ESTABLISHING A SYSTEM TO UNCOVER THE ROLE OF THE NEURAL CREST IN THE DOMESTICATION OF THE FOX *VULPES VULPES*

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ABSTRACT

The mechanisms underlying the gain and loss of traits in natural selection remains a central question in biology. A valuable model to examine how selection acts to modify the body plan is domestication. As far back as 1868, Charles Darwin had noted in *The Variation of Plants and Animals under Domestication* that in diverse cases of domestication of mammals, a striking set of similar phenotypes emerge. These changes were not limited to behavior, but also included morphological changes in coat color, teeth size, craniofacial structures, and tail and ear development. In the 1950s, Dmitry K. Belyaev sought to explore domestication dynamics by breeding the silver fox (*Vulpes vulpes*) under stringent selection of a single trait, friendliness. Interestingly, the same domestication phenotypes re-appeared. Tame foxes showed higher instances of loss of pigmentation at the extremities of the body, a reduction in size of the snout and teeth, and a three- to five-fold reduction in adrenal cortisol. While domestication traits may appear unrelated, studies in developmental biology have revealed that many of these morphological features share a common embryonic origin. Much of the skull, the adrenal medulla, head and trunk pigment melanoblasts, and teeth precursors are derived from neural crest cells. The neural crest is an embryonic, multipotent stem cell population that originates from the dorsal section of the neural tube, migrates, and gives rise to dozens of cell-types in vertebrate embryos. While the neural crest has been speculated to be the link between the emergent traits of domestication syndrome, the molecular, cellular, and genomic changes underlying these phenotypes remain unresolved. To tackle this, I utilized cell reprogramming strategies to begin creating an *in vitro* model to molecularly and cellularly explore the role of the neural crest in domestication.

BIOGRAPHICAL SKETCH

Noura Maziak was born in Aleppo, Syria, where she received her primary education until moving to the United States in 2007. From 2014-2018, she attended the University of Miami where she majored in biology and minored in chemistry and art history – ultimately graduating with a Bachelor of Science. During her Bachelor's, she received the President's Scholarship of the University of Miami and participated in the NIH Initiative for Maximizing Student Development (IMSD) Scholarship Program as well as the Leadership Alliance Summer Research-Early identification Program (SR-EIP). As an NIH IMSD Student Researcher, she worked on characterizing the genetic players underlying the physiology of *Drosophila melanogaster* respiratory activation under the supervision of Dr. James Baker. Furthermore, as a SR-EIP Fellow, she worked on regulation of chemotaxis in *Dictyostelium discoideum*. She began her graduate work on August 2018 at Cornell University where she worked on early neural development, fox domestication, and epithelial to mesenchymal transition in neural crest under the supervision of Dr. Marcos Simoes-Costa.

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INTRODUCTION

While the advent of modern biology is concomitant with the 19th century when Wallace and Darwin proposed their theory of evolution, the central mechanism of inheritance eluded both (2, 16). This would not get clarified until Mendel's work resurfaced at the start of the 20th century when Hugo DeVries, Carl Correns, and Erich von Tschermak independently rediscovered his work (73). Yet, while the mechanism was missing, Charles Darwin still had a comprehension on the dynamics of inheritance beyond his time acquired through his thorough observations abroad and at home. For example, in *On the Origin of Species* he speculates that, "If man goes on selecting, and thus augmenting, any peculiarity, he will almost certainly unconsciously modify other parts of the structure… The result of the various, quite unknown, or dimly seen laws of variation is infinitely complex and diversified." This observation was later expanded upon in his work *The Variation of Animals and Plants Under Domestication* in which he notes that while domesticated animals belonged to separate and distant lineages of species, a very similar set of physical attributes emerged (17, 18). These emergent traits ranged numerous changes in craniofacial structures, the appearance of white molting in animal coats, changes of body size, and the drooping of ears amongst others. This set of characteristics would be termed "domestication syndrome" (17, 74, 75). What adds to the peculiarity of domestication syndrome is that domesticated animals are not only distantly related (e.g. horses, caniforms, and ruminants split some 85 mya) but were also domesticated through differing trajectories and timelines as shown in Figure 1 (38, 52). Recently, the neural crest, a multipotent embryonic cell population, has been implicated

as the underlying driver of domestication syndromes (52, 74, 75). To address this, I have set out to establish a model in which we can explore the neural crest driven mechanisms underlying the morphological and physiological changes in domestication syndrome.

Domestication of the silver fox *Vulpes vulpes*

One of the most remarkable and extensive efforts to understand domestication was that of the taming of the silver fox, *Vulpes vulpes*. The biological riddle underpinning domestication captured the attention of Dmitriy K. Belyaev, a geneticist in the Soviet Union who would defy the rise of Lysenko and his non-Darwinian theories, to explore the same topic that had inspired Darwin a century back. With the aid of Lyudmila Trut, he began the decades long fox-domestication experiment in 1959 (6, 7, 9, 10, 62, 63). Belyaev believed that the unifying factor of all these "remote systematic groups" which varied not only in genera but also across orders was based on the "selection for the domesticated type of behavior at the very beginning of selection." (8) And thus, in the fox experiment, domesticated foxes were derived through selective breeding based on the tameness from a farmed-fox population (29, 36). While initial selection was based on a lack of aggression or fear from caretakers, upon successive generations, selection on tameness began to expand due to the appearance of novel behaviors including the desire to seek caretakers' attention. Alongside the founding of tame foxes, an aggressive population and an F_1 population (an experimental cross between tame and aggressive foxes) were derived (36, 62, 64).

Most peculiar, while the selection was purely based on behavior, the physiological and physical traits associated with domestication were recapitulated in

tame populations. This was a prime example of Darwin's observation. While seemingly augmenting a behavioral trait, in this case tameness, an "unconscious" modification in "other parts of the structure" appeared once more (16). For example, selection for domestication decreases the adrenal stress response. Tame foxes showed a significant drop in blood cortisol and ACTH levels, and interestingly, even upon stress, tame foxes experience a spike similar to the basal levels in unselected unstressed foxes (65, 60). Morphologically, within two decades, it was observed that a frequency of more than 10^{-2} of de novo piebald spotting, termed the *Star* (S) phenotype, was established in silver-black foxes selected for domestic behavior (9). The mutation in *Star* manifests pleiotropically with a visible depigmentation mark (from which "star" is derived) at the forehead and can extend to blue eyes and spotting at the belly, tail, paws and jaw (9, 61, 62). Of interest, it was foxes with the highest degree of tameness undergoing changes such as extra-seasonal sexual activation and conformational changes which gave rise to the majority of *star* fox litters. The parental group also did not display the star phenotype. Additionally, homozygotes, *SS*, deviate sharply from heterozygotes (those which were initially identified). In homozygote foxes, the star spotting extends over the whole face with white markings at the collar, the iridis displays heterochromia at all instances, and deafness is common (9). In development, it was noted that foxes which carry an mutant allele of the *Star* gene, experience a delay in melanocyte migration by an average of two days (61, 62). Melanocytes, a neural crest derivative, give rise to pigmentation explaining certain aspects of the visible phenotypes as well as implicating a change in neural crest behavior. Furthermore, other mutations which drastically alter pigmentation have been characterized to take place at higher instances in tame foxes,

including the Georgian white and platinum foxes (7). Foxes heterozygous for the platinum allele show for example pervasive white spotting alongside a light silver coat, while homozygosity is embryonic lethal. This allele was called the *W* allele and was shown to be distinct from the *S* allele (7, 9).

Recently, *EDNRB*, which has an exonic SNP in some foxes has been characterized as a putative gene underlying the *Star* phenotype (personal communication with Dr. A. Kukekova, UIUC). This is in agreement with the observation of delayed melanocyte migration in development as well as the myriad of traits exhibited by Star foxes which are evident in patients with Waardenburg syndrome, a neural crest pathology caused by mutations in a diverse set of genes including *EDNRB* (75). Furthermore, the *W* locus in platinum foxes was characterized to be a mutation in the *KIT* gene (33). *KIT* codes for a class III tyrosine kinase receptor with a broad biological reach partaking in processes including gametogenesis, hematopoiesis, and melanogenesis (21). The mutation present in platinum foxes is an A substitution of a G at the first nucleotide of intron 17 consequently leading to a loss of exon 17, a highly conserved region that is identical from humans to canids and a component in the kinase activity of KIT (12, 33).

Alongside these genetic explorations, recently the genome of the red fox, *Vulpes vulpes,* was assembled and regions concomitant with selection on tame and aggressive behavior were characterized (37). Genes within these regions were associated with known behavioral disorders such as autism spectrum disorder and bipolar disorder as expected. However, some genes at significant regions were peculiarly located at the border of the Williams-Beuren syndrome deletion occurring in humans, a syndrome

associated with hypersociability and friendliness as well as craniofacial abnormalities. Variants in Williams-Beuren syndrome genes have also been impacted in domestication of dogs and have been linked to hyper-sociability (69, 70). While the syndrome has clear cognitive and behavioral traits, patients also display well-defined neural crest-related craniofacial dysmorphisms. This link has also been made through developmental studies where the Williams-Beuren syndrome transcription factor, WSTF, plays a significant role in neural crest migration in *Xenopus laevis* (5). Interestingly, recent work exploring human "self-domestication" has also implicated WSTF as a master regulator which gave rise to the anatomically modern human face (80). Furthermore, transcriptomic studied of the hypothalamus and brain tissue in domestic foxes have identified differential expression of cell adhesion proteins such as protocadherins and putative WNT regulators DKKL1 (50, 72). While protocadherins have potential migratory implications on the neural crest, possible altered WNT signaling offers another mechanism by which selection on behavior in domestication is affecting a central signaling pathway in the neural crest developmental program.

Mutations in *KIT*, *EDNRB*, genomic changes at Williams-Beuren syndrome deletion, and putative altered regulation of WNT signaling all link to a change in neural crest biology. The neural crest is a principal synapomorphy from which derivatives partake in the founding of vertebrate-specific features (30, 51). Neural crest precursors form in the ectoderm of vertebrate embryos on the lateral edges of the neural plate during mid-gastrulation. Upon the folding and closure of the neural tube, this multipotent stem cell population undergoes epithelial to mesenchymal transition, migrates extensively, and differentiates to ultimately give rise to a diverse set of

derivatives. These include cells which comprise the adrenal medulla, craniofacial structures, pigmentation cells, and the peripheral nervous system (42, 51, 55). Thus, from the examination of the genetic and genomic changes which appear in domestic foxes and the derivatives which arise from the neural crest, "the neural crest hypothesis" – a hypothesis set forth implicating the neural crest as directly giving rise to domestication syndrome – gains traction (52, 74, 75).

Implications on the neural crest in other domesticates

Additionally, support of the neural crest hypothesis continues to emanate from a myriad of other domesticates. Multiple genes relevant in neural crest development and some even implicated in fox domestication showcase parallel effects in other species. For example, KIT which has been characterized as the cause of platinum fur phenotype in foxes is also implicated as the driver of piebaldism in mice, dogs, pigs, horses, and even humans (12, 20, 22, 23, 24, 26, 32, 75, 78). In horses, much like foxes, a loss of exon 17 causes a distinct pattern – Sabino spotting (12). In pigs, a duplication of the receptor gene causes a dominant white phenotype. Alternatively, pigs which show spotting also have loss of exon 17 (21). Peculiarly, the loss of exon 17 is caused by the same substitution experienced in foxes (33). Additionally, studies utilizing minks where selection was also based on behavior showed that while tame minks displayed a higher instance of spotting and piebaldism, this change was only slight in nature from non-selected minks. However, minks selected for aggression display a marked decrease (over 20%) in instances of white spotting and piebaldism (59). Molecular-genetic analysis has characterized a large duplication of genomic regions in aggressive minks

which contain genes responsible for neurotensin, a neuropeptide linked to aggression, and the gene for the KIT ligand, once again implicating altered development of melanocytes and the neural crest (21). Interestingly, while domestication syndrome is typically affiliated with mammals, marine domesticates show similar phenotypes to their mammalian counterparts. For example, in the carp *Cyprinus carpio*, another neural crest gene, FGFR1, has been characterized as the key driver of loss of pigment and scale variation in domesticated carp (77, 49). In fact, domestic carp underwent not one but two independent events of loss of function alleles of *Fgfr1a1* (49).

Alongside the genetic characterization of neural crest genes in domesticates, a new onslaught of studies has begun to illuminate many of the processes underlying domestication from an epigenomic and genomic standpoint. These new insights are once more not limited to mammals, but also extend to avian and marine domesticates (1, 13, 71). For example, changes in facial structures such as the jaw, abnormalities in pigmentation, and behavioral shifts have been well documented in domestic fish. When looking at European sea bass (*Dicentrarchus labrax*), the farmed population experiences positive selection for genes coding glutamate receptors similar to other mammalian domesticates, displays reduced aggression than their wild counterpart, and exhibits morphological changes mirroring domestication syndrome (1, 44). Remarkably, due to the novelty of fish farming and domestication, these populations share a low fixation index ($F_{ST} < 0.011$), and thus little genomic variation, yet display clear changes in their epigenetic landscape through DNA methylation. Peculiarly, the top differentially methylated regions include genes such as protocadherins which have also been characterized to be differentially regulated in domestic foxes through

transcriptomic studies of the forebrain and the hypothalamus (44, 50, 72). This line of work also showed that even in fish domestication where epigenetic changes are the core drivers of variation, the neural crest developmental program is majorly implicated (1). The bulk of the epimutations which farmed fish experience are at genes which are embryonically enriched and partake in cell processes such as cell migration and neural crest development, further supporting the putative central role of the neural crest in domestication (1).

Additionally, extensive work sampling worldwide wild jungle fowl species and red jungle fowl subspecies revealed that regardless of episodic gene flow and divergent lineages of jungle fowls, domestic chickens underwent positive selection for genes associated with neural crest developmental pathways (71). These include *MYC-C*, *ERBB4*, *BMPs*, and fittingly *FGFR1*. In parallel, genome sequencing of 14 horses from the Bronze and Iron Ages (over 2000-4000 years ago) showed that while early stages of directed domestication varied from prey-based domestication, horses also experienced positive selection on neural crest genes such as *FGFR1* and others taking part in developmental programs of ear, head, and neuronal derivatives (25, 41, 71). These findings further bolster the neural crest hypothesis.

Establishing a multifaceted exploration of the neural crest hypothesis

Ultimately, while we have a strong foundational understanding of the genetic, genomic, epigenomic, and organismal changes underlying domestication, we still lack a system in which functional characterization of the neural crest hypothesis can be explored. To resolve this, I began to establish an experimental system utilizing the fox

domestication model to explore the neural crest changes manifested in domestication. Due to the neural crest being limited to embryonic development, we have set out to utilize reprogramming strategies from which we can obtain induced neural crest cells from tame and aggressive fox lineages. This will not only give us access to an otherwise embryonic cell type but will also allow us to examine how this progenitor cell population is affected by selection under domestication. The establishment of such a system will pave the way to transcriptomic studies of a previously unattainable cell-type in foxes, migration and differentiation potential studies of neural crest across fox lineages, and genomic and epigenetic analysis of changes that might be altering gene expression and the cis-regulatory landscape. These strategies will aide in further defining the role of neural crest in the emergence of the domestication traits.

MATERIALS AND METHODS

Lentivirus Production and Transduction

Basic growth medium was made with Dulbecco's Modified Eagle Medium (Thermo Fisher; Cat. # 11965084) supplemented with 15% Fetal Bovine Serum (Thermo Fisher; Cat. # 10437028) and 1X antibiotic (Thermo Fisher; catalog # 15240062). Then, 4-5 x 10^6 HEK 293 T cells were seeded in a 10 cm plate with 8 mL of growth media and incubated at 37°C overnight at 5% CO_2. Cells were left to reach 80-90% confluency before transfection. Once desired confluency was reached, in a sterile microfuge tube, 7 μg of lentiviral vector plasmid DNA (Table 2) was diluted with sterile water to reach a final volume of 600 μL. DNA solution was then thoroughly mixed by vortexing. Diluted DNA solution was added to the tube of Lenti-X Packaging Single Shots (Takara Bio; catalog # 631847), cap was tightly closed, and the tube was vortexed at high speed until pellet was fully dissolved (~30-40 seconds). Samples were then incubated for 10 minutes at room temperature to allow nanoparticle complexes to form followed by a quick centrifugation to bring sample to the bottom. The entire 600 μL of nanoparticle complex solution was then added dropwise to the HEK 293 T cells with fresh 8 mL of basic growth medium. Plate was shaken gently up and down and side to side to make sure transfection reagent is well distributed. Media did change color slightly from the nanoparticle complex solution, but this is typical and won't affect viral production. The next day, 6 mL of fresh basic growth medium was added to the HEK 293 T cells and cells were allowed to rest at 37°C, 5% CO_2, for 48 hours. The following day, fresh fox growth media was made with MEM α (Thermo Fisher; catalog #

32571036) supplemented with 15% Fetal Bovine Serum, 1X GlutaMAX (Thermo Fisher; catalog # 35050061) and 1X antibiotic and 8 x 10^5 fox fibroblast cells (Table 1) were plated in a 10 cm dish in 10 mL of fox growth media and left to rest overnight. After overnight incubation, lentiviral supernatant was harvested and filtered through a 0.45-uM filter (Nalgene; catalog #725-2545) to remove cellular debris and replaced with 8 mL of fresh basic growth medium. Fox growth media was aspirated, and lentiviral supernatant was directly used to infect pre-plated fox fibroblasts (1:1 OKSM virus and transactivator virus; Table 2). For the most efficient infection, fresh basic growth media (3 mL) should be added to lentiviral media be to minimize cell death. Double infections with a 24-hour rest period also increased cell death. Thus, it is best to infect cells once with a lentiviral GoStick Value (GV) of ~1500 with 3mL fresh growth medium added. A day after infection, the medium was changed to fresh basic growth media and left to rest overnight before being plated on feeder cells at 40% confluency (trial 1; Figure 2). Alternatively, for the second trial, a day after infection, cells were left to rest once more, and a second infection was carried out overnight. After overnight incubation, fresh basic growth media was once more added, and cells were left to rest for 48 hours at 37^{o}C and 5% CO_2 before they were plated on feeder cell (30% confluency, except aggressive cell line which was plated at 10 % confluency).

Preparation of Feeder Cell Plates

Cell culture plates were first coated with 0.1% gelatin (Sigma; G-1890). When diluting and dissolving gelatin, it is essential to use double distilled sterilized water or

PBS and autoclaved for 30 minutes to avoid risk of contamination. A 2% solution can also be made and store at 4°C from which further dilutions can be made from. For a 24 well-plate, 200-400 μL of 0.1% gelatin solution was added per well and 6 mL were added for 10 cm plates. Plates were then placed at 37°C for at least 15 minutes, gelatin was then aspirated and plates were set to dry for 2 hours at room temperature. When drying it is best to do so in cell culture hood with UV irradiation to avoid contamination since most cell reprogramming occurs in antibiotic free conditions. Once plates were made, γ irradiated feeder cells were plated at 90% confluency using growth media. Feeder cells can last for up to two weeks but should be replaced after.

Cell Reprogramming

Induced pluripotent stem cell (iPSC) media was prepared with Knockout DMEM (Thermo Fisher; catalog # 10829018) supplemented with 20% embryonic stem cell qualified fetal bovine serum (Thermo Fisher; catalog # 10439016), 1X GlutaMAX, 1X non-essential amino acids (Thermo Fisher; catalog # 11140050), 1X sodium pyruvate (Thermo Fisher; catalog # 11360070), 1X 2-Mercatoptoethanol (Thermo Fisher; catalog # 21985023), 10 ng/ml basic fibroblast growth factor (bFGF; Peprotech; catalog # 100-18B), and 12.5 ng/mL human leukemia inhibitory factor when necessary (hLIF was added in the same amount to mTeSR1 plates tested for LIF dependence, EMD Millipore; catalog # LIF1010). Basic growth media of transfected fox fibroblasts on feeder cells was replaced with iPSC media or mTeSR1 (STEMCELL Technologies; catalog # 85850) one day after plating on feeders (Trial 1; Figure 2) in unison with 1

ug/mL doxycycline induction of our tetracycline responsive transactivator and OKSM casettes. For the second trial, basic growth media was replaced three days after induction (Trial 2; Figure 2). Throughout both trials, iPSC and mTeSR1 media and doxycycline were renewed daily. It is important to note that doxycycline has a short half-life (approximately 24 hours) and should not be premixed in media batches but added daily when media is being changed.

Lentivirus Quantification

Protocol was based on Lenti-X GoStix Plus (Takara; catalog # 631280). Briefly, 20 µl of viral supernatant was pipetted on GoStix cassette and allowed to rest for 10 minutes. After ten minutes, bands appeared from the presence of lentiviral p24. Images were taken through a Takara app in smartphone, quantifying the lentivirus titer through comparison of control and supernatant band intensities. A GoSTix Value (GV) was then used to compare virus amounts between preparations.

Live Alkaline Phosphatase (AP) Staining

Live Alkaline Phosphatase (AP) staining was done using manufacturer instructions (Thermo Fisher; catalog # A14353). Briefly, iPSC or mTeSr1 media was removed, and cells were quickly washed with pre-warmed DMEM/F-12 (Thermo Fisher; catalog # 11320033) for 3 minutes. Media was then aspirated and a second wash was conducted. Pre-prepared 1X AP Live Stain diluted in a 500X stock solution of DMEM/F-12 (6 µL of AP Live Stain in 3 mL of DMEM/F-12 for a 10 cm plate) was

then applied to the cell culture dish. Cells were then incubated for 30 minutes and washed again twice with DMEM/F-12 for 5 minutes to remove background signal. Once washes were done, fresh pre-heated DMEM/F-12 was added and cells were imaged with a fluorescent microscope using a standard FITC filter. Imaging should take place within 30-90 minutes of staining. Most robust fluorescent colonies were either marked to allow further expansion or picked.

Induction of neural crest cells from human embryonic stem (ES) cells

First, human embryonic stem cells (Table 1) were thawed 4 days prior to neural crest induction and allowed to expand to 70% confluency on Matrigel coated plates (STEMCELL Technologies; catalog # 07181) in mTeSR1. Then, basal neural crest induction media was prepared with DMEM/F-12 supplemented with 1% B-27 (Thermo Fisher; catalog # 17504001), 0.5% bovine serum albumin (Thermo Fisher; catalog # AM2616), 1X GlutaMAX, and 10 um of ROCK Inhibitor (Millipore Sigma; catalog # SCM075). Once desired confluency was achieved, mTeSR1 media was aspirated and plates were washed with DPBS (Thermo Fisher; catalog # 14190250). Cell were then treated with 0.4 mL of Accumax (Innovative Cell Technologies; catalog # AM-105) per well for a 6-well plate and incubated at 37^{o}C until they began to detach (~5 minutes). Once detached, 1.5 mL of basal neural crest induction media was added to each well, and cells are transferred to a 15 mL falcon tube to be spun down at 200 rcf for 5 minutes at room temperature. Supernatant was then aspirated and 2 mL of fresh NC induction media was added this time containing 3 uM CHIR99021 (Tocris; catalog # 4423).

Embryonic stem cells were then resuspended into single cell suspension and counted to be plated at a concentration of 2 x 10^4 /cm^2. Embryonic stem cell suspension is further diluted in neural crest induction media with CHIR, plated and left to rest for 24 hours. The following day cells were washed and new neural crest media with CHIR but without ROCK inhibitor was added (same media will be used in the following two days). Media was changed daily. At day 4, media is replaced but now without the addition of CHIR and left to rest again for 24 hours. Cells can then be used at the fifth day post induction.

Immunostaining of induced neural crest cells

Growth media was removed from each well and cells were gently washed twice with PBS. PBS volume was decided based on complete covering of the cells, without overfilling the wells (~200 ul/ per well in a 24-well plate; this will be the same volume which will be used for everything downstream). Once washed, 4% PFA was added to each well (same volume as PBS), and cells were incubated at room temperature for 10 minutes without shaking. Once fixation is complete, cells are washed once more with PBS three times with gentle shaking. Cells are then permeabilized with 0.1% NP40 (Thermo Fisher; Cat #) in PBS for 30 minutes. Permeabilization solution is then washed three times with PBS for 5 minutes at room temperature with gentle shaking. Cells were then blocked in 1% BSA solution (made in PBS and lyophilized fraction V BSA) and incubated at 37ºC for 30 minutes. Blocking solution was then removed, and 75 ul of diluted primary antibody in blocking solution was added to each well (1:200 dilution in blocking solution for Abcam brand antibodies, and 1:10 dilution for DSHB brand

antibodies; Table 3). Cells were then left to incubate for 1 hour at 37ºC. Once incubation was completed, cells were washed four times with PBS for 10 minutes at room temperature while gently shaking. Once washes were complete, an appropriate dilution of secondary antibody in blocking solution was added (1:2500), and cells were incubated for 30-45 minutes in the dark and washed three times once more with PBS for 10 minutes at room temperature while shaking. DAPI () was added at a 1:1000 dilution to last PBS, and fresh PBS was added after 10 minutes of DAPI incubation. Cells were then ready to image.

RESULTS

Experimental Rationale

Because of the limitation of obtaining fox neural crest cells in a manner that is fit for continuous experimental availability, I have set out to utilize cell reprogramming and *in vitro* differentiation to ultimately derive induced neural crest cells from fox fibroblasts. The expansion of our cell reprogramming capacity and methods of attaining neural crest cells in a dish have already brought incredible insights. Studies utilizing these methods have shed light not only on neural crest pathologies, but also on the evolutionary changes of neural crest between man and chimp and more recently the biology underlying human "self-domestication" (4, 34, 40, 45, 47, 48, 58, 80). While these novel methodologies are beginning to become more utilized in human systems, they have not yet been implemented in other species beyond chimps to explore broad evolutionary processes.

Here, I began to utilize a similar approach to query how an embryonic cell type – the neural crest – is being differentially altered under the selective pressure of domestication in the fox. To begin this, I attempted to reprogram fibroblast cells from seven different lines of aggressive, tame, and F1 foxes (Table 1) to induced pluripotent stem cells (iPSCs) which will allow for downstream induction into neural crest cells. Since no previous work had been done on the derivation of fox induced pluripotent stem cells, I adopted and implemented methods from the reprogramming of canine induced pluripotent stem cells (3, 35, 39, 43, 54, 68). My method was based on the canid iPSC methods which showed complete and successful reprogramming of adult derived

fibroblasts mirroring our fox fibroblast age and passage (39, 43, 54). Furthermore, lentiviral transduction was used to transcribe pluripotency factors Oct4 , Klf4, c-Myc, and Sox2 (OKMS) under a Tet-On promoter through a transactivator upon induction with doxycycline. I chose a polycistronic OKMS plasmid to minimize the number of differing viruses needed for successful reprograming. In this system, we only need a virus carrying the transactivator and a virus which has all pluripotency factors under the control of a single promoter. My primary goal of first obtaining fox induced pluripotent stem cells rather than a direct induction of fibroblasts to induced neural crest cells was based on the desire to achieve a starting population of pluripotent stem cells from which we could continually derive induced neural crest cells through the method described by (4, 26, 34). This would mean that induced neural crest cells would be made from a single clonal population of iPSCs from each cell line. This grants us a more controlled experimental setup, where variations which would take place in a protocol that normally lasts up to three weeks with a restricted output is instead replaced with a setup where experimental variation is limited to a 5-day long protocol.

Lentivirus production

Two trials were carried out for cell reprogramming into iPSCs. In order to achieve successful reprogramming, a high viral titer is necessary of a virus containing the transactivator and another with the inducible polycistronic 2A spaced Oct4-KLF4-MYC-SOX2 (OKMS) cassette. However, on the first trial of virus production, I attained a low titer for the virus carrying the transactivator (GV value of 436 for transactivator,

GV of 682 for OKMS virus). This seems to have been due mostly to the use of tetracycline-free fetal bovine serum (FBS) (Takara; catalog # 631107) during viral production. HEK 293 T cells cultured in tetracycline-free FBS and transfected to make virus experienced growth arrest throughout viral production (a 72 hour period). Upon the second trial, basic FBS was used for HEK 293 T cells during viral production. Under these conditions, no growth arrest was visible, and viral production was increased considerably to a GV value of 1290 for the transactivator and a GV of 1489 for the OKMS carrying virus. Additionally, a virus with an hUBC-driven GFP cassette was also successfully made (GV = 1964) in order to test infection efficacy in fox fibroblast cells.

Reprogramming experiments

During the first and second trials of reprogramming two medias were tested – mTeSR and iPSC media – as well as the inclusion or exclusion of hLIF (Figure 2). Work in canine cell reprogramming showed that the majority of the methods which achieved successful reprogramming used media supplemented with bFGF in combination with LIF (3, 35, 39, 43, 54). This deviates from human and mouse reprograming where only one is necessary respectively. During the first trial, more stem cell-like colonies appeared in plates with LIF, but no visible difference was seen between mTeSR or iPSC media plates with LIF. During this trial, altered cell morphology and putative colonies which were alkaline phosphatase positive (Figure 3A) also appeared. However, upon expansion, most colonies experienced cell-death and

differentiation, with loss of ES cell-like morphology regardless of continual treatment of doxycycline. Furthermore, the first trial revealed that even with serum deprivation (mTeSR media), fox fibroblasts divided and expanded at an exuberant rate. While they were initially plated at 40% confluency, within less than a week cells reached full confluency. This proved inconvenient due to the necessity of having to split cells during the reprogramming phase.

For the second trial, a third eGFP integrating virus was made to investigate which titration was most robust at infecting fox fibroblast cells (Figure 3). From this I derived that a double infection with a high titer of virus (GV= 1383) had the brightest GFP expression and could help optimize reprogramming. Thus an additional infection was conducted and cells were left to rest for 72 hours before cells were replated on feeders and induced with doxycycline. While added second infection and viral titer was comparable to the eGFP virus, (transactivator virus GV= 1290, OKMS virus GV= 1489), some cell death was visible. This could be caused due to a larger cassette size of the OKSM virus. Upon the second trial, fox fibroblast cells were also plated at a lower confluency on feeder cells (10-30%). Once more, plates not supplemented with LIF showed little to no promising colonies within two weeks. While the seeding confluency was reduced, a similar issue of vast cell division was visible. Yet, the aggressive fox cell-line, which was seeded at 10% confluency, began to show promising colonies, with mound like shapes and clear borders resembling that of mouse embryonic stem cells (Figure 4; Trial 2). Furthermore, these colonies were alkaline phosphatase positive making them promising reprogrammed candidates. However, their expansion proved to

be hard when it came to enzymatic dissociation. This remains an issue to be resolved due to its ability to hinder large-scale culturing. Moreover, these and future putative colonies should be tested for retroviral gene silencing, a marker of fully undifferentiated iPSC colonies (31), the expression of endogenous pluripotency markers TRA-1-60, TRA-1-80, and SSEA-4, as well as differentiation into the three germ layers and the capacity to form teratomas.

Induction of neural crest like cells from human embryonic stem (ES) cells

During cell reprogramming, we also set out to establish a protocol for creating induced neural crest cells from stem cells. We did this by utilizing human ES cells (Table 1) and treating them with neural crest induction media and CHIR for three days following the protocol set out by Gomez *et al.* (26) The attained induced neural crest cells had a distinct morphology from their stem cell derivatives. Furthermore, I carried out immunostaining of these cells which displayed many neural crest factors (Figure 5A). Additionally, work from my lab-mate Debadrita Bhattacharya, showed that these induced neural crest cells are dynamic in their transcription of known modulators of neural crest development (Figure 5B). This provides us with grounds on which we can derive fox induced neural crest cells upon the confirmation of the putative reprogrammed colonies.

Another method for the derivation of induced neural crest cells described by Bajpai *et al*. (4) should also be considered. Briefly, in this protocol, induced neural crest cells are derived from stem cells by stem cell differentiation in suspension to

neuroectodermal spheres which comprise of radial arrangement of neuroepithelial cells termed as “rosettes”. These rosettes have also been shown to give rise to neural crest cells by Lee *et al*. (4) Bajpai *et al.* however, improve on this finding by enriching first for the rosettes which will begin to adhere at about a week post induction. From these adhered rosettes some cells with stellate morphology will begin to migrate outwards from the cluster. These were characterized as induced neural crest cells. What this method allows beyond the derivation of neural crest cells is a convenient *in vitro* model of the early programs which the neural crest must undergo – delamination and migration. First, induced neural crest cells begin to spread outwards in a continuous monolayer from the attached neural sphere recapitulating the sheet-like migration which cranial neural crest undergo when delaminating from the dorsal neural tube (45). Second, the induced NCC at the outermost periphery will begin to disperse from each other, much like the streams of migration following the initial sheet like migration *in vivo*.

DISCUSSION

The processes which underlie domestication syndrome traits are complex and biologically rich, making the attenuation of a system in which cell types such as the neural crest can be explored in various domesticates a worthwhile undertaking. Here, I show promising early results at the possibility of deriving fox induced pluripotent stem cells and the downstream establishment of a method from which we can ultimately derive fox induced neural crest stem cells in vitro. Further analysis of putative reprogrammed colonies will prove essential, as well as our new knowledge on potential pitfalls in reprogramming such as a high division rate and viral titer. The successful derivation of these induced neural crest cells will also expand our opportunities to test a myriad of other systems of domestication as well as general neural crest peculiarities including, for example, dog breeds with hyper-exaggerated craniofacial features. Last, it allows us to probe not only cellular behavior, but also the amalgam of epigenetic and genomic changes modifying neural crest biology under selective pressure on behavior.

While the neural crest developmental program is clearly implicated in the process of domestication, it is vague how or why that is the case. Generally, in vertebrate evolution, the neural crest has a rich history of elaborations where progressive co-option of transcription factors has expanded ectomesenchymal neural crest as seen in cyclostomes to become regionalized with well-defined axial levels exemplified in amniotes (18, 42, 51, 55). This also hints to a dynamic cis-regulatory landscape, where temporal and spatial resolution is continually being altered for neural crest gene regulatory networks. This rich evolutionary history also presents us with a view of the

neural crest as a malleable cell population, that for one reason or another, contains a certain plasticity. This could also imply that the cis-regulatory aspect of neural crest development has the ability to "respond" to environmental changes. This phenomenon has been hypothesized to drive much of the facial alterations experienced by electric fishes where "mosaic evolution" – a pattern wherein traits experience varying rates of change – is visible wherein the lower jaw experiences higher rates of transformation when compared to other cranial structures (19). This ability for the neural crest to thus "respond" to environmental stimuli, or in this case selection, provides us with an interesting possibility that the changes which are needed (e.g. tamability) in domestication might also necessitate changes in neural crest development. This is especially interesting when analyzing areas with a high degree of alteration in domestic animals such as seen in regions and genes underlying Williams-Beuren syndrome, a pathology where behavioral traits are intimately intertwined with neural crest biology (69, 70, 80). This also provides us with multiple avenues of thought on how farmed fish experience epimutations at neural crest genes. One avenue of thought has been that domesticating and generally farming an animal, places the animal in a novel environment. For example, farmed fish must survive at a considerably denser environment that that in the wild. Intriguingly, one way to resolve this new pressure, is by means of dampening the stress response (1, 29, 60). Fittingly, glucocorticoids regulate DNA methylation and could provide a mechanism by which the stress response drives initial epigenetic changes in domesticates. Farmed foxes also must have experienced a similar environmental change from going to fields to then needing to survive in densely packed cages. This could also mean that farmed foxes have already

experienced a first selective sweep which destabilized the population, giving rise to variation in stress responses from which further selection was conducted to achieve tamability. In comparison, a population with little to no variation of stress-response due to intense selection on a fine-tuned flight or fight response, as seen in zebras, could explain the inability to domesticate certain species. Belyaev also noted the possibility of epigenetic changes being drivers of the *Star* phenotype. While EDNRB might be the driver of the emergent traits linked to *Star* foxes, Belyaev noted that *Star* has a rate of heritable change in either direction which is too high for conventional mutation (9, 61). While this remains a hypothesis of interest, how epimutation might become fixed is unclear. Alternatively, gene ontology enrichment analyses have also implicated altered DNA repair response in certain domesticates (12, 71). This could also provide us with a model in which farmed and domesticated animals experience higher rates of mutations, and that different developmental systems might be more forgiving for such mutagenesis.

These observations lead us to two hypotheses. First, that the neural crest is directly being selected for in domestication. Evidence favoring this hypothesis includes that the initial selection of the flight or fight response and the neural crest derivative, the adrenal medulla. Second, that the neural crest is being altered as an indirect cosequence. This could be due to changes in the central nervous system which shares an intimate developmental history with the neural crest, or through broad organismal changes which are altering behavioral and morphological changes in domesticates as a consequence. Yet, due to the complexity which might arise from any of these systems,

I believe that these hypotheses are not strictly mutually exclusive. For example, primary epigenetic responses in early domestication can be broad in nature, yet their role in affecting the neural crest, while not inherently direct, could be necessary to further drive consequent stages of domestication. Thus, a muddled admixture of evolutionary processes is likely.

Neural crest as a central figure in domestication

When hypothesizing that selection on tameness could be a direct selection on the neural crest, there are multiple possibilities. For example, craniofacial changes are especially unique. Studies using grafting experiments have not only identified mechanisms which influence skeletal size and shape across species but have also shed light on the intimate relationship of the neural crest with the central nervous system (6, 14). For example, when grafting duck cranial neural crest cells into a chicken host, regardless of the change in environment and the signaling of the epithelium, the host chicken will develop a duck beak on the side of the graft (77). This has some important implications. At least in avian organisms, these results indicate that to a certain extent, cranial neural crest mesenchyme is autonomous. This could also indicate that domestic animals undergoing diverse facial changes have a neural crest population that is being intrinsically affected rather than extrinsic changes. Second, signals such as FGF8 emitted from cranial neural crest cells have also been implicated in forebrain patterning. When ablated, the forebrain region experiences massive cell death, and adding a bead soaked in FGF8 is sufficient to rescue in part forebrain morphology (14). This is

especially of interest since the forebrain region often experiences changes in domestication (65). These findings pave a way to hypothesize that the neural crest might be altered directly with a possibility of being a driver of behavioral changes, thus holding a central role in selection for tameness.

Another affected derivative which might support a hypothesis where tameness is a product of the neural crest are the changes experienced in the adrenal glands. The adrenal glands of selected foxes are smaller, and their function reduced (65). Tame foxes also experienced three to fivefold reduction in blood-cortisol levels when stressed. These changes are especially interesting when thinking of how primary foxes were selected based on a reduced flight or fight response. As afore mentioned, the inside of the adrenal gland is also formed by the adrenal medulla, which is derived from trunk neural crest. The adrenal medulla takes in neural signals and creates a hormonal signal through the release of epinephrine (67). An affected adrenal gland thus is intimately correlated to the flight of fight response and possibly being altered by selection on the neural crest derived adrenal medulla.

The neural crest as a bystander in domestication

Transcriptomic studies and genomic comparison of the tame foxes compared to farm-bred and aggressive foxes could also support that the neural crest is being affected as a bystander. For example, transcriptomic data of the forebrains of tame and aggressive foxes have shown that some of the mis-regulated genes between these

populations include genes such as DKKL1, a putative WNT inhibitor (37, 50, 72). WNT signaling is essential for proper neural crest induction, and mis-regulation through altered expression of inhibitors in tissue adjacent to neural crest could lead to changed development. Furthermore, broad changes in metabolism endocrine release (1, 41, 71) could be creating a global affect in domesticates rendering the emergence of similar traits, from altered neural crest development, to altered body and brain size. These various possibilities on the underlying mechanisms of attenuating of neural crest-related emergent traits in domestication provide us with ample biological and evolutionary questions to be explored.

Future directions

Thus, for further exploration, establishing an experimental system utilizing the fox domestication project will aid in gaining a mechanistic understanding of neural crest biology which can allow us to functionally explore the epigenetic and genomic changes and their roles in neural crest behavior. Such a system will pave-way to transcriptomic studies where we can ask which molecular players are being altered across cell lines, functional tests on migration and differentiation potential of neural crest across fox lineages, and genomic and epigenomic analysis to probe the cis-regulatory landscape. These strategies will define the role of neural crest in the emergence of the domestication traits.

In order to develop such a system and due to the current inefficacy of lentiviral driven reprogramming the derivation of induced neural rest could and should be

explored through alternative approaches (Figure 6). These include utilizing an episomal vector allowing us to avoid genomic aberrations which can be caused from lentiviral integration (68, 78). This nonintegrating plasmid holds a multitude of optimized reprogramming factors as well as positive and negative selection markers which could make the attenuation of pluripotent stem cells more streamlined and specific. Alternatively, a direct induction of neural crest cells from fox fibroblasts could also be attempted. Sox10 which has been characterized as a central player in neural crest specification, has also been shown to be sufficient, alongside WNT signaling to induce adult somatic cells to reprogram into induced neural crest cells (34). A possible drawback for direct neural crest induction stands in the limitations of this alternate method. The first being the production of a finite and limited amount of induced neural crest cells and the extent of further expansion (34), as well as the timeline which could add experimental noise between replicates. Furthermore, once the putative colonies are expanded and tested for their in vitro differentiation capacity and their ability to form teratomas in immunodeficient mice, two main methodologies could be approached to create induced neural crest cells, including the system which has been established in the lab. Thus the attenuation of pluripotent stem cell lines provides us with a more versatile tool-kit from which we can explore neural crest biology.

Once induced neural crest cells are obtained, it would be of interest to conduct RNAseq to identify which genes might be altered through differential regulation in these fox populations. This will shed light on which genes and pathways are being altered and whether they have broad cellular, neural and neural crest, or neural crest-specific

ontologies. Also, obtaining fox iPSCs opens the possibility to derive not only induced neural crest cells but also early neural progenitor-like cells which could be utilized for further comparative transcriptomic studies (32).

The characterization of such differentially expressed genes in the fox neural crest cells will also allow for further functional probing of the processes underlying domestication. This can be achieved by testing migration and differentiation capacity. These assays can be approached through multiple paths. First, one can conduct xenograft experiments to measure migratory abilities of fox neural crest cells in the avian embryo. Second, to examine differentiation potential, a neural crest single-cell clonal analysis can be conducted. In this experiment, fox neural crest cells would be cultured in sparse conditions and tracked for their potential in forming clonal colonies of differentiated derivatives. Eventually, genomic techniques such as Cleavage Under Targets and Release Using Nuclease (CUT&RUN) and 3C-base methods can be utilized to begin characterizing putative enhancer regions active in derived fox neural crest cells and test their activity across cell lines (28, 56) . Furthermore, it would be interesting to see if any enhancer regions have experienced SNPs between lines which might alter their activity as seen between human and chimp cis-regulatory regions (47). These exploratory studies will shed light on cis-regulatory regions and their role in evolution by means of selection.

Closing remarks

Ultimately, the study of emergent traits in domestication has played an integral role in our understanding of evolutionary processes. However, much work remains to

be done in understanding the biology at the core of the emergence of novel traits. Here, I began to establish a model which will provide a robust and attainable system to approach the cellular mechanisms underlying the advent of domestication syndrome traits. Furthermore, by understanding the mechanisms underlying these traits, we can tackle the question of how domestication phenotypes emerge over a few generations. These assays will clarify which mechanisms are under selection in neural crest development, helping us further understand how multiple traits simultaneously emerge in domestication.

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Table 1. Cell lines used

Fox cell line ID	Gender	Animal name and fur coloration	Population	LCA animal number	Passage
VVU-83	Female	Dunyasha, silver-black	F1	2007-0009	5
VVU-84	Female	Asyiya, platinum	Tame	2007-0010	5
VVU-85	Male	Arni, silver-black	Tame	2007-0011	5
VVU-86	Male	Zhum, georgian white	F1	2007-0012	5
VVU-89	Male	6817, silver-black	Aggressive	2007-0015	6
-	Male	Star	Tame	2019-0250	3
-	Female	Star	Tame	2019-0251	3
Human Cell line	**Gender**	**Cell type**	**NIH approval**		
WA01	Male	Human Embryonic Stem cells	NIHhESC-10-0043		

Table 2. Plasmids used for lentiviral production

Plasmid	Addgene #	Description
pLVX-TetOne-Puro Vector	Takara Bio; catalog # 631849	Vector containing transactivator with puromycin resistance cassette
FUGW	14883	3rd generation lentiviral plasmid with hUbC-driven EGFP
FUW-tetO-hOKMS	51543	Inducible expression of polycistronic 2A spaced Oct4-KLF4-MYC-SOX2

Table 3. Antibodies used for cell immunostaining

Antibody	Animal	Company
SOX5	Rabbit	Abcam
SLUG	Rabbit	Abcam
TFAP2A	Mouse	DSHB
FOXD3	Rabbit	Abcam
ETS1	Rabbit	Abcam

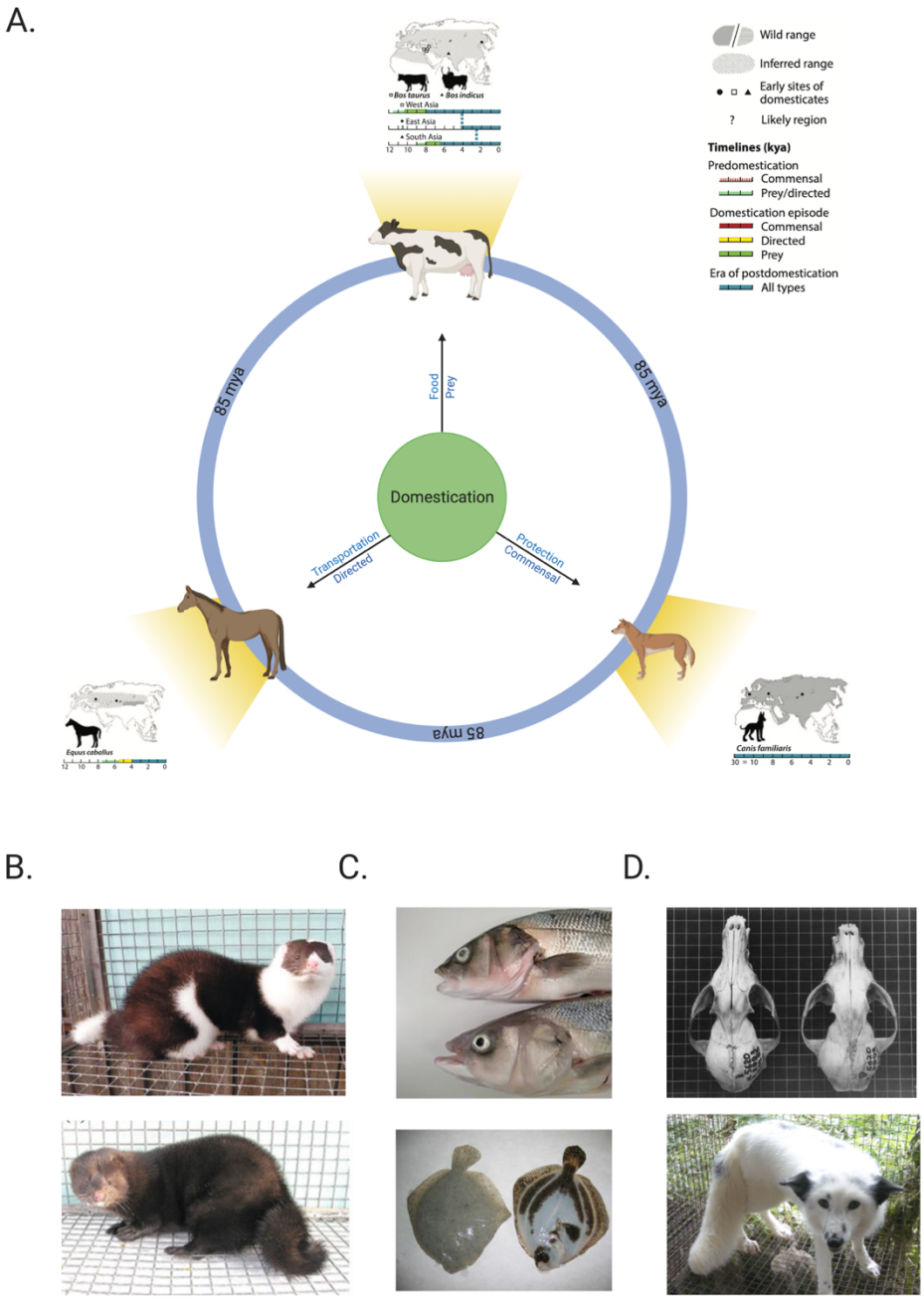

Figure 1. Domestication across taxa. A. Domesticates come from different regions, were domesticated through differing manners (commensal, prey, directed) and for differing motives (protection, food, transportation). Moreover, the timelines at which domestication was carried through vary vastly across species – from ~7 thousand years ago in horses to ~30 thousand years ago in dogs. Data adapted from Larson *et al.* 2014. B. While species vary, a similar set of traits appear. In minks, increased spotting and curling of the tail is found in tame populations. In farmed fish, there is higher instances of an altered lower jaw and de-pigmentation. And tame foxes experience mutation that lead to altered coat colorations and shorter and wider snouts. Images adapted from Anastasiadi *et al*. 2019 and Trut *et al.* 1999.

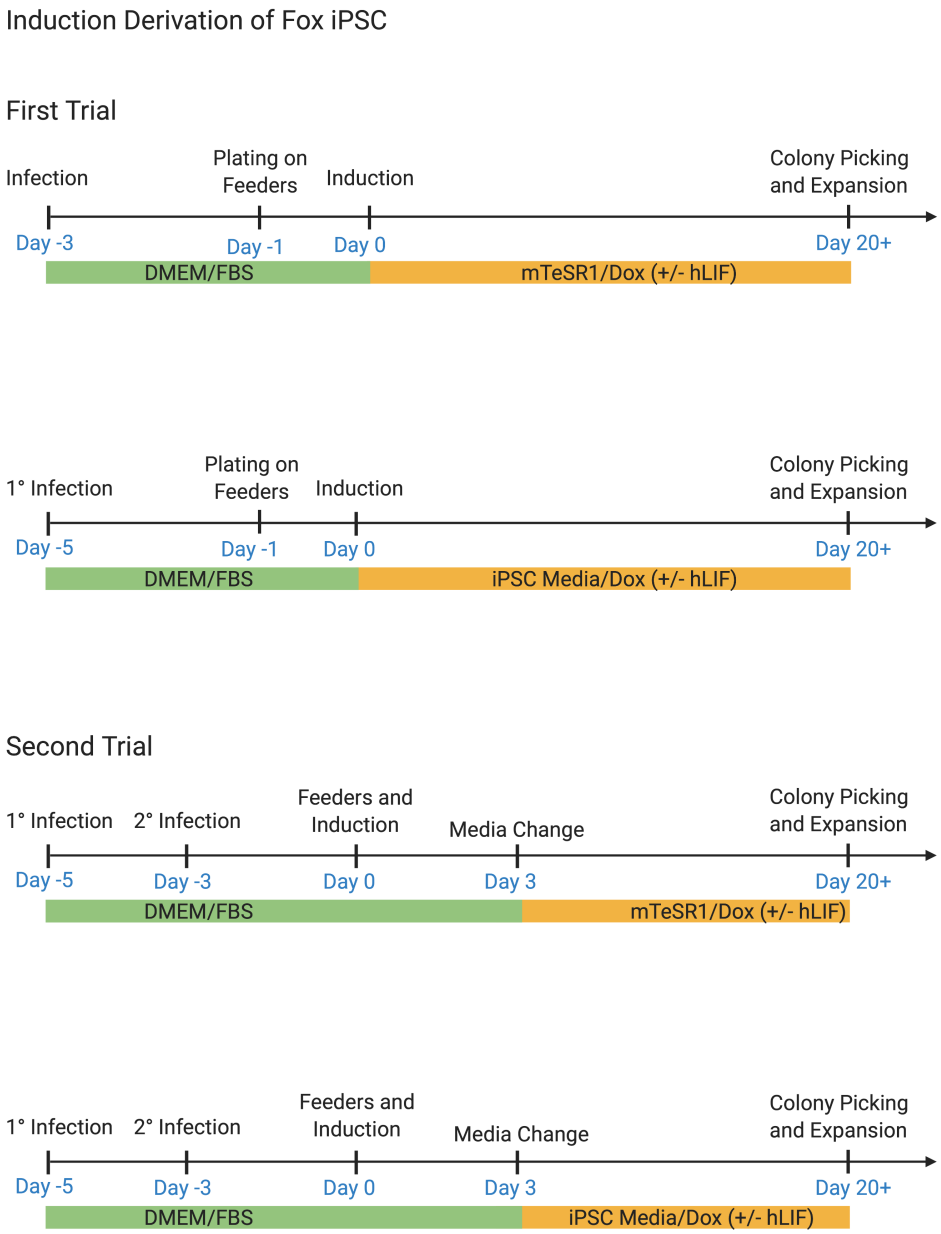

Figure 2. Differing culture conditions in cell reprogramming trials. In the first trial, a single infection was carried out and fox fibroblasts were plated on feeders after 48 hours. Media change and induction took place 24 hours after cells were plated on feeders. In the second trial, two infections were carious out with a 24-hour rest period in between. After 72 hours after the secondary infection, cells were plated on feeders in the morning, and induced with doxycycline at night. Cell continued to be cultured in basic growth media for 72 more hours to allow time for pluripotency markers to get transcribed.

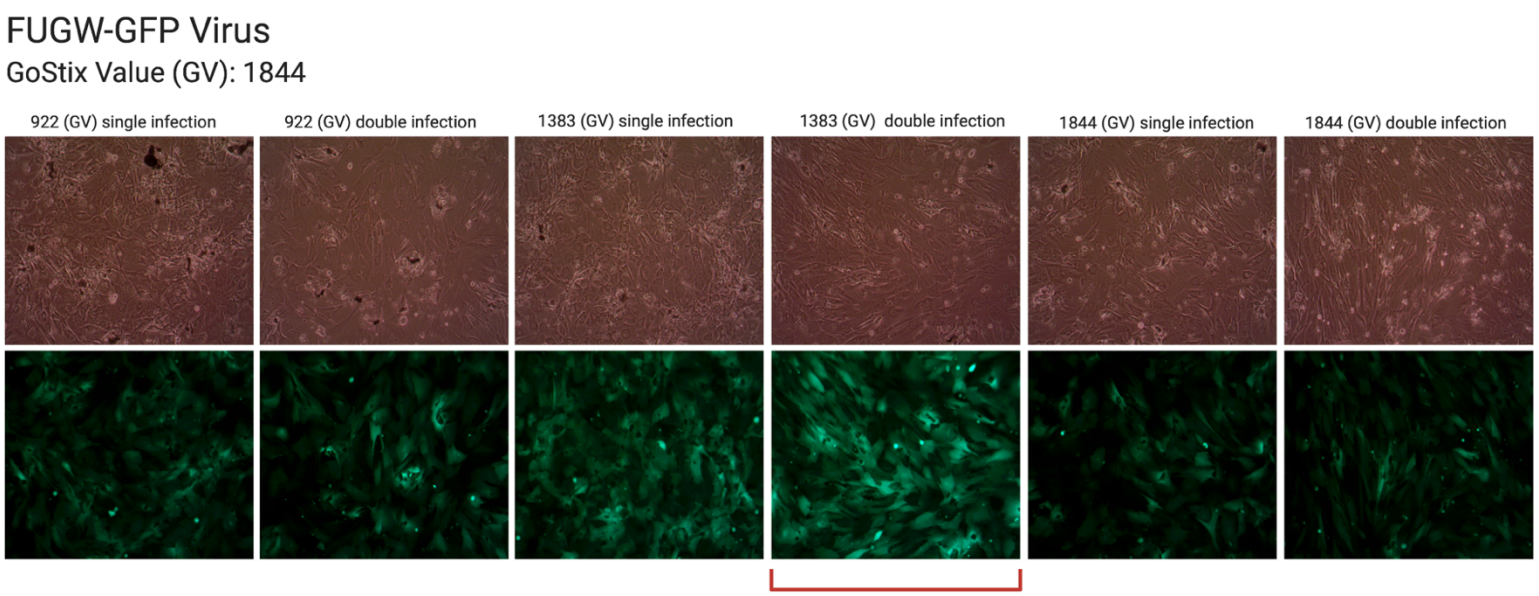

Figure 3. Qualifying viral titer and efficiency of transduction. Various dilutions and infection numbers were tested with known GV values. Cells infected twice with a viral titer quantified at ~1400 GV showed brightest GFP fluorescence.

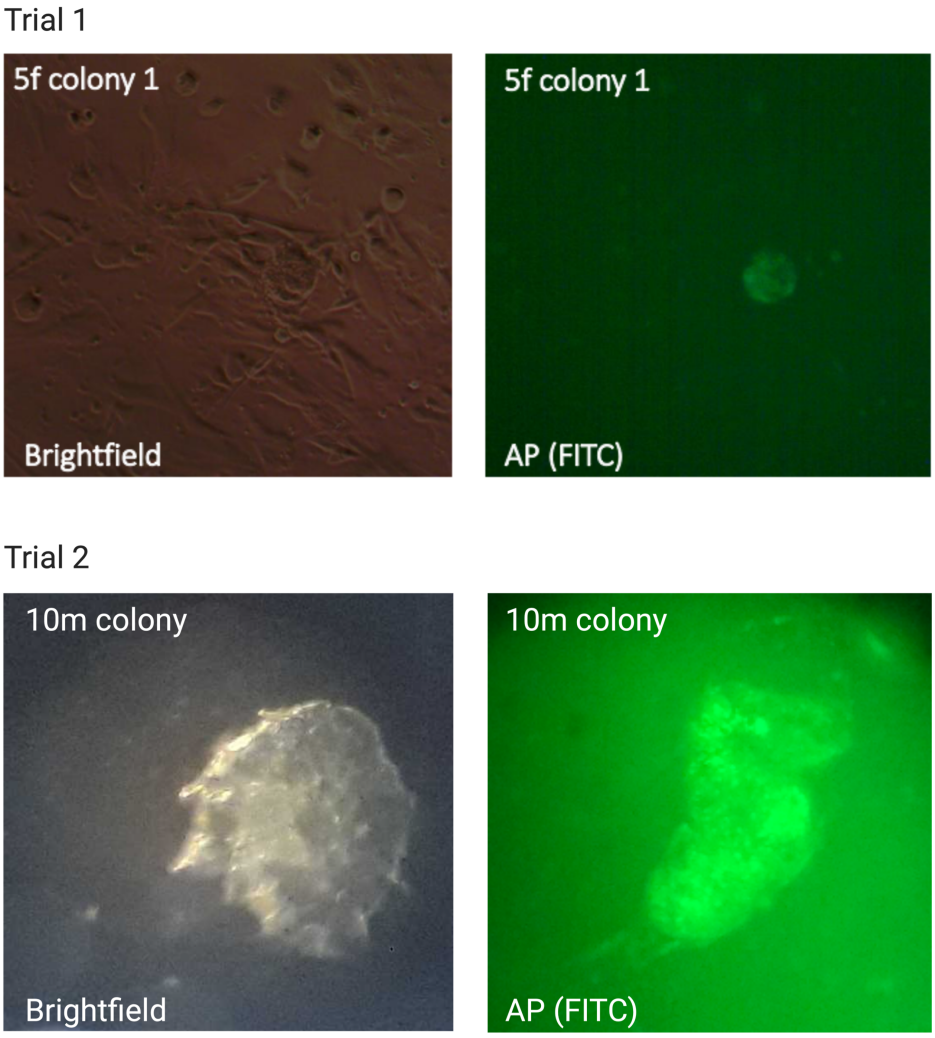

Figure 4. Putative reprogrammed colonies are positive for live alkaline phosphatase staining. Colonies which appeared upon the second trial were generally larger and showcased more promising stem cell morphology.

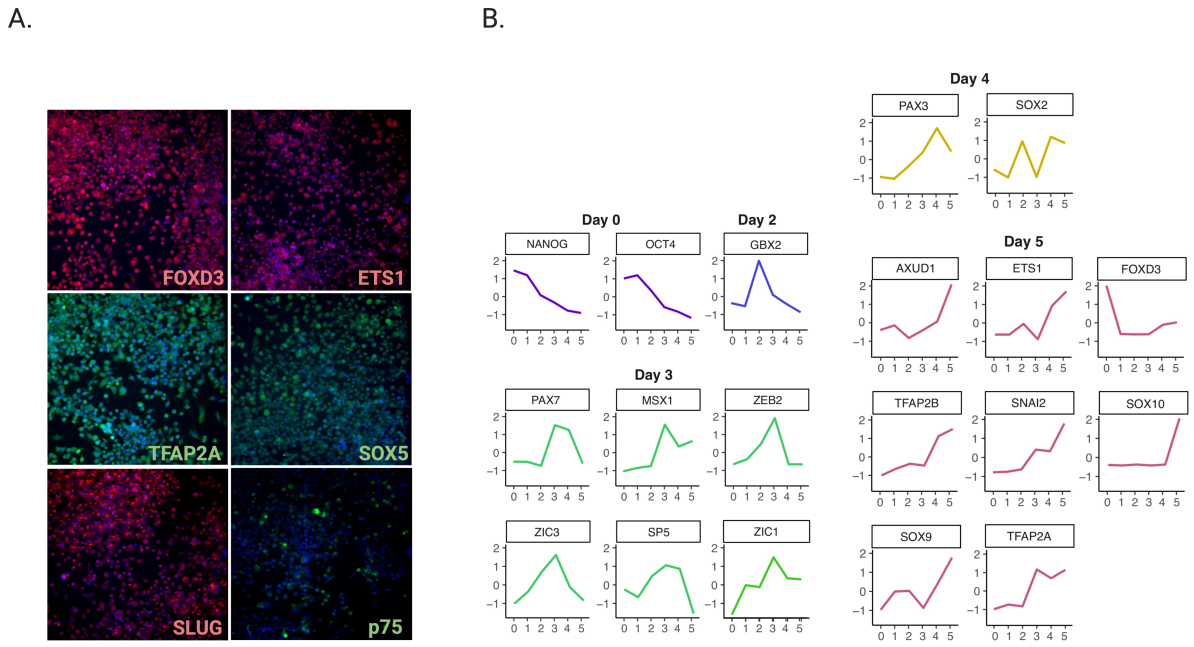

Figure 5. Induced neural crest cells display neural crest markers and have dynamic profiles for neural crest factors throughout induction. A. Induced neural crest cells immunostain for known neural crest transcription factors (Foxd3, Ets1, Tfap2a, Sox5 and Slug) and marker (p75). B. qPCR analysis of neural crest genes validated induction from stem cell identity to a more neural crest identity where pluripotency markers decrease throughout induction (e.g. Nanog and Oct4), and neural crest factor transcription increases (e.g. Tfap2a and Sox9/10).

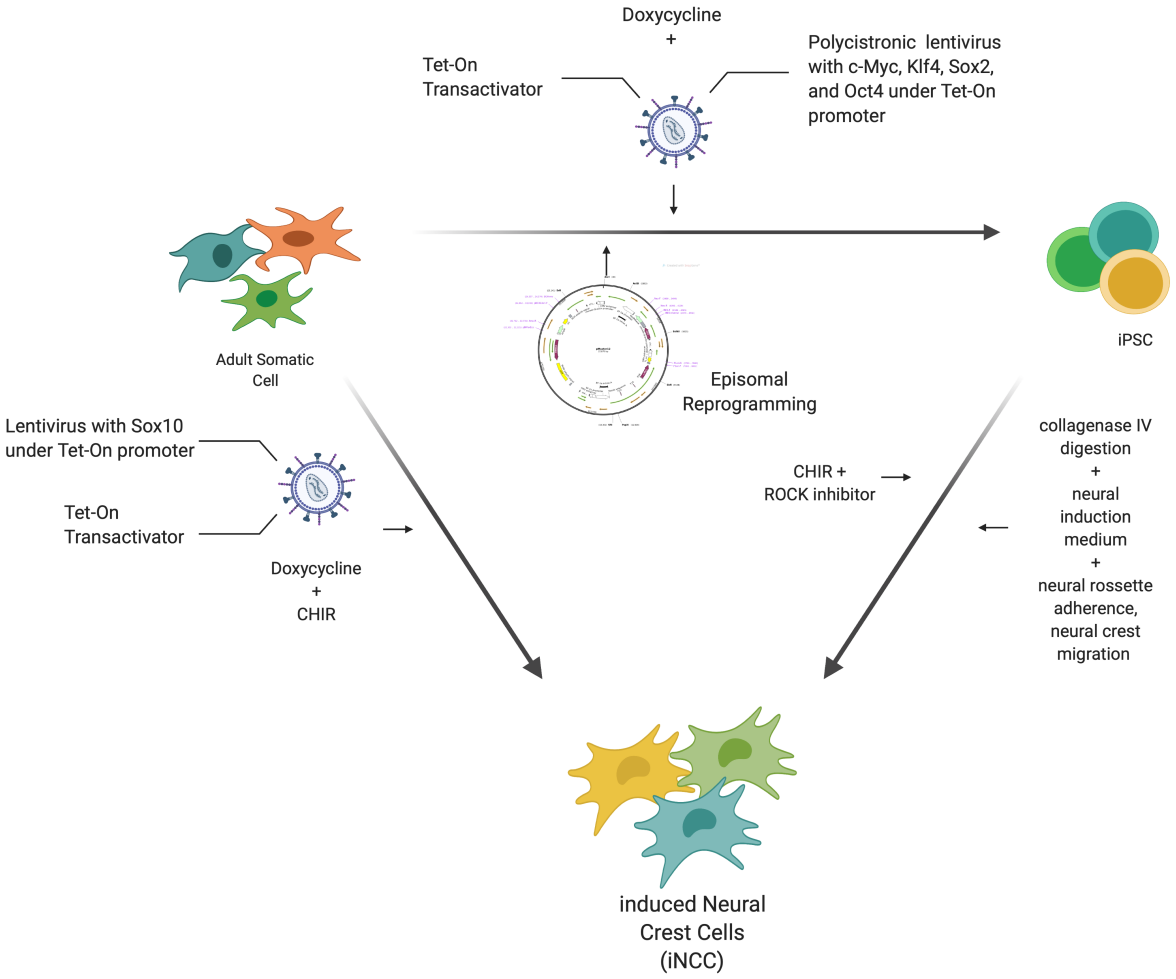

Figure 6. Alternative methods for the derivation of induced neural crest cells (iNCCs). There's a multitude of protocols by which neural crest could be made. Fox fibroblasts could first be reprogrammed to iPSCs and then differentiated into induced neural crest cells, or alternatively, fibroblasts could be directly reprogrammed into induced neural crest cells.