Jingyi Ding

BIO-450

Dr.Krogan

Lit Review #2:Significant Limb Development Factor: Hox Genes

Introduction:

In the field of developmental biology, the vertebrate limb is a significant and valuable model for studying the genetic coordination of a complex developing structure. In general, the tetrapod limb is made out of a proximal-to-distal series of long bones, the stylopod, zeugopod in the arm, and the digits in the hand, and the digits are separated from the former two by the mesopodium, 'an articulation based on an array of small roundish bones' (Woltering et al. 2010). Studies show that the tetrapod limb most likely evolved during the Devonian as an adaptation to the buoyancy-lacking environment of the land (Coates et al. 2002). The fossil record demonstrates that limbs evolved from fins via successive steps of distal elaboration, which further caused the formation of the autopod as a tetrapod-specific evolutionary innovation, and distal fin radials were defined as putative evolutionary precursors of digits (Boisvert et al. 2008). The tetrapod limb development is an incredibly complex process, and many factors/genes are involved in this process. Hox genes encode transcription factors belonging to the broad family of homeodomain-containing proteins. In the process of organism development, as a subset of homeotic genes, Hox genes control the body and limb plan of an embryo along the head-tail axis; and after the formation of embryonic segments, Hox genes function as determining the type of appendages or the different kinds of vertebra that will form on a segment (Salsi et al.2008). Hox genes are the big players in the limb development.

Meanwhile, studies point out that the patterning of the skeletal element, in both fin and limb, largely relies on the action of signaling centers operating from the onset of their outgrow: the apical ectodermal ridge (AER) and the zone of polarising activity (ZPA) (Delgado et al. 2016). However, significant differences are observed in the morphology and development of the most distal part of fins and limbs. In tetrapods, the influence of the AER is maintained throughout limb development, and it plays a vital role

in differentiating stylopod, zeugopod, and autopod, but in fish, AER is quickly converted into a fin-fold during fin development and further gives rise to the apical dermoskeleton (Mariani et al. 2008). The experiment performed six years ago confirmed that 'the overexpression of hoxd13a gene induces a phenotype that mirrors two of the major morphological alternations marked as crucial in limb evolution by the fossil record: endochondral expansion and dermoskeleton retraction' (Freitas et al. 2012). This study suggests the significance of Hox genes in contributing the evolution of tetrapod.

Therefore, this lit review will specifically focus on the Hox genes, and what roles it plays in the process of tetrapod limb development. At the same time, the significance of Hox genes in the tetrapod evolution aspect will be mentioned as well.

Lit review:

#1: Distal limb patterning requires modulation of cis-regulatory activities by HOX13

The combinatorial expression of Hox gene along the body axes help to determine the cell fate and generate the body plan. The previous study shows the HOXA13, and HOXD13 knockout caused the digit agenesis in mice. However, how HOX13 proteins regulate transcriptional outcomes and confer identity to the distal-most limb cells has remained unknown. Thus, this study focuses on investigating how HOX13 regulate the transcriptional program in the distal-most limb cells by using the developing mice limb bud as a model system. The genomic sites bound by HOXA13 and HOXD13 in vivo were generated and analyzed first; the changes in chromate marks and gene expression during the transition from the early to the late-distal limb program were observed as well; authors also noted the changes in chromatin marks and gene expression in late-distal limb cells without HOX13 functioned. In this study, chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing of 'post-translationally modified lysine (K) 27 residues of histone H3' can help to reveal the genome-wide pattern of active and polycomb-mediated repressed chromatin states (Rushikesh et al. 2016). Combing genome-wide analyses of histone modification and transcription factor occupancy is used to identify transcription factor-mediated regulatory networks (Rushikesh et al. 2016).

Results show that HOX13 primarily bind to putative CRMs that associated with either activation or repression in late-distal WT limb buds, and the loss of HOX13 results in the functional chromatin state changes at CRMs in distal cells. These results further indicate that in the absence of HOX13, the early limb program fails to terminate, while the implementation of the late-distal regulatory program is compromised. The substantial interaction between altered chromatin state and gene expression changes in HOX13 knockout limb buds indicates that HOX13 dependent establishment and maintenance of the functional chromatin state regulates transcriptional outcomes in presumptive digit cells. Taking together, this study shows by binding at Hox-regulatory regions, HOX13 plays a critical role in setting the cis-regulatory networks that control the digit-specific development; and the unique alternation of chromatin state at CRMs and the corresponding changes in gene expression provide a foundation for understanding the tissue-specific regulatory driven by HOX13 proteins (Rushikesh et al. 2016).

#2: Hoxd13 and Hoxa13 directly control the expression of the EphA7 Ephrin Tyrosine kinase receptor in developing limbs

The first article talks about the role of HOX13 in regulating the digital specification during the process of limb development. So what are other roles HOX13 plays in the limb development? This article specifically focuses on the HOX downstream target genes called Ephrins and ephrin tyrosine kinase receptors. Eph and ephrins are expressed in various regions of the vertebrate embryo in dynamic patterns, and they were identified play significant roles in the ‘control of cell shape, cell migration, cell sorting, wiring of neurone in the nervous system, and the formation of boundaries between structures’ (Amparo et al. 2003). Recent studies show that Eph-ephrin signals limb development and the expression of the EphA7 gene in developing limbs was observed to correlate with the expression of Hoxa13 and Hoxd13, but its direct regulation by these genes has never been addressed. Thus, this study further investigates the regulation of EphA7 by HOX13, focuses explicitly on whether EphA7 is indeed a direct target of HOXD13 and/or HOXA13. The experimental method like chromatin immunoprecipitation (ChIP) was used in this study. Results show that the EphA7

promoter region contains multiple potential binding sites for HOX13, but only one of these sites is bound in vivo by the HOXA13 and HOXD13. At the same time, they found out that a mutation of the HOXA13/HOXD13 binding site was sufficient to prohibit activation of transcription from the EphA7 promoter (Salsi et al. 2005). Taking together indicates that EphA7 is a direct downstream target of the HOXA13 and HOXD13 during the process of limb development. This finding provides additional evidence that Hox genes play a critical role in controlling the limb formation, in this case, by regulating the ephrin tyrosine kinase receptor.

#3: Hoxd13 binds in vivo and regulated the expression of genes acting in key pathways for early limb and skeletal patterning

As the most 5'located HoxD gene, Hoxd13 plays an essential role in the limb development, for example, as the previous article revealed, it regulates the expression of ephrin tyrosine kinase receptor. However, due to the limited understating on Hoxd13 downstream target genes, the mechanisms underlying the control of developmental processes by Hoxd13 are still elusive. Thus, this study took advantage of the ChIP-on-chip technology to determine direct downstream target genes for the Hoxd13 protein. The study specifically focused on analyzing Hoxd13-bound genes known to play relative roles in limb patterning, chondrogenesis, and skeletogenesis. Authors isolated genomic sequences bound by Hoxd13 invivo via crosslinking and ChIP (Salsi et al. 2008). In addition, this study analysed by ChIP the binding of Hoxd13 to genomic sequences within three different candidate direct target loci: SFPR1,BMP2, and BMP4 (Salsi et al. 2008), where SFRP1 is involved in limb development (Satoh et al. 2006); BMP2 was indicated to be a direct target of the paralogous Hoxa13 (Knosp et al. 2004) while the BMP4 was shown to be bound invitro by Hoxd13 and Hoxa13 (Suzuki et al. 2003). In all, there were 248 gene loci identified by using ChiP-on-chip that bound invivo by the Hoxd13 protein. Study further indicates that 'Hoxd13 binds in vivo, in developing limbs, the loci of Hand2, a gene crucial to limb AP axis patterning, of Meis1 and Meis2, involved in PD patterning, of the Sfrp1, Barx1, and Fbn1 genes, involved in skeletogenesis, and of the Dach1, Bmp2, Bmp4, and Emx2 genes (Salsi et al. 2008).

Further, by performing loss-and-gain-of-function experiments in chick, the misexpression of Hoxd13 in developing chick limbs changes the expression of the majority of these genes. Taking together, one can tell that Hoxd13 directly regulated the transcription of critical genes for early limb AP and PD axis patterning, and essential genes involved in skeletal patterning at later stages.

#4: Key pathways regulated by HoxA9,10,11/HoxD9,10,11 during limb development

Previous three articles have explicitly been the focus on investigating the role of Hox13 plays in the limb development. However, it is essential to know that the 39 mammalian Hox genes show problematic patterns of functional overlap. Thus, it is necessary to remove multiple combinations of paralogous and flanking genes to better define the developmental roles of Hox genes, and this study focuses on HoxA9,10,11 and HoxD9,10,11. Unlike previous studies to use Cre/LoxP to remove an entire Hox cluster, this study used a different strategy. Authors examined limb development in mice with the frameshift mutation in six Hox genes (Hoxa9,10,11 and Hoxd9,10,11). A novel recombineering method that allows the 'simultaneous targeting of frameshift mutations into multiple flanking genes' was performed to generate mice. They found out that the Hoxa9,10,11 −/− /Hoxd9,10,11 −/− mutant mice show a reduced ulna and radium that is more severe than seen in Hoxa11 −/−/Hoxd11 −/− mice, which indicate the role for the flanking Hox9,10 genes in limb development. Further, there was the significant reduction of Shh expression observed in the ZPA while the decreased Fgf8 expression detected in the AER (Anna et al., 2015). The laser capture microdissection was coupled with RNA-Seq to characterize the gene expression programs in both wild-type and mutant limbs (Anna et al., 2015). Hox mutants mice showed significantly altered expression of 'Pknox2, Zfp467, Gdf5, Bmpr1b, Dkk3, Igf1, Hand2, Shox2, Runx3, Bmp7 and Lef1' and all of them had been previously identified to play important roles in bone formation (Anna et al., 2015). In general, this study indicates HoxA9,10,11/HoxD9,10,11 regulate critical pathways during limb development.

#5: Hoxd13 contribution to the evolution of vertebrate appendages

Despite functions of Hoxd13 discussed in the previous three articles, another critical role Hoxd13 plays in the field of biology is it contributes the evolution of vertebrate limbs. Fossil data suggests that 'limbs evolved from fish fins by sequential elaboration of their distal endoskeleton, giving rise to the autopod close to the tetrapod origin' (Renata et al. 2012). Modulation of 5'Hoxd gene transcription (through tetrapod-specific digit enhancers), has been considered as a possible evolutionary mechanism caused such morphological transformations. Thus, by over-expressing Hoxd13a in zebrafish, it allows authors to investigate the influence of increasing 5'Hoxd expression in fin development. As the results, increased proliferation, distal expansion of chondrogenic tissue and fin-fold reduction were observed. At the same time, authors observed similar expression in zebrafish fins and mouse limbs that promoted by 'tetrapod-specific 5'Hoxd enhancer CSC' (Renata et al. 2012). Taking all these finding together, study shows that in the manner of fin-to-limb evolution and the origin of tetrapod limbs, Hoxd13 plays a significant role.

#6: HoxA and HoxD expression in a variety of vertebrate body plan features reveals an ancient origin for the distal Hox program

Similar to article #5, this article focuses on investigating other genes from Hox family that also contribute morphological transformation from fin-to-limb in vertebrates. The unique place about this study is this is the first time people demonstrate that the distal phase expression pattern occurs with the posterior HoxA genes, meaning the DP expression is no longer only associated with HoxD gene cluster (Sophie et al. 2014). Further, the DP expression got observed in a variety of body plan features, including the former Hox-free zone ('sensor adornments that develop from the first mandibular arch') (Renata et al. 2012). This study also presented the first evidence for DP expression of the HoxA genes in the 'hindgut and vent of three ray-finned fishes' (Renata et al. 2012). Overall, the Hox DP expression pattern observed in this study appears to be an ancient module and can be provided as a shared genetic program that suggests deep homology of a variety of distally elongated structures, and it has played a crucial role in the evolution of morphological diversity in vertebrates.

#7: Conservation and divergence of regulatory strategies at Hox loci and the origin of tetrapod digits

Despite the use of comparative gene expression analyses, critical aspects of the fin-to-limb transformation remain controversial, especially the origin of tetrapod digits (JoostM et al., 2014). In this study, authors specifically focus on investigating whether the two phases of Hox gene transcription (Hoxa and Hoxd) that observe during tetrapod limb development also appear during zebrafish fin development. The same bimodal chromatin architecture got found in zebrafish embryos, meaning that the regulatory mechanism used to generate tetrapod limb patterns may anticipate the divergence between fish and tetrapods (JoostM et al., 2014). However, when the genomic regions that control the expression of Hox gene in fish fins are introduced into mice, only the proximal limb segment got triggered instead of the presumptive digits (JoostM et al., 2014). Overall, these results indicate that although fish have the Hox genes to regulate and product digits, this potential is not promoted as it is in tetrapods, which further demonstrate that fin radials are to homologous to tetrapod digits.

Conclusion:

As one of the most critical factors involved in the limb development, scientists have been focused on the mechanism of how Hox genes regulate limb development for years. All these articles addressed in this lit review explicitly focus on the analyzing members of Hox family including Hoxa13, Hoxd13, HoxA9,10,11 and HoxD9,10,11, and how they functioned during the tetrapod limb development and how they contribute morphological transformation from fin-to-limb in the vertebrates. Overall, one can tell that Hox13 is the critical member of Hox family that responsible to set the cis-regulatory networks that control the digit-specific development. And Hoxd13 and Hoxa13 directly control the expression of the EphA7 ephrin tyrosine kinase in developing limbs while Hoxd13 regulates the expression of critical genes such as Sfrp1, Barx1, Bmp2 that play significant roles in early limb development and skeletal patterning. Meanwhile, one article in this lit review reveals that HoxA9,10,11/HoxD9,10,11 regulate critical pathways during limb development as well. Last three articles intend to address the role Hox genes

played in the aspect of fin-to-limb evolution and the origin of tetrapod limbs. As the major player involved in the process of tetrapod limb development, I believe more undiscovered potential roles of Hox genes need to be investigated in the future.

Reference list:

Woltering JM, Duboule D (2010) The origin of digits: expression patterns versus regulatory mechanisms. *Dev Cell.*18(4):526-32.

Coates MI, Jeffery JE, Rut M (2002) Fins to limbs: what the fossils say. *Evol Dev.*4(5):390-401.
Boisvert CA, Mark-Kurik E, Ahlberg PE (2008)The pectoral fin of Panderichthys and the origin of digits. *Nature.* 456(7222):636-8.

Valentina Salsi, Maria Alessandra Vigano (2008) Hoxd13 binds in vivo and regulates the expression of genes acting in key pathways for early limb and skeletal patterning. *Developmental Biology* 317(2): 497-507

Delgado I, Torres M Semin (2016) Gradients, waves and timers, an overview of limb patterning models. *Cell Dev Biol*. 49():109-15.

Mariani FV, Ahn CP, Martin GR (2008).Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. *Nature.*453(7193):401-5.

Freitas R, Gómez-Marín C, Wilson JM, Casares F, Gómez-Skarmeta JL (2012) Hoxd13 contribution to the evolution of vertebrate appendages. *Dev Cell.* 23(6):1219-29.

Rushikesh Sheth, Iros Barozzi, David Langlais et al. (2016) Distal limb patterning requires modulation of cis-regulatory activities by HOX13. *Cell Rep.* 17(11): 2913-2926.

Amparo Palmer and Rüdiger Klein (2003). Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. *Genes&Dev.* 17: 1429-1450.

Valentina Salsi&Vincenzo Zappavigna (2005). Hoxd13 and Hoxa13 directly control the expression of the EphA7 ephrin tyrosine kinase receptor in developing limbs. *The journal of biological chemistry.* 281: 1992-1999.

W. Satoh, T. Gotoh, Y. Tsunematsu, S. Aizawa, A. Shimono (2006). Sfrp1 and Sfrp2 regulate anteroposterior axis elongation and somite segmentation during mouse embryogenesis *Development*, 133: 989-999

W.M. Knosp, V. Scott, H.P. Bachinger, H.S. Stadler (2004). HOXA13 regulates the expression of bone morphogenetic proteins 2 and 7 to control distal limb morphogenesis *Development*, 131: 4581-4592

M. Suzuki, N. Ueno, A. Kuroiwa. (2003) Hox proteins functionally cooperate with the GC box-binding protein system through distinct domains. *J. Biol. Chem.,* 278: 30148-30156
Anna M. Raines, Bliss Magella, Mike Adam and S. Steven Potter (2015). Key pathways regulated by HoxA9,10,11/HoxD9,10,11 during limb development. *BMC developmental biology.* 15:28.

Sophie Archambeault, Julia Ann Taylor, and Katen D Crow (2015). HoxA and HoxD expression in a variety of vertebrate body plan features reveals an ancient origin for the distal Hox program. *EvoDevo.* 5:44

Joost M. Woltering, Daan Noordermeer et al. (2014). Conservation and divergence of regulatory strategies at Hox loci and the origin of tetrapod digits. *PLoS Biol.* 12(1): e1001773