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Fibroblast Growth Factor Diversity in the Developing Bat Limb

by

Katrina S. Hofstetter

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The members of the committee appointed to examine the thesis of Katrina S. Hofstetter find it satisfactory and recommend that it be accepted.

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Abstract

There are many theories pertaining to the evolution of mammalian flight. The most accepted is that mammals first glided before powered flight evolved. Since there is a limited fossil record for mammalian flight, a comparative genetic approach must be used to understand the evolution of powered flight. Using a bat model to study the evolution of flight gives a unique perspective because of the bat's unusual limb morphology. The bat model we are using, *Carollia perspicillata*, provides an extreme in limb development created by nature. We compare this limb to those of the common lab mouse. The mouse is the standard model for functional limb experiments in mammals, as it has particularly been well characterized limb development for a mammal. We are interested in the genetic differences between mice and bat limbs, and whether these genetic differences contribute to the unique limb morphology of the bat.

Chapter 1

Introduction

We are interested in the molecular mechanisms that regulate the process of evolution. This thesis attempts to explain how the *Fibroblast Growth Factors (Fgf)* genes are contributing in bat limb development. More broadly, we are interested in the differential regulation of *Fgf* genes during mammalian limb development. *Fgf*'s play a fundamental role in limb formation, because they are thought to initiate and maintain a positive feedback loop with *Sonic Hedgehog* (*Shh*), which promotes outgrowth of the limb bud (Laufer, et al., 1994; Farin et al., 2013). This thesis describes progress toward three different goals. First, the relative expression of *Fgf4,8,9* and *19* were quantified by qPCR to identify what *Fgf* genes were expressed at the highest levels in bats. Second, RNA *in situ* hybridization was performed to determine the mRNA expression pattern of *Fgf19* and *Fgf4* in both bat and mice limbs. Our last goal was to perform a bioinformatic assay to determine if *Fgf19* was under adaptive evolution.

Fibroblast growth factor importance

Fgf 's are thought to play a fundamental role in limb formation because they initiate and maintain a positive feedback loop with *Sonic Hedgehog* (*Shh*). *Sonic hedgehog* is a gene essential for anterior, posterior pattering of the limb bud (Mass and Fallon, 2005). *Fgfs* function by up-regulating *Shh.* *Shh* in turn up-regulates a gene called G*remlin*, an antagonist of BMP (Supplemental figure 1; Merino et al., 1999). BMP in the limb bud functions to promote differentiation of tissue. An increase in BMP will down-regulate the amount of *Fgf* present (Laufer, et al., 1994; Farin et al., 2013). This feedback loop

promotes outgrowth of the limb bud by increasing *Fgfs, Shh* and *Grelmin.* An increase in *Grelmin* will decrease the amount of BMP, less BMP would increase the amount of *Fgfs* and prolong the differentiation of tissue. A lack of *Fgf* in the limb bud prematurely shuts off the positive feedback loop, resulting in truncated limbs, and a lack of digit formation (Niswander et al., 1992). In the bat, this regulatory loop is re-induced at later stages of limb development in the inter-digit region of the hand plate (Hockman et al., 2008). This re-induction is proposed to be the reason that bat digits are elongated, and why they maintain webbing between the digits (Hockman et al., 2008).

We have obtained preliminary evidence that the *Fibroblast growth factor (Fgf)* gene family has evolved differently in bats relative to other mammals (Cretekos et al., 2007; CJC unpublished data, and Susan Mackem pers. comm.). We speculate that changes in the gene expression of *Fgf* limb developmental control genes could account for the adaptation of limbs into bat wings, and may have, therefore, played an important role in the evolution of powered flight in mammals. Our collaborator, Susan Mackem (NIH/NCI), carried out whole-transcriptome shotgun sequencing (RNAseq) of RNA-derived cDNA purified from developing *Carollia perspeciliata (Carollia)* embryo forelimb tissue. This technique provides detailed information on differential expression of genes at the resolution of the number of transcripts for all of the genes expressed in the tissue sample. We noticed that the expression profiles of the *Fgf* gene family showed some striking differences compared with what has been published for mouse, the standard mammal model for limb development studies. Specifically, *Fgf19* was expressed at relatively high levels in developing *Carollia* forelimbs, but has not been reported to be expressed in developing mouse hand plates (Maruoka et al., 1998; Wright

et al., 2004). In contrast, *Fgf4* plays an important role in mouse limb development, but was not detected by RNAseq of developing *Carollia* forelimbs (Boulet et al. 2004).

Role of *Fgf15/19*

Fgf15 in mouse and human *Fgf19* both descended from a common ancestral *Fgf* making them orthologs of one and another (Ornitz and Iton, 2001; Wright et al., 2004). The formation of *Fgf15* and *Fgf19* was thought to have come about by a double gene duplication event. The first duplication event is believed to have produced the *Fgf3/Fgf4* and the *Fgf19/15* lineages. A second duplication event split the *Fgf3/Fgf4* linage into two separate genes, *Fgf3* and *Fgf4*. Therefore, while *Fgf19* and *Fgf15* are orthologous, theyhave had more time to accumulate changes (Wright et al., 2004). Normally *Fgf's* are 90% identical in their amino acid composition, while *Fgf19* and *15* are on average only 51% identical in amino acid composition (Ornitz and Iton, 2001).

Both *Fgf15* and *Fgf19* have relatively similar functions in mammals. Specifically, looking at the differences between mice and chicks shows that *Fgf15* and *Fgf19* can be optic inducers (Wright et al., 2004). Spatially in both the mouse and the chick they have similar expression patterns. *Fgf19*, however, in the chick is found in the early optic placode directly under the thickened ectoderm, while *Fgf15* in mouse is found near the optic placode, but in the surface ectoderm ventral to the optic placode (Wright et al., 2004).

In the developing chick *Fgf15* is expressed in the neurectoderm along the rostro-caudal axis and is thought to be needed for nervous system development. Both *Fgf's* are expressed in the primitive streak in chicks (Wright et al., 2004).

Most important to our research is the observation that *Fgf15* in mice is not expressed in the Apical Ectodermal Ridge (AER), while *Fgf19* in chicks shows expression in the AER during limb bud stages (Kurose et al., 2004). These differences in the expression of the orhologs could be important in producing the unique limb phenotype of the bat, as well as suggesting that some transcriptional control elements have evolved over time (Wright et al., 2004).

Role of *Fgf4*

Fgf4 was first thought to be involved in limb development when RNA *in situ* experiments indicated that *Fgf4* was present in the developing AER (Niswander et al., 1992). The AER is a layer of ectodermal cells that forms on the outer region of each limb bud. The AER is a major source of signaling molecules needed to ensure proper formation of the limb (Gilbert, 2006). The presence of *Fgf4* during the development of the AER indicates that *Fgf4* is a signaling molecule that may be needed to maintain the AER. This was demonstrated by creating single and double mutate knockouts of *Fgf4* and *Fgf8*. The function of *Fgf8* appears to be maintaining the AER and limb bud outgrowth. Creating a single knockout mutant of *Fgf8* created defects in the forelimb, however, the distal region of the limb still formed. Indicating that another *Fgf* may have a redundant function and be able to somewhat compensate for a lack of *Fgf8* (Boulet et al., 2004).

Double mutants of *Fgf8* and *Fgf4* lacked both forelimbs and hind limbs, further indicating the importance of *Fgf4* in development (Boulet et al., 2004).

quantitative Polymerase Chain Reaction (qPCR)

The relative importance of *Fgfs* in mouse indicated by transgenetics implies that *Fgf8* is the most important *Fgf* during mouse limb bud development (Boulet et al., 2004). *Fgf4* is the *Fgf* of second most importance, since it's the only other gene found that when knocked out in tandem with *Fgf8* produces an even more severe phenotype than *Fgf8* alone. RNAseq data indicates that there is a lack of expression of *Fgf4* during *Carollia* limb development, and that *Fgf19* is expressed at the highest level in later stages of bat limb development (Susan Mackem pers. Comm.).

RNA *in situ*

The expression of *Fgf4* and *Fgf19* genes was characterized with RNA *in situ* hybridization. These patterns of messenger RNA expression will be examined in *Carollia* embryos and well as mice. Characterizing the *Fgf4* pattern of expression may confirm the importance of *Fgf4* in limb development. A lack of *Fgf4* expression indicated by RNAseq data could allude to a change occurring in the in the regulatory region (CJC unpublished; S. Mackem pers. comm.).

Bioinformatic analysis of *Fgf19*

We have identified a likely candidate for adaptive selection in *Fgf19*. We believe that since the *Fgf19* amino acid sequence is not well conserved (figure 1), especially when comparing rodents (*Fgf15*) and other mammals, that *Fgf19* may be under adaptive selection. Examples of genes under adaptive selection or that contain mutations in the regulatory region provide support to two different ideas about the process of evolution. The literature currently suggests that regulatory mutations can cause alterations in developmental control genes. These mutations can lead to changes in the morphology and physiology of mammals (Cretekos et al., 2008, Carroll, 2008). Specifically in bats, it has been shown that the *Prx1* limb enhancer region promotes limb skeletal elongation. Generating transgenic mice that contained the *Prx1* bat enhancer produced mice that had 6% longer forelimbs than their littermates (Cretekos et al., 2008). These experiments support the widely accepted hypothesis that mutations in the regulatory region of genes are important in evolution.

Although adaptive evolution has contributed to the development of animal vision, respiration, digestive metabolism, host defense, and human speech (Jessen et al., 1991; Hughes, 2002; Yokoyama, 2002; Zhang et al., 2002(a); Zhang et al., 2002(b)), whether variation in the proteins of mammals generally contributes to changes in morphology and physiology is controversial. It has been put forth that altering the protein-coding region may change the conformational shape of the protein, altering its ability to bind to its target (Carroll, 2008).

We hypothesize that while regulatory regions are important in the control of timing and expression of proteins, mutations in the protein coding region would likely change the conformational shape of the protein. This new conformational shape could bind different receptors or bind the same receptor in a different way and cause a new signaling cascade. This new signaling cascade could contribute to changes in morphology and physiology.

References

Boulet AM, Moon AM, Arenkiel BR, Capecchi MR. 2004. The roles of Fgf4 and Fgf8 in limb bud initiation and outgrowth. Dev Biol. 273, 361-372.

Carroll SB. 2008. Evo-Devo and an expanding evolutionary synthesis: a genetic theory of morpholgical evolution. Cell. 134, 25-36

Cretekos CJ, Deng JM, Green ED, NISC Comparative Sequencing Program, Rasweiler JJ IV, Behringer RR. 2007. Isolation, genomic structure and developmental expression of Fgf8 in the short-tailed fruit bat, Carollia perspicillata. Int. J. Dev. Biol. 51, 333-338

Cretekos CJ, Wang Y, Green ED, NISC Comparative Sequencing Program, Martin JF, Rasweiler JJ IV, Behringer, R.R. 2008. Regulatory divergence modifies forelimb length in mammals. Genes Dev. 22, 141-151.

Farin HF, Ludtke T H-W, Schmidt MK, Placzko S, Schuster-Gossler K, Petry M, Christoffels VM, Kispert A. 2013. Tbx2 terminates Shh/Fgf signaling in the developing mouse limb bud by direct repression of Gremlin 1. PLoS Genet. 9, 1-13.

Gilbert SF. 2006. *Developmental biology*. 8. ed. Massachusetts: Sinauer. 505-526.

Hockman D, Cretekos CJ, Mason MK, Behringer RR, Jacobs DS, Illing N. 2008. A second wave of Sonic hedgehog expression during the development of the bat limb. PNAS. 44, 16982-16987.

Hughes AL 2002. Natural selection and the diversification of vertebrae immune effectors. Immunol. Rev. 190, 161-168.

Jessen TH, Weber R E, Fermi G, Tame J, Braunitzer G. 1991. Adaptation of bird hemoglobins to high altitudes: demonstration of molecular mechanisms by protein engineering. Pro. Natl. Acad. Sci. 88, 6519-6522.

Kurose H, Bito T, Adachi T, Shimizu M, Noji S, Ohuchi H. 2004. Expression of fibroblast growth factor 19 (Fgf19) during chicken embryogenesis and eye development, compared with Fgf15 expression in the mouse. Gene Expr Patterns. 6, 687-693.

Laufer E, Nelson CE, Johnson RL, Morgan BA, Tabin C. 1994. *Sonic hedgehog* and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and pattering of the developing limb bud. Cell. 79, 993-1003.

Maas SA, and Fallon JF. 2005. Single base pair change in the long-rang Sonic Hedgehog limb-specific enhancer is a genetic basis for preaxial polydactyly. Dev. Dyn. 232, 345-348

Maruoka Y, Ohbayashi N, Hoshikawa M, Itoh N, Hogan BM, Furuta Y. 1998.Comparison of the expression of three highly related genes, *Fgf8, Fgf17,* and *Fgf18,* in the mouse embryo. Mech. Dev. 74, 175-177

Merino R, Rodriguez-Leon J, Macias D, Ganan Y, Economides AN, Hurle JM. 1999. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. Dev. 126, 5512-5522

Niswander L, and Martin GR. 1992. Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. Development. 114, 755-766

Ornitz D M, and Itoh N. 2001. Fibroblast growth factors. Genome Biol. 2, 3005.1-3005.12

Wright TJ, Ladher R, McWhirter J, Murre C, Schoenwolf GC, Mansour SL 2004. Mouse Fgf15 is the ortholog of human and chick Fgf19, but is not uniquely required for optic induction. Dev. Biol. 26, 264-275.

Yokoyama S. 2002. Molecular evolution of color vision in vertebrates. Gene. 300,69-78.

Zhang J, Webb DM, Podlaha O.(a) 2002. Accelerated Protein Evolution and Origins of Human-Specific Features: FOXP2 as an Example. Genetics. 162, 1825-1835

Zhang J, Zhang YP, Rosenberg HF. (b) 2002. Adaptive evolution of duplicated pancreatic ribonuclease gene in a leaf-eating monkey. Nat. Genet. 30, 411-415.

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Chapter 2

Fibroblast Growth Factor Diversity in the Developing Bat limb

Fibroblast Growth Factor Diversity in the Bat Apical Ectodermal Ridge

Hofstetter, K.S., Harnsberger, A., Dean, M.J., .Tang, C. Y., Brown, E.A., Burnham, J.L., Rasweiler, J.J. IV, Behringer, R.R., Cretkeos, C.J.

Keywords

Adaptive Evolution, *Fgf4*, *Fgf* 19.

Abstract:

Morphological diversity of mammals has been hypothesized to be caused mainly by variations occurring within the regulatory regions of genes. Variations in orthologous proteins have been documented, and may contribute to morphological diversity. Variations in protein-coding have been proposed to be unimportant in evolution. Contrary to this proposal, we identified an orthologous protein under adaptive evolution that may contribute to the evolution of powered flight in bats. Characterization of bat *Fgf19* expression demonstrated that *Fgf19* is expressed along the apical ectodermal ridge of the limb bud in early stages of development, and is re-induced at later stages in the inter-digit tissue. RNA *in situ* experiments illustrate that the expression of the ortholog of *Fgf19*, *Fgf15*, was not expressed in the hand plate of mice. This up-regulated expression of *Fgf19* is likely the result of variation in the regulatory region. Analysis of *Fgf19* with Bayesian methods indicated that *Fgf19* is under adaptive evolution. This finding indicates that variation in the protein-coding region and variation in the regulatory region have contributed to the evolution of this gene. We propose that both protein-coding variation

and regulatory variation are important in the evolution of morphological diversity in mammals.

Introduction:

A number of members of the Fibroblast Growth Factor (*Fgf*) gene family are co-expressed in the limb bud during development (Mariani et al., 2008). *Fgf* 8, *Fgf4*, *Fgf* 9, and *Fgf17* are most abundant *Fgf*s expressed in the Apical Ectodermal Ridge (AER; Mariani et al., 2008; Pownall and Isaacs, 2010). We have identified a subset of *Fgf* genes localized to the AER that are differentially expressed between *Carollia perspicillata* (henceforth *Carollia*), the short tailed fruit bat, and *Mus musculus*, the common laboratory mouse. This finding indicates that bats are using a different set of *Fgf* genes for limb development. This outcome also may indicate that diversity in these genes is critical for the evolution of powered flight.

RNAseq performed on cDNA purified from developing forelimb tissue of *Carollia* embryo revealed some striking differences when compared with what has been published for mouse (CJC unpublished data, and S. Mackem pers. comm.). Specifically, *Fgf19* was expressed at a relatively high level in the developing hand plate of *Carollia* CS 16 (Carnegie Stage 16), but has not been reported to be expressed in developing mouse hand plates. *Fgf19* is expressed, however, in the developing wings of domestic chickens (Kurose et al., 2004). In contrast, *Fgf4* plays a crucial role in the development of the mouse limb (Niswander, 1992; Boulet et al. 2004; Mariani, et al., 2008), but was not

detected above background levels by RNAseq in the developing hand plates of *Carollia* CS 16 (CJC unpublished data, and S. Mackem pers. comm.).

Fgf 's play a fundamental role in limb formation, because they are thought to initiate and maintain a positive feedback loop with Sonic Hedgehog (*Shh*). *Shh* expressed in the posterior region of the developing hand plate is needed to maintain expression of *Fgf*s expressed in the AER. *Fgf*s function to increase cell division promoting the outgrowth of the limb bud (Laufer, et al., 1994; Sun et al., 2000; Farin et al., 2013). A lack of *Fgf* in the AER prematurely shuts off the positive feedback loop, resulting in truncated limbs, and a lack of digit formation (Niswander et al., 1992). In the bat, this regulatory loop is re-induced at later stages of limb development in the inter-digit region of the hand plate (Hockman et al., 2008). This re-induction is proposed to be the reason that bat digits are elongated, and why they maintain webbing between the digits (Hockman et al., 2008).

Fgf15 in mice has been identified as the homolog of human *FGF19* (Ornitz and Iton, 2001). Normally, orthologous *Fgf* genes among mammalian species are well conserved in their amino-acid composition (90%), whereas *Fgf19* and *Fgf 15* are not well conserved and on average only 51% identical in amino-acid composition (Figure 1; Ornitz and Iton, 2001). *Fgf15* and *Fgf19* have relatively similar functions in mammals, are spatially similar in their expression patterns, and are located on syntenic regions of their respective chromosomes (Ornitz and Iton, 2001; Kurose et al., 2004; Wright et al., 2004). *Fgf15* in mice is not expressed in the AER during limb development, but *Fgf19* in chicks shows expression in the AER during limb development (Kurose et al., 2004). This difference of expression could be important in producing the unique limb phenotype of

the bat, as well as indicating that some transcriptional control elements have evolved over time. The lack of sequence conservation could indicate that the *Fgf19* coding sequence is under adaptive evolution. If *Fgf19* has undergone adaptive selection, *Fgf19* would be one of only a handful of genes identified to be under adaptive evolution, as well as the first gene identified to be under adaptive evolution contributing to morphological diversity.

RNA *in situ* experiments indicate that *Fgf4*, *8,* and *9* are expressed in the developing AER in mouse (Niswander and Martin, 1992; Colvin et al.,1999). Presence of *Fgf8* has been established as essential for limb-bud development in mice (Lewendoski et al., 2000; Moon and Capecchi, 2000; Sun et al., 2000; Sun et al., 2002; Boulet et al., 2004). The function of multiple *Fgfs* appears to be maintaining the AER and limb-bud outgrowth (Boulet et al., 2004; Mirani et al., 2008). With an *Fgf8* knockout, the distal region of the limb still formed, indicating that additional *Fgfs* may have a redundant functions, and be able to partially compensate for a lack of *Fgf8* (Boulet et al., 2004). Double mutants of *Fgf8* and *Fgf4* lacked both forelimbs and hind limbs; indicating that other *Fgfs* (*Fgf9)* is not able to compensate for a lack of both *Fgf4* and *Fgf8* in mice. (Boulet et al., 2004, Mariani, et al., 2008). In addition, removal of the AER and placement of an *Fgf4* soaked bead either apically or posterior to the developing limb bud decreased the severity of the limb phenotype produced by removal of the AER (Niswander et al., 1993). These experiments further indicated the importance of *Fgf4* in limb-bud development. To find what *Fgfs* are expressed at the highest level in *Carollia* a quantitative Polymerase Chain Reaction (qPCR) experiment was designed. Based on the RNAseq data we predict that *Fgf19* will be expressed at a higher level that *Fgf8* and that

there will be no *Fgf4* expression. Indicating that *Fgf19* is the most essential *Fgf* for bat limb development and suggesting that *Fgf19* has replaced for *Fgf4* in the bat.

The expression of *Fgf4* and *Fgf19* genes will be characterized with RNA *in situ* hybridization. These patterns of messenger RNA expression will be examined in *Carollia* embryos and well as in mouse. Characterizing the *Fgf4* pattern of expression may confirm the importance of *Fgf4* in limb development. On the other hand, a lack of *Fgf4* expression, as determined by RNAseq data, could indicate that a change has occurred in the regulatory region (CJC unpublished; S. Mackem pers. comm.). This variation, if identified, could provide support to the hypothesis that regulatory regions are important for morphological change (King, 1975; Caroll, 2005; Cretekos, 2008) The current literature provides support that variation in regulatory regions of the genome influence limb morphology (Cretekos et al., 2008). There is no current evidence, however, that changes in the protein-coding region will affect limb morphology in bats. There is debate as to whether changes in the protein-coding region of genes can cause morphological and physiology changes in mammals. Although adaptive evolution has contributed to the development of animal vision, respiration, digestive metabolism, host defense, and human speech (Jessen et al., 1991; Hughes, 2002; Yokoyama, 2002; Zhang et al., 2002(a); Zhang et al., 2002(b) whether variation in proteins in mammals generally contribute to changes in morphology and physiology remains controversial.

Regulatory mutations can cause alterations in developmental control genes by affecting the timing and amount of genes present during development. Those mutations can lead to changes in the morphology and physiology of mammals (Cretekos et al., 2008, Carroll, 2008). The *Prx*1 limb enhancer region promotes limb skeletal elongation

in bats. Generating gene-targeted mice that replaced the mouse *Prx*1 limb enhancer with the limb enhancer of the bat produced mice that had 6% longer forelimbs than their wild-type littermates (Cretekos et al., 2008).

We hypothesize that morphological diversity can be caused by variations in both the regulatory and protein coding regions of genes, and seek to identify examples that may be contributing to morphological diversity among mammals. Identifying genes that contain variation in the protein-coding region will provide candidates for bioinformatics analysis to test for genes under adaptive evolution. Identifying such variations will challenge the hypothesis that regulatory region are the most important source of variation for morphological evolution.

Methods

Animals

Bat embryos (*Carollia*) were collected from a wild population on the island of Trinidad, West Indies and staged according to Cretekos et al. (2005). Bat embryos were collected and exported with the permission of the Wildlife Section, Forestry Division of the Ministry of Housing and the Environment, Republic of Trinidad and Tobago. Mouse embryos were generated from timed matings using out bred mice with a mixed background of Swiss, C57Black6, and SJL. All embryo collections and husbandry procedures for mice were approved by the Idaho State University Institutional Animal Care and Use Committee (IACUC).

Cloning *Carollia Fgf19*

The *Myotis Fgf19* sequence obtained from Ensembl (http://www.ensembl.org; accessed June 2012) was used in a MegaBLAST search against the nucleotide collection database to find orthologous mammalian *Fgf19* sequences. Sequences were chosen to represent diverse mammals. *Fgf19* sequences chosen: *Ailuropoda melanoleuca* (panda), *Bos taurus* (cow), *Homo sapiens* (human), *Mus musculus* (house mouse), *Myotis* (little brown bat), *Pongo abelii* (orangutan), *Sus scrofa* (pig), *Rattus norvegicus* (rat), and *Rhesus macaque* (rhesus monkey). Highly conserved regions were identified and used to design primers to amplify *Fgf19* from Stage 13-16 *Carollia* embryo cDNA (Cretekos et al., 2005). A *Carollia Fgf19 in situ* probe was amplified using the primers F2 R2 (supplemental table 1). The sequences 5' and 3' of the *in situ* probe region of *Fgf19* were amplified using the primers F1 R1 and F3 R3 respectively (supplemental table 1). *Carollia* embryo cDNA was generated using BluePrint First Strand cDNA Synthesis Kit (Takara, Ostu Shiga, Japan) according to the manufacturer's instructions. Amplified products were cloned using the pGEM®-T Easy Vector System I kit (Promega, Madison, WI) and sequence verified, using BLAST (NCBI).

qPCR

Two *Carollia* CS 14 limb buds were dissected from the bat embryos and stored in RNAlater (Ambion, Grand Island, NY). Tissue was homogenized in stabilized phenol using a mortar and pestle. RNA was extracted using RNeasy Plus Universal mini kit (Qiagen, Venlo, Limburg). From the RNA, cDNA was made using BluePrint 1st strand cDNA synthesis kit (Takara, Ostu Shiga, Japan). Primers for qPCR were designed using

Primer-Blast (NCBI; supplemental table 2). PCR products were run on a 1% agarose gel, and bands were extracted using a MiniElute Gel Extraction Kit (Qiagen, Venlo, Limburg), sequenced and the resulting sequences subjected to BLAST (NCBI) to the confirm the identity of the gene in question. qPCR was carried out using a a Dyad DNA Engine thermocycler (BioRad) with an EvaGreen kit (MidSci, St. Louis, MO). Primer concentrations were determined by first performing a primer gradient from 150 nmol per reaction to 350 nmol per reaction. Primer concentrations that gave the lowest Cq value were used (supplemental table 2). A 2x dilution curve starting with 80ng/ml of cDNA was used to calculate efficiencies. Data was analyzed using ΔΔCt method (Pfaffal, 2001). Paired t-test were done comparing *Fgf4* to each of the other *Fgf's,* an alpha of >0.01 was adopted for significance. Genes were run in triplicate and the average Cq values for each triplicate set were used to calculate the standard error.

Whole mount in situ hybridization

The *Carollia Fgf19 in situ* probe plasmid was digested with *Eco*R1 and transcribed in vitro using T7 RNA polymerase in the presence of digoxigenin-11-UTP (Roche Diagnostics Corporation, Indianapolis, IN). For bat embryos, whole-mount *in situ* hybridization was performed on *Carollia* embryos between stages CS 14-16 as described (Rasweiler et al., 2009) with the following modification - Proteinase K was used for one-half the recommended time to reduce tissue degradation. The *Fgf19* riboprobe was used at a final concentration of 2 µg/ml in hybridization buffer with a 50 mg/ml concentration of Denhardt's reagent (Fisher Scientific). *Fgf4* riboprobe was used at a final

concentration of 1µg/ml in hybridization buffer. Hybridization and post-hybridization washes were performed at 65°C. For mouse embryos, whole mount *in situ* hybridization was performed between 10.5- 12.5 days postcoitum (dpc) using an *Fgf15* riboprobe (Wright et al., 2004).

Embryos were imaged on a 1% agrose cushion made with 50% glycerol in phosphate buffered saline (PBS), using a Leica MZ6 stereo dissecting microscope and V-Lux 1000 fiber optic light source. Images were taken with a Lecia DMC1200 microscope camera.

Phylogenetic analysis

The cDNA alignment for generating a phylogenetic tree was aligned with Clustal Omega, and converted into Nexus format with the Segret tool (Galaxy, www.pig.egg.isu.edu). The cDNA alignment was analyzed with Model test software (Galaxy, www.pig.egg.isu.edu) (Yang, 1997; Posada, 2008). *Fgf19* Multiple Sequence Alignment best fit with the HKY model of evolution. HKY takes into account different nucleotide substitution, as well as different transversion and transition rates. HKY is unique because it allows for a different rate for pyrimidine to purine and purine to pyrimidine substitutions (Hasegawa et al., 1985). A phylogenic tree was generated with Bayesian probability and BEAST v1.7.5 software. The suggested parameters for a coding-sequence tree was used (Drummond and Rambaut, 2007). The BEAST xml file was generated with BEATui v1.7.5 software (Drummond and Rambaut, 2007). We used the SRD06 model

for partition into codon positions. The SRD06 model allowed us to have a different rate of mutation than positions 1 and 2 for the nucleotide in the third position of the codons (Drummond and Rambaut, 2007).

A lognormal-relaxed clock was used as opposed to a strict clock. A random start tree was selected for the tree model as opposed to a UPGMA tree. The BEAST tree file was generated in Galaxy; 4.1 million trees were generated and Tree Annotator was used to score the best tree (BEAST v1. 7.5.). The phylogenic tree was visualized with FigTree. FigTree also was used to reformat the tree into Newik format. We used TranSeq to generate the amino-acid sequence for *Carollia;* all combinations of the reading frames were generated, and all sequences were compared with the Multiple Sequence Alignment before the most conserved sequence was selected. A Phylip format cDNA alignment was generated with TranAlign.

Branch-model Analysis of the evolution of the phylogenic tree and was revealed with the PAML software available on the Galaxy website (www.pig.egg.isu.edu). A dN to dS ratio (ω) was calculated for each branch with Codonml branch tool from PAML. A Likelihood ratio test was used to test if the branch in the phyolegenic tree leading up to the Chiropteran branch was under adaptive selection. We adopted a alpha of 0.05 for tests of significance. The Branch-model was selected, and all other parameters were set to default, expect the branch model. A 2 or 3 ratio model was used for the alternative hypothesis. This allowed for individual ω parameters to be set for each branch. The Branch model was used for the null hypothesis, this allowed for the branches to have the same ω value. This analysis will reveal if the branch in the phylogeny ancestral to the Chiropteran is undergoing a faster rate of mutation than the other branches. We can

assume that the branch is evolving faster than the rest of the tree with a significant p-value, and that the branch may be under adaptive selection. Because the power of a dN to dS ratio was low, a Likelihood ratio test was needed calculate tests of significance (Xiong, 2006; Bielawski and Yang, 2004).

Site-model analysis of the evolution of each amino acid was conducted to examine if there were any codons under adaptive evolution. An ω value for each site of the *Fgf19* amino-acid sequence was calculated by with Codonml site software from PAML 4.0. The site model used an M2a selection model, and the fixed alpha value was set to zero. Those parameters were selected with the suggestions from PAML manual (Yang, 1997). The alternative branch-site model (model A) and the null-branch site model were tested, leaving all other default parameters the same. A Likelihood ratio test was calculated and compared against a chi-squared able to test for statistical significance. An alpha of ≤ 0.05 adopted (Bielawski and Yang, 2004).

Results:

Fgf19 was confirmed to be poorly conserved amongst different mammalian species. When compared across a diverse group of mammals the amino-acid composition of *Fgf19* showed an approximate 42% conservation. *Carollia Fgf19* was compared to *Ailuropoda melanoleuca* (panda), *Myotis lucifugus* (little brown bat), *Bos taurus* (cow), *Sus scrofa* (pig), *Homo sapiens* (human), *Pong oabelii* (orangutan), *Rhesus macaque* (rhesus monkey), *Rattus norvegicus* (rat), and *Mus musculus* (mouse; Figure 1). Specifically, nucleotide and amino acid alignments between the *Fgf* genes of human,

Carollia, and mouse illustrated how poorly *Fgf19/15* is conserved when compared to other *Fgfs. Fgf8* is 95% conserved between *Carollia* and mouse at the nucleotide level and 100% conserved at the amino acid level. This is in stark contrast to *Fgf19/15,* which is 46% conserved at the nucleotide level and only 32% conserved at the amino acid level (Table 1; Table 2).

RNAseq data indicates that *Carollia Fgf19* also differs in the expression level reported for mouse. To confirm the RNAseq data a quantitative PCR (qPCR) reaction was performed. RNA was extracted from bat forelimb buds collected from Trinidad. This RNA was converted to cDNA and used to analyze the expression level of *Fgf4, 19, 9,* and *8.* From the whole forelimb bud *Fgf19* was expressed at the highest level, 30 fold higher than *Fgf8* (Figure 2). *Fgf9* was expressed at slightly higher levels than *Fgf4*, which was expressed at the lowest level (Figure 2). Genes of interest were normalized to two housekeeping genes; *Protein Kinase G 1 (Pkg1)* and *Hypoxanthie-guanie phosphoribosyltransferase (Hpr)t).* qPCR reactions were done in triplicate. Standard error was calculated between the average of the triplicates. *Fgf19, 8* and *9* are all expressed at a significant level above *Fgf4* (Figure 2).

At stage CS 14 in *Carollia, Fgf19* was expressed along the outer ridge of the developing hand plate in the Apical Ectodermal Ridge (AER). At stage CS 15, the expression of *Fgf19* occurred in a wider strip along the AER. At both CS 16 and CS 17, *Fgf19* appears to be expressed in the mesenchyme connecting the forelimb digits. Throughout those stages, equivalent stages of mouse Embryonic day (E) 9- E12 *Fgf15* was expressed in the developing nervous system (Figure 3). *Fgf15* was expressed in the brain, spinal cord, somites, and tail bud (Figure 3). *Fgf15* was not expressed, however, in

the limb buds of mice at theses stages (Figure 3; Figure 4). *In situ* experiments with mouse RNA indicated that *Fgf4* was expressed in the brain, and in a thin strip along the AER. RNA *in situ* preformed on *Carollia* embryos at CS 14 lacked expression in the developing AER, but expression in the brain was present (data not shown).

This lack of conservation between bat *Fgf19* and *Fgf19* in other mammals called into question if *Fgf19* would be under adaptive selection. This was tested by generating a phylogenic tree with BEAST software and a diverse set of *Fgf19* sequences (*Ailuropoda melanoleuca* (panda), *Myotis lucifugus* (little brown bat), *Bos taurus* (cow), *Sus scrofa* (pig), *Homo sapiens* (human), *Pong oabelii* (orangutan), *Rhesus macaque* (rhesus monkey), *Rattus norvegicus* (rat), and *Mus musculus* (mouse)). Figure 6 depicts the genetic distance between the selected mammalian *Fgf19* genes. The Likelihood Ratio Test on the Phylogenic tree generated by BEAST for *Fgf19* determined that the Codomnl branch analysis was $\chi^2 = 0.667$, p =0 .80 (df = 2). Codonml-site analysis documented that 80.2% of the amino acids were under purifying selection, 16.2% were neutral, and 3.6% may have been under adaptive evolution. The Likelihood Ratio Test for the branch site model was $\chi^2 = 11.69$, p >0.01 (df = 2). The Bayes Empirical Bayes Analysis (BEB) noted the probability of an $\omega>1$ in the foreground linage. The amino acid at site 188 (proline) was under adaptive evolution with a probability of 98.7%. Other amino acids that were possibly be under adaptive evolution, but were not statistically significant, included site 8 (Glutamine) probability of 82.2%; site 25 (Serine) probability of 62.3%, site 28 (Glycine) probability 54%; site 99 (Asparagine) probability of 53%; and site 125 (Phenylalanine) probability 50.7.

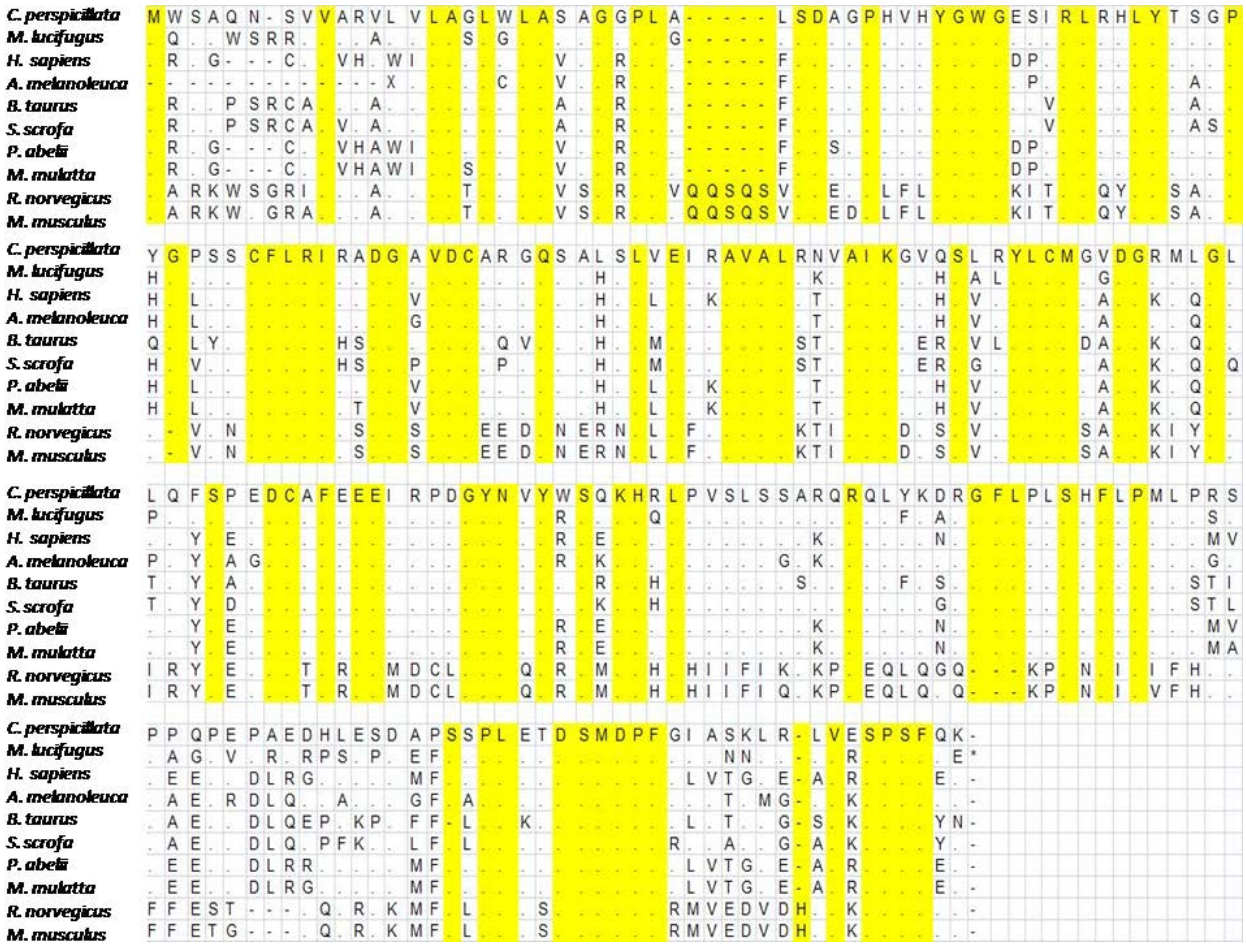

Figure 1. Clustal W alignment of *Fgf* 19 codons for taxa of mammals was generated with MEGA. Yellow columns illustrate conserved codons.

Table 1. The percent of nucleotides that are conserved when compared. Alignments were made with MEGA software. Conservation percentages were found by computing the pairwise distance with MEGA.

Gene	Bat to Mouse	Bat to Human	Mouse to Human
Fgf8	95%	94%	94%
FGf4	79%	92%	82%
Fgf9	91%	93%	92%
Fgf15/19	46%	78%	55%

Table 2. The percent of amino acids that are conserved when compared. Alignments were made with MEGA software. Conservation percentages were found by computing the pairwise distance with MEGA.

Gene	Bat to Mouse	Bat to Human	Mouse to Human
Fgf8	100%	100%	100%
FGf4	86%	92%	86%
Fgf9	99%	100%	99%
Fgf15/19	32%	75%	36%

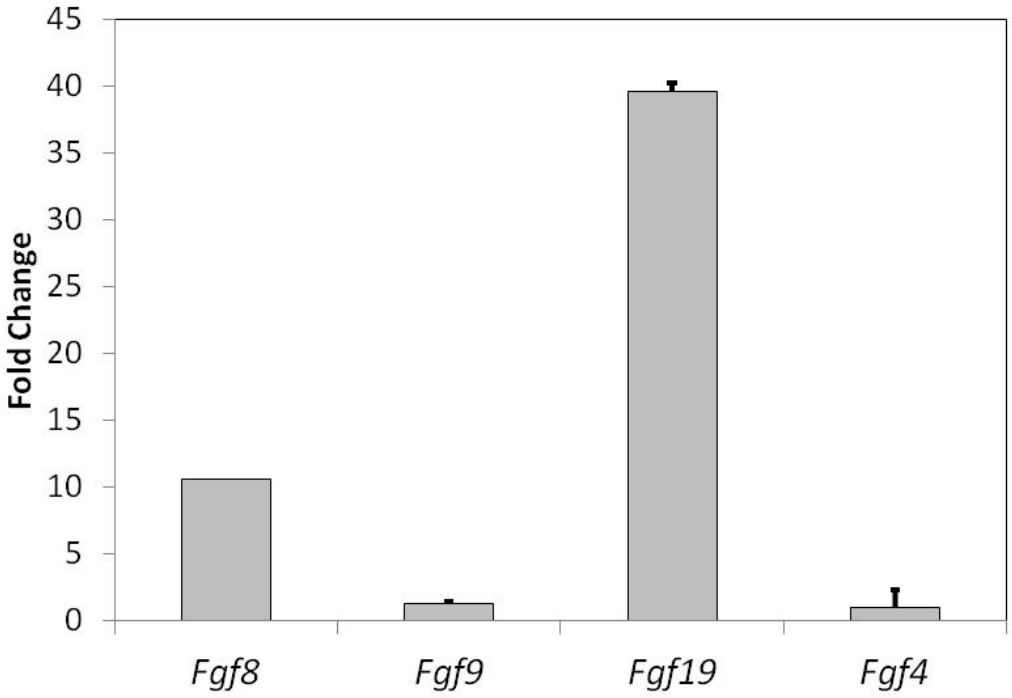

Figure 2. qPCR analysis using cDNA from CS 14 bat forelimbs. *Fgf4* was used for the control value since it was expressed at the lowest level. Data was analyzed using a paired T-test; p-value of <0.01 was considered significant. * depict gene expression levels that were significantly different from *Fgf4* levels. Samples were done in triplicate and standard error was calculated from the average of the triplicates run for each gene.

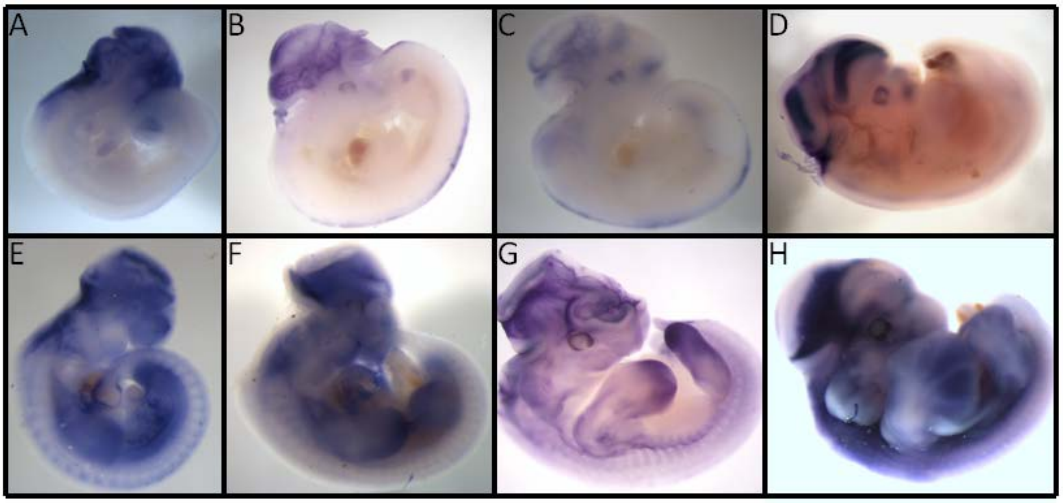

Figure 3. Expression of *Fgf15* in mice and *Fgf1*9 in bats by whole-mount RNA *in situ* hybridization. [A-D]. *Fgf15* expression in mouse, embroyic day (E) 9.5 - 12.5 expression is localized to the brain, spinal cord, and tail bud. [E-H] *Fgf19* expression in bat, Carnegie stage (CS) 13-16. [E-G] CS 13-15 expression of *Fgf19* is located in the developing limb bud in the AER and appears to be in the meshemchyme under the AER, in the somites, and brain. [H] CS 16 *Fgf19* is expression is located in the interdigit mesenchyme, and brain.

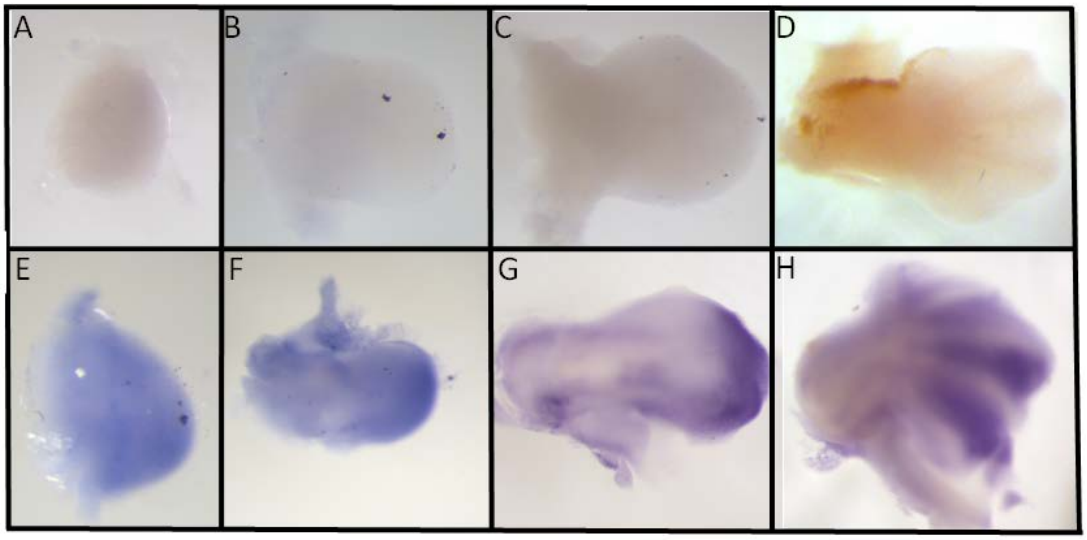

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Figure 4. Expression of *Fgf15* in mice and *Fgf1*9 in bats by whole-mount RNA *in situ* hybridization. . [A-D] *Fgf15* expression in mouse limb embryoic day (E) 9.5-12.5. Mouse limb bud lack any expression of *Fgf15.* [E-H] *Fgf19* expression in bat, Carnegie stage (CS) 13-16. [E-G] CS 13-15 expression of *Fgf19* is located in the developing limb bud in the AER and appears to be in the meshemchyme under the AER. [H] CS 16 *Fgf19* is expression is located in the interdigit mesenchyme.

MWSAQNWSVVARVLVLAGLWLASAGGPLA------SDAGPHVHYGWGE
SIRLRHLYTSGPYGPSSCFLRIRADGAVDCARGQSALSLVEIRAVALR
NVAIKGVQSLRYLCMGVDGRMLGLLQFSPEDCAFEEEIRPDGYNVYWS
QKHRLPVSLSSARQRQLYKDRGFLPLSHFLPMLPRSPPQPEPAEDHLE
SDAPSSPLETDSMDPFGIASKLRLVESPSFQK*

Figure 5. Amino acid alignment of *Fgf19* (*Fgf1*5 in mice) for *Carollia perspicillata.* The amino acid at site 186 (proline) was under adaptive evolution with a probability of 98.7% using PAML (Yang, 1997). This is highlighted with an * Other amino acids that were possibly be under adaptive evolution, but were not significant at the 95% confidence interval: site 7 (Trypotophan, 82.2%); site 23 (Serine, 62.3%), 26 (Glycine, 54%); site 97 (Asparagine, 53%); and site 123 (Phenylalanine, 50.7%).

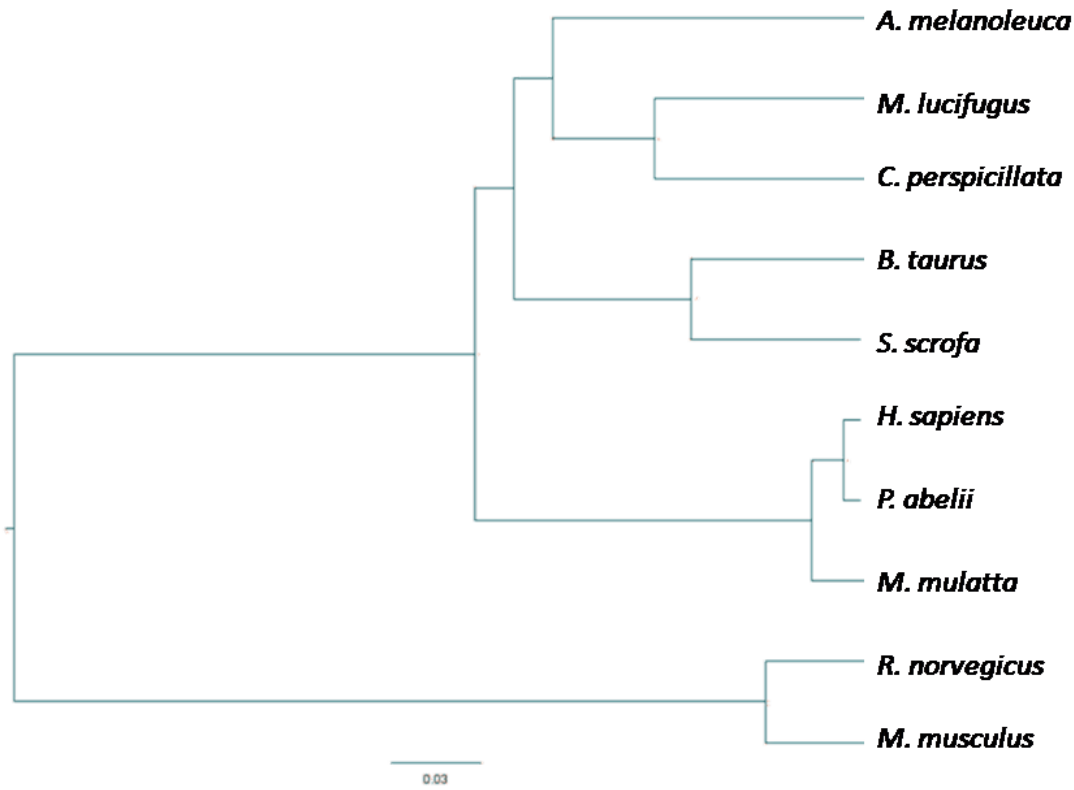


Figure 6.Evolutionary relationships of 10 diverse mammals. Sequences were aligned with Clustal omega, whereas trees were generated using BEAST software, utilizing Bayesian methods.

Discussion:

Fgf8 is the most critical *Fgf* for limb development in mouse (Lewendoski et al., 2000; Moon and Capecchi, 2000; Sun et al., 2000; Sun et al., 2002; Boulet et al., 2004). *Fgf4* is also necessary in mouse for normal limb formation (Niswander et al., 1993; Boulet et al., 2004). A mouse lacking expression of *Fgf8* produces a truncated limb (Lewandoski et al., 2000; Boulet, et al. 2004). An *Fgf4* mutant knockout alone lacks a phenotype indicating *Fgf8* or another *Fgf* can compensate for the lack of *Fgf4* (Moon et al., 2000; Boulet et al., 2004; Mirani et al., 2008). Lack of *Fgf4* expressed in the *Carollia* AER indicates another *Fgf* is potentially compensating for the lack of *Fgf4*. In the mouse *Fgf4* is able to compensate for *Fgf8*, and from this we hypothesize that in *Carollia Fgf19* has replaced *Fgf4*.

When compared to other *Fgfs*, *Fgf19/15* is very much less conserved among species. *Fgf8* is 100% conserved at the amino acid level, in contrast to *Fgf19* which is only 32% conserved. A lack of sequence conservation between *Fgf19* of *Carollia* and other mammals indicates that *Fgf19* may be under adaptive selection (Figure 1; Table 1: Table 2).

qPCR demonstrates that *Fgf19* is expressed at the highest level in the limb bud, and that *Fgf4* is expressed at the lowest level (Figure 2). This indicates that *Fgf19* and *4* have undergone a regulatory change during the evolution of powered flight. *Fgf4* and *Fgf19* are in close proximity on Chromosome 11, separated by approximately 70,000 base pairs. One explanation for why *Fgf19* is expressed in bat, but *Fgf15* is not expressed

in mice, is that the regulatory region between *Fgf4* and *Fgf* 19 may have inverted. It is possible that the regulatory region for *Fgf19* is being up-regulated. Given the structure of sententic region of *Fgf19* and *Fgf4* we hypothesize that there is a limb enhancer region in the area of *Fgf19* and *Fgf4* that has flipped. In the mouse the enhancer functions to repress *Fgf19* and up-regulate *Fgf4*, but in the bat the enhancer region has flipped as to up-regulate *Fgf19* and repress *Fgf4.*

Fgf19 expression does not occur in mouse limbs as demonstrated by RNA *in situ* experiments (Figure 3; Figure 4). *Fgf19* is expressed in the Apical Ectodermal Ridge (AER) in a similar pattern as *Fgf4* in mice (Boulet et al 2004, Wright et al., 2004). Bioinformatics analysis of *Fgf19*, however, indicates the gene is under adaptive evolution. We purpose that both regulatory and coding-sequence variation may contribute to the evolution of *Fgf19* gene in bats. Current thought is that changes in the protein-coding region do not change functional morphology, because altering the protein shape would likely cause the protein not to bind to the receptor (Carroll, 2004). We postulate that changes in the shape of the proteins could be essential for changes in functional morphology in mammals. Changes in protein shape could alter how the protein interacts with its receptors, which in turn, could affect the level of the downstream signal cascade. If the downstream signaling cascade is changed, a different phosphorylation pattern in target transcription factors in the limb could result. In addition, *Fgf* receptors bind to many different types of *Fgf* ligands, and each *Fgf* ligand favors a particular *Fgf* receptor (Martin, 1998). *Fgf19* has a unique affinity for FGFR4 (Xie et al., 1999), which has not been shown to be expressed in the limb (Xu et al., 1999). We hypothesize that the protein-coding region of *Fgf19* has evolved to use another Fgf receptor found in the limb.

Fgf19 unique up-regulation and protein-coding evolution poses an additional alternative for the notion that only regulatory regions are important in the evolution of functional morphology. We propose that regulatory variation along with protein-coding variation are both important in changes in morphology, and may be selected in the same gene at the same time.

References:

Bielawski JP, Yang Z. 2003. Maximum Likelihood Methods for Detecting Adaptive Protein Evolution. J. Struct. Funct. Genomincs. 3, 201-212.

Boulet AM, Moon AM, Arenkiel BR, Capecchi MR. 2004. The roles of Fgf4 and Fgf8 in limb bud initiation and outgrowth. Dev Biol. 273, 361-372.

Carroll SB, Grenier JK, Weatherbee SD. 2001. From DNA to diversity: molecular genetics and the evolution of animal design. Evol Dev. 3, 364-365.

Carroll SB. 2005. Evolution at two levels: on genes and form. *Plos Biology*. 3, 1159-1166

Carroll SB. 2008. Evo-Devo and an expanding evolutionary synthesis: a genetic t heory of morpholgical evolution. Cell. 134, 25-36

Colvin JS, Feldman B, Nadeau JH, Goldfarb M, Ornitz DM. 1999. Genomic organization and embryonic expression of the mouse fibroblast growth factor 9 gene. Dev. Dyn. 216,72-88

Cretekos CJ, Weatherbee SD, Chen CH, Badwaik NK, Niswander L, Behringer RR, Rasweiler JJ IV. 2005. Embryonic staging system for the short-tailed fruit bat, *Carollia perspicillata*, a model organism for the mammalian order chiroptera, based upon time pregnancies in captive-bred animals. Dev Dyn. 233, 721-738.

Cretekos CJ, Deng JM, Green ED, NISC Comparative Sequencing Program, Rasweiler JJ IV, Behringer RR. 2007. Isolation, genomic structure and developmental expression of Fgf8 in the short-tailed fruit bat, Carollia perspicillata. Int. J. Dev. Biol. 51, 333-338

Cretekos CJ, Wang Y, Green ED, NISC Comparative Sequencing Program, Martin JF, Rasweiler JJ IV, Behringer RR. 2008. Regulatory divergence modifies forelimb length in mammals. Genes Dev. 22, 141-151.

Drummond AJ, and Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214

Farin HF, Ludtke T H-W, Schmidt MK, Placzko S, Schuster-Gossler K, Petry M, Christoffels VM, Kispert A. 2013. Tbx2 terminates Shh/Fgf signaling in the developing mouse limb bud by direct repression of Gremlin 1. PLoS Genet. 9, 1-13.

Hasegawa M, Kishino H, Yano T, 1985. Dating of the Human-Ape Splitting by a Molecular Clock of Mitochondria DNA. J. Mol. Evol. 21,160-174

Hockman D, Cretekos CJ, Mason MK, Behringer RR, Jacobs DS, Illing N. 2008. A second wave of Sonic hedgehog expression during the development of the bat limb. PNAS. 44, 16982-16987.

Hughes AL. 2002. Natural selection and the diversification of vertebrae immune effectors. Immunol. Rev. 190, 161-168.

Jablonka E, and Lamb MJ. 1998. Epigenetic inheritance in evolution. J. Evol. Biol. 11, 159-183.

Jessen TH, Weber RE, Fermi G, Tame J, Braunitzer G. 1991. Adaptation of bird hemoglobins to high altitudes: demonstration of molecular mechanisms by protein engineering. Pro. Natl. Acad. Sci. 88, 6519-6522.

King MC, Wilson AC. 1975. Evolution of two levels in humans and chimpanzees. Science. 188. 107-116.

Kurose H, Bito T, Adachi T, Shimizu M, Noji S, Ohuchi H. 2004. Expression of fibroblast growth factor 19 (Fgf19) during chicken embryogenesis and eye development, compared with Fgf15 expression in the mouse. Gene Expr Patterns. 6, 687-693.

Laufer E, Nelson CE, Johnson RL, Morgan BA, Tabin C. 1994. *Sonic hedgehog* and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and pattering of the developing limb bud. Cell. 79, 993-1003.

Lewandoski M, Sun X, Martin GR. 2000. Fgf 8 signalling from the AER is essential for normal limb development. Nat. Genet. 4, 460-463.

Niswander L, and Martin GR. 1992. Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. Development. 114, 755-766

Niswander L, Tickle C, Vogel A, Booth I, Martin GR. 1993. Fgf-4 replaces the AER and directs outgrowth. Cell. 75, 579-587.

Martin GR. 1998. The roles of FGFs in the early development of vertebrate limbs. Genes Dev. 12, 1571-1586.

Mariani MV, Christina PA, Martin GR. 2008. Genetic evidence that FGFs play an instructive role in limb proximal-distal patterning. Nature. 453, 401-405.

Moon AM, Boulet AM, Capecchi MR. 2000. Normal limb development in conditional mutants of Fgf4. Development. 127. 989-996.

Moon AM, and Capecchi MR. 2000. Fgf 8 is required for the outgrowth and patterning of the limbs. Nat. Genet. 26, 455-459.

Ornitz D M, and Itoh N. 2001. Fibroblast growth factors. Genome Biol. 2, 3005.1-3005.12

Posada D. 2008. jModelTest: Phylogenetic Model Averaging. Mol. Biol. Evol. 25, 1253-1256.

Pownall ME, Issacs HV. 2010. Limb development. In *FGF signalling in vertebrate development*. (ed. D. Kesseler). California: Morgan and Clyapool Life Sciences.

Rasweiler JJ IV, Cretekos CJ, Behringer RR. 2009 The Short-Tailed Fruit Bat Carollia perspicillata: A Model for Studies in Reproduction and Development. Cold Spring Harbor Protocols.

Sun X, Lewandoski M, Meyers EN, Liu YH, Maxson RE, Martin GR. 2000. Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. Nat. Genet. 25, 83-86

Sun X, Mariani FV, Martin GR. 2002. Functions of FGF singalling from the apical ectodermal ridge in limb development. Nature. 418, 501-508.

Tamura K, Peterson D, Perterson N, Stecher G, Nei M, Kumar S. 2011. MEGA: molecular evolutionary genetics analysis using maximun likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731-2739.

Todt WL, and Fallon JF. 1984. Development of the apical ectodermal ridge in the chick wing bud. J. Embryol. exp. Morph. 89, 21-41

Wright TJ, Ladher R, McWhirter J, Murre C, Schoenwolf GC, Mansour SL. 2004. Mouse Fgf15 is the ortholog of human and chick Fgf19, but is not uniquely required for optic induction. Dev. Biol. 26, 264-275.

Xie MH, Holcomb L, Deuel B, Dowd P, Huang A, Vagts A, Foster J, Liang J, Brush, J, Gu Q, Hillan K, Goddard K, Gurney AL. 1999. FGF-19 a novel fibroblast growth factor with a unique specificity for FGFR4. Cytokine. 11, 729-735

Xiong J. 2006. Essential Bioinformatics. Cambridge University Press. Cambrige, UK.

Xu X, Weinstein M, Li C, Deng CX. 1999. Fibroblast growth factor recpetors (FGFR) and their roles in the limb development. Cell.Tissue. Res. 296,33-43

Yang Z. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl. Biosci. 5, 555-560.

Yokoyama S. 2002. Molecular evolution of color vision in vertebrates. Gene. 300,69-78.

Zhang J, Webb DM, Podlaha O. (a) 2002. Accelerated Protein Evolution and Origins of Human-Specific Features: FOXP2 as an Example. Genetics. 162, 1825-1835

Zhang J, Zhang YP, Rosenberg HF. (b) 2002. Adaptive evolution of duplicated pancreatic ribonuclease gene in a leaf-eating monkey. Nat. Genet. 30, 411-415.

Chapter 3

Conclusion and Future Directions

There are two differing hypotheses on how evolution of morphology can occur. One, supported largely by Carroll, is that change in the protein-coding region of genes are unimportant. Carroll's rationale is that changes in the protein-coding region are selected against because such changes would lead to a different protein shape, which in turn would prevent the ligand from binding to the receptor. Several papers were published in 2002 in response to this argument, focusing on genes under adaptive evolution. Even though several different genes were found to be under adaptive evolution, and those genes affected a large range of morphologies, the debate continues.

We have shown that *Fgf19* is under adaptive selection, and the up-regulation from mouse to bats provides substantial evidence that the regulatory region of *Fgf19* has also undergone an evolutionary change. What *Fgf19* demonstrates is that both protein-coding changes and regulatory changes are important in the evolution of morphology.

qPCR analysis still needs to be completed. Several sets *Fgf3, 17* and FGFR4 qPCR primers have been designed for qPCR. Due the low levels of expression in any tissue type of *Carollia,* qPCR efficiencies were too high to analyze. This high efficiency is likely due to primers randomly binding to each other due to the lack of target DNA. cDNA pools from tissue and stage specific *Carollia* embryos will be used in an attempt to lower the primer efficiencies. qPCR analysis should also be performed on hind limb tissue of CS 14 *Carollia* embryos. Since bat hind limb formation is similar to that of mouse, confirmation of high levels of *Fgf19* and low levels of *Fgf4* would confirm a regulatory change.

A lack of sequence conservation between mammals in the 5' end of *Fgf4* made *Fgf4* exceptionally hard to clone. I propose to use a Rapid Amplification of cDNA ends (RACE) protocol and kit to amplify the 5' end of *Fgf4.* After the complete sequence is obtained bioinformatic analysis should be preformed on the protein-coding region of *Fgf4.* Bioinformatic analysis should be done to confirm that *Fgf4* is not under adaptive evolution.

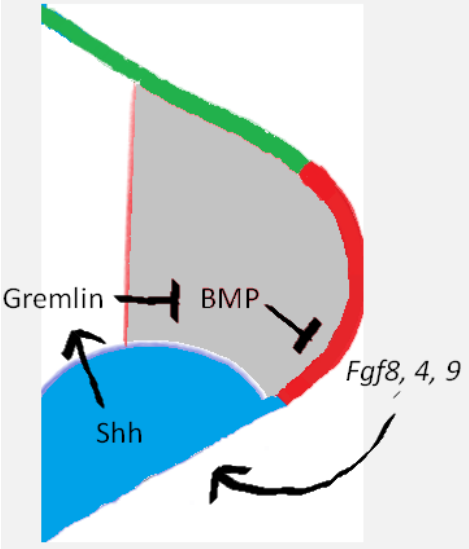
Supplemental Tables:

Table 1. Primers used to clone *Fgf19* and *Fgf4.* Primer set number 2 was used to make RNA *in situ* probes.

Gene	F1	R1
Fgf19 1 (5')	GGAGGCGTGGTGTGAGTG	CCAGCCGTAGTGCACGTG
Fgf19 2	CACGTGCACTACGGCTGG	ATCCCAAAAGGGTCCATGCTG
Fgf19 3 (3')	AGGCACAGCCAATGTCAAAC	CAGCATGGACCCTTTTGGGAT
Fgf4 2	TGGAGGCCGAGCTGGAGCG	GGGGAGGAAGTGGGTGACCT
Fgf4 3 (3')	ATGTTCATTGCCCTGAGCA	GAACCATAAATAATTTGGTGG

Table 2. qPCR primers used for amplification. Concentrations listed are the amount of each primer per reaction

Gene	F1	Concentration F1	R1	Concentration R1
Fgf8	GGCCAACAAGCGCATCAACGC	350nmol	GCACGATCTCCGTGAAGACAC	350nmol
FGF19	GTTTGGTGGAAATCAGGGCA	300nmol	CTCCTCGAAGGCGCAGTCCTC	350nmol
FGF9	CAGGAAAGACCACAGCCGAT	350nmol	CCCCTTCTCATTCATGCCA	300nmol
FGF4	CTACTGTAACGTGGGCATTGG	350nmol	GTGAAGAAAGGCGAGCCGTAC	300nmol
PGK1	CCTGTTGGAGAACCTTCGCT	150nmol	GTGAGCAGTGCCAAAAGCAT	250nmol
HPRT	GGGGACATAAAAGTGATTGGT	150nmol	CTTGACCAAGGAAAGCAAGGT	250nmol



Supplemental Figure 1. Limb regulatory loop showing the interaction of *Fgf*, Shh, Gremlin, and BMP during limb outgrowth. Adapted from Gilbert SF. 2006. Developmental Biology. 8. ed. Massachusetts: Sinauer.