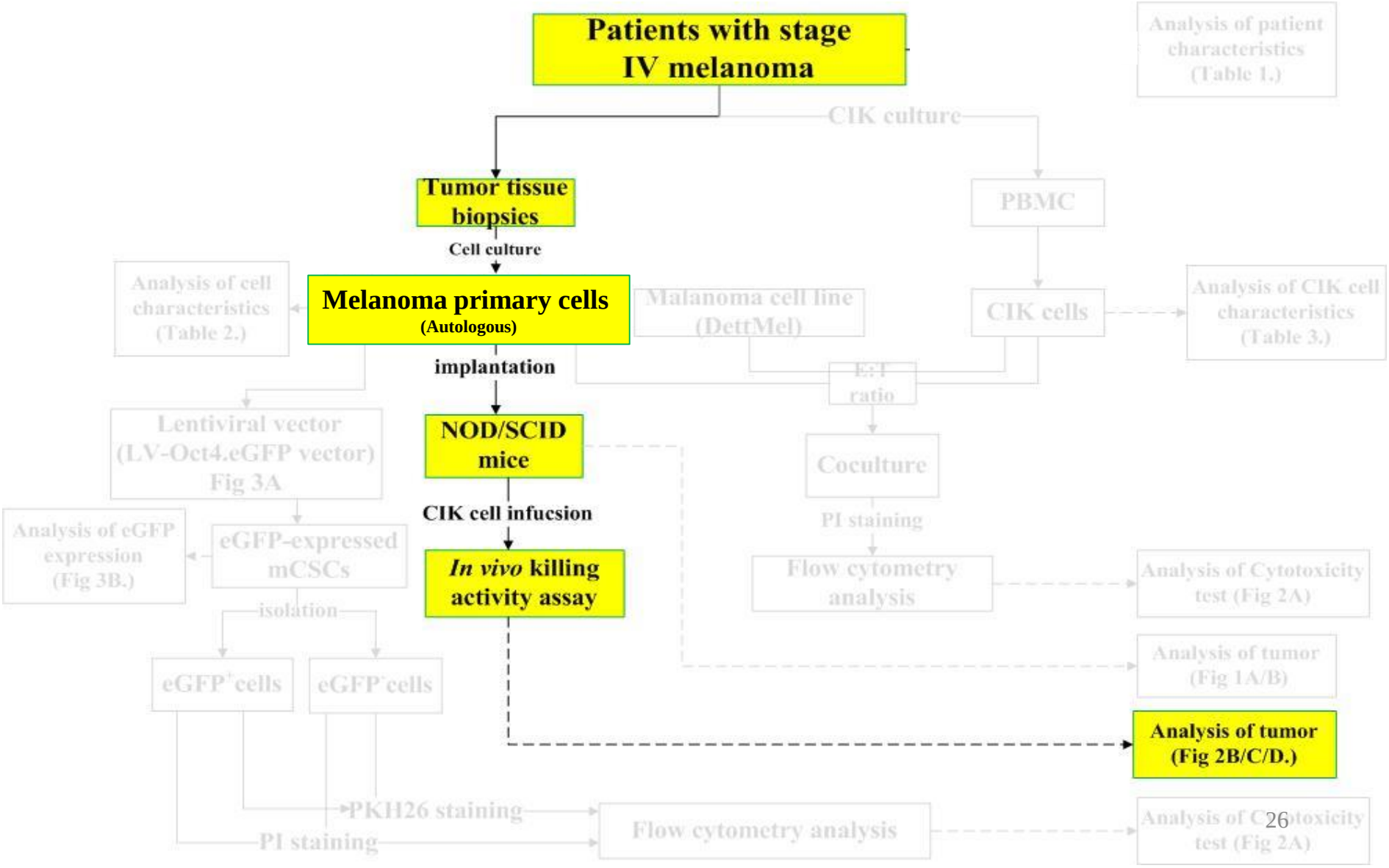
In vitro killing activity of CIK cells against metastatic melanoma cells



Comparing	P-value
Autologous or Allogenic	0.9991

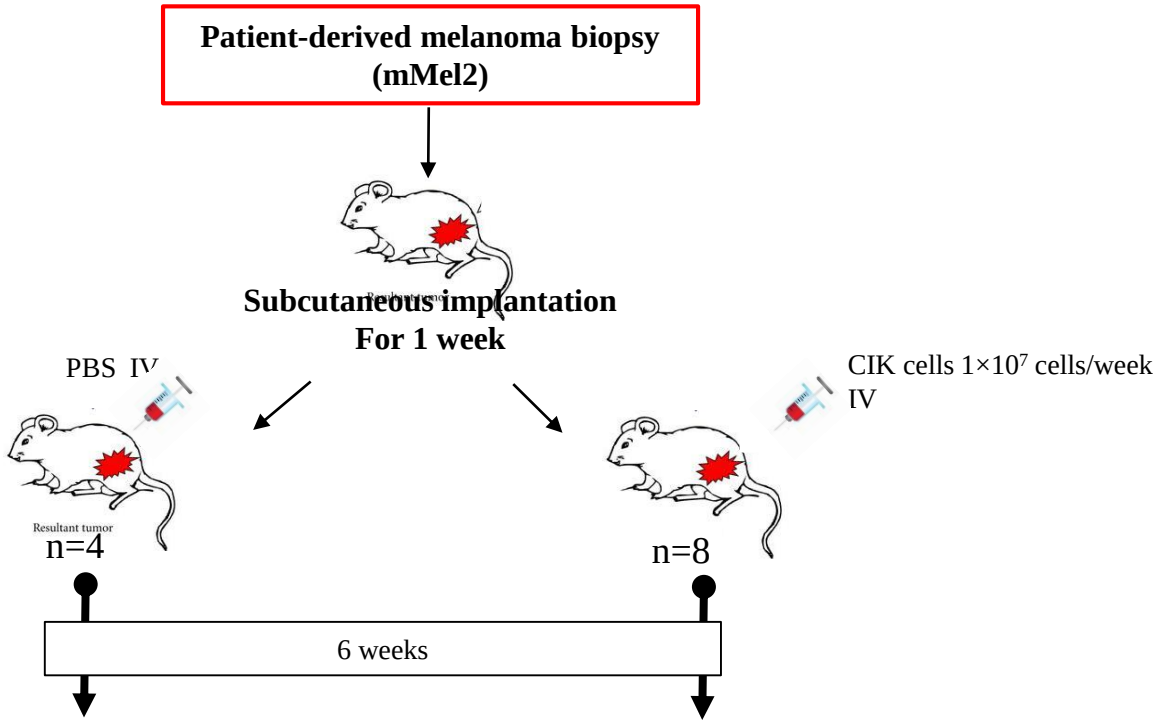
Figure 2 A: A patient-derived CIK cells efficiently killed in vitro all bulk autologous melanoma target (n=35;left); results were comparable with those obtained with allogeneic CIK cells assessed in parallel versus the same tumor cells (n=20; right). Tumor killing was evaluated by flow cytometry assay conducted after coculturing mature CIK cells with PKH-26-stained targets for 4 hours.

In vivo killing activity of CIK cells against autologous metastatic melanoma cells



In vivo killing activity of CIK cells against autologous metastatic melanoma cells

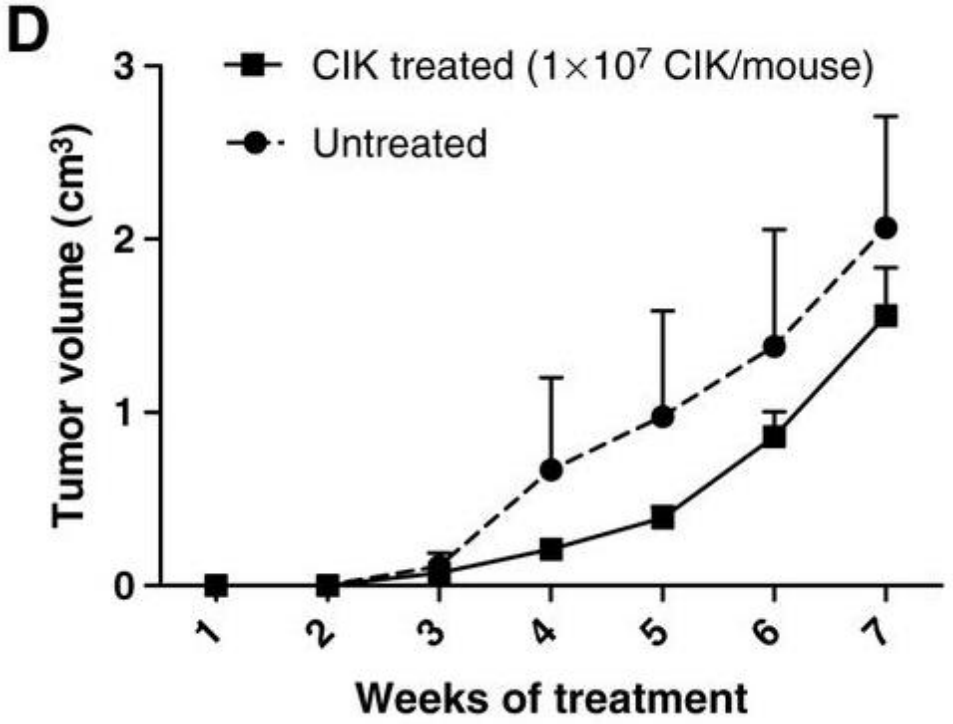
To evaluate the activity of patient-derived CIK cells in vivo against autologous metastatic melanoma.



Analysis

- Necrotic tissue areas
- Lymphocytic infiltration
- Tumor growth

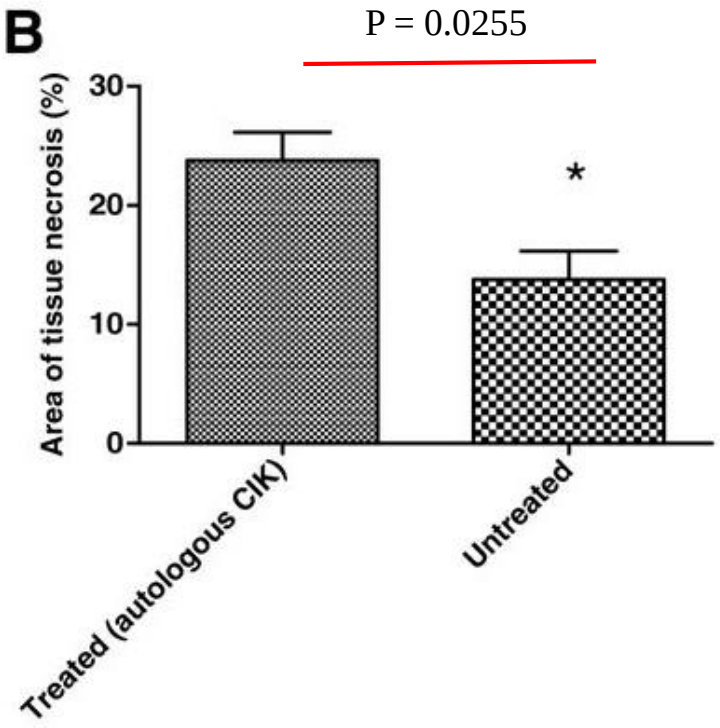
In vivo tumor size analysis



$P = 0.0308$

Figure 2D. A significant delay in tumor growth was observed in NOD/SCID-treated mice compared with the controls (*n*=4). Tumor volume increments were expressed as mean ± SEM and CIK activity was analyzed by two-way ANOVA (P = 0.0308)

In vivo necrotic tissue areas and lymphocytic infiltration analysis



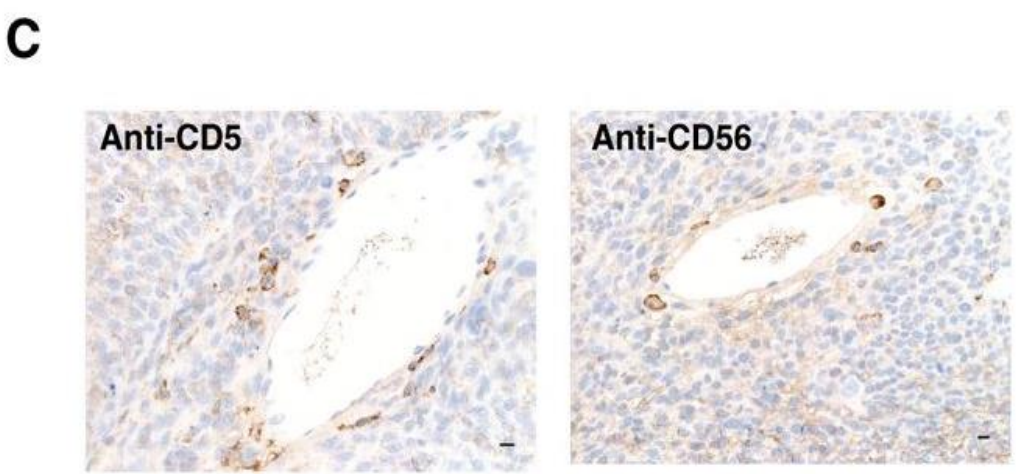


Figure 2B. Percentage of tissue necrosis on tumor growth was calculated at the end of the experiment on paraffin-embedded histologic sections. The results were expressed by mean ± SEM and the extension of necrotic areas was analyzed by an unpaired, two-tailed t test (*, *P = 0.0255*).

Figure 2C. At the end of the infusions, infiltration of CIK cells at tumor sites were shown by immunohistochemistry using antibodies against CD5 and CD56.

Discussion

- CIK cells efficiently killed autologous metastatic melanoma cells.
- Although CIK cells have the killing capacity, the linear projection and quantification of prospective **clinical efficacy** is difficult to be predicted.

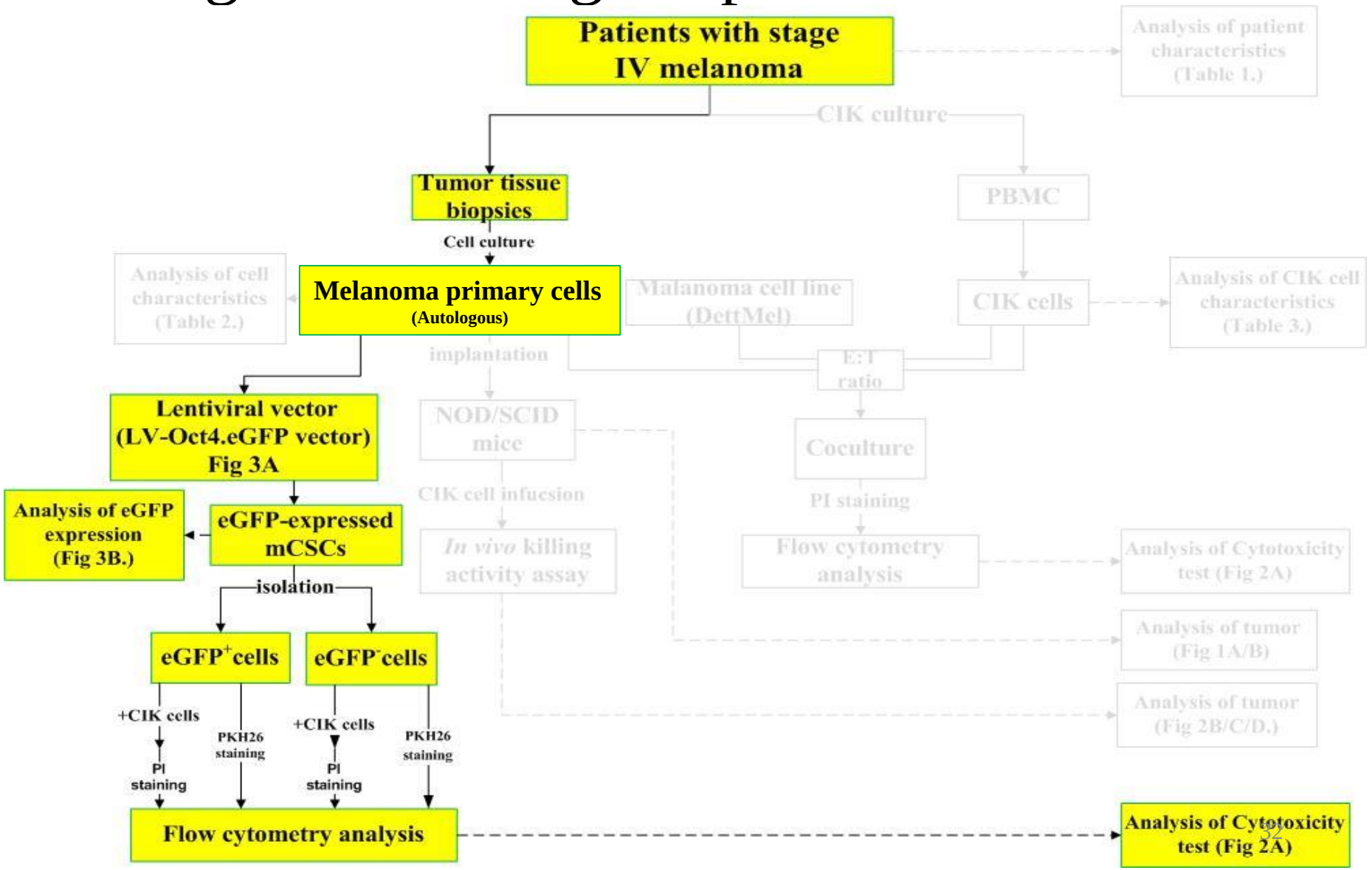
Discussion

Table 2. Phenotype characterization of primary melanoma cell cultures

Subject number	% MIC A/B	% ULBP1	% ULBP2	% ULBP3	% NGFR	% MCSP	% Oct4	% ALDH bright	% ABCG2	% Mitf-	% VEGFR1 bright	% CD34	% CD117	% HLA-ABC
mMel1	33.7	0	62.8	0	74.2	64.7	15.5	8.3	11.8	16.8	19.3	7.6	neg	99.9
mMel2	15.2	1.6	40.7	1.9	58.3	71.4	11.5	8.7	12.4	16.9	10.0	18.0	neg	99.5
mMel3	90.1	1.6	97.9	0	93.0	81.5	8.3	28.9	12.2	14	12.5	25.1	neg	99.6
mMel4	64.9	0	60.6	7.4	19.9	95.0	10.1	8.2	1.3	10.7	10.6	6.3	neg	99.2
mMel5	7.3	1.1	40.3	1.3	82.4	88.9	10.7	11.6	7.3	6.8	12.3	12.3	neg	99.7
mMel6	32.4	0	55.0	0	50.8	95.1	7.3	2.8	11.2	6.5	10.7	6.4	88.8	99.1
mMel7	20.5	0	64.5	0	39.1	78	10.7	8.9	14.1	9.8	5.0	3.1	30.1	99.4
mMel8	53.5	0	52.7	0	28.9	74.2	11.4	3.3	17.5	15	4.4	3.1	neg	99.6
mMel9	7.4	0	65.5	0	82.3	99.7	8.3	6.0	10.2	3.4	2.7	2.1	neg	neg
mMel10	14.2	1.17	62.1	16.6	33.6	73.6	15.2	9.9	13	8.3	5.6	7.0	70.3	neg

- CIK cells were effective against two MHC class I negative melanoma samples, which confirmed their potential in melanomas with immunogenicity alterations.

Activity of CIK cells against autologous putative mCSCs



Lentiviral vector transduction

- Delivery of transgenes directly into a variety of cells in vitro and in vivo
- Transgene
  - *Oct4* gene (LV-Oct4.eGFP)
    - Dedifferntiation of melanoma cells to CSCs
    - Only mCSCs are able to activate the Oct4 promoter to express eGFP
  - Phospho Glycerato Kinase (*PGK)* gene (LV-PGK.eGFP)
    - Most abundant mRNA and protein species in the cells

Lentiviral vector transduction

- Gene as a reporter of expression
  - Green fluorescent protein gene (eGFP)
  - Expression of interested gene
    - Bright green fluorescence
      - Exposure to light in the blue to ultraviolet rang
    - Fluorescent microscopy

LV-Oct4.eGFP putative mCSCs

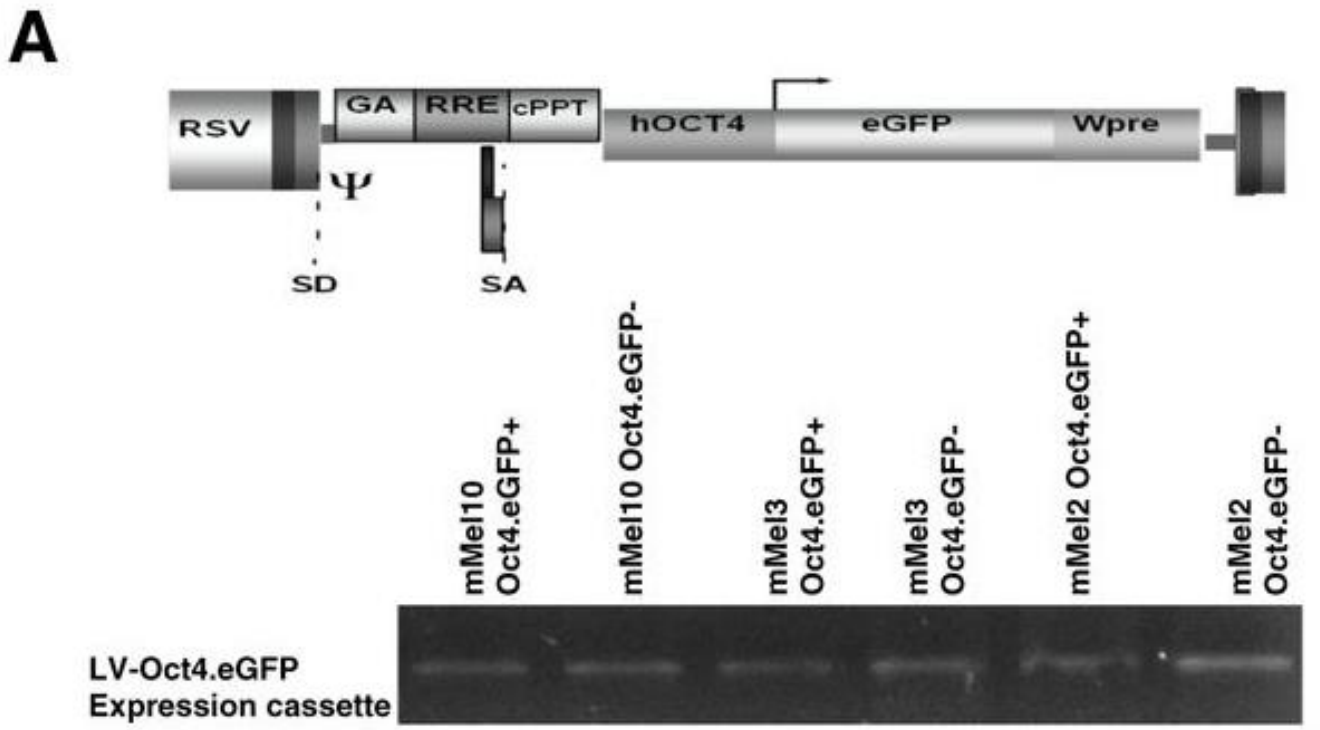
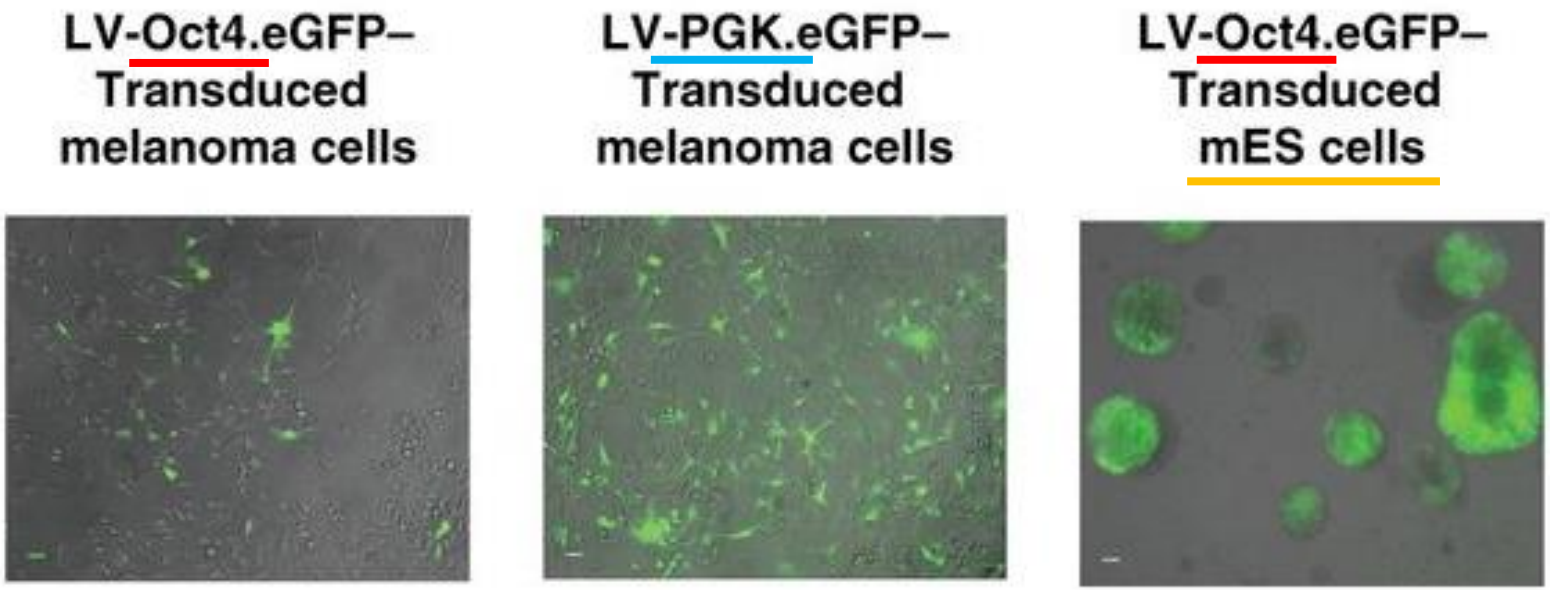


Figure 3A. Picture shows representative PCR electrophoresis gel with primers annealing on the lentiviral vector backbone upstream and downstream the Oct4.eGFP expression cassette.

eGFP expression in melanoma cells

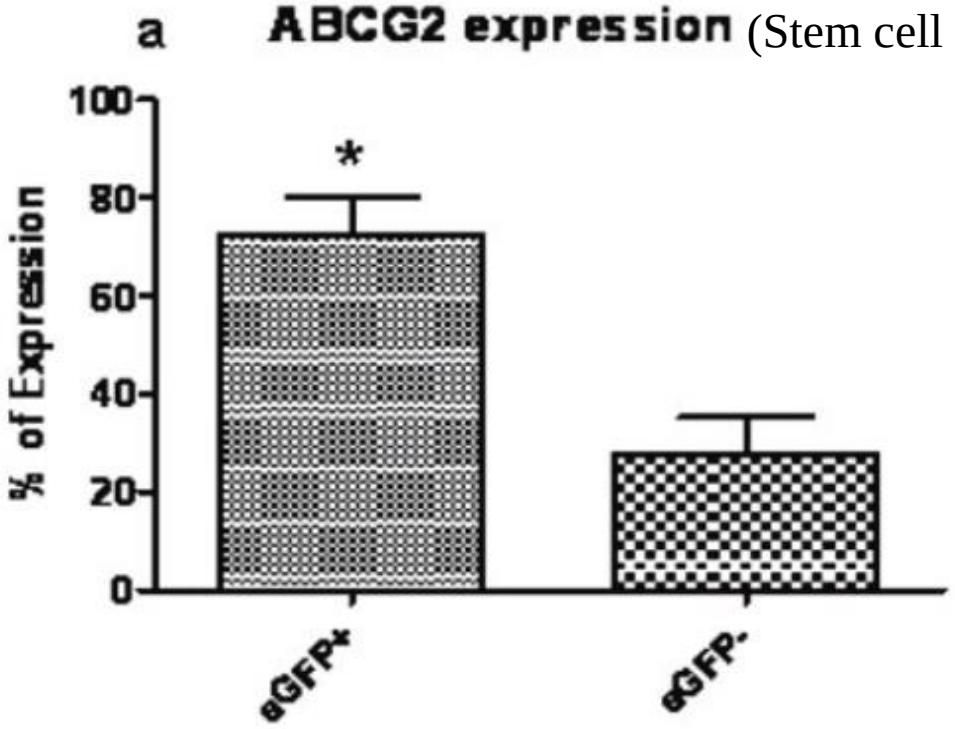
To confirmed that both primary melanoma cells and murine embryonic (mES) stem cells could be transduced efficiently



Samples	Control of eGFP expression	Positive control
11.5%±2.5%	>90%	>90.5%

Figure 3B. Representative eGFP expression in melanoma primary cell cultures transduced with LV-Oct4.eGFP or LV-PGK.eGFP.

LV-Oct4.eGFP putative mCSCs

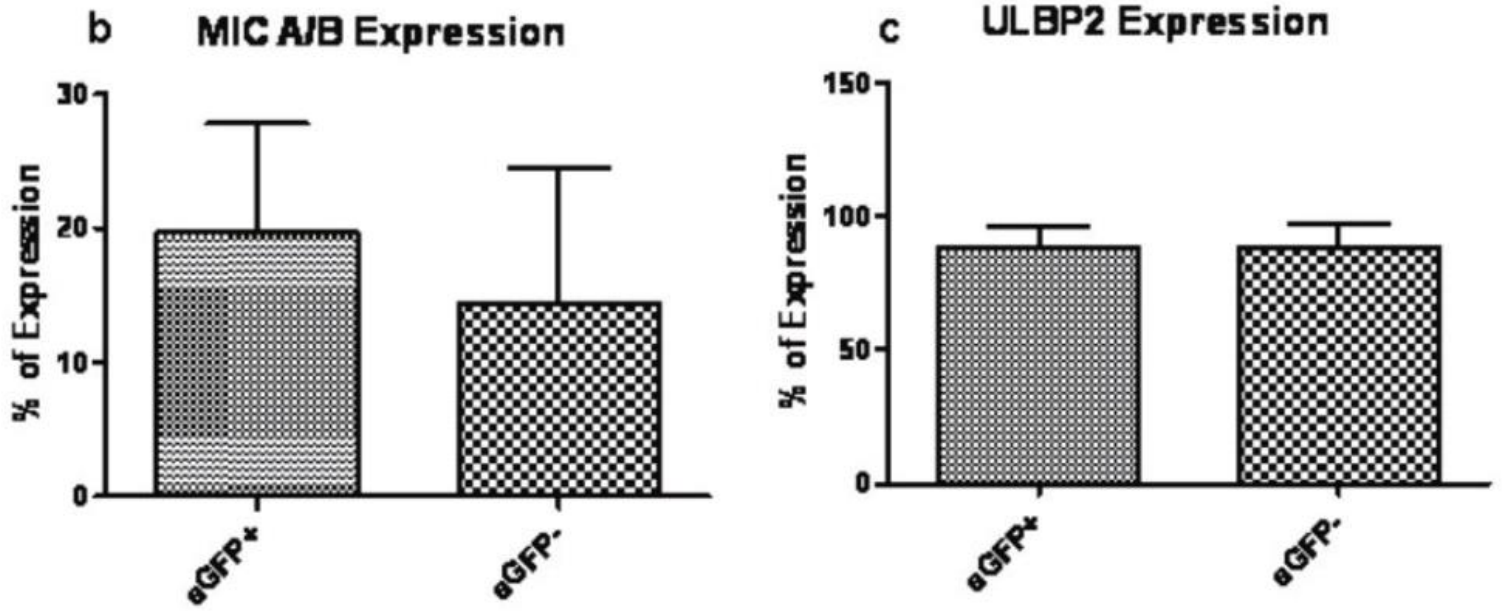


(Stem cell marker)

Condition	Mean ± SEM	n	P-value
ABCG2/eGFP+	72.4± 4%	4	0.0002
ABCG2/eGFP-	27.6± 4%		

Supplement Figure 3. Picture shows the expression levels of ABCG2 in eGFP pupulation

LV-Oct4.eGFP putative mCSCs



	Mean ± SEM	n	P-value
MIC A/B in eGFP+	19.7± 5%	3	0.5185
MIC A/B in eGFP-	14.3± 6%		
ULBP2 in eGFP+	88.3± 5%	3	1.000
ULBP2 in eGFP-	88.3± 5%		

Supplement Figure 3. Picture shows the expression levels of MIC A/B and ULBP2 in eGFP population

In vitro proliferation of putative mCSCs

To evaluated the proliferation ability of putative mCSCs

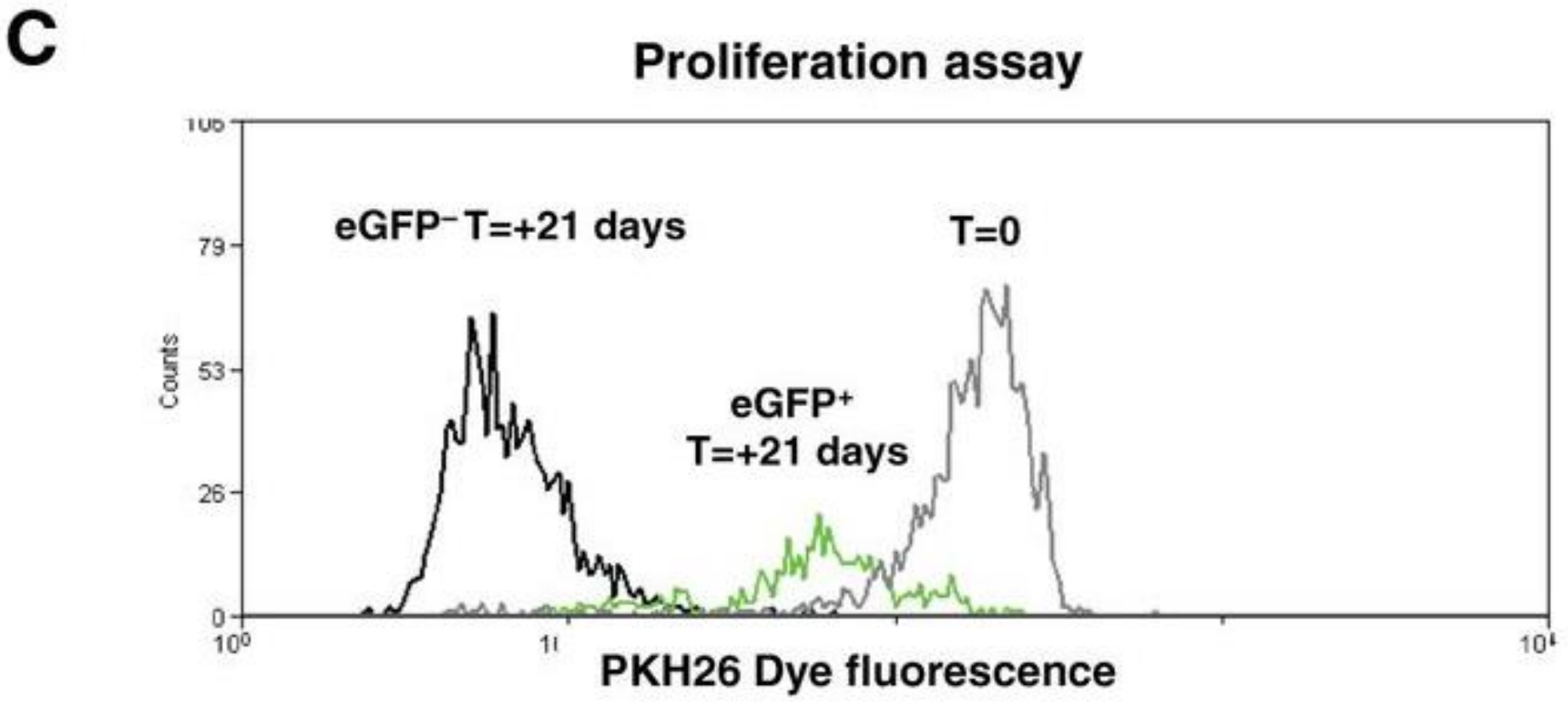


Figure 3C. Proliferation assay *in vitro* was evaluated by staining LV-Oct4.eGFP-transduced tumor cells with the vital dye PKH26 and assessing the fluorescence intensity decrement over time. *n*=5

Discussion

- eGFP$^+$ melanoma cell fraction was enriched in putative mCSCs.
- The cell fraction endowed with stemness features is stably detectable and retained in different samples.
- This is consistent with the decade-old CSC theory that tumors contain a subset of cells that both self-renew and generate differentiated progeny.

In vitro killing activity of CIK cells against eGFP$^+$ and eGFP$^-$ melanoma cells

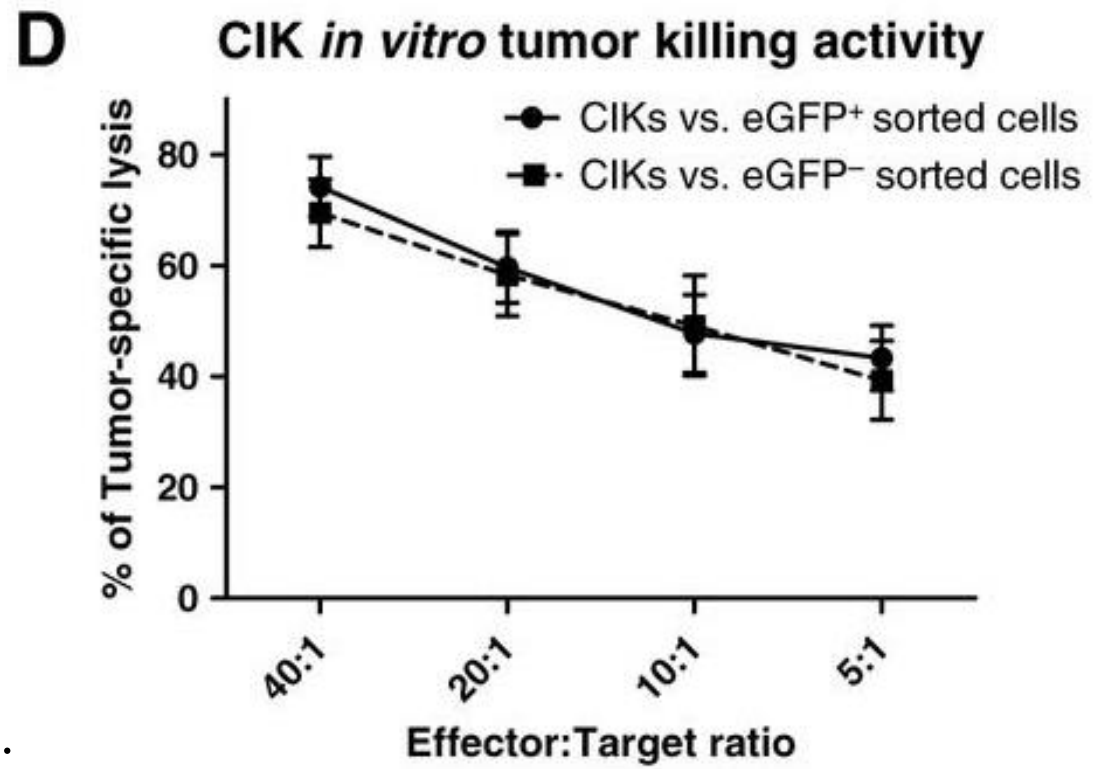


Figure 3D.

Condition	n	P-value
CIK/eGFP$^+$	4	0.8224
CIK/eGFP$^-$ cells		
CIK/both eGFP$^+$ and eGFP$^-$ cells	5	0.6286
CIK/total tumor cells		

Discussion

- CIK cells kill a subset autologous metastatic melanoma cells able to activate *Oct4* that reliably defines a subpopulation of tumor cells with stemness features.

Conclusion

- The MHC-unrestricted tumor killing mechanism of CIK cells showed in this study may advantage it over other immunotherapy approaches because it addresses the difficult quest of targeting mCSCs and also HLA-negative tumors.
- This strategy could reduce resistance occurrence by improving the odds of targeting the crucial CSC subset from which tumor regrowth is speculated to start.

Comments

- Table 1. it was not addressed about the criteria that used to collect tissue biopsy from subcutaneous or lymph nodes.
- This article did not address why they chose mMel1-6 for the assessment of tumorigenic capacity *in vivo* NOD/SCID mice.
- Figure3B, it did not show the negative control and which instrument was used to evaluate the transducted cells.

Thank you for your attention