Statement of Research E. M. De Robertis

From the renewal of an HHMI Investigatorship (October 2009)

Molecular Mechanisms of Embryonic Self-regulation

During the period under review, we discovered a biochemical pathway of interacting extracellular proteins that explains, for the first time, the molecular mechanism of self-regulation of a morphogenetic field. Three new principles emerged. First, we found that long-range cell-cell communication during dorsal-ventral (D-V) patterning occurs through the action of extracellular regulators of growth factors that control the flow of BMPs, which are then released by Tolloid proteinases at a distance from where they were produced\textsuperscript{1-4}. Second, we discovered that the D-V BMP and the anterior-posterior (A-P) Wnt signaling gradients are integrated via phosphorylations of the transcription factor Smad1/5/8 by BMP receptor (BMPR) and GSK3, a protein kinase regulated by Wnt\textsuperscript{5,6}. Third, we uncovered unexpected connections between cell division and protein degradation\textsuperscript{7} that open new cell biological directions of great promise for the next funding period.

Within the organism, cells proliferate, differentiate and die as part of groups of hundreds or thousands of cells, called morphogenetic fields, that have the remarkable property of self-regenerating after perturbations. Fields were first identified by Ross Harrison in amphibian embryos\textsuperscript{8}. He
found that half of the mesoderm of a future forelimb would induce a whole limb - not half a limb - after transplantation\textsuperscript{8,9}. The \textit{Xenopus} blastula also forms a morphogenetic field, which can be bisected and still give rise to well-patterned identical twins\textsuperscript{10,11}. Understanding self-regulation has been the long-term goal of my research\textsuperscript{12}.

**Chordin as the key to Spemann’s Organizer**

Embryonic cell differentiation is regulated by a D-V activity gradient of BMPs (Bone Morphogenetic Proteins, part of the TGF-β superfamily of growth factors), transduced through phosphorylation of the transcription factors Smad1/5/8. This morphogen gradient determines embryonic tissues\textsuperscript{13-15}. For example, in the ectoderm, BMP inhibition induces Central Nervous System (CNS) while high BMP signals induce epidermis. The precise allocation of tissue types requires a reproducible and resilient molecular mechanism.

In 1924, Hans Spemann and Hilde Mangold performed a transplantation experiment that started a new era in embryology. They discovered that a small group of dorsal cells, called the organizer, was able to respecify the host’s embryonic field, inducing a Siamese twin including a complete CNS\textsuperscript{16}. Spemann received the 1935 Nobel Prize in Physiology for this discovery of embryonic
induction by organizer. Using a *Xenopus* Spemann organizer cDNA library, we isolated many secreted molecules expressed in this tissue, such as Chordin, Frzb, Crescent, and Cerberus. Surprisingly, most of these proteins were novel and functioned as inhibitors of growth factors, rather than as growth factors as we had expected.

The key molecule for understanding embryonic induction by organizer tissue is Chordin, a BMP antagonist secreted in prodigious amounts by the organizer (estimated at 33 nM in the gastrula extracellular space if uniformly distributed, but reaching much higher levels on the dorsal side). As shown in the previous period, transplanted organizers depleted of Chordin (using antisense morpholino oligos) lose all embryonic inductive power. Chordin and a co-factor called Twisted gastrulation (Tsg) form a ternary complex with BMPs, via the Cysteine-rich (CR) modules in Chordin. This inhibits binding to BMPR and the complex can diffuse in the intercellular space. Inhibition is reversible, for Zinc metalloproteinases of the Tolloid (Tld) family can cleave Chordin at two specific sites, releasing BMPs for signaling. Parallel work by other researchers in *Drosophila*, zebrafish, and various
organisms has established that the Chd/Tsg/BMP/Tld biochemical pathway constitutes the ancestral embryonic D-V patterning mechanism.

Self-regulation is mediated by dorsal and ventral signaling centers

During the period being considered, we showed that embryonic self-regulation requires a ventral signaling center. A duel between the dorsal organizer and the ventral center - which secrete distinct sets of antagonists and BMPs under opposite transcriptional control – explains self-regulation. A molecular see-saw, in which BMP signals activate ventral genes and repress dorsal ones, generates a self-adjusting gradient of BMP activity. We elucidated the extracellular biochemical pathway illustrated nearby, in which blue arrows indicate transcriptional regulation by Smad1/5/8, black arrows show the direct protein-protein interactions demonstrated biochemically in our laboratory, and red arrows denote the flux of Chordin/Bmp complexes towards more ventral regions, as discussed below.
Self-regulation through opposite transcriptional control of BMPs

The ventral center expresses BMP4 and BMP7 and the dorsal center BMP2 and ADMP25-27. Expression of BMPs in dorsal tissue is paradoxical, because this is the region of the embryo where BMP signaling is lowest. Bruno Reversade and I found that when all four BMPs are depleted simultaneously, the embryonic field collapses and the entire embryo becomes covered in CNS1. By grafting wild-type tissues into BMP-depleted embryos, we demonstrated that both the dorsal and ventral centers serve as sources of BMPs that diffuse over long distances in the embryo, rescuing CNS (marked by Sox2) and epidermal (cytokeratin) patterning. These two sources of BMP signals originating from opposite poles of the embryo generate a self-regulating gradient1.

Extracellular D-V communication via Sizzled, Crossveinless-2 and Tolloid

During this period we also found that the BMP gradient is established and self-regulated through three novel extracellular protein-protein interactions.

**Sizzled** is a *Xenopus* sFRP (secreted Frizzled-related protein) expressed ventrally28. With Hojoon Lee we discovered that Sizzled binds to tolloids via its
frizzled domain. In enzyme kinetic analyses, Sizzled was a competitive inhibitor of Chordin cleavage, with a $K_i$ in the same range (20 nM) as the $K_m$ of the Tolloid enzyme for its substrate. This reaction provides self-regulation, for when BMP levels increase, Sizzled expression is upregulated, causing Chordin levels to increase, inhibiting BMP and restoring the gradient.

**Crossveinless-2** (CV2) is a ventral center secreted protein that contains five Chordin-like CR modules, first cloned in *Drosophila* by Seth Blair and subsequently by us in the mouse. CV2 has pro-BMP effects in *Drosophila* wing cross veins. However, overexpressed CV2 is a strong BMP inhibitor that binds BMPs preventing BMPR binding. CV2 remains tethered via glypicans/Dally to the surface of the cells that secrete it, mediating endocytosis of BMPs. With Andrea Ambrosio we found the molecular explanation for the pro-BMP effects of CV2: it binds with high affinity (1-2 nM) to Chordin protein. CV2 binds with even higher affinity to Chordin/BMP complexes or Chordin fragments cleaved by Tolloid protease. Thus, CV2 functions as a molecular sink, concentrating Chordin/BMP complexes diffusing from more dorsal regions. Once in the ventral side, BMPs are released from Chordin by Tolloid, allowing maximal BMP signaling.
Tolloid chordinase activity is critical to D-V patterning, so we developed a new fluorogenic substrate, mimicking a Chordin cleavage sequence, for enzyme kinetic studies. We discovered that BMP4 is a non-competitive inhibitor of Tolloid or BMP1 (an alternatively spliced form of this enzyme). BMPs were initially purified as bone-inducing proteins from bone matrix. BMP2-7 were TGF-β-related, but why the BMP1 proteinase co-purified as well had remained a mystery. We found that BMP4 binds specifically to the “CUB” domains of BMP1/Tolloid with high affinity (K_D 15-20 nM), establishing an enzymatic negative feedback loop in the ventral side.

These three novel protein-protein interactions – Sizzled-Tolloid, CV2-Chordin, and Tolloid-BMP4 – help explain how the dorsal and ventral centers communicate over long distances to maintain a self-adjusting signaling gradient.

MAPK activation inhibits Smad1, explaining “heterologous” neural inducers

We became interested in Smad1 after finding that neural induction by IGF and FGF is caused by MAPK phosphorylations in the central “linker” region of Smad1 that inhibit its nuclear activity. During the 1930s, amphibian experimental embryology ground to a halt when it was found that dead organizer tissue, or non-specific substances such as nucleoproteins, sterols, and even sand
particles, could cause CNS differentiation when placed in contact with salamander ectodermal explants\textsuperscript{10,11}. We found that the neural induction caused by dissociation of \textit{Xenopus} ectodermal cells is due to sustained activation of MAPK and Smad1/5/8 inhibition\textsuperscript{39}. Using \textit{Ambystoma maculatum} embryos over three breeding seasons (Jan.-Feb.), Cecilia Hurtado and I showed that “heterologous” neural inductions, such as culturing ectoderm attached to a glass surface or sandwiched around sand particles, were caused by MAPK/Erk activation\textsuperscript{40}. The heterologous inducers that had brought down Spemann’s edifice have finally found a molecular explanation: the activation of MAPK linker phosphorylations that inhibit Smad1/5/8.

**Integrating A-P and D-V positional information via Smad1 phosphorylations**

When twins are generated, the D-V and A-P body axes are seamlessly integrated. Christof Niehrs (Heidelberg) has proposed that the A-P axis is established by a gradient of Wnt, maximal in the posterior\textsuperscript{41}. During our investigations on neural induction, we noticed GSK3 sites in Smad1/5/8 that could be primed by the MAPK linker sites\textsuperscript{5} (GSK3 phosphorylates substrates containing Serine or Threonine four amino acids upstream of a pre-phosphorylated residue\textsuperscript{42}). This was very exciting, because the canonical Wnt pathway signals by regulating the activity of GSK3\textsuperscript{43}.
With Luis Fuentealba and Edward Eivers, we generated high-titer phospho-specific antibodies for human pSmad1\textsuperscript{GSK3} and pSmad1\textsuperscript{MAPK}, and Drosophila pMad\textsuperscript{GSK3} and pMad\textsuperscript{MAPK}. This allowed us to identify a novel cell biological pathway that terminates Smad1 signaling\textsuperscript{5,6}. After activation of BMPR, Smad1 is sequentially phosphorylated at C-terminal, MAPK, and GSK3 sites. Phosphorylation by GSK3 is required for polyubiquitylation by Smurf1 E3 ligase, transport along microtubules to the centrosomes, and proteasomal degradation\textsuperscript{5}. Epistatic experiments in Xenopus\textsuperscript{5} and Drosophila\textsuperscript{6} showed that Wnt/Wg signaling inhibits Smad1/Mad phosphorylation by GSK3, and that Smad1/Mad are required for many aspects of Wnt signaling. Thus, we have discovered a new branch of the “canonical” Wnt pathway (previously believed to signal exclusively by increasing β-Catenin levels), in which Wnt increases the duration of the BMP/Smad1 signal.

Embryonic positional information may be encoded by a system of Cartesian coordinates in which the D-V BMP and the A-P Wnt gradients are integrated at the level of Smad1/5/8 phosphorylations\textsuperscript{5}. In this view, the BMP gradient determines the intensity (or amplitude) and the Wnt
gradient the duration of Smad1/5/8 signals. Smad1/5/8 are transcription factors that coordinate the expression of hundreds of downstream target genes; this hard-wired system of signaling integration may provide resilience to the embryonic body plan.

Conclusions

During the period under review we discovered a biochemical pathway for the self-regulation of the *Xenopus* embryonic field. Self-regulation is mediated by extracellular signaling between dorsal and ventral signaling centers that secrete cocktails of BMPs and their regulators. These centers self-adjust to signaling changes in one another because they are under opposite transcriptional control by BMPs. The D-V BMP gradient is integrated with the A-P Wnt gradient intracellularly through phosphorylations of Smad1/5/8 by GSK3, such that Wnt increases the duration of the BMP signals.
References


