POOLED IN-VIVO CRISPR/CAS9 SCREENING TO STUDY THE ROLE OF IMMUNE CHECKPOINTS IN THE REGULATION OF LEUKEMIA STEM CELLS

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INTRODUCTION

BACKGROUND

- **Leukemic Stem Cells (LSCs)** initiate and sustain Acute Myeloid Leukemia (AML)
- LSC persistence during treatment makes **targeting of LSCs** important for a complete cure
- **Immune checkpoint** receptors and ligands regulate T-cell activation, but have also been found to be expressed on cancer cells in different cancer entities
- **Signalling in these immune checkpoints** is complex: bidirectional signalling has been shown for some, and others induce inhibitory or stimulatory effects depending on the cellular context

HYPOTHESIS & QUESTIONS

- Immune checkpoints regulate LSCs in a cell autonomous fashion or via interacting with the stem cell niche

? **How** can we screen for immune checkpoints of importance for AML LSCs in a systematic and functional way?

? **Where** are possible hits from the screening and their ligand/receptor counterpart expressed?

? **What**'s the function of possible hits in hematopoiesis and leukemia development?

RESULTS – FIG. 1

FIG. 1 - ADDITION OF SCF, TPO AND GM-CSF TO CULTURE MEDIUM PROMOTES AML LSC *IN VITRO* SURVIVAL WHILE KEEPING COLONY FORMING CAPACITY INTACT

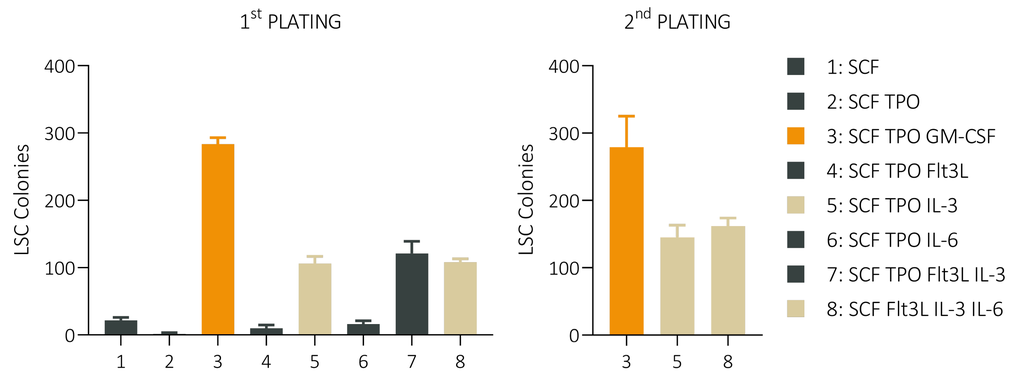
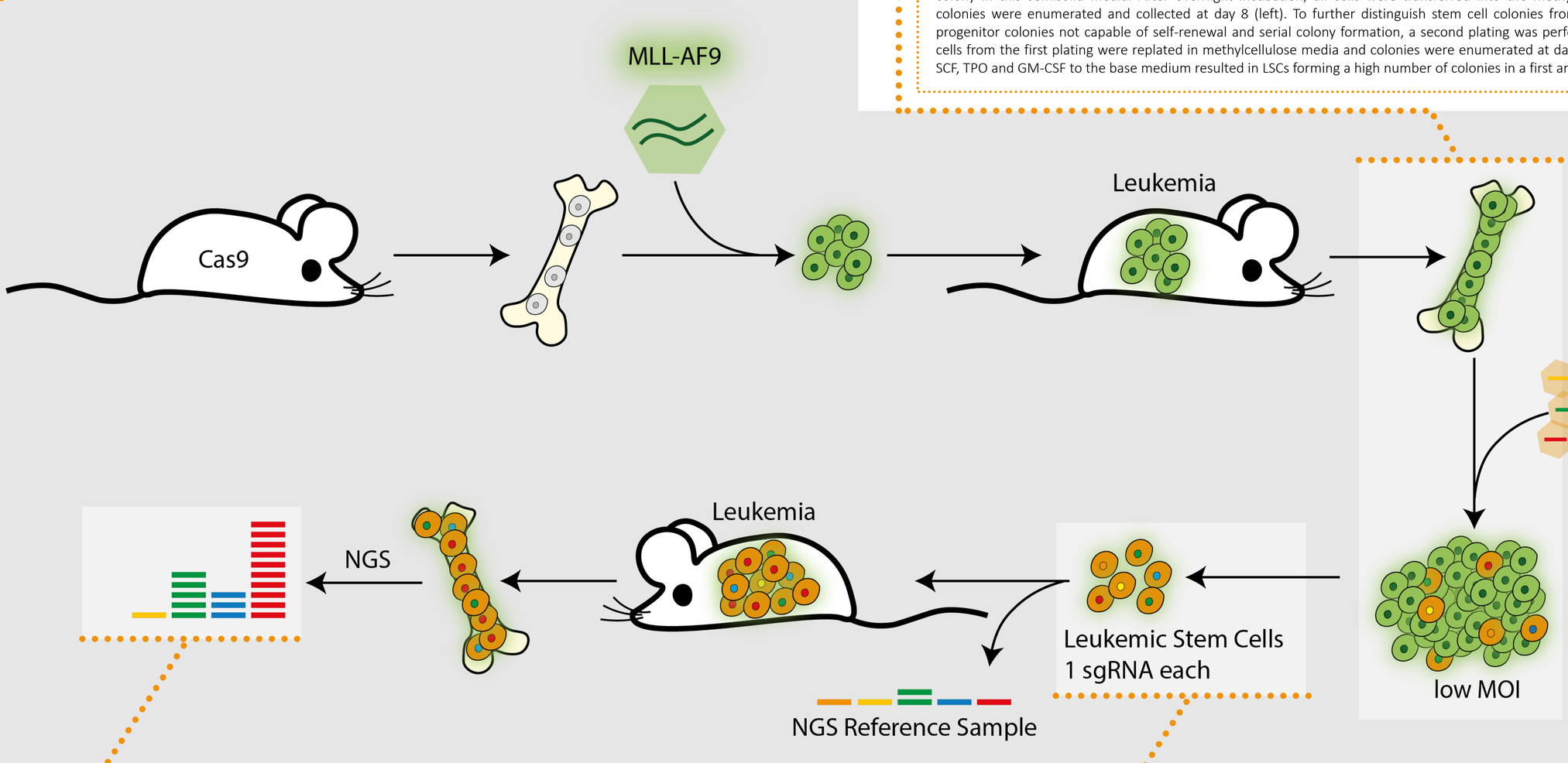


Fig. 1 Optimization of AML LSC *in vitro* Culture. 1'000 AML LSCs (leukemic GMPs, lin$^-$sca-1$^-$c-kit^{hi}FcγRII/III$^+$GFP$^+$) were incubated overnight in the base medium RPMI 10% FCS + 1mM L-Glu + 1x Penicillin/Streptomycin, supplemented with indicated cytokines (condition 1-8). Survival and colony forming capacity were assessed by methylcellulose assay, where only stem cells can form a colony in this semisolid media. After overnight incubation, all cells were transferred into the methylcellulose media, and colonies were enumerated and collected at day 8 (left). To further distinguish stem cell colonies from more differentiated progenitor colonies not capable of self-renewal and serial colony formation, a second plating was performed. For this, 1'000 cells from the first plating were replated in methylcellulose media and colonies were enumerated at day 6 (right). Addition of SCF, TPO and GM-CSF to the base medium resulted in LSCs forming a high number of colonies in a first and second plating.



RESULTS – FIG. 2

FIG. 2 - OVERNIGHT PUROMYCIN SELECTION ENRICHES FOR AML LSCs HAVING RECEIVED AN sgRNA-LENTIVIRAL PARTICLE WITHOUT INTRODUCING DETRIMENTAL STRESS

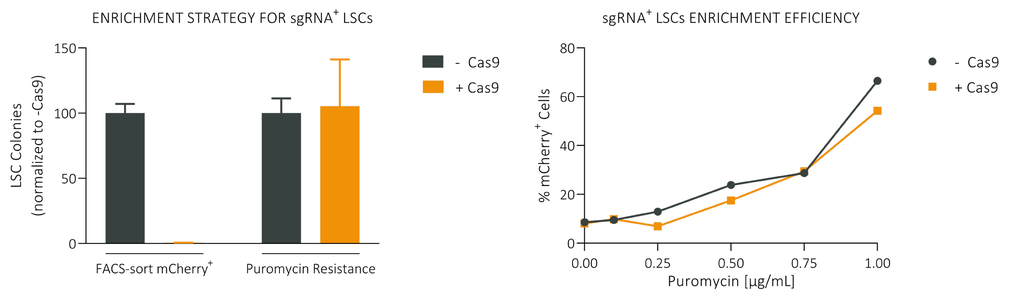


Fig. 2 Optimization of Enrichment Strategy for LSCs Having Received an sgRNA-Lentiviral Particle. Low MOI transduction of AML LSCs with the lentiviral sgRNA library requires sgRNA$^+$ LSCs enrichment. Two selection markers are present on the sgRNA-plasmid: the mCherry-fluorophore, or a puromycin resistance gene. mCherry$^+$ FACS-sorting of LSCs was detrimental to LSCs expressing the Cas9 protein, possibly due to the combined stress of FACS-sort and Cas9-induced DNA-double strand breaks. Overnight selection with puromycin at 1µg/mL yielded similar LSC colony numbers between -Cas9 and +Cas9 LSCs, indicating that enrichment with puromycin is less stressful to LSCs undergoing Cas9-induced DNA double strand breaks (left). Overnight selection with puromycin at different concentrations strongly enriches for sgRNA$^+$ LSCs, assessed by frequency of mCherry$^+$ cells after colony formation at day 7 (right).

RESULTS – FIG. 3

FIG. 3 - *IN VITRO* COLONY SCREENING OF 25 IMMUNE CHECKPOINT GENES SHOWS TECHNICAL FEASIBILITY AND IS ABLE TO RETRIEVE POSITIVE DROPOUT CONTROL GENES

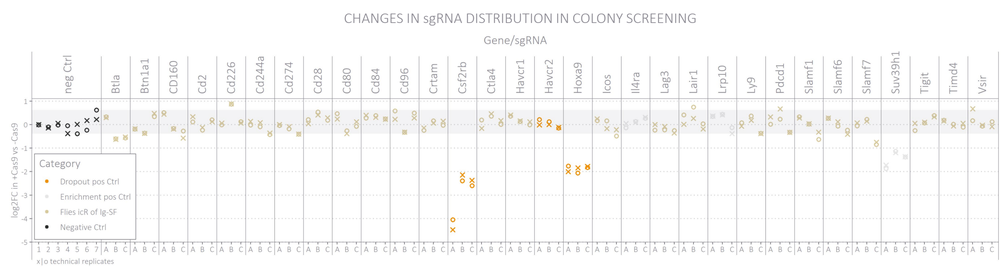


Fig. 3 *In vitro* Colony Screening Retrieves Dropout Positive Control Genes in AML LSCs. To show feasibility of the screening, optimize next generation sequencing setup, and screen for immune checkpoints on LSCs exerting their effect in an autocrine fashion, an *in vitro* colony screening was performed. AML LSCs were transduced with an sgRNA library targeting 25 immune checkpoints at 3 sgRNAs per gene (A-C). The library also contained 7 negative control sgRNAs with no effect on LSC function to show biological variance inherent to the screening. In a first library, 6 positive control genes were included with an expected effect: 3 of them are expected to drop out (orange), and 3 are expected to be enriched (grey). While 2/3 positive dropout control genes were found with a strong impact on LSC colony forming capacity, none of the positive enrichment control genes showed the expected effect. Positive dropout control genes include Csf2rb (involved in GM-CSF receptor) and Hoxa9 (known to be essential for AML LSCs). None of the targeted 25 immune checkpoint genes showed statistical significance when analyzed with the MAGeCK algorithm (N=1).

DISCUSSION

- *In vitro* optimizations are done to a level where the **screening is functional**
- 2/3 positive control dropout genes were found with a **strong expected effect**
- Positive enrichment genes were not found *in vitro.* Literature only provides weak hints for them to be involved in our AML model LSCs.
- **Ligands** from microenvironment are missing in the *in vitro* colony assay - signalling is only functional in autocrine loops on LSCs. Completely functional screening needs to be performed in the *in vivo* mouse model.

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