Comparing the general principles of antero-posterior patterning of the limb and dorso-ventral patterning of the neural tube.

From the times of Aristotle to the modern age, the study of developmental biology has delivered more questions than answers. The understanding of body plan patterning was advanced greatly in the 1960's by the work of Lewis Wolpert with his proposition of the French Flag Model (Wolpert, 1969). Work with the fruit fly *Drosophila melanogaster* (Nussleinvolhard and Wieschaus, 1980) vindicated Wolpert's French Flag Model with the discovery of Hox genes; it is now widely accepted that segmental gene activation leads to localized morphogen gradients and these play a large part in determining the positional value of cells in many parts of an organism. Although developmental biology has advanced in the 21st century, the exact mechanisms for specification of limb structures are still ambiguous. Through the examination of the neural tube, it can be seen that many similar processes occur as in the limb; noticeably, Sonic Hedgehog (*Shh*) has been identified as the main gradient forming molecule. This essay will discuss the mechanisms of cell specification and differentiation in both the neural tube and limb with the aim of showing that many general principles can be found in body patterning.

The discovery of a polarising region at the posterior margin of the chick limb bud (Saunders and Gasseling, 1968) led to a repeat of Spemann and Mangolds classic grafting experiments. The chick wing was used as a model due to the favourable properties of a chick egg; easy grafting and external development allowing easy visualisation. The conclusion was that a group of posterior mesenchymal cells could determine the fate of other cells in a dose dependant way, as indicated by transplanting reduced or increased numbers of these cells to the anterior margin of the wing bud. A morphogen gradient was the best explanation for the presence of this region (Wolpert, 1969). Work by Tickle showed that Retinoic acid, a vitamin A derivative, had the potential to create a full duplication of

posterior digits on the anterior side of the wing (Tickle et al., 1982). Retinoic acid is now known to regulate *Meis* gene expression, which can lead to the transcriptional activation of *Shh* (Riddle et al., 1993).

Shh is one of three mammalian homologues to the drosophila *hedgehog* gene. *Shh* is synthesised as a 45kDa preproprotein, and undergoes a series of cleavages and modifications (as shown in Figure 1) leading to the production of a 20kDa signalling protein, *Shh-np,* derived from the N-terminus of the original molecule. The signalling molecule undergoes palmitoylation at an N-terminal cysteine, and cholesterol is also added to a C-terminal position before secretion. These modifications have been shown to increase signalling efficacy 30 fold; this is due to large molecular weight complexes being formed (Buglino and Resh, 2008). This protein complex is formed with the help of Dispatched1 (*Disp1*), a transmembrane protein which is required for effective diffusion of *Shh-np*; *Disp1*$^{-/-}$ mouse mutants show reduced ventralisation of neural progentitors, suggesting *Shh* is not reaching target cells (Caspary et al., 2002). Its role is possibly to aid the formation of a high molecular weight complex enabled by the post translational modifications. Without post-transcriptional modification, complex formation, and extracellular accessory proteins, the efficacy of *Shh* is greatly reduced (Chen et al., 2004).

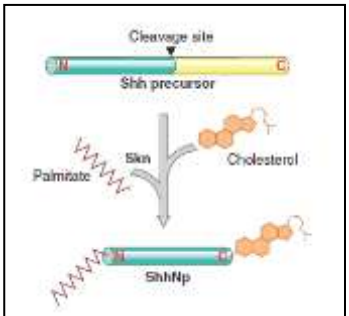

Fig. 1. *Shh* undergoes multiple post-translational modifications which aid in signalling efficacy

The neural tube cell identities are determined by the level of *Shh* expressed in that area; a distinct threshold must be reached for a different cell type to be produced. From the Floor plate, *Shh* spreads dorsally to produce ventral nerve precursor's dependant on the level and time of *Shh* expression. Interaction with a range of molecules aids its diffusion over the target area. Increased sulfation of Heparin sulphate proteoglycans (HSPG) increases the accumulation of *Shh* in the ventral

lumen of the neural tube. Alongside HSPGs, another class of *Shh*-binding molecules enhance signalling; *Cdo, Boc* and *Gas1* act cell-autonomously to potentiate signalling (Allen et al., 2007) whilst *Ptch1* and *Hhip1* inhibit signalling (Jeong and McMahon, 2005). Although functional compensation exist between these proteins, total loss results in severe phenotypes in mice; $Gas1^{-/-}Cdo^{-/-}$ double mutant embryos form no floor plate, p3 or PMN domains in the neural tube (Allen et al., 2007). Once *Shh* reaches its target the intracellular signalling pathway involved with transduction has shown to be active whether or not the ligand is present. Recent work (Zhao et al., 2007) has uncovered the fact that a graded concentration of extra cellular *Shh* leads to a graded reduction in repression of the intracellular signalling pathway, rather than a simple on or off situation. In the absence of *Shh,* the 12 pass trans-membrane protein Patched1 (*ptch1*) actively represses the seven pass trans-membrane protein smoothened (*Smo*). This repression prevents the movement of *Smo* to the primary cilia. The role of primary cilia in *Shh* signalling has only recently been uncovered. Striking similarities between mouse mutants deficient in intraflagellar transport and centrosomal proteins and chick *Talpid*[3] mutants (which have a range of neural tube and limb defects) suggested a link between hedgehog signalling and primary cilia (Yin et al., 2009, Corbit et al., 2005). Mutations inducing *Shh* or *Smo* activity causes localization of *Smo* to the cilia in both the neural tube and in the limb, however Invertebrate hedgehog signalling investigations have shown no requirement for cilia, indicating this could be restricted to vertebrates (Bangs et al., 2009). Translocation of *Smo* from the cytosol into the cilia leads to blockage of breakdown of Gli3A into Gli3R; the Gli family are zinc finger domain transcription factors involved in the expression of *Shh* gene targets. Their role appears to be multifunctional and graded; in response to increasing amounts of *Shh* they act as transcription factors, actively transcribing genes such as 5' Hox*d* genes and members of the TBox (*Tbx*) family (Matise and Joyner, 1999). *Tbx* over expression will create more posterior digits in chick legs and they have also been implicated in congenital digit defects in humans. In the absence of *Shh* GliR is produced in greater volumes and translocates to the nucleus where it represses gene expression.

Gli2 is also catalysed without *Shh* present, but unlike Gli3, it is broken down entirely. Gli1 appears to only be produced in the presence of Gli2 and 3 (Pan et al., 2006). From the dissection of the intracellular pathway we can show that varying the concentration of the extracellular signal *Shh* will produce a graded cellular response. This provides a mechanism for morphogen gradients.

To better understand the processes occurring in human appendages, an animal model with five digits was needed (Niswander, 2003). The mouse is a good model due to the similarity of its limbs to ours, and also due to its well characterised and mapped genetics. However, genetic manipulation of the mouse limb has not simplified the understanding of patterning; two or more models now exist which could feasibly explain the formation of unique digits in humans. The temporal model was proposed by the work from the Tabin research group and draws from two studies (Harfe et al., 2004, Scherz et al., 2007). The temporal expansion model suggests that posterior digits are derived from polarizing region cells with time-dependant direct *Shh* signalling providing patterning. As 3 digits would have to be produced from the same group of cells, a longer period of profileration would be required; this is feasible due to *Shh* being expressed for 60 hours in the mouse. Lineage mapping of *Shh*-expressing cells show that they contribute to digits 4 and 5 and part of digit 3. This process is independent of long range *Shh* diffusion as reduction in *Disp1* activity, required for *Shh* diffusion did not alter digit 4 and 5 development (Scherz et al., 2007). The proposed mechanism of anterior patterning relies on a more traditional morphogen concentration gradient.

The second model, termed the biphasic model, suggests that *Shh* has a dual role depending on time in development. Early on, *Shh* specifies limb digits in a concentration dependant manner, and then later it acts as a growth promoting mitogen to stimulate proliferation of cells in individual digits. Conditional rapid *Shh* inactivation showed digits did not form in the traditional Anterior to posterior sequence; instead digits 4 and 1 were formed first, followed by 5 and then 3. Visualization of cartilage condensation fitted this alternating sequence (Zhu et al., 2008). The point at which the

above models collide is in the identity of posterior digits; work aimed at clarifying the models (Scherz et al., 2007) also gives an ambiguous answer, which has been disputed by the two main research groups and could therefore lend evidence to either theory. Currently position and morphology are used to judge identity of digits. With the increasing complexity of manipulations taking place, a molecular marker would be extremely beneficial. A newer model which aims to integrate these theories is termed the Alternating Specification Theory (Fig. 2.). It suggests that digits 2 and 3 are specified as in the chick limb with a growth dependant morphogen gradient, whilst digits 4 and 5 rely on lengthened exposure to *Shh.* This model avoids the assumptions of early specification of anterior digit positions made by the temporal model, and still fits with evidence uncovered by the biphasic model (Towers and Tickle, 2009). Clinical evidence from the phenotype of patients with Feingold's syndrome also suggests that two separate growth mechanisms may be involved in mammalian patterning.

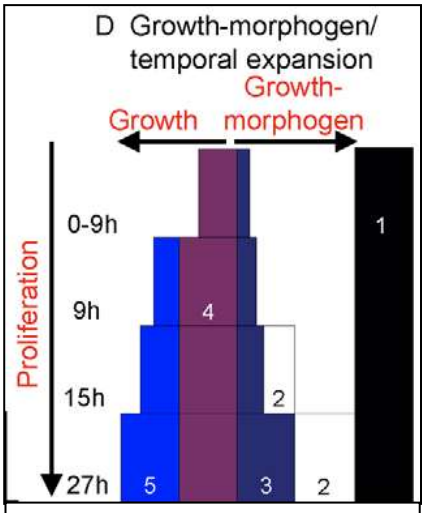

Fig. 2. Alternating specification may explain limb digit patterning.

Whichever theory is deemed most accurate, each one shows that growth plays a key role in digit patterning; *N-myc* and *Cyclin D* genes are regulators of the cell cycle, which can be under *Shh* control in the chick limb (Towers et al., 2008), But the exact mechanisms are still to be uncovered. The use of molecular markers and manipulations of the chick limb (where each digit can be distinguished by phalange number) will aid this process.

Growth is not thought to be a major contributing factor to progenitor development in the neural tube. The main problem to be uncovered here lies with the spread of *Shh* throughout the lumen. Using GFP linked *Shh* it was shown that floor plate derived *Shh* does not play a role in induction of ventral nerve progenitors. Its role may instead be secondary; neuronal guidance and glial cell

specification. This finding means that the gradient of *Shh* is derived solely from notochord *Shh,* but questions still remain over how the morphogen gradient is maintained. Recent studies have also shown that activation of the signalling pathway is dependent on the duration of time *Shh* is available to a cell; the 'temporal adaptation' model proposes that desensitisation leads to the need for increasing *Shh* to activate the same amount of Gli transcription factors (Dessaud et al., 2007). Time sensitivity is likely to be controlled through negative feedback loops mediated by the many extracellular accessory proteins described earlier. Together, these processes allow for a small group of signalling cells to produce a defined and exact molecular gradient.

More than just *Shh* is involved in patterning in the neural tube; ectoderm overlying the roof plate of the neural tube secretes multiple members of the BMP and Wnt families of signalling molecules (see Fig.3.A). Later in development, the dorsal most cells of the roof plate also secrete Wnts. The study of the Gli3 gene has shown highly conserved non coding DNA on either side of the coding region, which has a high binding specificity to Wnt molecules. This could indicate that Wnt actively represses Gli3 in dorsal areas of the neural tube, leading to a defined area of expression. Integration of signalling pathways is also present in the limb (Fig 3.B.); unlike in the neural tube, however, the contrasting signals come from an area adjacent to the polarizing region, the apical ectodermal ridge (AER). FGF from the AER, Wnt signals from dorsal ectoderm and BMPs from the ventral ectoderm all interact and influence *Shh* signalling,

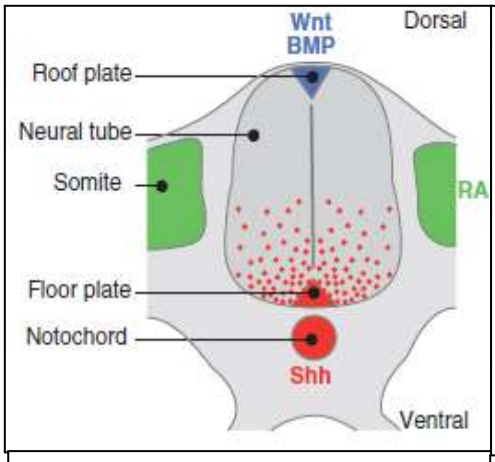

Fig. 3.A. *Shh* does not act alone in neural tube patterning; Wnt signalling from the roof plate acts to restrict Gli expression

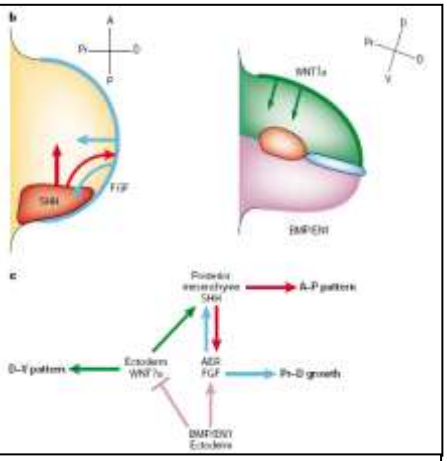

Fig. 3.B. correct patterning of the distal limb structures requires Integration of signalling systems

The neural tube and distal appendage both rely on *Shh* in combination with other signals to produce a morphogen gradient. In each case, downstream signalling pathways effect a change of gene expression leading to distinct and local changes in morphology. The two regions differ in the fact that limbs require growth to amplify the morphogen gradient, whilst the neural tube simply regulates the diffusion of the morphogen. The presence and activity of extracellular modulators in the limb also needs to be quantified.

As work progresses on the subject of morphogen mediated patterning in both the neural tube and the limb, more complex methods of study are being employed. Limb studies are still relying widely on visual experiments, for example grafts and fluorescence, to give clues as to the molecular identity of controlling elements. In the neural tube such experiments are impossible. Techniques such as microarray assay and gene chip experiments have been used. In the neural tube it is now possible to hybridise DNA or RNA to a chip and show which genes are up or down regulated under certain experimental conditions. Similar experiments have taken place in the limb, but due to ambiguity of digit identities within and between species, much work is still to be done.

Words: 2,953

Figures:

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