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Investigating the Reproductive Biology of *Monodelphis domestica* to Understand the Evolution of Pregnancy

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One can learn about people in two opposite ways: meet many different people and witness the diversity of thoughts, habits, and ambitions, or develop a committed relationship and learn the depth of human experience. Realizing that the second way fits me better not only with people but also with labs, I continued my work in the Wagner lab that I began in the summer of 2013.

Organisms are contingent products of history, not necessary theorems of physical laws. There is beauty in the lack of inevitability behind organismic forms, and I want to understand how phylogenetic history constrains and makes possible subsequent evolutionary developments. I studied the cis-regulatory evolution of a transcription factor called Forkhead Box O1 (FoxO1). Previous research in the Wagner lab identified FoxO1 as a key player in pregnancy. Successful pregnancy requires decidual stromal fibroblasts, whose important function is to mediate fetal-maternal interaction during gestation. FoxO1 is one of the "identity genes" of decidual stromal fibroblasts; the cells lose their identity, such as the capacity to express characteristic genes, under conditions that deprive them of FoxO1. Evolutionarily, decidual stromal fibroblasts emerged in the most recent common ancestor of placental mammals. They are absent in marsupials and monotremes, who also lack full pregnancy. Hence a key question regarding the evolution of pregnancy is how decidual stromal fibroblasts emerged.

Physiologically, decidual stromal fibroblasts differentiate out of endometrial stromal fibroblasts. While marsupials lack the former, they do have the latter. This suggests that endometrial stromal fibroblasts, being common to all mammals, acquired an ability to differentiate into decidual stromal fibroblasts in the most recent common ancestor of placental mammals. Importantly, the endometrial stromal fibroblasts of marsupials lack FoxO1, which is necessary for them to differentiate into decidual stromal fibroblasts. Therefore, I hypothesized that underlying the emergence of decidual stromal fibroblasts is the cis-regulatory recruitment of FoxO1.

Previously, I have synthesized the 1-kbp promoter regions of *foxO1* genes from human, mouse, manatee, and opossum (our model organism *Monodelphis domestica*), measured their transcriptional outputs using reporter constructs, and found that placental mammalian promoters exhibit significantly higher activities than that of opossum. This finding was consistent with the hypothesis that the *foxO1* locus underwent positive cis-regulatory evolution that led to the emergence of decidual stromal fibroblasts in the most recent common ancestor of placental mammals. Over the summer, I compared the promoter sequences of *foxO1* genes from 18 placental mammals and 2 marsupials. Interestingly, I found 4 stretches of nucleotides, each 10-15 bps long, which were extremely well conserved in all 20 mammalian species. Immediately flanking these ultra-conserved regions were eutherian-specific regions that were conserved among placental

mammals but divergent in marsupials. This pattern hinted at an interesting evolutionary narrative: new transcription factor binding-sites evolved in the vicinity of preexisting sites to increase the transcriptional output of the *foxO1* promoter. If this were the case, it would indicate a peculiar feature of promoter architecture, in which several ultra-conserved sites enable the emergence of novel transcription factor binding-sites in the vicinity.

I mutated these regions and found that while ultra-conserved regions are functional both in the manatee and the opossum promoters, eutherian-specific regions are functional only in the manatee promoter. This was consistent with the idea that eutherian-specific regions newly gained functional significance in the most recent common ancestor of placental mammals. Further, to see whether the putative transcription factor binding to the ultra-conserved region is actually interacting with that binding to the adjacent eutherian-specific region, I inserted 1-, 2-, 3-, 4-, 5-, and 10-bp fragments between the two sites, expecting that while the first 5 insertions will sequentially disrupt the hypothetical protein-protein interaction, thereby decreasing the transcriptional output of the mutated promoter, the 10-bp fragment will restore it. This indeed was the case. I am currently planning to insert fragments with different base compositions to make sure that the restoration of the transcriptional output upon 10-bp-insertion is not due to the specific sequence of the fragment I had used. Also, I hope to be able to identify the two hypothetical transcription factors binding to these conserved regions, though that has proved to be rather difficult.

I originally planned to carry out immunohistochemistry on *M. domestica* tissues, looking for the absence of HIF-1α, OSR2, and JAZF1 proteins, because like FoxO1 these transcription factors were predicted to be important for establishing decidual stromal fibroblast identity. However, they turned out to be rather functionally dispensable, so I did not proceed with the plan. I am currently investigating the effect of canonical WNT-signaling pathway on the expression of FoxO1, focusing on their evolutionary relationship. So in the near future I expect to perform immunohistochemistry on *M. domestica* tissues looking for component proteins of canonical WNT-signaling pathway, such as ß-catenin. I will submit the protocol as soon as I carry it out.

This summer has been an invaluable experience. I learned that anything that *could* go wrong in experiments *would* go wrong eventually. More things did not work than things that worked, and even trivial things suddenly stopped working with no clue as to why. I realized that laboratory life is an indefinite extension of problem solving, and much pain and pleasure lies in it. However, I believe it is precisely the failures and trials-and-errors, rather than blind successes, which make me grow. I am grateful to EDEN Undergraduate Fellowship for making my fertile summer possible.