The University of Michigan Transgenic Animal Model Core

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CRISPR/Cas9 and Mouse Embryonic Stem (ES) Cell Training

The purpose of the class is to provide training in building CRISPR/Cas9 reagents, all aspects of ES cell culture manipulation, and to provide the scientific background needed to make genome edited/gene targeted ES cells and mice. You will learn both the methods and the principles behind the methods.

The CRISPR/Cas9 ES Cell Training Course is designed to instruct researchers in the art of design, construction, and validation of CRISPR/Cas9 reagents and in pluripotent mouse ES cell culture and the methodologies of gene targeting. The training includes daily laboratory sessions on molecular biology techniques such as DNA cloning, DNA sequencing, PCR, testing Cas9 for endonuclease activity, and all aspects of mouse embryonic stem cell culture. Daily seminars cover key papers in the field. Trainees are expected to read papers, participate in discussions, and present at least one seminar. Special topics specific to trainee research are welcome. The course will take place in room 2578 Medical Science Research Building II, and will last two weeks. In order to provide as much hands-on experience as possible, the training class size is limited to four or fewer trainees.

A. Purpose: To instruct personnel in the art of CRISPR/Cas9 design and application, in mouse ES cell culture, and applications of Cas9 and ES cells to gene targeting in mice and in ES cells.

B. Training Overview: Discussion and lab experience in molecular biology techniques required to construct CRISPR/Cas9 reagents and in tissue culture techniques required to maintain ES cells in an euploid pluripotent state. This includes DNA cloning; electroporation of Cas9 plasmids into mouse ES cells; purification of DNA from Cas9 treated ES cells and evaluation of Cas9 gene editing; ES cell cloning; and ES cell cryopreservation and recovery. A basic familiarity with cell culture and molecular biology are pre-requisites for the training.

C. Preparation:

C1. DNA cloning procedures described in Ran et al. (2013) will be taught in the class. Students should read Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. 2013. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 8:2281-2308.

C2. For trainees new to molecular biology, an introductory text in DNA Cloning such as “Gene cloning and DNA analysis an introduction” by T.A. Brown (2010) Wiley-Blackwell that is available as an e-book from the University of Michigan Library is recommended.
https://mirlyn.lib.umich.edu/Record/011585851

C3. Mouse ES cell culture will be carried out as described: Hughes ED, Saunders TL. 2011. “Gene Targeting in Embryonic Stem Cells” in Advanced Protocols for Animal Transgenesis: An ISTT Manual. S Pease and TL Saunders (eds) Springer-Verlag, Berlin. pp. 291-325.

C4. Novices in tissue culture are expected to read "Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications." 6th ed. 2010. Freshney. Wiley-Blackwell. Trainees are expected to be familiar with basic tissue culture procedures.

D. Example schedule (subject to revision):

Week One

Mon. ES Cells: Thaw MEFs, media preparation, observe passage R1 ES cells, observe electroporation of ES cells with CRISPR/Cas9 reagents
CRISPR: Resuspend & quantify oligonucleotides for CRISPR cloning

Tues. ES Cells: Passage R1 ES cells, set up R1 96-well plate,
CRISPR: Phosphorylate and anneal oligos, ligate

Wed. ES Cells: Passage R1 ES cells, set up P100 @ 3000, set up FBS test
CRISPR: transform bacteria

Thurs. ES Cells: Prepare feeder layers, ES cell instructional video
CRISPR: change electroporated cell medium

Fri. ES Cells: Split/freeze R1 96-well plates,
CRISPR: clone pick bacteria colonies for PCR, set up broths for minipreps

Sat. ES Cells & CRISPR: Renew ES cultures with fresh media

Sun. ES Cells: Passage ES cells for chromosome spread preparation
CRISPR: change electroporated cell medium

Week Two

Mon. ES Cells: Chromosome preparation, thaw cryopreserved cells from 96-wells
CRISPR: harvest ES cells from CRISPR electroporation, prepare genomic DNA, set up indel test PCR

Tues. ES Cells: Photograph and count chromosomes
CRISPR: plasmid minipreps, purify PCR products, Qubit DNA quantitation, submit for sequencing (RUSH)

Wed. ES Cells: Pick ES cell colonies
CRISPR: Perform T7 endonuclease test on CRISPR PCR products

Thurs. ES Cells: Fix and stain FBS test, start 96-well DNA prep
CRISPR: Evaluate sequence data from miniprep plasmids and indel test PCR products

Fri. ES Cells: Evaluate FBS test, finish 96-well DNA prep
CRISPR: crRNA + trRNA directed Cas9 digestion of a purified PCR product

E. Training Follow-up: This consists of the ability of the trainee to produce genome edited and gene-targeted germline competent embryonic stem cells. This is beyond the scope of a two-week training session. Please let us know your results as you proceed in your project.

Sample Bibliography: Papers Discussed Subject to Revision
Special Topic Suggestions Welcomed

Class Reading List

As part of the ES class, you will receive a course packet when you arrive for class containing references and protocols. We will discuss a number of relevant papers, please read them in advance. You will be assigned to present the papers for one of the days below. If you have interest in a special topic, please let us know. If you have any questions, comments or concerns please feel free to contact us. We will have some books available for you on the shelf during the class. Please sign them out if you remove them from the lab. We look forward to seeing you Monday morning at 8:30am, in the Stem Cell Lab (Room 2578, MSRB II).

Discussion Papers:

Day 1: Overview of CRISPR/Cas9 and Gene Targeting in ES Cells

Capecchi MR. 1994. Targeted gene replacement. Sci Am.270:52-59.

Doudna JA, Charpentier E. 2014. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science. 346:1258096.

Day 2: ES Cell Biology

Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC.1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A. 1993 Sep 15;90(18):8424-8.

Fedorov LM, Haegel-Kronenberger H, Hirchenhain J. A comparison of the germline potential of differently aged ES cell lines and their transfected descendants. Transgenic Res. 1997 May;6(3):223-31.

Longo L, Bygrave A, Grosveld FG, Pandolfi PP. 1997. The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism. Transgenic Research 6:321-328.

Day 3: CRISPR/Cas9 Cloning and Testing

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. 2013. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 8:2281-2308.

Sakurai T, Watanabe S, Kamiyoshi A, Sato M, Shindo T. 2014. A single blastocyst assay optimized for detecting CRISPR/Cas9 system-induced indel mutations in mice. BMC Biotechnol. 2014 Jul 21;14:69.

Day 4: CRISPR/Cas9 Design Workshop

Haeussler M, Schönig K, Eckert H, Eschstruth A, Mianné J, Renaud JB, Schneider-Maunoury S, Shkumatava A, Teboul L, Kent J, Joly JS, Concordet JP. 2016. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol. 17:148.

University of Michigan Post-Docs: download and install Lasergene software on your laptop from this URL:

https://wiki.umms.med.umich.edu/display/UMHSHELPDESK/Lasergene

If needed, contact H.I.T.S. (734-936-8000) and ask for help from Eric Weimer.

A cloud based alternative to Lasergene software is the package provided by Benchling.com.

Workshop objectives: download genomic sequence for gene of interest; identify critical exon, submit DNA sequence to sgRNA selection algorithm; design oligonucleotides for cloning; design PCR screen for targeted gene; design knockin donors for point mutations, knockins, and conditional alleles.

Day 5. Gene Targeting Pitfalls in Mouse Models

Koscielny G, Yaikhom G, Iyer V, Meehan TF, Morgan H, Atienza-Herrero J, Blake A, Chen CK, Easty R, Di Fenza A, Fiegel T, Grifiths M, Horne A, Karp NA, Kurbatova N, Mason JC, Matthews P, Oakley DJ, Qazi A, Regnart J, Retha A, Santos LA, Sneddon DJ, Warren J, Westerberg H, Wilson RJ, Melvin DG, Smedley D, Brown SD, Flicek P, Skarnes WC, Mallon AM, Parkinson H. 2014. The International Mouse Phenotyping Consortium Web Portal, a unified point of access for knockout mice and related phenotyping data. Nucleic Acids Res. 42(Database issue):D802-9.

Pan Y, Zhang L, Liu Q, Li Y, Guo H, Peng Y, Peng H, Tang B, Hu Z, Zhao J, Xia K, Li JD. 2016. Insertion of a knockout-first cassette in Ampd1 gene leads to neonatal death by disruption of neighboring genes expression. Sci Rep.;6:35970.

Goodwin LO, Splinter E, Davis TL, Urban R, He H, Braun RE, Chesler EJ, Kumar V, van Min M, Ndukum J, Philip VM, Reinholdt LG, Svenson K, White JK, Sasner M, Lutz C, Murray SA. 2019. Large-scale discovery of mouse transgenic integration sites reveals frequent structural variation and insertional mutagenesis. Genome Res. 29:494-505.

Vanden Berghe T, Hulpiau P, Martens L, Vandenbroucke RE, Van Wonterghem E, Perry SW, Bruggeman I, Divert T, Choi SM, Vuylsteke M, Shestopalov VI, Libert C, Vandenabeele P. 2015. Passenger Mutations Confound Interpretation of All Genetically Modified Congenic Mice. Immunity. 43:200-209.

Day 8: CRISPR/Cas9-mediated Genome Editing Part I: Cultured Cells

Canver MC, Bauer DE, Dass A, Yien YY, Chung J, Masuda T, Maeda T, Paw BH, Orkin SH. 2014. Characterization of Genomic Deletion Efficiency Mediated by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 Nuclease System in Mammalian Cells. J. Biol.Chem. 289:21312-21324.

Liang X, Potter J, Kumar S, Ravinder N, Chesnut JD. 2017. Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. J Biotechnol. 241:136-146.

Vakulskas CA, Dever DP, Rettig GR, Turk R, Jacobi AM, Collingwood MA, Bode NM, McNeill MS, Yan S, Camarena J, Lee CM, Park SH, Wiebking V, Bak RO, Gomez-Ospina N, Pavel-Dinu M, Sun W, Bao G, Porteus MH, Behlke MA. 2018. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. Nat Med. 2018 24:1216-1224.

Day 9: CRISPR/Cas9-mediated Genome Editing Part II: ES Cells

Gennequin B, Otte DM, Zimmer A. 2013. CRISPR/Cas-induced double-strand breaks boost the frequency of gene replacements for humanizing the mouse Cnr2 gene. Biochem Biophys Res Commun. 441:815-819.

Schick JA, Seisenberger C, Beig J, Bürger A, Iyer V, Maier V, Perera S, Rosen B, Skarnes WC, Wurst W. 2016. CRISPR-Cas9 enables conditional mutagenesis of challenging loci. Sci Rep. 6:32326.

Rezza A, Jacquet C, Le Pillouer A, Lafarguette F, Ruptier C, Billandon M, Isnard Petit P, Trouttet S, Thiam K, Fraichard A, Chérifi Y. 2019. Unexpected genomic rearrangements at targeted loci associated with CRISPR/Cas9-mediated knock-in. Sci Rep. 9:3486.

Day 10: CRISPR/Cas9-mediated Genome Editing Part III: Mouse Zygotes

Boroviak K, Doe B, Banerjee R, Yang F, Bradley A. 2016. Chromosome engineering in zygotes with CRISPR/Cas9. Genesis. 2016 Feb;54(2):78-85.

Quadros RM, Miura H, Harms DW, Akatsuka H, Sato T, Aida T, Redder R, Richardson GP, Inagaki Y, Sakai D, Buckley SM, Seshacharyulu P, Batra SK, Behlke MA, Zeiner SA, Jacobi AM, Izu Y, Thoreson WB, Urness LD, Mansour SL, Ohtsuka M, Gurumurthy CB. 2017. Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. Genome Biol. 18:92.

Chu VT, Weber T, Graf R, Sommermann T, Petsch K, Sack U, Volchkov P, Rajewsky K, Kühn R. 2016. Efficient generation of Rosa26 knock-in mice using CRISPR/Cas9 in C57BL/6 zygotes. BMC Biotechnol. 16:4.

Day 11. CRISPR/Cas9 Gene Editing Pitfalls

Boroviak K, Fu B, Yang F, Doe B, Bradley A. 2017. Revealing hidden complexities of genomic rearrangements generated with Cas9.Sci Rep. 7:12867.

Kosicki M, Tomberg K, Bradley A. 2018. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. Nat Biotechnol. 36:765-771.

Simeonov DR, Brandt AJ, Chan AY, Cortez JT, Li Z, Woo JM, Lee Y, Carvalho CMB, Indart AC, Roth TL, Zou J, May AP, Lupski JR, Anderson MS, Buaas FW, Rokhsar DS, Marson A. 2019. A large CRISPR-induced bystander mutation causes immune dysregulation. Commun Biol. 2:70.

Day 12. CRISPR/Cas9 Off Target Hits

Anderson KR, Haeussler M, Watanabe C, Janakiraman V, Lund J, Modrusan Z, Stinson J, Bei Q, Buechler A, Yu C, Thamminana SR, Tam L, Sowick MA, Alcantar T, O'Neil N, Li J, Ta L, Lima L, Roose-Girma M, Rairdan X, Durinck S, Warming S. 2018. CRISPR off-target analysis in genetically engineered rats and mice. Nat Methods. 15:512-514

Iyer V, Boroviak K, Thomas M, Doe B, Riva L, Ryder E, Adams DJ. 2018. No unexpected CRISPR-Cas9 off-target activity revealed by trio sequencing of gene-edited mice. PLoS Genet. 2018 Jul 9;14(7):e1007503.

Thomas M, Burgio G, Adams DJ, Iyer V. 2019. Collateral damage and CRISPR genome editing. PLoS Genet. 15:e1007994.

Montoliu L, Whitelaw CBA. 2018. Unexpected mutations were expected and unrelated to CRISPR-Cas9 activity. Transgenic Res. 27:315-319.

Supplemental Reading:

Adams DJ, van der Weyden L. 2008. Contemporary approaches for modifying the mouse genome. Physiol Genomics. 34:225-238.

Branda CS, Dymecki SM. 2004. Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Dev Cell. 6:7-28.

Camper SA, Saunders TL, Kendall SK, Keri RA, Seasholtz AF, Gordon DF, Birkmeier TS, Keegan CE, Karolyi IJ, Roller ML. 1995. Implementing transgenic and embryonic stem cell technology to study gene expression, cell-cell interactions and gene function. Biol Reprod 52:246-57.

Carlson CM, Largaespada DA. 2005. Insertional mutagenesis in mice: new perspectives and tools. Nat Rev Genet. 6:568-80.

Copeland NG, Jenkins NA, Court DL. 2001. Recombineering: a powerful new tool for mouse functional genomics. Nat Rev Genet. 2:769-79.

Culture of Animal Cells: A Manual of Basic Technique. Freshney, RI. 1994. Wiley-Liss. New York.

SCIENCE Book Stacks - 4th floor--Call No: QH 585 .F741 1994

Embryonal Stem Cells: Introducing Planned Changes into the Animal Germline. Hooper, ML. 1992. Harwood Academic Publishers. Philadelphia.

SCIENCE Book Stacks - 4th floor--Call No: QH 442 .H651 1992

Farley FW, Soriano P, Steffen LS, Dymecki SM. 2000. Widespread recombinase expression using FLPeR (Flipper) mice. Genesis 28:106-110.

Fiering S, Epner E, Robinson K, Zhuang Y, Telling A, Hu M, Martin DIK, Enver T, Ley TJ, Groudine M. 1995. Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper

regulation of the beta-globin locus. Genes and Development 9:2203-2213.

Guide to Techniques in Mouse Development. 2010. Wassarman, PM, Soriano P, eds. Methods in Enzymology, vol. 476. Academic Press, New York.

Kranz A, Fu J, Duerschke K, Weidlich S, Naumann R, Stewart AF, Anastassiadis K. 2010. An improved Flp deleter mouse in C57Bl/6 based on Flpo recombinase. Genesis. 48:512-520.

Manipulating the Mouse Embryo: A Laboratory Manual, Fourth Edition. Behringer, R. Gertsenstein, M, Vintersten, K, Nagy, A. 2014. Cold Spring Harbor Press. New York.

Mouse Genetics: Concepts and Applications. Silver, LM. 1995. Oxford University Press. New York. SCIENCE Book Stacks - QH 432 .S561 1995

Nagy A. Cre recombinase: the universal reagent for genome tailoring. Genesis. 2000 Feb;26(2):99-109.

Online at http://www.informatics.jax.org/mgihome/resources/online_books.shtml

Soriano P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 21:70-1.

Teratocarcinomas and Embryonic Stem Cells; A Practical Approach. Robertson EJ, ed., IRL Press at Oxford University Press, 1987.

Lobe CG, Koop KE, Kreppner W, Lomeli H, Gertsenstein M, Nagy A. Z/AP, a double reporter for cre-mediated recombination. Dev Biol. 1999 Apr 15;208(2):281-92.

Novak A, Guo C, Yang W, Nagy A, Lobe CG. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis. 2000 Nov-Dec;28(3-4):147-55.

Olson EN, Arnold HH, Rigby PW, Wold BJ. Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. Cell. 1996 Apr 5;85(1):1-4.

O'Shea KS. 2004. Self-renewal vs. differentiation of mouse embryonic stem cells.Biol Reprod.71:1755-65.

Vasquez KM, Marburger K, Intody Z, Wilson JH. Manipulating the mammalian genome by homologous recombination. Proc Natl Acad Sci U S A. 2001 Jul 17;98(15):8403-10.

Wolfer DP, Lipp HP. 2000. Dissecting the behavior of transgenic mice: is it the mutation, the genetic background, or the environment? Exp Physiol. 85:627-34.

Nuclease mediated genome editing:

Aida T, Chiyo K, Usami T, Ishikubo H, Imahashi R, Wada Y, Tanaka KF, Sakuma T, Yamamoto T, Tanaka K. 2015. Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice. Genome Biol. 16:87.

Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug RG 2nd, Tan W, Penheiter SG, Ma AC, Leung AY, Fahrenkrug SC, Carlson DF, Voytas DF, Clark KJ, Essner JJ, Ekker SC. 2012. In vivo genome editing using a high-efficiency TALEN system. Nature. 491:114-118.

Carbery ID, Ji D, Harrington A, Brown V, Weinstein EJ, Liaw L, Cui X. 2010. Targeted genome modification in mice using zinc-finger nucleases. Genetics. 186:451-459.

Chen S, Lee B, Lee AY, Modzelewski AJ, He L. 2016. Highly Efficient Mouse Genome Editing by CRISPR Ribonucleoprotein Electroporation of Zygotes. J Biol Chem. 291:14457-67.

Chen F, Pruett-Miller SM, Huang Y, Gjoka M, Duda K, Taunton J, Collingwood TN, Frodin M, Davis GD. 2011. High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. Nat Methods. 8:753-755.

Cheng AW, Wang H, Yang H, Shi L, Katz Y, Theunissen TW, Rangarajan S, Shivalila CS, Dadon DB, Jaenisch R. 2013. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. Cell Res. 10:1163-1171.

Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S, Kim JS. 2014. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Res. 24:132-141.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. 2013.

Multiplex Genome Engineering Using CRISPR/Cas Systems. Science. 339:819-823.richards

Cui X, Ji D, Fisher DA, Wu Y, Briner DM, Weinstein EJ. 2011. Targeted integration in rat and mouse embryos with zinc-finger nucleases. Nat Biotechnol. 29:64-67.

Frock RL, Hu J, Meyers RM, Ho YJ, Kii E, Alt FW. 2015. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. Nat Biotechnol. 33:179-186.

Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol. 31:822-826.

Fujii W, Kawasaki K, Sugiura K, Naito K. 2013. Efficient generation of large-scale genome-modified mice using gRNA and CAS9 endonuclease. Nucleic Acids Res. 41:e187.

Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Ménoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R. 2009. Knockout rats via embryo microinjection of zinc-finger nucleases. Science. 325:433.

Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS. 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell. 154:442-451.

Hashimoto M, Takemoto T. 2015. Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing. Sci Rep. 5:11315.

Joung JK, Sander JD. 2013. TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol. 14:49-55.

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Li D, Qiu Z, Shao Y, Chen Y, Guan Y, Liu M, Li Y, Gao N, Wang L, Lu X, Zhao Y, Liu M. 2013. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nat Biotechnol. 31:681-683.

Li W, Teng F, Li T, Zhou Q. 2013. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. Nat Biotechnol. 31:684-686.

Ma Y, Zhang X, Shen B, Lu Y, Chen W, Ma J, Bai L, Huang X, Zhang L. 2014. Generating rats with conditional alleles using CRISPR/Cas9. Cell Res. 24:122-125.

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