

## Lecture 3 - Molecular Regulation of Development. Growth factor signaling, Hox genes and the body plan

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### Lecture Objectives

To understand how cell differentiation and morphological change are regulated in molecular terms during development. Two questions will be examined:

- 1) How is tissue differentiation in the **dorsal-ventral (D-V) axis** regulated by **gradients of growth factor signals**? **Morphogen gradients** secreted by inducing cells in organizer centers can affect long-range cell communication.
- 2) How do **Hox genes** orchestrate pattern formation in the **antero-posterior (A-P) axis**? We will analyze the colinearity between the order of the Hox genes in the DNA and their expression in embryonic regions. Particular emphasis will be placed on the molecular mechanism of Hox gene transcriptional activation by **retinoic acid nuclear receptors**. The study of Hox genes has made important contributions to our understanding of the Evolution of Development (Evo-Devo).

### Key Words:

- **Morphogen:** A signaling molecule that can diffuse in the embryo, inducing multiple cell fates at different threshold concentrations. Usually emanates from an organizing center.
- **Growth factors:** Secreted proteins that bind to cell surface receptors and cause changes in cell proliferation and differentiation. Examples are Epidermal growth factor (**EGF**), Fibroblast growth factor (**FGF**), Insulin-like growth factor (**IGF**), **Insulin**, and the **TGF $\beta$**  superfamily.
- **Growth factor antagonists:** Secreted proteins that can bind growth factors and prevent them from signaling. Antagonists can regulate morphogen gradients.
- **Transforming growth factor beta (TGF $\beta$ ) superfamily:** The largest family of growth factors in humans (30 different ones). Frequently they inhibit cell proliferation and induce cell differentiation. Examples are **Bone Morphogenetic Proteins (BMP)** and **TGF $\beta$ -1 to 3**.
- **Dorsal-ventral (D-V) axis.** The back to belly direction of the embryo.
- **Antero-posterior (A-P) axis.** Rostral to caudal differentiation of the body.
- **Homeodomain:** A 60 amino acid DNA-binding domain present in Hox and other DNA-binding proteins.
- **Homeobox:** Nucleic acid sequence encoding a homeodomain. Homeobox sequences have been highly conserved through evolution, and can be detected by

nucleic acid hybridization across species. There are many homeobox genes (170 in the human genome, of which only 39 are Hox genes (present in clusters).

- **Hox gene complexes:** Clusters of about 10 coordinately expressed Hox genes related to the *Drosophila Antennapedia* homeobox. Four gene complexes present in humans, each Hox cluster is in a different chromosome.

## 1) A GRADIENT OF GROWTH FACTOR SIGNALING REGULATES DORSAL-VENTRAL DEVELOPMENT.

- Cells influence each other through cell-cell signals mediated by growth factors.
- During development, **organizer signaling centers** generate gradients of morphogen signals. Cells can respond to different threshold concentrations of a morphogen by adopting multiple differentiated fates (Fig. 2).
- One of the best examples of morphogen gradients is provided by **Dorsal-Ventral (D-V)** cell differentiation of the frog (*Xenopus*) embryo (Figs. 3 and 4).
- Ventral mesoderm secretes **BMP4**, a TGF $\beta$  family member that induces ventral tissues (Fig. 5). BMPs were discovered by UCLA orthopedic surgeon Marshall Urist, who showed that demineralized bone matrix could induce ectopic bone when transplanted subcutaneously into rats.
- The dorsal (Spemann) organizer tissue specifically expresses a secreted protein called **Chordin** that induces neural tissue in dorsal ectoderm (Figs. 6 and 7).
- **Chordin** is a **BMP antagonist** that binds to **BMP4** and prevents it from binding to its receptors (Fig. 8), generating a gradient of BMP4 signals as it diffuses in the embryo. **Noggin** is another BMP antagonist, which functions in a similar way.
- **How does BMP signal to turn genes on or off? BMP4 receptors**, part of the **TGF $\beta$  receptor** family, are transmembrane Serine-Threonine protein kinases that signal by phosphorylating transcription factors (called Smads) in the cytoplasm that then translocate into the nucleus, bind to DNA, and activate transcription. This process of **signal transduction** mediates the effects of a protein bound on the cell surface to the gene transcription level (Fig. 9 and movie 10).
- Phosphorylated Smad has low affinity for DNA, so it also needs additional **DNA-binding partners** (other transcription factors) to activate gene transcription.
- The **BMP gradient** can be visualized in the *Xenopus* gastrula as a gradient of phosphorylated Smad1 transcription factor which is maximal in the ventral side (Fig. 11).

- At gastrula, graded BMP activity in ectoderm, mesoderm and endoderm is established by secretion of BMP antagonists (Chordin and Noggin) in the dorsal side that oppose BMP4 signals from the ventral side (Fig. 12). Are there one or multiple gradients?
- Endogenous Chordin protein forms a gradient that diffuses long-range from the Spemann organizer where its mRNA is produced (Fig. 13). Cells in both the ectoderm and mesoderm can sense BMP concentrations from a single morphogen gradient established in a narrow extracellular matrix region that separates ectoderm from mesoderm (Fig. 13).
- The Chordin/BMP morphogen gradient induces differentiation of different tissues in mesoderm and ectoderm (Fig. 14).
- In the mesoderm, a single gradient gives rise to multiple mesodermal fates (**notochord, somite, lateral plate**) at gastrula. Low BMP4 signaling levels lead to dorsal mesoderm formation.
- In the ectoderm, low BMP levels produce Central Nervous Tissue (**CNS**), high BMP levels give rise to **epidermis** and intermediate levels to **neural crest** (Fig. 14). BMP inhibition causes CNS to be induced in the embryo.
- Different germ layers have specific **DNA-binding partners** for phosphorylated Smad1, explaining the different cellular responses. For example, the Smad1 binding partner in mesoderm is called Brachyury.
- Cells can communicate with each other over very long distances through diffusible morphogen gradients (Fig. 15).
- In conclusion, a morphogen gradient can be generated by a source of growth factor (such as BMP) or by a localized source of inhibitor (such as Chordin in Spemann's organizer). Both mechanisms are used in the embryo.
- **Cells utilize a surprisingly small number of cell-cell signaling pathways to communicate with each other** (Fig. 16). These pathways transduce signals from the extracellular medium to turn genes on and off in the nucleus, as will be explained by Prof. John Colicelli. The principal signaling pathways are:
  - 1) **Receptor Serine/Threonine kinases** such as TGF- $\beta$  (transforming growth factor- $\beta$ ) and BMP (bone morphogenetic proteins) receptors;
  - 2) **Receptor Tyrosine kinases** (RTKs, such as EGF, FGF, IGF, and Insulin receptors;

- 3) **G protein-coupled receptors**, also known as 7-transmembrane serpentine receptors, which transduce a multitude of small molecule and polypeptide signals such as **epinephrine (adrenalin)**, **odorants**, **histamine**, **prostaglandins**, and **gonadotropins** (such as **FSH** and **LH**);
- 4) **Wnt growth factors**, which signal through LRP6 (lipoprotein-receptor related protein 6) and Frizzled receptors and cause the stabilization of a nuclear protein called  $\beta$ -Catenin and many other proteins;
- 5) **Hedgehog** proteins, which signal through the Patched and Smoothed transmembrane proteins and prevent the cleavage of a transcriptional activator called Gli;
- 6) **Notch**, a membrane receptor that is activated by membrane-bound ligands leading to the proteolytic release and nuclear translocation of its intracellular domain;
- 7) **Nuclear hormone receptors**, which are transcription factors that are activated by hydrophobic ligands such as steroid hormones, thyroid hormone and retinoic acid (to be explained today).

You do not need to remember any of the names above right now, but these pathways will come up in your studies as they explain how human cells interpret the multitude of signals they receive from their extracellular milieu. Each one of these **signal transduction pathways** has been linked to human cancer. The only important thing to remember today is that in the embryo the same signals can trigger different cell differentiation responses in cells of diverse developmental history because of different combinations of DNA-binding partners.

2) **HOX GENES** (Fig. 17) -

- a) **Colinearity**
- b) **Activation by Retinoic Acid**
- c) **Hox genes in Evolution and Development**

- **Homeotic transformations** are changes of one body part into the likeness of another. Early evolutionists were very interested in these intriguing morphological changes. Cervical ribs (found in 0.5% of humans) are examples of homeotic transformations (Fig. 18). In humans, 1-3% of us may have a lumbar rib (13 thoracic vertebrae instead of the “normal” 12).
- In *Drosophila*, **homeotic mutations** that change a balancer organ into a wing (bithorax 4-winged fly), or antenna into a leg (Antennapedia mutation) were identified (Fig. 19).
- Prof. E. B. Lewis started working in *Drosophila* homeotic genes in 1946 at Caltech, and continued until he passed away in 2004. For his work, he received the Nobel Prize for Medicine in 1995. I will tell you the story of the discovery of

the homeobox.

- Lewis' genetic studies predicted homeotic genes would be duplicated (Fig. 20).
- When the *Antennapedia* gene was cloned in the laboratory of Walter Gehring in Switzerland, a short sequence was found to be conserved in several other *Drosophila* homeotic genes in Southern blots by low-stringency nucleic acid hybridization (achieved by lowering temperature or increasing salt concentration).
- The *Drosophila* homeobox probe was used to isolate vertebrate Antennapedia-like homologues (called **Hox genes**) from **vertebrate** genomic DNA libraries (Fig. 21).
- The **homeobox** was sequenced and found to consist of a conserved region of 180 nucleotides that encoded a **DNA-binding domain** of 60 amino acids (Fig. 22).
- The **homeodomain** forms a helix-turn-helix domain that fits into the major groove of the DNA, recognizing a 6-8 base pair DNA sequence (Fig. 22). DNA-binding domains of the helix-turn-helix type are found in large numbers of proteins, including the lac repressor of *E. coli*. The Hox homeodomain also interacts with a transcriptional co-activator (called mediator) perhaps explaining its sequence conservation.
- The name Hox genes is reserved for homeodomains that resemble the sequence of Antennapedia and are present in the Hox complexes. There are only 39 Hox genes in humans (but our genome contains 170 homeodomain-containing DNA binding proteins).

**2a) Colinearity:** Remarkably, Hox genes are arranged in the genome in clusters in the same order in which they are expressed in the body (Fig. 23).

- In the fruit fly the *labial* homeobox gene is expressed in anterior and *abdominal-B* in posterior segments. Their closest Hox homologues in the mouse are expressed with the same A-P relationships (Fig. 23).
- The exceptional conservation of this arrangement between species suggests that a gene system that controls the A-P axis arose very early in evolution.
- In vertebrates, two whole-genome duplications have occurred. These duplications may be the secret of our evolutionary success. The duplications resulted in **four Hox complexes** (Figs. 24, 25).
- Each **Hox complex** has about 10 genes. By comparing their sequences, they can be aligned in 13 "**paralogous**" (duplicated) groups according to their homeodomain amino acid sequences.
- Our chordate ancestor the amphioxus has a single Hox complex containing about 13 Hox genes. Humans have only 39 Hox genes instead of  $13 \times 4 = 52$ . This is because some Hox paralogues were lost (Fig. 25). Gene loss is very common during evolution.
- The degree of conservation of the complexes between *Drosophila* and humans is

amazing. A system this complicated could not have arisen independently twice in evolution. Even microRNAs (miRs) located in between the homeodomain genes have been conserved. This complex regulatory gene system was present in the last common ancestor of *Drosophila* and mammals.

- The most striking feature of Hox genes is their **colinearity**, both **spatial** and **temporal** (Figs. 25 and 26).
- **Spatial colinearity**: The most anteriorly expressed genes are at one end, and they are expressed in progressively posterior regions towards the other end of the complex (Fig. 25). All paralogues share the same A-P border of expression in the embryo.
- **Temporal colinearity**: genes on the anterior end of the complex (paralogue 1, labial-type) are transcribed first; those on the other end (paralogue 13) are expressed last. This sequential “opening” of the chromatin of Hox complexes takes several days in the mouse embryo (Fig. 26).
- A big mystery is why these genes have stayed arranged together in the genome during evolution. Perhaps due to common regulatory elements that regulate the chromatin structure of all genes in the complex? The genes might have to stay together to ensure the spatial and temporal colinearity.
- Extensive studies with **mouse gene knockouts** support the view that cells “know” their position along the A-P axis based on the **combination** of Hox genes (“**Hox code**”) they express; e.g., knockout of *Hoxc8* produces an extra rib (homeotic transformation) (Fig. 27). Exposure of pregnant mice to the vitamin A derivative **retinoic acid (RA)** can also cause lumbar ribs (as in your case this week).

## 2b) Hox gene expression is activated sequentially by retinoic acid

- When human teratocarcinoma cells (a model for the embryonic epiblast) are treated with **retinoic acid (RA)**, homeobox genes are activated **sequentially**. The anterior genes (Hox B1 and B2) are the most responsive to RA (Fig. 28). A possible interpretation is that the chromatin of Hox complexes is opened up for transcription in an orderly way.
- **Retinoic acid receptor (RAR)** is not a cell surface receptor but rather a ligand-activated transcription factor. **Nuclear receptors** work by entirely different principles than the growth factor receptors discussed earlier. Nuclear receptors contain a DNA-binding domain (DBD) and a ligand-binding domain (LBD) (Fig.

29).

- **Nuclear receptors** are molecular machines that activate transcription only when bound to ligands. When bound to their ligand, nuclear receptors bind to DNA and to co-activator proteins that switch transcription on.
- The same molecular design is used by **RA, cortisone, estrogen, progesterone, androgen, vitamin D** and **thyroid hormone receptors**. All these ligands are hydrophobic and can enter cells by diffusing through the cell membrane (Fig. 30).
- **Nuclear receptors** are very important in medical practice because administration of their ligands or of antagonists of these receptors allows one to regulate gene transcription, as in the case of RU-486 explained earlier (progesterone receptor antagonist).
- RAR binds to specific sequences in DNA called **RA response elements (RARE)**.
- The Hox complexes have a RARE in the DNA preceding paralogue 1 (Fig. 31). This RARE functions as a **DNA enhancer** element utilized for the expression of multiple Hox genes. The gene closest to the RARE enhancer is expressed first.
- This type of control of multiple genes by a common regulatory enhancer may explain why Hox gene complexes have stayed together in the genome during evolution.
- Normally, the anterior-most border of Hox gene expression is **rhombomere 3** (a hindbrain segment) (Fig. 32).
- When embryos are treated with retinoic acid, the anterior boundaries of Hox gene expression move anteriorly (ectopic expression).
- Exposure to RA causes Hox genes to be expressed ectopically in more anterior regions. The effect of RA is particularly devastating in regions of the head that do not express any Hox genes normally, such as pharyngeal arch 1 (maxilla and mandible) and the midbrain (Fig. 32).
- The ectopic activation of Hox genes explains to a large degree the teratogenicity of RA (Accutane, Isoretinoin), a vitamin A derivative used for the treatment of cystic acne. Despite label warnings, Accutane has been taken by at least 160,000 women of childbearing age. Common abnormalities found are cleft palate (maxilla), micrognathia (underdevelopment of the lower jaw), microtia (and deafness) and CNS malformations, as in your case on acne this week.

## 2c) Molecular regulation of Evolution and Development (Evo-Devo). Developmental Constraints.

- Genes that control development also control the evolution of body shapes between different species.
- The study of developmental control genes - such as the Hox genes that control the A-P axis and the Chordin/BMP system that controls the D-V axis - has revolutionized Evolutionary Biology.
- The bilateral invertebrates are called **protostomes** (mouth-first) because the blastopore gives rise to the mouth. The chordates, which include the vertebrates, are part of the **deuterostome** (mouth-second) group in which the blastopore becomes the anus (Fig. 33).
- The last common ancestor of vertebrates and invertebrates is called **Urbilateria** (Ur = primeval) and must have been a very complex animal, which used the Hox gene system to pattern the A-P axis and the Chordin/BMP system for D-V development. (Fig. 33).
- The Urbilateria gave rise to 30 bilateral animal phyla (out of the grand total of 35 animal phyla, each defined by a distinct body plan).
- From sequencing studies of several genomes it can be concluded that Urbilateria had a Hox complex containing at least seven Hox genes (Fig. 27).
- The deep conservation of development-controlling gene systems suggests that the possible outcomes of evolution were constrained by the use of these gene networks. Natural selection selected variations that had to be compatible with the ancestral developmental gene systems that determine body shape. Thus, not all potential evolutionary outcomes were possible.
- In summary, developmental gene networks such as Hox genes probably channeled the outcomes of evolution.
- **Conclusion:** The study of developmental control genes has revolutionized Evolutionary and Developmental Biology, helped explain the pathogenesis of human congenital malformations, and how cell-cell signaling by morphogen gradients is regulated.