DEPARTMENT OF
ANIMAL & AVIAN SCIENCES

UNIVERSITY OF MARYLAND
College of Agriculture and Natural Resources

Rational selection of traits using site-specific nucleases

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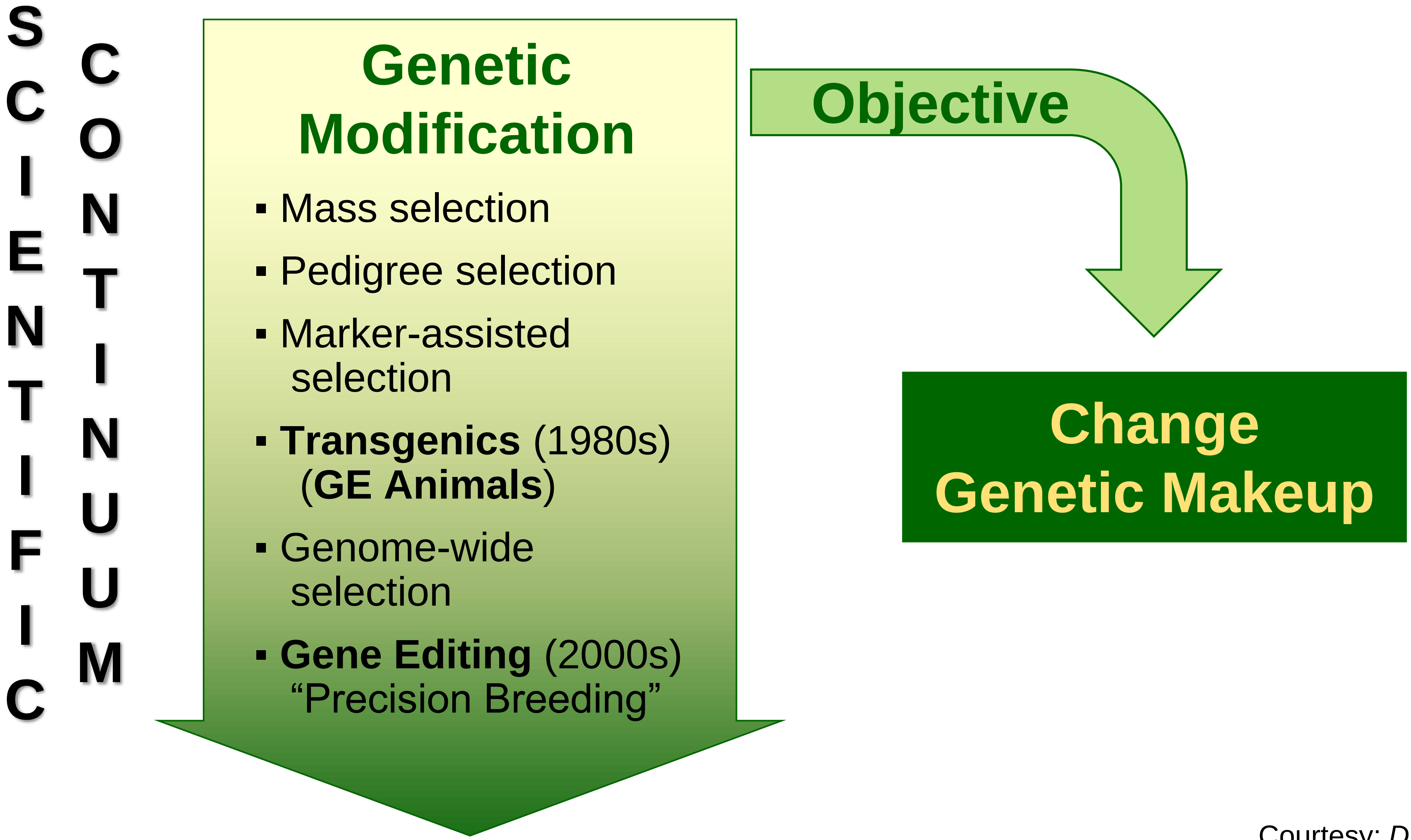
USDA ars

RENOVATE

“Precision breeding” using genome editors



SCIENTIFIC CONTINUUM

Genetic Modification

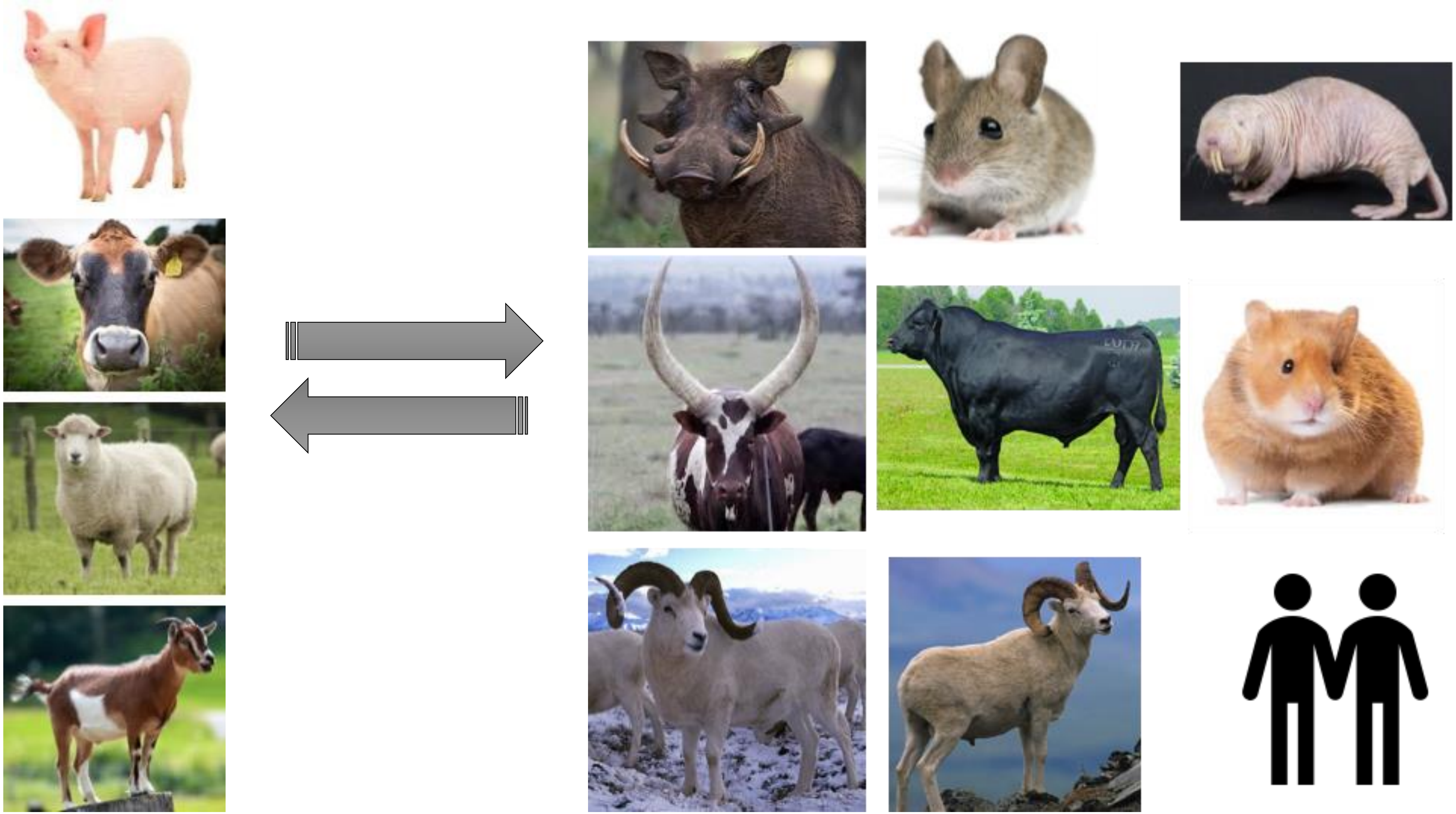
- Mass selection
- Pedigree selection
- Marker-assisted selection
- **Transgenics** (1980s) (**GE Animals**)
- Genome-wide selection
- **Gene Editing** (2000s) “Precision Breeding”

Objective

Change Genetic Makeup

Courtesy: *Dr. Diane Wray-Cahen*

Harnessing genetic diversity using genome editors



Project 1: Introducing novel human Prion variants into cattle for generating Mad cow resistant cattle

Objective :

1) Optimize reproductive technologies

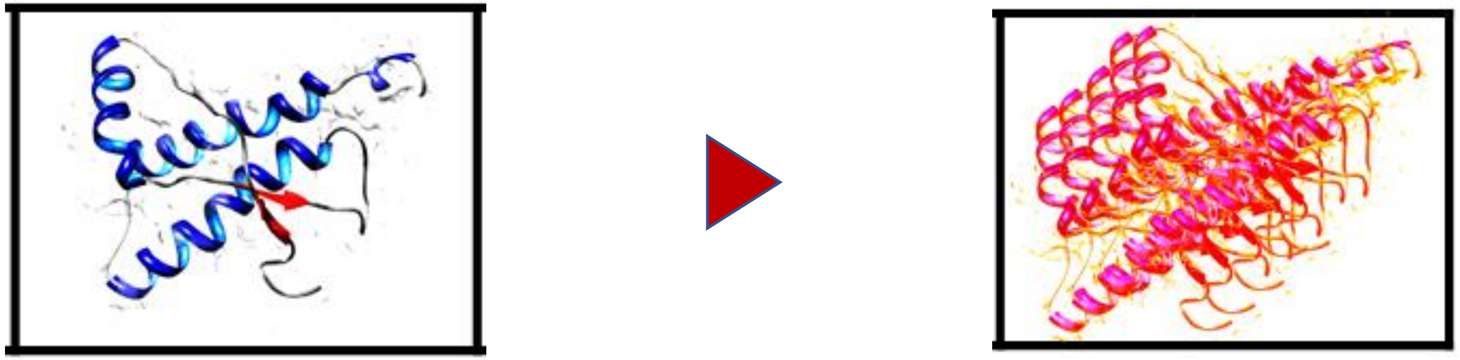
2) Optimize methodology for delivery of editors, and screening

Rationale:

1. Prion- Prp knockout animals are viable. A range of mutations are tolerated.

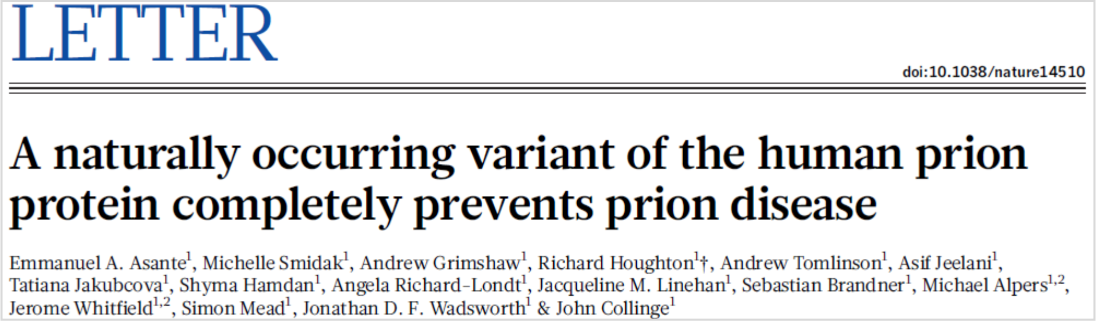
2. A natural variant in humans have been identified that provides resilience

Background: Misfolded Prion diseases



Human:	CJD (Creutzfeldt-Jakob Disease) Fatal Familial Insomnia Kuru
Cow:	Bovine Spongiform Encephalopathy Cow (“Mad Cow” Disease)
Sheep/ Goats:	Scrapie
Deer/Elk/ Moose	Chronic Wasting Disease

Generating Prion (Mad-cow) resistant cattle



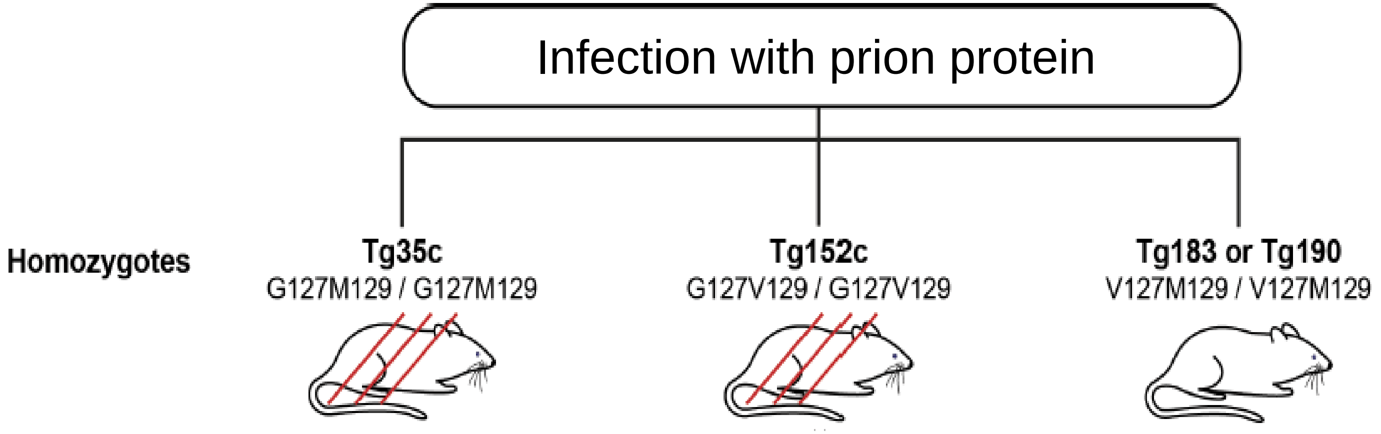
LETTER

doi:10.1038/nature14510

A naturally occurring variant of the human prion protein completely prevents prion disease

Emmanuel A. Asante[1], Michelle Smidak[1], Andrew Grimshaw[1], Richard Houghton[1]†, Andrew Tomlinson[1], Asif Jeelani[1], Tatiana Jakubcova[1], Shyma Hamdan[1], Angela Richard-Londt[1], Jacqueline M. Linehan[1], Sebastian Brandner[1], Michael Alpers[1,2], Jerome Whitfield[1,2], Simon Mead[1], Jonathan D. F. Wadsworth[1] & John Collinge[1]

A variant (G –V) was found in the human prion protein that conferred resistance to Kuru, a prion disease found in tribal groups in Papua New Guinea.



Infection with prion protein

Homozygotes

Tg35c
G127M129 / G127M129

Tg152c
G127V129 / G127V129

Tg183 or Tg190
V127M129 / V127M129

Reagent optimization (Guide/Oligo)

Goal:

ATGGTGAAAAGCCACATAGGCAGTTGGATCCTGGTTCTCTTTGTGGCCAT….///

F guide: GCAGUGGUAGGGGGCCUUGG GTATAC(AccI)

CTGGAGCAGTGGTAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAG

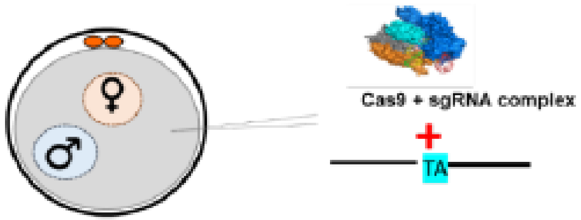
ACCACCGAUGUACGACCCUU :R guide

…//..CCTCATTTTTCTCATAGTAGGATAG

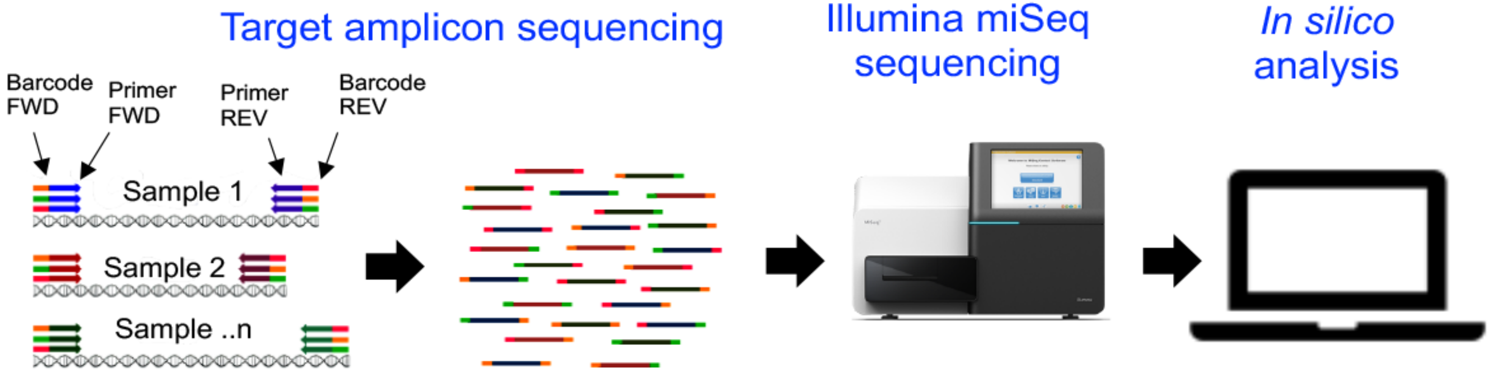
Glycine > Valine

GGCTAC > GTATAC

Process:



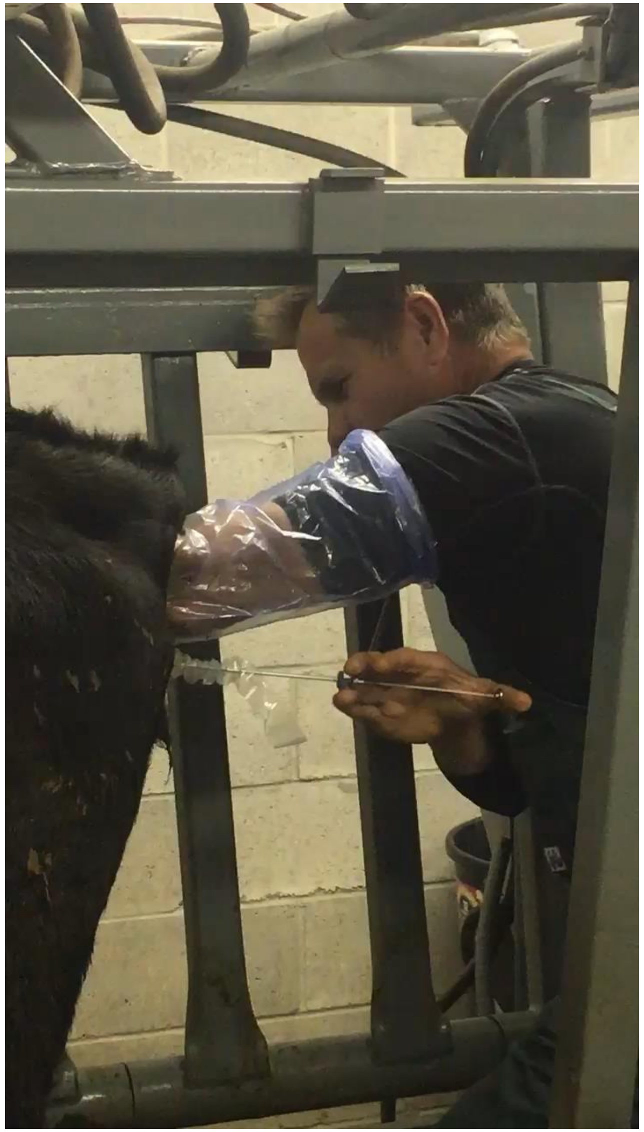
Results:



		Symmetrical						Asymmetrical						Reverse-asymmetrical					
	Allele	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
WT	gcagtggtagggggccttggtggctacatgctgggaagtgcc	65.6	10	82.2	52	0.45	18.1	70.85	14.4	36	88.4	52.6	82.8	2.84	24.5	22.8	67.4	87.4	30.17
HDR	gcagtggtagggggccttggtgtatacatgctgggaagtgcc	30	84.6	9.77		3.21	4.22	6.342	5.93	3.44	11.6	47.4	12.3	55.9	58.3	77.2	8.21	12.6	65.4
NHEJ	gcagtggtagggggccttgg-ggctacatgctgggaagtgcc	4.46						4.074						41.3					
	gcagtggtagggggcct---tggctacatgctgggaagtgcc				48												18.1		
	gcagtggtagggggccttggTtggctacatgctgggaagtgcc					12.5	24.4	18.73											
	Other		5.37	8.06		83.9	53.3		79.7	60.5			4.91		17.2		6.35		4.424
	Reads in Fastq	3634	3430	3911	687	3224	3278	2813	3135	3134	2795	2804	1521	2645	3569	3618	3654	3468	2959

Park et al., accepted (The CRISPR Journal)

Embryo transfers (7/12 recipients are pregnant)



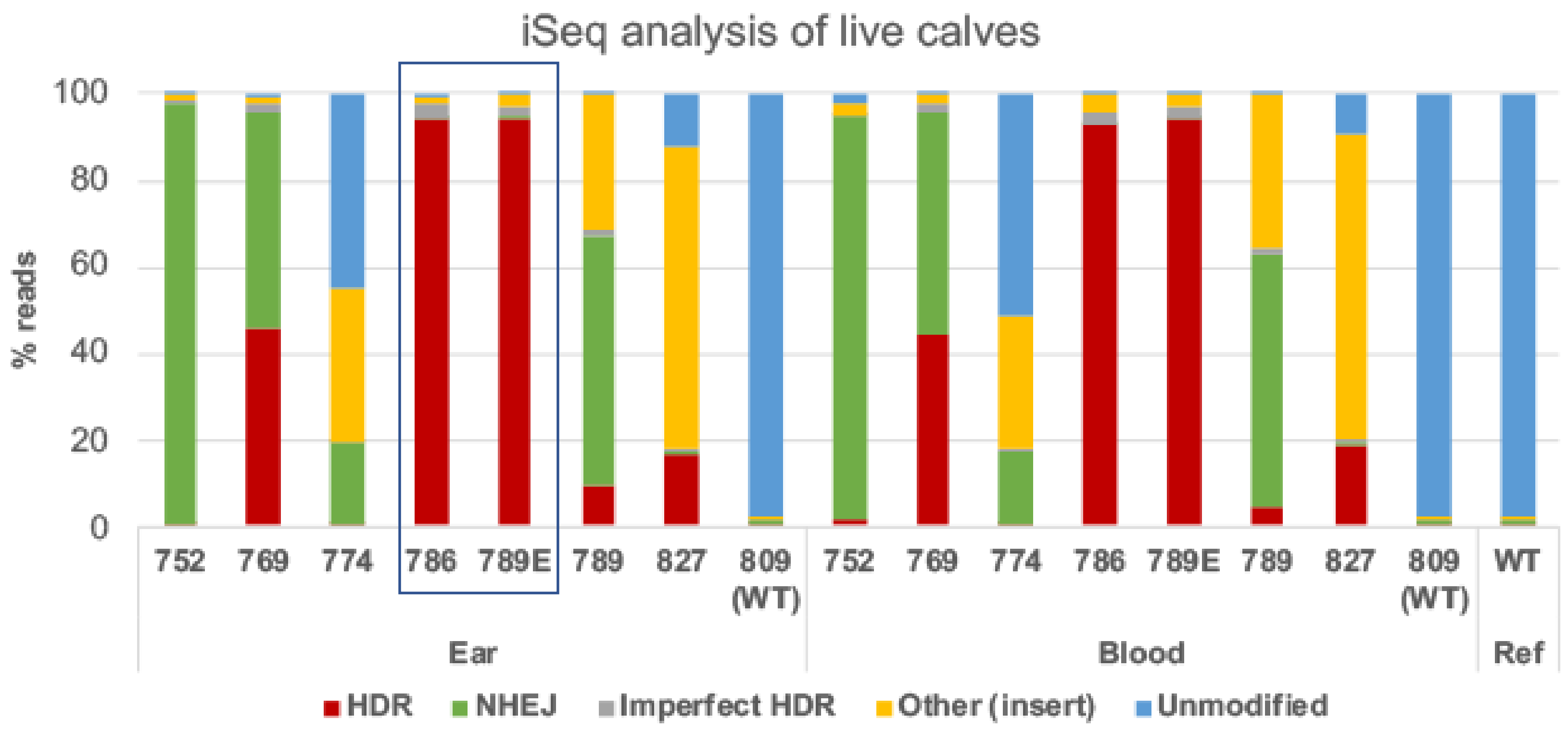
Recipients	Treatment	Animal ID	Pregnancy outcome	Calving data	Sex of offspring
1	bPRNP	752	Pregnant	Twins (1 normal+ 1 stillborn^{SB})	Male Female^{SB}
2	bPRNP	769	Pregnant	1 calf	Male
3	bPRNP	772	Non-pregnant		
4	bPRNP	774	Pregnant	1 calf	Female
5	bPRNP	786	Pregnant	1 calf	Male
6	bPRNP	789	Pregnant	2 calves (1 euthanized)	Male
7	bPRNP	804	Non-pregnant		
8	bPRNP	819	Non-pregnant		
9	bPRNP	827	Pregnant	1 calf	Male
10	Control	806	Non-pregnant		
11	Control	809	Pregnant	1 calf	Male
12	Control	829	Non-pregnant		
			Total live calves at birth	6 edited calves; 1 WT control calf	

Park et al., accepted (The CRISPR Journal)

Genome edited calves

Park et al., accepted (The CRISPR Journal)

Frequency of targeted alleles in edited calves



		Ear								Blood								Ref
	Reference sequence	752	769	774	786	789E	789	827	809 (WT)	752	769	774	786	789E	789	827	809 (WT)	WT
HDR	GCAGTGGTAGGGGGCCTTGGTGTATACATGCTGGGAAGTG	0.22	45.49	0.05	94.2	94.55	9.53	16.81	0.08	1.44	44.3	0.42	92.64	94.14	4.33	18.85	0.25	0.01
NHEJ		97.93	50.32	19.51	0.14	0.13	57.69	0.39	1.73	93.29	51.24	17.2	0.08	0.1	58.91	0.33	1.47	0.08
	GCAGTGGTAGGGGGCCTTGG-GGCTACATGCTGGGAAGTG		31.4	12.18							30.87	10.09						
	GCAGTGGTAGGGGGCCTTG----CTACATGCTGGGAAGTG						30.98			62.59					36.96			
	GCAGTGGTAGGGGGCCTTGG---CTACATGCTGGGAAGTG	68.41					5.9								2.41			
Imperfect HDR		0.24	1.98	0.01	3.21	2.48	1.16	0.72	0.01	0.24	2.14	0.11	3.29	3.24	1.03	0.77	0.04	1.61
Other (insert)		1.44	1.83	35.32	2.05	2.54	31.51	70.07	0.42	3.12	2.01	30.68	3.83	2.28	35.65	70.87	0.5	0.41
Unmodified	GCAGTGGTAGGGGGCCTTGGTGGCTACATGCTGGGAAGTG	0.17	0.38	45.1	0.4	0.3	0.11	12	97.76	1.92	0.32	51.59	0.15	0.24	0.08	9.17	97.75	97.89
Reads aligned		8683	16979	15016	11622	16523	19670	24618	8417	417	14660	15199	18410	9689	15827	14116	25008	14671

Park et al., accepted (The CRISPR Journal)

Significance/Highlights:

- Feasibility of introducing targeted genetic modifications and introgression of novel variants by direct injection of editing reagents into the zygotes

- We <u>report for the first time</u> the use of CRISPR/Cas genome editors to introduce novel allelic variants in cattle by direct modification in zygotes.

Park et al., accepted (The CRISPR Journal)

Project 2: Introducing novel rat variants into pigs for eliminating boar tain

Objective :

1) Eliminate boar taint in pigs by genome editing

2) Rats don’t exhibit boar taint. Introgress rat variants into pigs in steroidal enzymes

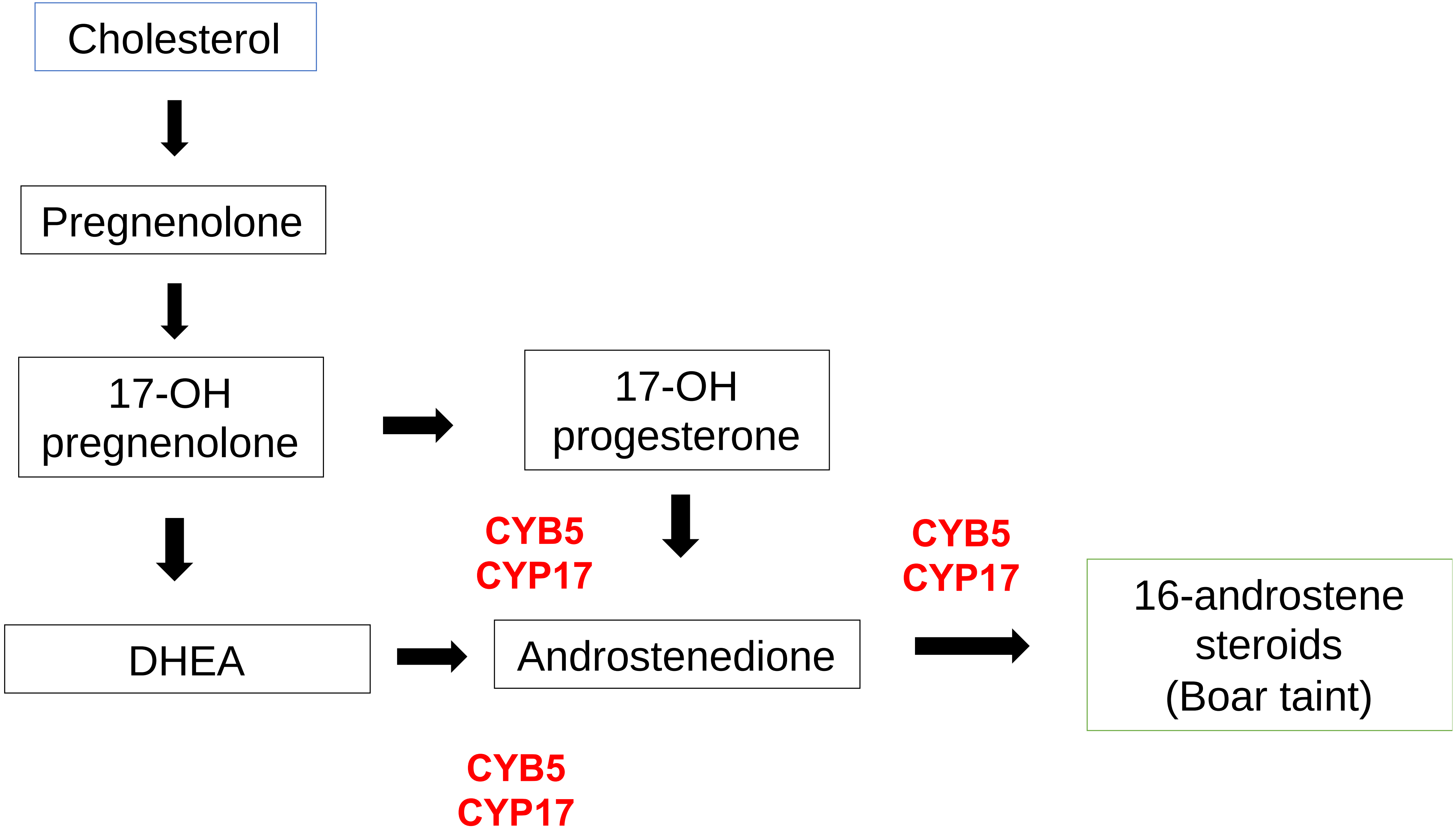
Rationale:

1) Boar taint is an offensive odor in uncastrated pigs

2) Requires physical castration

3) Vaccine trials were unsuccessful

Overview: Boar taint etiology



Cholesterol

Pregnenolone

17-OH pregnenolone

17-OH progesterone

DHEA

Androstenedione

16-androstene steroids (Boar taint)

CYB5
CYP17

CYB5
CYP17

CYB5
CYP17

Rationale: Boar taint etiology

- A rare polymorphism in the porcine **CYB5** gene just upstream of the translational start site results in **decreased** production of CYB5 and decreased synthesis of androstenone (Peacock et al., 2008).

- A **CYB5** knockout mouse model has a dramatically **low levels of testicular androgens** (McLaughlin et al., 2010). Therefore, totaling eliminating the expression of CYB5 is not an option.

Comparative genomics

Steroid binding pocket of CYB5A

	1	11	21	31	41
Rat CYB5	MAEQSDKDVK	YYTLEEIQKH	**K**DSKSTW**V**IL	HHKVYDLTKF	LEEHPGGEEV
human CYB5	MAEQSDEAVK	YYTLEEIQKH	**N**HSKSTW**L**IL	HHKVYDLTKF	LEEHPGGEEV
pig CYB5	MAEQSDKAVK	YYTLEEIQKH	**N**NSKSTW**L**IL	HHKVYDLTKF	LEEHPGGEEV

Steroid binding pocket of CYP17A1

	80	90	100	110	120
Human	QLAKEVLIKK	GKDFSGRPQM	A**TL**DIA**S**NNR	K**GIAFAD**SGA	HWQL
Pig	QLAKEVLLKK	GKEFSGRPRV	M**TL**DIL**S**DNQ	K**GIAFAD**HGT	SWQL
Rat	QLAREVLIKK	GKEFSGRPQM	V**TQ**SLL**S**DQG	K**GVAFAD**AGS	SWHL

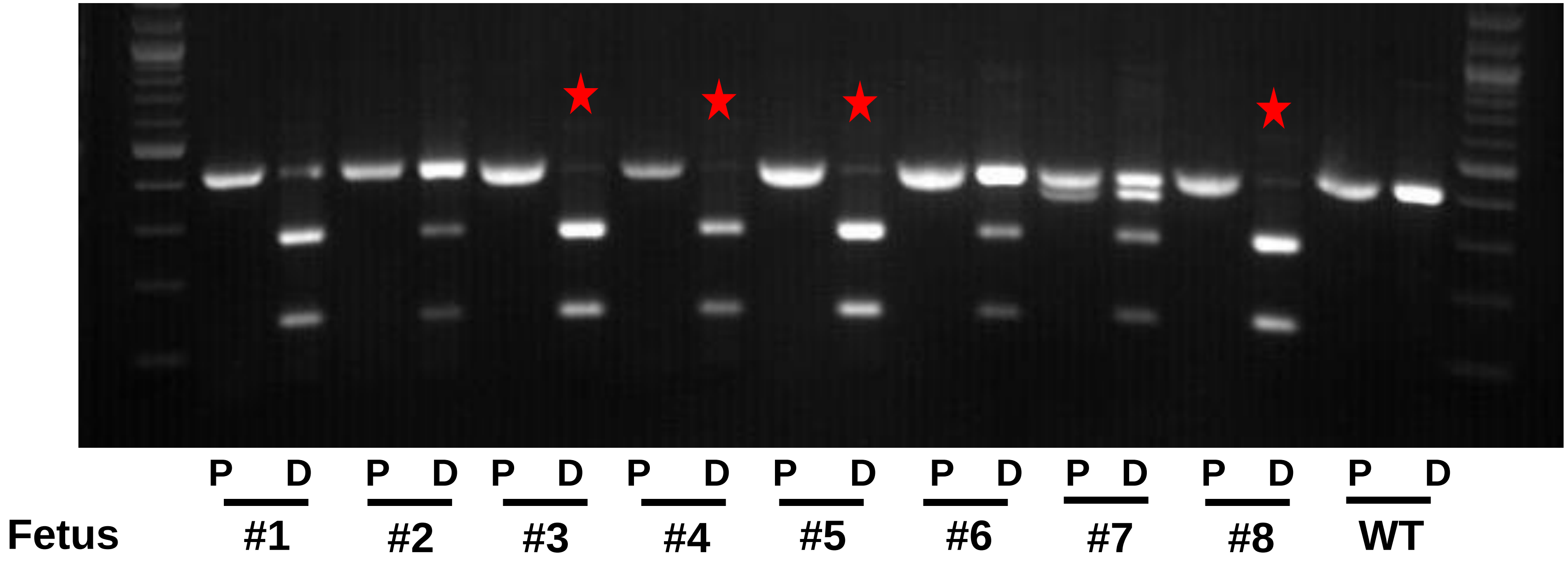
In vitro genetic screen validated the mutants

CYB5 mutations with CYP17 mutations	17OHP	DHEA	16A	16A/DHEA ratio
R52M +L102Q	1.174	0.699	0.607	1.032
R52M +I112V	1.257	0.566	0.282	0.567
R52M +L102Q/I112V	1.500	1.162	0.750	0.282
R52M/D103S	1.282	0.761	0.457	0.600
R52M/S106A	1.484	0.529	0.616	1.167
R52M/NQ108QG	1.176	0.861	0.563	0.653
N62S + D103S	0.897	1.166	0.912	0.787
N62S + 104L	0.904	1.317	1.760	1.484
N62S + S106D	1.252	0.071	0.399	2.042
N62S +L102Q/I112V	1.032	0.963	0.748	0.765
R52M+N62S/D103S	**1.195**	**0.827**	**0.534**	**0.645**
R52M+N62S/S106A	1.437	0.546	0.799	1.462
R52M+N62S/NQ108QG	1.130	0.877	0.771	0.881
R52M+N62S + L102Q/D103S/I112V	1.130	0.839	0.333	0.426
R52M/G57R/N62S/T70S + L102Q/D103S/I104L/NQ108QG/I112V	1.536	0.257	0.503	1.979
G57R + D103S	0.950	1.085	0.905	0.836
G57R + NQ108QG	0.833	1.255	1.231	0.983
T70S + D103S	0.947	1.087	0.937	0.863
T70S + NQ108QG	0.855	1.221	1.201	1.180
N21K + D103S	1.132	0.835	0.490	0.585
L28V + D103S	1.068	0.924	0.643	0.693
N21K/L28V + D103S	1.110	0.867	0.588	0.677

Screening of CYP17A1 targeted fetuses by restriction enzym (BstZ17I) digestion

D103S

```
      GAGTCTGATG GCTGTGTCCT TTCCTGCTTT CCAAAGATGA CTCTAagtAT   New BstZ171 site
10951 aCTGTCAGAC AACCAAAAGG GGATTGCCTT CGCCGACCAT GGTACCTCCT
11001 GGCAGCTGCA TCGGAAGCTG GCACTGAGCA CCTTTTCCCT GTTCAAGGGT
11051 GGCAACCTGA AGCTGGAGAA CATCAGTGAG TGCCCAGCCG GCCCTGGGCT
```



P: PCR product
D: Digest with BstZ17I

Summary: Boar taint project

- Edit *CYB5* locus on *CYP17*^{mut} background.

- Screen for steroid profile at weaning and at puberty

Conclusion: Advantages of Genome editing vs Conventional Breeding

- Increase precision and efficiency of introducing desirable traits (*conventional breeding is random*)
- Introduction of novel traits not possible with conventional breeding
- Separate “linked” genes(“Hitchhiker effect”)
- Increase heritability of traits

Acknowledgements

UNIVERSITY OF MARYLAND 1856

USDA ars
United States Department of Agriculture
Agricultural Research Service

Ki-Eun Park
Anne Powell

Chi-Hun Park
Juli Frey

RENOVATE

Sean Simpson
Jerel Waters
Bhanu Telugu

Centre for Tropical Livestock Genetics and Health

Simon Lillico
Bruce Whitelaw
Djikeng Appolinaire

Genus

Mark Cigan
Jonathan Lightner

THOMAS D. MORRIS INCORPORATED

Chris Coutu
Tom Morris

Acknowledgments- Funding sources

BILL & MELINDA GATES *foundation*

Genus

USDA

NIFA

NATIONAL INSTITUTES OF HEALTH

MSCRF
MARYLAND STEM CELL RESEARCH FUND

UNIVERSITY OF MARYLAND
COLLEGE PARK
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