Generation of a *L. mexicana* 'super knockout' cell line using CRISPR-Cas9 deletion of the genes encoding nucleoside transporters NT1.1, NT1.2 and NT2

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Background

Equilibrative nucleoside transporters (ENTs) are essential to the life cycle of Leishmania mexicana, allowing for the salvage of nutrients needed for survival of the parasite. To date, five ENT genes have been identified for *L. mexicana*, only three of which (NT1.1, NT1.2, NT2) are known to be involved in nucleoside salvage; NT3 and NT4 are nucleobase transporters of promastigotes and amastigotes, respectively. Deletion of the NT1.1, NT1.2 and NT2 genes should provide a favourable expression system for the heterologous expression and characterise the activity of (protozoan) nucleoside and nucleobase transporters.

CRISPR-Cas9 technology is a simple powerful tool for editing genomes. It allows researchers to easily cut of double stranded DNA.It is faster, cheaper and more accurate than previous techniques of editing DNA.CRISPR stands for Clustered *R*egularly *I*nterspaced Short Palindromic Repeats. The CRISPR Cas9 strain was developed and described by the group of Eva Gluenz of the University of Oxford, who generously made it available (Beneke et al., 2017).

GENE ID	GenBankTM accession number	Size b.p.	The author's designated name	Homology to syntenic L. donovani gene
LmxM.15.1230(NT1.1)	XM_003873593	1476	LmexjNT1A	LdBPK_151230
LmxM.15.1240(NT1.2)	XM_003873594	1476	LmexjNT1B	LdBPK_151240
LmxM.36.1940(NT2)	XM_003874493	1500	LmexjNT2	LdBPK_362040.1

Table 1: Shown Gene IDs of NT 1.1., NT1.2 and NT2 of the *L. mexicana* strain.

Transporter	Purine substrates	Pyrimidine substrates
NT1.1	(ADO, ADE)[2017]	URD, URA[2017]
NT1.2	ADO[2017]	TMD, URD[2017]
NT2	INO, GUO[2000]	None
NT3	ADE, XAN, HYP, (GUA)[2003]	None
NT4	ADE[2007]	None
U1	None	URA, (URD)[2005]

Table 2: Purine and pyrimidine transport activities of Leishmania spp..

Objective of the study

This study aimed to create a new 'super-knockout' cell line (SUPKO) by generating a null background cell line that does not take up any purine or pyrimidine nucleosides, surviving through the salvage of purine nucleobases and biosynthesis of pyrimidines. Using CRISPR-Cas9 technology, four different *L. mexicana* Cas9 T7 cell lines were generated: NT1-sKO, NT1-dKO, NT2-sKO and NT2-dKO

Method

To generate the `**MJN Super-KO**` cell line, we will start by deleting the *L. mexicana* NT1.1/NT1.2 locus while replacing it with blasticidin and Tubrocidin selection cassette to generate a DKO of *L. mexicana* NT1.1/NT1.2. The antibiotic and the drug will be added to the medium to delete the both allele, to select *L. mexicana* NT1.1/NT1.2 null parasites. To delete *L. mexicana* NT2 from the *L. mexicana* NT1.1/NT1.2 null parasites, This cell line will be transfected with cas9 guide RNAs. The NT2 will be removed by transfecting with the same resistant cassette with puromycin and formycin B selection pressure.

Results

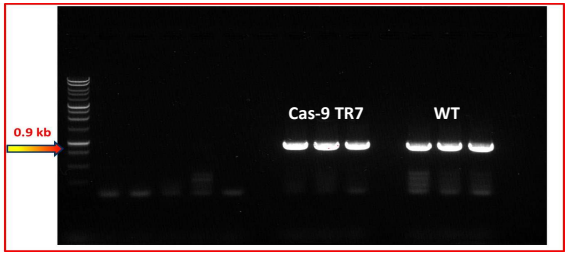

Figure 1: Agarose gel electrophoresis of DNA fragments produced by PCR of DKO of NT1 & NT2 confirmation of 'MJN Super-KO

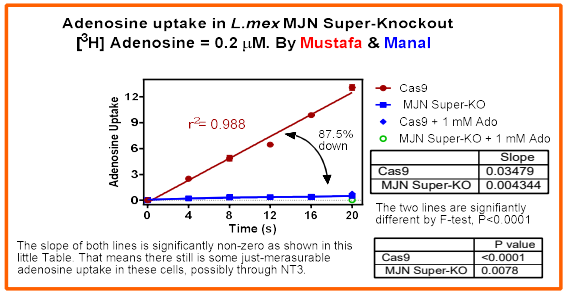

Figure 2: Time course of Cas-9 and *L. mex* 'MJN Super-KO' to confirm deletion of NT1.

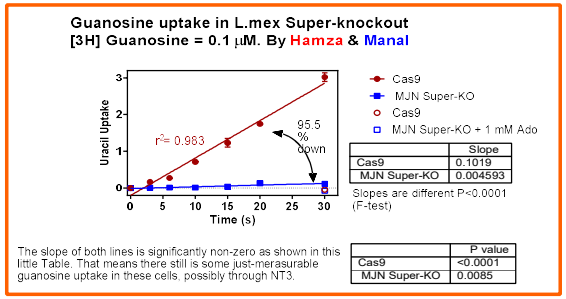

Figure 3 Time course of Cas-9 and *L. mex* 'MJN super-KO' to confirm deletion of NT2.

Summary of Results

Transport Substrate	Conc.	Cell line1	Cell line 2	Percentage inhibition
Adenosine	0.05µM	CAS-9	SUPKO	87.5%
Guanosine	0.1µM	CAS-9	SUPKO	95.5%
Guanosine	0.1µM	CAS-9	SUPKO	95.5%
Uridine	100nM	CAS-9	SUPKO	98.6%
Thymidine	0.1µM	CAS-9	SUPKO	99.7%
Hypoxanthine	**0.1µM**	**CAS-9**	**SUPKO**	**23.0%**
Thymidine	0.05µM	CAS-9	SUPKO	91.3%
Adenosine	0.2µM	CAS-9	NT1-KO C2	99.4%

Table 3 Summary of the percentage inhibition in SUPKO of radiolabel of purine and pyrimidine by uptake assay.

Transport Substrate	Conc.	Cell line1	Cell line 2	Percentage inhibition
Adenosine	0.2µM	CAS-9	NT1-KO	99.4%
Guanosine	0.2µM	CAS-9	NT2-KO	83.7%

Table 4 Summary of the percentage inhibition in NT1 (Adenosine radiolabel) and NT2 (Guanosine radiolabel) by uptake assay using radiolabel.

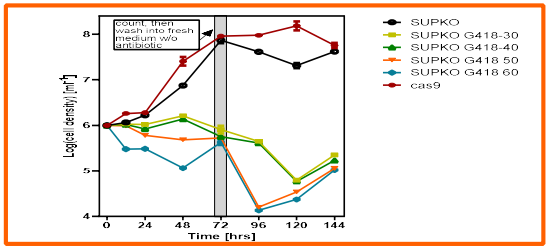

Figure 3: Growth Curve of SUPKO with treated G418 antibiotic at different concentration counting for 6 days.

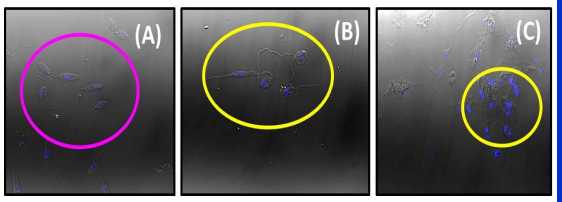

Figure 4: DAPI staining of CAS-9 T7 cell line testing the morphology of the cell shape after 24hrs (A) control, (B) Blasticidin + Tubercidin, and (C) Puromycin + Formycin B treated.

Conclusions

We successfully created a *L. mexicana* (SUPKO) cell line that is null for NT1.1, NT1.2 and NT2, and is suitable for use in further studies to identify any additional ENTs utilised by the parasite. The identification of these transporters will not only improve our understanding of how ENTs work but also potentially allow for therapeutic targeting.

Acknowledgement

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