Advanced Review

Neural induction and early patterning in vertebrates

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In vertebrates, the development of the nervous system is triggered by signals from a powerful 'organizing' region of the early embryo during gastrulation. This phenomenon—neural induction—was originally discovered and given conceptual definition by experimental embryologists working with amphibian embryos. Work on the molecular circuitry underlying neural induction, also in the same model system, demonstrated that elimination of ongoing transforming growth factor-β (TGFβ) signaling in the ectoderm is the hallmark of anterior neural-fate acquisition. This observation is the basis of the 'default' model of neural induction. Endogenous neural inducers are secreted proteins that act to inhibit TGFβ ligands in the dorsal ectoderm. In the ventral ectoderm, where the signaling ligands escape the inhibitors, a non-neural fate is induced. Inhibition of the TGFβ pathway has now been demonstrated to be sufficient to directly induce neural fate in mammalian embryos as well as pluripotent mouse and human embryonic stem cells. Hence the molecular process that delineates neural from non-neural ectoderm is conserved across a broad range of organisms in the evolutionary tree. The availability of embryonic stem cells from mouse, primates, and humans will facilitate further understanding of the role of signaling pathways and their downstream mediators in neural induction in vertebrate embryos.

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ESTABLISHMENT OF THE NEUROECTODERM IN VERTEBRATES

In all vertebrates, the fertilized egg divides to generate a blastocyst (or blastula). Three different territories called embryonic germ layers, ectoderm, mesoderm, and endoderm, emerge in the blastula. In the amphibian embryo, where the dorsal (D) and ventral (V) sides of the embryo are specified during fertilization, each germ layer has a distinct D–V polarity and is fated to generate different tissues as the embryo matures (Animation 1, Supporting Information). Subsequently during gastrulation, the primitive ectoderm (called epiblast) covers the outside of the embryo and forms different tissue derivatives depending on position along the embryonic D–V axis. The central nervous system (CNS) derives from the most dorsal region of the ectoderm, which thickens and flattens after gastrulation to form the neural plate. During subsequent stages, the plate rolls into a tube, separates from the overlying epidermis, and goes on to form the brain at the anterior, and spinal cord at the posterior end. In contrast, on the ventral side, most of the remaining ectoderm forms the epidermis. The neural crest forms where the dorsal and ventral boundaries meet at the edge of the neural plate. This progenitor cell population detaches and migrates throughout the embryo to form the peripheral nervous system, cranium, and cartilage of branchial arches. Ectodermal cells at the most anterior edge of the neural–epidermal boundary give rise to placodal areas that will form sensory organs—such as the ear and nose—as well as some cranial sensory ganglia (Figure 1). At the start of gastrulation, cells from any part of the ectoderm can still develop as either epidermis or neural tissue, but by the end of gastrulation commitment has occurred.[1] These events are characteristic of all vertebrates although the timing

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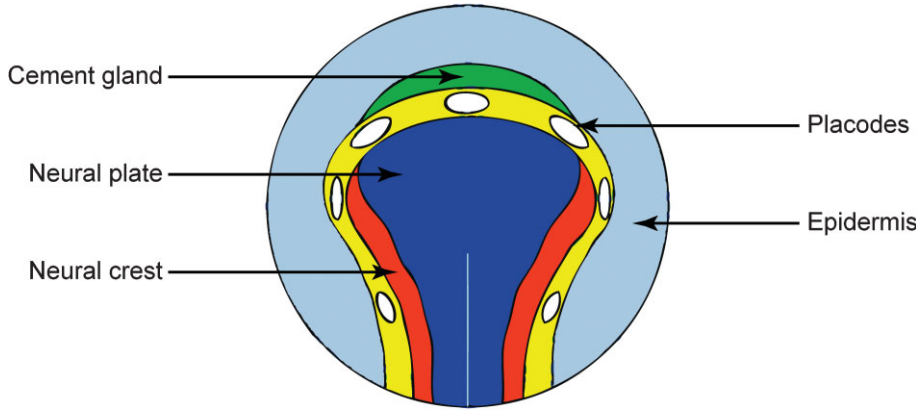

FIGURE 1 | Fate map of the anterior border of the neural plate in *Xenopus* embryos. Schematic of dorsal–anterior (head-on) view of a *Xenopus* neurula (the ventral side is up, and the dorsal side is down).[2] Different colors highlight different fates.

and geometry vary across phylogeny. Thus, the first step in the establishment of the nervous system in vertebrates involves the partition of the ectoderm into epidermal and neuroectodermal primordia during gastrulation.

LESSONS FROM EXPERIMENTAL EMBRYOLOGY

The Mangold and Spemann Experiments

The fundamental insight into how the neural plate is established came from the famous experiment of Mangold and Spemann, in which tissue from the dorsal blastopore lip (located in the dorsal mesoderm) of an early newt gastrula was grafted to the ventral side of a second embryo.[3] The host embryo developed a second set of dorsal axial structures on the ventral side, including a well-organized second nervous system. This experiment suggested that signals from the dorsal lip region, which became known to amphibian embryologists as 'Spemann's organizer', were responsible for diverting nearby ectoderm to a neural fate (Animation 2, supporting information). In normal development, cells of the organizer involute into the embryo during gastrulation, giving rise to dorsal structures in the mesoderm such as muscle and the notochord that underlie the future neural plate. Lineage tracing experiments[4] demonstrated that while the entire mesodermal derivative of the secondary axis was derived from the progeny of the grafted cells, the entire nervous system (with the exception of the floor plate) was derived from the host. This confirmed that signals from the organizer caused ventral ectodermal cells - that normally would have given rise to epidermis - to convert instead to neural fate. These results were also reproduced in fish by Oppenheimer, where grafting pieces of organizer (called the shield in fish) were able to induce a secondary axis in the host fish.[5,6] Analogous grafting experiments carried out in the chick and the mouse embryos (where the organizer is called the node) led to similar results,[7,8] highlighting the evolutionary conservation of the 'organizer' as source of signal(s) that is sufficient to generate the entire nervous system.

Development of the Animal Cap Explants and Assays

The organizer graft experiments subsequently led to an early form of tissue culture, where the ectoderm of the blastula, called the animal cap, was explanted and cultured in simple pond water. By itself, the isolated animal cap only formed epidermal tissue[1] (Animation 3, supporting information), but when recombined with explants derived from another portion of the embryo, the same explant generated other cell types. Mesodermal derivatives, for example, arose in animal cap explants after exposure to early endoderm, whereas neural tissue arose after exposure to dorsal mesoderm of different ages, including organizer tissue.[9,10] This work demonstrated the remarkable potential of animal cap cells to form an array of mesodermal and ectodermal derivatives, depending on the inductive interactions that were encountered over the course of early development. In addition, these experiments reinforced the view from the organizer transplant experiments that the ectoderm forms epidermis as a default state. The obvious line of experiments that followed was to substitute the inducing tissue (the vegetal pole for mesoderm induction and the organizer for neural induction) with cocktails of extracts or factors that would elicit an inductive response from the animal cap followed by a morphological and molecular diagnostic of the induced fate.

Decades after the discovery of the organizer, however, the identification of the molecules underlying neural induction remained elusive. Limitations in existing techniques thwarted biochemical approaches to identify the endogenous inducers, while the animal cap had the capacity to non-specifically convert to neural fate in response to a variety of materials, often from rather exotic sources (such as guinea pig bone marrow, blue jay liver, and boiled dead organizer). More unexpected and surprising was the fact that simple cell dissociation of the animal cap led to conversion of cells from epidermal to neural fate *directly*, without previous or concomitant induction of mesoderm (Animation 4, supporting information). To explain these results, neural inducers were proposed to be widely distributed and under negative control in the animal cap by factors that could be lost by dissociation, but the nature of either the inducer or its inhibitor remained undefined. Thus, many decades after the discovery of the organizer, the study of neural induction had reached a virtual impasse.

LESSONS FROM MOLECULAR EMBRYOLOGY

The field of embryonic induction was invigorated in the early 1990s by the introduction of modern molecular techniques to complement classical experimental embryology in *Xenopus*. Conversion of the animal cap cells into mesodermal or neural tissue could now be unambiguously scored using fate-specific molecular markers to diagnose induced cell types, and potential inducers could be tested as purified peptide growth factors or by microinjection of synthetic RNA. These approaches soon led to the discovery that physiological amounts of polypeptide growth factors of the fibroblast growth factor (FGF) and transforming growth factor-β (TGFβ) family were sufficient to impose mesodermal fates in animal caps. For instance, animal caps treated with increasing thresholds of Activin - a member of the TGFβ family - responded by forming ventral, lateral, and dorsal mesoderm (including the organizer).[11] Work in a variety of vertebrate model systems has irrevocably established the pivotal role of these growth factors in the formation of mesodermal and organizer tissues in the embryo.[12] Animal caps also formed some neural tissue when treated with high concentrations of a mesodermal inducer such as Activin, suggesting that neural tissue was also induced by growth factor action. However, neural induction in this case was likely to be indirect. Experimentally, the difference between *indirect* versus *direct* neural induction in animal cap explants can be assessed by examining tissue-specific markers, where direct induction is characterized by the expression of neural markers (*NCAM*) in the *absence* of mesodermal/organizer-specific molecular markers (*brachyury* and *goosecoid*). Expression of both markers, however, is a strong indication of an indirect cascade where one signaling factor induces responding cells to release additional inducing factors, a phenomenon that continues to confounding inducer studies today (see below). It was only when the field of mesodermal induction turned to the study of the Activin receptor - one of the first TGFβ family receptors cloned in mammals and in *Xenopus* -[13] that the nature of direct neural inducers began to emerge.

Molecular Basis of Neural Induction

To ask if Activin signaling was necessary for mesoderm induction and also to validate the *in vivo* relevance of these findings, a synthetic RNA encoding a dominant-negative mutant form of the Activin receptor (DN-ActRIIB) was engineered to antagonize the inducing activity of Activin. Like many dominant-negative mutants, DN-ActRIIB is now known to be broad acting, and capable of blocking all TGFβ ligands, including Nodals, bone morphogenetic proteins (BMPs), and growth differentiation factors (GDFs) (i.e., much of the TGFβ pathway shown in Figure 2). When expressed in early embryos, DN-ActRIIB completely inhibited endogenous mesoderm induction, in line with the idea that TGFβ signaling through the ActRIIB receptor is necessary for mesoderm induction *in vivo*. DN-ActRIIB expression in animal caps also completely blocked mesoderm induction by Activin. Unexpectedly, however, when expressed alone in control animal caps in simple pond water (i.e., not exposed to any ligand), it led to a strong conversion of fate directly from epidermal to neural (Animation 5, supporting information), in the absence of neural-inducing signals from Spemann's organizer.

THE 'DEFAULT MODEL' MODEL OF NEURAL INDUCTION

That neural tissue is induced by cell dissociation or by expression of DN-ActRIIB were disparate observations with one common denominator: they both made sense if traditional thinking about neural induction was inverted. In this revised view, the default fate for animal caps would not be epidermal but anterior neural. Ongoing signals in the explants repress the natural tendency of the cells to become neural by inducing the epidermal fate. When this signaling was interrupted (by either expression of DN-ActRIIB or cell dissociation), cells assumed a forebrain fate. The model

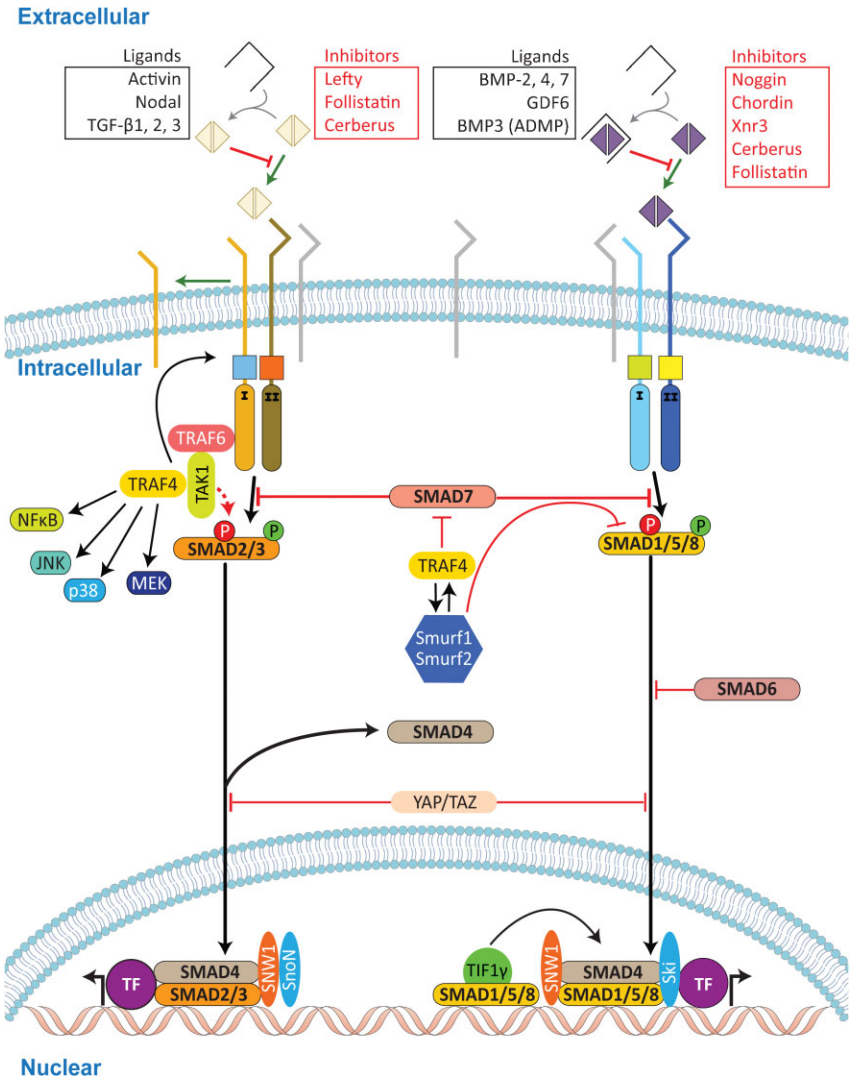

FIGURE 2 | The transforming growth factor-β (TGFβ) pathway. More than 30 different TGFβ ligands are encoded in the vertebrate genome. They include members of BMPs, GDFs, Activins, Nodal, and TGFβs, all of which activate the TGFβ pathway. The activity of these ligands is regulated by a large number of secreted inhibitory factors (Table 1) that inhibit TGFβ signaling extracellularly. Upon secretion, homodimer or heterodimer of TGFβ ligands that escape inhibition bind to TGFβ receptors at the cell membrane. Ligands act as morphogens exerting diverse cellular responses based on the levels and duration of signaling. Dimeric TGFβ ligands bind type II receptors that phosphorylate and activate type I receptors in a heterotetrameric complex. Receptor activation, in turn, leads to the propagation of signaling by at least two pathways involving Smad (in the canonical pathway) or Traf/TGFβ-Activated-Kinase-1 (TAK1, in the non-canonical pathway). In the canonical pathway, a type I receptor propagates the signal by phosphorylating serine residues located at the C-terminus of receptor-Smads (R-Smads). Two groups of R-Smads transduce signals: R-Smads 2/3 (from Activins/Nodals and TGFβ1/2/3) and R-Smad1/5/8 (from BMP2/4/7 and some GDFs). R-Smads are part of a trimeric complex with a common mediator Smad—called co-Smad4—that translocates to the nucleus to regulate transcription via transcription factors. As in the extracellular space, a series of inhibitors influences input from TGFβ signaling inside the cell at multiple levels. At the membrane level, coreceptors, such as Bambi, EGF-CFCs, and Tomoregulins, regulate the activity and selectivity of TGFβ receptor transduction. Downstream of receptor activation, inhibitory influences on R-Smads occurs by linker phosphorylation via MAPK, GSK3β, and CDKs, providing connections between TGFβ and other signaling pathways. TGFβ signaling itself also has the ability to phosphorylate the R-Smad linker. Linker phosphorylation leads to either degradation via ubiquitination by Smurf1/2 or changes in R-Smad specificity of gene regulation. Smad6 and Smad7 provide another level of inhibition. Smad6 acts in a BMP-dependent manner to compete with Smad4 binding and inhibit nuclear translocation of Smad1/5/8, whereas Smad7 acts in a ligand-independent manner to inhibit the pathway at multiple levels, including downstream of the activated type I receptor. Finally, dephosphorylation of the C-terminal end of R-Smads, by phosphatases such as small C-terminal domain phosphatases, has also been shown to downregulate the signal. The YAP/TAZ complex regulates Smad nuclear translocation and connects to the Hippo pathway. The non-canonical TGFβ pathway is not as well understood; however, type II TGFβ receptors have been shown to signal through the Traf/TAK1 proteins. TAK1, in turn, activates JNK, p38, and MEK and the NF-$\kappa\beta$ pathway. As TAK1 can also be activated by a variety of cytokines, the WNT pathway, and the MAPK pathway, it provides yet another integration site for crosstalk amongst different signaling pathways.

proposed furthermore that neural inducers from the organizer might work by locally antagonizing these epidermal-inducing signals, allowing dorsal ectoderm to follow its 'default' anterior neural fate. These considerations led to the formulation of a new model of neural induction called the default model,[14,15] which was initially controversial because it implies that vertebrate embryonic cells will become nerve cells of the forebrain unless told otherwise.[16] However, subsequent work on the endogenous epidermal inducing signal(s) also shed light on the inhibitory nature of the organizer-derived signal.

Epidermal Induction

The nature of the epidermal-inducing signals was revealed by experiments in which dissociated animal cap cells were treated with purified proteins. As animal cap cells are neuralized upon dissociation, candidate factors could be tested for the ability to suppress neuralization and restore epidermal specification, thus replacing endogenous signals lost on dispersion. Treating these cells with Activin blocked neuralization, but it did so by inducing mesoderm.[17] However, another member of the TGFβ superfamily, BMP4, not only suppressed neuralization but also proved to be a potent epidermal inducer (Animation 6, supporting information). Significantly, the dominant-negative Activin receptor blocks signaling not only by BMP4 but also by related molecules, BMP2 and BMP7. These also happen to be epidermal inducers in this assay.[18] The expression pattern of the BMPs is in accord with their proposed role as neural inhibitors: BMP4 RNA is found throughout the ectoderm at the start of gastrulation, subsequently disappearing from the prospective neural plate.[19–21] Epidermal differentiation is also blocked in animal caps after inhibiting endogenous BMP signaling using dominant-negative BMP receptors,[21–23] dominant-negative BMP4 or BMP7 ligands,[24] or antisense BMP4 RNA,[22] suggesting further that the BMP family members are essential epidermalizing factors *in vivo*.

Endogenous Neural Inducers

Three independent approaches in *Xenopus* led to the identification of the endogenous neural inducers. The first was based on screening cDNA libraries for their neural-inducing activity. This led to the discovery of the first *bonafide* endogenous direct neural inducer: *noggin*.[25] The second involved isolating organizer-specific genes. This led to the identification of *chordin*.[26] Finally, testing the activity of candidate TGFβ inhibitors led to the characterization of *follistatin*. All three genes are secreted proteins, specifically expressed in the organizer, and with direct neural-inducing ability. This established that the organizer was indeed the source of signals that could induce neural tissue. At the time, the fact that one of them, follistatin, was a known extracellular inhibitor of a few TGFβ ligands was in agreement with the default model.[27]

Convergence and Reconciliation for Neural Induction

The identification of *noggin*, *chordin*, and *follistatin* localized in the organizer led at first to the search for receptors that could instructively transduce their activity during neural induction. However, biochemical characterization of these neural inducers established that they are all potent extracellular inhibitors of TGFβ family signaling (the different arms of the TGFβ pathway are shown in Figure 2). They bind with high affinity to the ligands, thus preventing them from activating their cognate receptors.[28,29] These observations suggested that high morphogen thresholds of BMP signaling on the ventral side of the ectoderm promote epidermal fate, whereas on the dorsal side BMP signaling is kept low by organizer-generated BMP inhibitors, thus promoting a neural fate (Figure 3). There is now an extensive list of secreted TGFβ inhibitors, some of which are expressed in organizers isolated from a variety of species (Table 1). Every member of this list that has been tested in the animal cap assay has been shown to act as a direct neural inducer. In addition to these natural inhibitors, a number of small molecules that block the different branches of the TGFβ signaling have been characterized (Table 2). As with endogenous inhibitors, they have been shown to act as direct neural inducers when tested in the context of animal cap explants or in mammalian pluripotent stem cells, as discussed below.

Animal cap cells pass through two competence phases sequentially: first, in the mid and late blastula stages when they respond to Activin/Nodal signaling by forming mesendodermal derivatives. This is followed by a second phase in gastrula and early neurula when they respond to BMP signaling by differentiating into epidermis. In the default model therefore, a neural fate ensues only when animal cap cells avoid both mesoderm- and epidermal- inducing signals. Perhaps this explains why coinhibition of both SMAD1/5/8 and SMAD2/3 branches of the canonical pathway induces a neural fate more potently than each alone[89] in a manner similar to DN-ActRIIB, which interferes with both Activin/Nodal and BMP signaling.

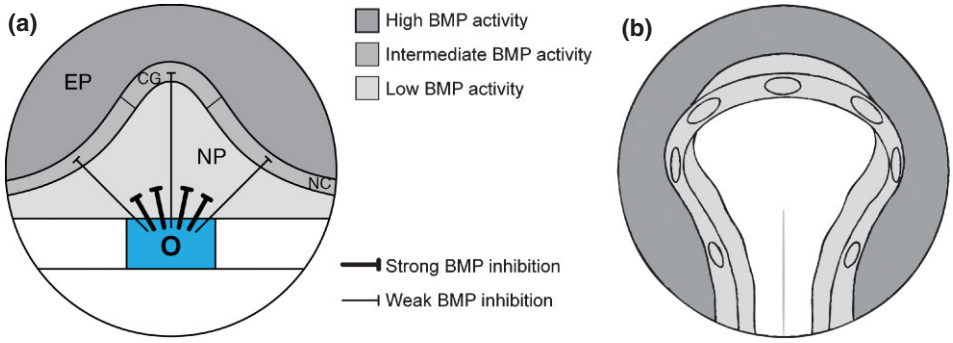

FIGURE 3 | Schematic of graded BMP activity in the gastrula and neurula ectoderm. (a) A schematic fate map of the early gastrula shows the approximate positions of the future neural plate (NP), border region, and epidermis, viewed from the dorsal side. The cement gland (CG) and sensory placodes form in the anterior border region mid-dorsally, whereas the neural crest arises more laterally. Diffusible antagonists produced in the organizer region of the mesoderm, including noggin, chordin, and follistatin, result in a graded distribution of BMP signaling in the neighboring ectoderm. The relative position of epidermis (EP), NP, organizer (O, in blue), CG, and neural crest (NC) is shown. Sensory placodes form at various positions in the border region but are not shown here for simplicity. (b) Correlation with neurula fate map shown in Figure 1.

Evolutionary Conservation of Molecular Circuitry Underlying Neural Induction

Inhibition of ongoing TGFβ signaling to delineate neural and non-neural ectoderm has been conserved evolutionarily. In the fruit fly *Drosophila* for example, *short gastrulation* (*sog*) is a homolog of the organizer-specific BMP inhibitor *chordin*. *Sog* was identified in a systematic screen for genes involved in patterning the *Drosophila* embryo along the D–V axis.[90] As in vertebrates, the dorsal and ventral regions of the ectoderm of the *Drosophila* embryos generate different fates. However, as the embryonic axis is flipped in Arthropods compared to Chordates, the epidermis forms in the dorsal regions, whereas the neural tissue arises from a ventral position. Nonetheless, the molecular circuitry involving inhibition of BMP in segregating dorsal from ventral ectoderm operates in precisely the same manner as in vertebrates.[91,92] *Drosophila* counterparts of the BMP signaling branch of the TGFβ pathway, including ligands, receptors, and inhibitors such as *Sog*, generate an activity gradient of *Dpp*, a BMP-like ligand, from high dorsal to low ventral, thus specifying epidermal and neural tissue, respectively.[93] Indeed, *Sog* has been shown to directly promote neuroectoderm specification in blastoderm drosophila embryos by inhibiting the anti-neurogenic and dorsalizing activity of *Dpp*.[94] This activity of *Sog* is also shared by other annelids, such as spider and beetles.[95] Similarly, inhibition of HrBMPb, the ascidian homolog of BMP, is required for induction of rostral neural lineages in sea squirts (urochordates), and its overexpression results in a fate switch of the presumptive neural cells to epidermal lineages.[96] A notable exception to this rule is found in Acorn worms (hemichordates), which lack both, an organized CNS as well as segregation of the ectoderm into neurogenic and epidermal territories. Exposure of these embryos to exogenous BMPs does not repress neural markers, and conversely, BMP knockdown does not promote neuralization, even though it has a role in D–V patterning in these embryos.[97] Taken together, these observations perhaps suggest that D–V patterning by the BMP pathway is an ancient mechanism that evolved early in metazoans and was subsequently utilized by many metazoans that have a CNS as a means of establishing different ectodermal fates in the early embryo.[95] The conservation of this neural induction mechanism has also been observed in mammalian embryos and has now been demonstrated in human embryonic stem cells (hESCs) as well (see below).

MOLECULAR REDUNDANCY IN NEURAL INDUCTION

As with most signaling pathways, the BMP patterning system that underlies neural induction in vertebrates is notable for extensive redundancy in gene function that has made loss-of-function approaches problematic (Table 1). Thus, genetic tests of the putative neural inducers in other species were initially unimpressive because mutations that eliminate only one of these inhibitors tend to have relatively mild phenotypes on their own. For example, a loss-of-function mutation in Zebrafish *chordin* (the *chordino* mutant) causes only a reduction in the size of the neural plate, while mouse embryos that lack just one of the BMP antagonists, *chordin* or *noggin*, by knockout mutations have a relatively normal nervous system. However, the full potential of these antagonists becomes apparent when several of them are removed

TABLE 1 | Secreted Inhibitors of the BMP Pathway

Gene	Inhibits	Species	Gastrula Expression†	Features-Comments	References
Chordin	BMP-2,4,7	Mouse *Xenopus* Zebrafish Chicken	Node (m) Organizer (x,z) Node and rostral mesendoderm (c)		26 30 28 31
CHL/chordin-like	BMP-4,5,6	Mouse	No	3 CR domains	32
Noggin	BMP-2,4,7 GDF-5	Mouse *Xenopus* Zebrafish	Node (m) Organizer (x,z) Axial mesendoderm (c)	3 noggin-like genes found in Zebrafish	25 33 34 35 36 37
Follistatin	BMP-2,4,7,11 GDF-8,11 Activin	Mouse *Xenopus* Chick	Node (m) Organizer (x) Node, mesendoderm, caudal neural plate (c)		27 38 39 40 37
FSRP proteins: FLRG, Flik	BMP-2,6,7 Activin	Mouse Chicken	FLRG: e7.0 by Northern (m) Flik-1: node (c)	Follistatin related	41 42 43 44 45
Cerberus	BMP-4 xNr-1,2 Wnt-8	*Xenopus* Mouse (Cer1) Chicken	Anterior endoderm (x) Anterior visceral endoderm (m) Hypoblast, Ant. Endoderm, Prechordal plate (c)		46 47 48 49 50
Coco	BMP-4 Activin xNr-1 Wnt-8	*Xenopus*	Gradient from animal to vegetal Strongest expression in ectoderm	Cerberus/dan related	51
Dan	BMP-2,4,7 GDF-5,6,7	Mouse *Xenopus*	No No		52 50 53 54
Caronte	BMP-4,7	Chicken	Mesoderm flanking the node		55 56
Lefty1 Lefty2	Nodal	Mouse Chicken	Notochord/midline (Lefty1; m,c) Mesoderm (Lefty2; m,c)		57,58
Dante	ND	Mouse	Node	No full-length cDNA reported	53
PRDC	ND	Mouse	ND	Cerberus/Dan-like	59
Drm/Gremlin	BMP-2,4	Mouse *Xenopus*	No No		50 60 53
Neuralin-1	BMP-4,5 TGF-β1,2	Mouse	Emerging neural plate	3 CR domains	61 32
CTGF	BMP-4 TGF-β1	*Xenopus*	Weak expression	1 CR domain	62
Kielin	ND	*Xenopus*	Axial mesoderm	27 CR domains	63

TABLE 1 | Continued

Gene	Inhibits	Species	Gastrula Expression†	Features-Comments	References
TSG	BMP-4	*Xenopus* Mouse	Ventral region (x) ND	Reported to act as both an antagonist and an agonist of BMP signaling	64 65 66 67 68
Amnionless	ND	Mouse	Visceral endoderm	1 CR domain 1 TM domain	69
CRIM-1	ND	Mouse	No	6 CR domains 1 IGFBP motif 1 TM domain	70
Nell family	ND	Mouse (NELL1,2) Chicken	No ND	Multiple CR domains. Multiple EGF domains. Some contain TM domains	71 72 73
Xnr3	BMP-4	*Xenopus*	Organizer	Nodal-related gene	74
Sclerostin/SOST	BMP-5,6	Mouse	No		75,76 77
Sclerostin-like	ND	Mouse	ND		76
Jiraiya	BMPRII	*Xenopus*	Dorsal ectoderm		78
Cross Veinless 2	BMP4,5,7	*Xenopus*, Mouse, Drosophila	Primitive streak, Precardiac mesoderm, Tailbud	5 CR domains, 1 VWD domain, 1 TIL domain. Reported to act as both an antagonist and an agonist of BMP signaling	79–81
xNorrin	Xnr1 BMP4 Fzd-4/Lrp	*Xenopus* Zebrafish Chick Mouse Human	Oocyte to late blastula Animal pole	Cystine-knot domain Binds to Fzd-4 and acts as a WNT ligand	82,83

Abbreviations: ND, not determined; CR, cysteine rich; EGF, epidermal growth factor; IGFBP, insulin-like growth factor binding protein; TIL, trypsin-inhibitor like; TM, transmembrane; VWD, von Willibrand factor type D.
†Expression as measured by RNA localization. Species expression domains are described as follows: (m) mouse; (c) chicken; (x) *Xenopus laevis*; (f) zebrafish.

TABLE 2 | Small Molecules Shown to Block Different Branches of the TGFβ Signaling

Small Molecule Inhibitors	Target Receptors	*In Vitro* Concentration	References
SB431542	ALK4, 5, 7	10 μM	84
A083-01	ALK4, 5, 7	0.5 μM	85
Dorsomorphin	ALK2, 3, 6 (non-specific: VEGFR, AMP Kinase)	1–2 μM	86
LDN-193189	ALK2, 3, 6 (non-specific: VEGFR, AMP Kinase)	100 nM	87
DMH1	ALK2, 3 (highly specific)	0.5–5 μM	88

at the same time. For example, a complete loss of neural tissue is observed when all three BMP antagonists—*chordin*, *follistatin*, and *noggin*—are simultaneously targeted using morpholinos, both in *Xenopus*[98] and Zebrafish.[99] Similarly, loss of *noggin* and *chordin* alone in mouse embryos have no severe phenotypes, while the double *noggin/chordin* mutant lacks all anterior neural structures.[100] Conversely, multiple BMP ligands are required for epidermal differentiation: at least three of the four BMPs, BMP2/4/7, need to be disrupted by morpholinos in *Xenopus* embryos to expand the neural plate, but even then, some ventral epidermal tissue remains. Thus, neural induction *in vivo* may depend on multiple ligands and inhibitors as a means to ensure robustness of BMP signaling inhibition during early patterning of the embryo.

FGF SIGNALING AND NEURAL INDUCTION

Differential BMP signaling fulfills the expectation of an instructive mechanism for determining why neural tissue forms in one place in the embryo but not another.[101] The default model, however, leaves open the possibility that other factors are involved in neural induction, including those operating in a more permissive fashion to alter the competence of the ectoderm both spatially and temporally. The best evidence for a factor in this category are ligands of the FGF and insulin-like growth factor (IGF) family, both of which bind to tyrosine kinase receptors and signal via the MAPK cascade. Significantly, FGF signaling has also been shown to inhibit BMP signaling in the early embryo by several mechanisms, thus potentially influencing the response of tissue to the activity of the BMP inhibitors produced by the organizer during neural induction. FGF/MAPK signaling, for example, can promote phosphorylation of the linker domain and degradation of SMAD1, thereby reducing the efficacy of BMP signaling.[102] FGF signaling can also inhibit BMP activity indirectly by inducing the expression of a protein called ZEB2, a zinc-finger homeodomain transcription factor (also known as SIP1 or ZFHX1b), which binds to and represses the transcriptional activity of SMADs.[103] For much of the neural plate, the role of FGF signaling is likely to be minor, because neural induction by the BMP inhibitors occurs readily in *Xenopus* in the absence of FGF signaling.[104] As discussed in the next section, this has also been shown to be the case in mammalian pluripotent cells.

NEURAL INDUCTION AND EARLY NEURAL PATTERNING

The early neural plate is already specified to form different parts of the nervous system as it arises following neural induction. For example, the wider part of the neural plate at the anterior end of the embryo will form brain tissue, whereas the narrow part posteriorly will form the spinal cord. A complex set of inductive signals generated from different parts of the organizer as well as neighboring epidermis is known to pattern the neural plate into different regions along the embryonic axes. Strikingly, in the absence of these additional signals, neural tissue induced by inhibiting BMP signaling leads to anterior forebrain-like tissue as a default state, whereas more posterior regions of the nervous system require additional WNT, FGF, and Retinoic acid signaling.[105–107] Thus, a hallmark of the default model is that ectoderm will form neural tissue with forebrain character in the absence of instructive signals. More posterior regions of the nervous system such as spinal cord are thought to be induced in two steps, by inhibiting BMPs, followed by a posteriorizing signal, even if both steps are mediated by the same factor such as FGF.

NEURAL INDUCTION IN MAMMALIAN EMBRYONIC STEM CELLS

About 30 years ago, mouse embryonic stem cells (mESCs) were derived from the blastocysts of the preimplantation mouse embryo.[108,109] These cells provided the functional *in vitro* definition of ESCs: unlimited proliferation (self-renewal) with retention of the capacity to differentiate into cells from each of the three embryonic germ layers—ectoderm, mesoderm, and endoderm (pluripotency). The formal test of ES cell pluripotency was provided by the ability to contribute significantly to all tissues in the morula aggregation assay.[110] This advance provided the technical means to manipulate the mouse germline and formally demonstrated that mESCs, reintroduced into the context of implantation development, were able to give rise to all cells of the embryo. Even more stringently, mESCs have been shown to generate entire mice in the tetraploid embryo complementation assay.[111]

Human embryonic stem cells were derived thereafter from human blastocysts.[112] These hESC lines demonstrated the hallmark characteristics of self-renewal and pluripotency. While the gold standard pluripotency assays of morula aggregation or tetraploid embryo complementation are ethically impermissible using human cells, hESCs have passed all the standard tests for pluripotency including embryoid body (EB) formation teratoma assays and contribution to the embryonic germ layers of the mouse embryo.[113]

The cardinal translational promise of stem cell biology is that these cells can be used to generate novel *in vitro* models of intractable and poorly understood diseases and potentially for regenerative cell replacement strategies. From a developmental perspective, however, mouse and human ESCs provide an *in vitro* platform to test hypotheses and investigate mechanisms controlling embryonic fate determination. For mouse, this system is a complement to *in vivo* approaches; however, for human it constitutes only the experimental window into early human embryogenesis. As with *Xenopus* pluripotent animal cap cells, a composite picture of the necessity and sufficiency of TGFβ/BMP inhibition for neural induction in mammalian pluripotent cells is also emerging.

Similarities and Differences in the Pluripotent State of Mouse and Human ESCs

Both mouse and human ESCs express identical embryonic transcription factors (TFs) such as OCT4 (POU5F1), SOX2, and NANOG during pluripotency, and form teratomas as xenografts.[114] However, there are also important differences—in signaling requirements, X-chromosome status, and growth characteristics—between human and mouse ESCs, which can be explained by the current view that mESCs represent an earlier stage of development than hESCs.

Mouse ESCs require LIF, BMP, and WNTs for the maintenance of a naïve (or 'ground') state of pluripotency.[115] Treatment with FGFs or WNT inhibitors induces a conversion of mESCs to epiblast stem cells (EpiSCs) that can self-renew and maintain pluripotency, but acquire the gene expression signature of postimplantation epiblast cells.[116,117] This suggests that FGFs and low levels of WNT signaling may also contribute to the transition from naïve to primed pluripotency *in vivo*. Mouse EpiSCs have distinct signaling requirements—Activin/Nodal and FGF—compared to mESCs and display the same phenotype when derived directly from both preimplantation and postimplantation embryos.

Human ESCs on the other hand are dependent on Activin/Nodal and FGF signaling for maintenance of pluripotency, similar to mEpiSCs and different from mESCs, even though they are derived from an equivalent embryonic source as mESCs: the inner cell mass of preimplantation blastocysts.[118–120] Human ESCs are therefore considered to represent a more advanced stage of pluripotency than mESCs and are closer to mEpiSCs in their developmental potential.[114,119] On the basis of functional assays, it seems likely—though not formally proven—that the pluripotent cells of *Xenopus* animal caps are closer to the primed pluripotent state of mEpiSCs and hESCs than to the naïve state of mESCs because they can give rise to all the germ layer derivatives in the absence of priming.[121–123] Somatic cells reprogrammed to a pluripotent state, called induced pluripotent stem cells (iPSCs), share identical signaling properties for maintenance and differentiation to the ESCs of the species from which they were derived. Hence, mouse iPSCs require LIF, BMP, and WNT for pluripotency, whereas human iPSCs require Activin/Nodal and FGF signaling (Figure 4).[124–126]

Neural Induction in Mouse ESCs/EpiSCs and the Role of FGF Signaling

Neural induction paradigms in ESCs have evolved over the past decade from culturing ESCs as EBs in serum- and retinoic acid-containing medium to coculturing ESCs with cell lines possessing neural-inducing activity, and now to defined culture conditions utilizing some combinations of growth factors or small molecules. We will only discuss the latter two protocols, as they are more informative with respect to the default model. A screen of feeder cell lines identified a bone marrow-derived stromal line that could strongly promote neuronal differentiation from mESCs without concomitant mesoderm induction.[128] The nature of this stromal cell-derived neural-inducing activity remains unknown, but this activity could be blocked by BMP4, which in turn promoted an epidermal fate. This study was therefore among the first to provide evidence suggesting that the same signaling mechanisms determining the fate of pluripotent *Xenopus* animal cap cells may be conserved in mammalian ESCs as well. Subsequently, it was shown that mESCs cultured under defined low-density conditions—mimicking the dissociated *Xenopus* animal cap experiments—promoted their conversion into nestin-expressing neural precursors.[129] In this paradigm, inhibition of BMP signaling with Noggin or Cerberus enhanced the appearance of neural colonies, as did Smad4 knockout mESCs, which are resistant to TGFβ/BMP signaling. A role for FGF in neuralization of mESCs was also suggested in this study, as well in separate studies utilizing defined media conditions in monolayer mESC cultures where the FGF pathway was either stimulated or repressed.[130–132] These studies, however, did not resolve whether FGF was acting directly or indirectly as a neuralizing factor.

Although not known at the time, these observations can be easily reconciled by the fact that mESCs require FGF signaling to progress to a primed state of pluripotency, i.e., the epiblast-like EpiSC state (also referred to as 'primitive ectoderm' in some studies), before they acquire the competence for neural induction.[116,133,134] Hence, FGF signaling conceivably regulates the competence of mESCs for germ layer differentiation, rather than neural induction *per se*.[134,135] In fact, FGF signaling has recently been shown to inhibit rather than promote neural induction in EpiSCs, as would be expected from the default model.[135,136] In addition, small-molecule inhibitors of TGFβ/BMP signaling promote rapid neural commitment from EpiSCs under defined conditions, providing direct evidence for the validity of the default model in the mouse system.[137] It is worth noting that FGF signaling can directly inhibit SMAD signaling by promoting the degradation of SMAD1 via linker phosphorylation, as has been suggested in animal caps, but this role has not been directly tested

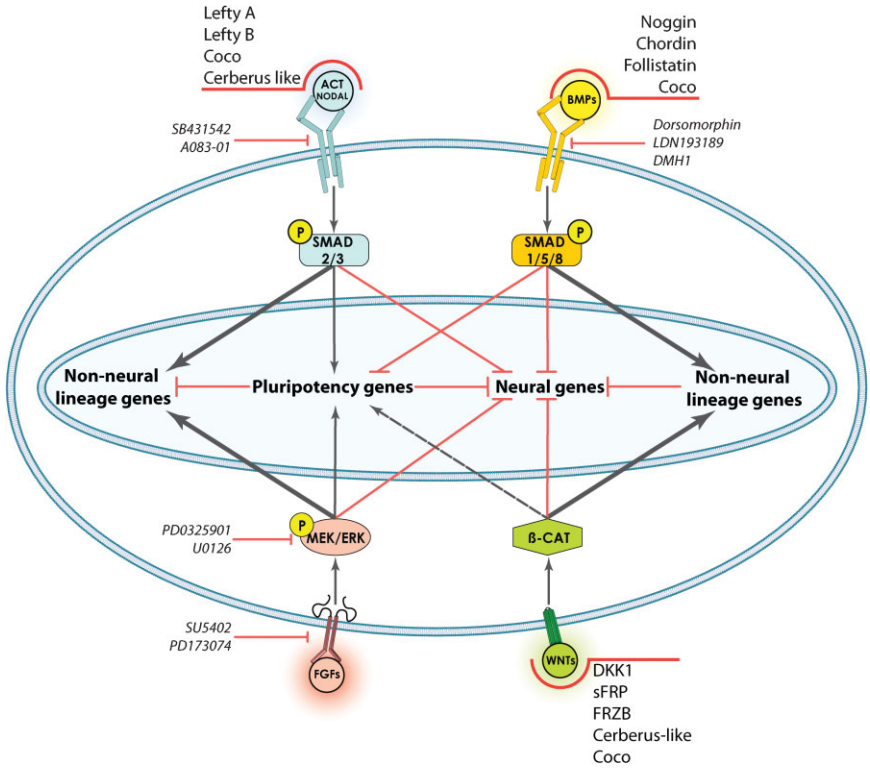

FIGURE 4 | Signaling pathways involved in pluripotency and induction of neural fate in human embryonic stem cells (hESCs) by a 'default' mechanism. The three pathways mediating pluripotency in primed pluripotent cells, i.e., Activin/Nodal-SMAD2/3, FGF-MEK, and WNT-β-catenin, may repress neural fate directly and indirectly via pluripotency genes like NANOG. In addition, all these pathways can promote alternate non-neural fates at higher thresholds of signaling, as denoted by thick lines. These non-neural fates in turn also repress neural fate genes. Inhibition of TGFβ and BMP signaling by secreted proteins (such as Lefty and Noggin) or small molecules (SB431542 and LDN193189) are sufficient to convert pluripotent hESCs to a neural fate.[127] Hence, the state of pluripotency requires overcoming of the default neural state. Arrows represent activation (shown as proportional to the thickness of the lines), whereas hatches represent inhibition. Dotted lines denote postulated mechanisms from evidence in non-human systems.

in the paradigms above.[102] Thus, the requirements for FGF may be largely explained by its role in the transition of mESCs to EpiSCs, which are then primed for differentiation and hence resemble the pluripotent cells of the *Xenopus* animal cap more closely.

Other well-characterized feeder- and serum-free protocols have also been developed for neural induction from mESCs that do not involve exogenous FGF signaling. For example, exposure of low-density mESC monolayer cultures to a sonic hedgehog inhibitor led to the generation of telencephalic neurons that recapitulated the temporal hierarchy of *in vivo* cortical development.[138] In this context, inhibition of sonic hedgehog prevented ventral patterning of the nascent neural progenitors, while the low-density culture condition promoted neuralization in a manner evocative of the *Xenopus* animal cap dissociation experiments. Similarly, EB differentiation with small-molecule inhibitors of TGFβ and WNT signaling also recapitulated major spatial and temporal milestones of cortical development and generated functional neurons with forebrain identities.[139,140] While the use of a TGFβ inhibitor falls in line with default neural differentiation, the WNT inhibitor in this paradigm likely facilitates the transition of mESCs to EpiSCs, as discussed above. Indeed, inhibition of endogenous WNT signaling in mESCs has been shown to readily promote their conversion to EpiSCs.[117] Furthermore, exogenous BMP4 completely abolished neural induction in this setting, supporting the default model's tenet that inhibition of both Activin/Nodal-SMAD2/3 and BMP-SMAD1/5/8 signaling is necessary for neural induction.[89]

Neural Induction in Human ESCs/IPSCs and the Role of FGF Signaling

Not surprisingly, many of the same protocols that have been used for neural induction in mESCs have also been adapted for neural differentiation of hESCs. As hESCs do not survive as single cells, most early studies have used EB differentiation approaches in the absence of exogenous factors. This approach showed that hESCs preferentially differentiate into

anterior (forebrain) neural derivatives, presumably reflecting a default pathway in the absence of exogenous signaling.[141,142] A role for endogenous FGF signaling was suggested as a requirement for neural induction in these studies, because small-molecule inhibitors of FGF signaling reduced the number of cells expressing PAX6.[143,144] However, it is important to note that FGF or FGF inhibitors were not added in the initial 4 days of differentiation before the appearance of PAX6.[141,144,145] This leaves open the possibility that FGF signaling is not directly promoting neural induction in these experiments, but rather has a survival and/or proliferative role in the early neuroepithelium. In support of this idea, exogenous FGF appeared to increase the size of neural colonies without changing the efficiency of neural induction.[142] In addition, neuralized hESCs displayed low levels of BMP-SMAD1/5/8 signaling, presumably because of the high-level expression of several soluble BMP antagonists such as Noggin, Follistatin, and Gremlin as well as intracellular inhibitors of BMP signaling such as SMAD6 and ZEB2 (SIP1/ZFHX1B). Several other EB-based protocols regularly include Noggin in serum-free medium to promote neuralization of hESCs.[146,147] Together, these studies suggest that in the absence of exogenous morphogens, hESC colonies take on a neural fate of anterior character in line with the default model.

Inhibition of Activin/Nodal-SMAD2/3 signaling has also been shown to be a prerequisite for neuroectodermal differentiation of hESCs either as EBs or as monolayer cultures.[148–151] Combining the classical observations made in *Xenopus* animal cap explants with these studies in hESCs, a feeder-free protocol for direct neural differentiation of hESCs utilizing small-molecule inhibitors of Activin/Nodal-SMAD2/3 and BMP-SMAD1/5/8 signaling demonstrated rapid and high efficiency conversion to neural fate (>80% of cells).[127] This system was made additionally tractable by use of a Rho-associated kinase inhibitor, which confers survival on hESCs as single cells[152] permitting single-cell plating of hESCs/hiPSCs and differentiation in adherent culture conditions. As expected from the default model, the neuralized cells were of anterior identity in this paradigm, expressing the forebrain TFs OTX2 and FOXG1. The primitive neuroepithelia could subsequently be patterned into multiple regional CNS derivatives, including midbrain, floor plate, and spinal cord. This dual-SMAD inhibition paradigm has now been adapted for chemically defined media as well as EB-based hESC and hiPSC differentiation protocols.[153,154] It is worth noting that while exogenous FGF was used during neural induction in the original protocol, it has since been shown that FGF signaling directly inhibits induction of the neural determinant PAX6.[155] This is in line with the inhibitory role of FGF in neural induction of EpiSCs derived from mouse embryos.[136] Interestingly, the inhibitory effect in hESCs was found to be restricted to a limited window, as continued FGF inhibition in the presence of TGFβ/BMP inhibition promoted a peripheral nervous system fate. Together, these studies provide the strongest evidence so far that the molecular mechanism underlying neural fate specification in hESCs is conserved from *Xenopus* and conforms to the default model (Figure 4).

Other protocols have also been developed for neural induction in hESCs/hiPSCs and show a requirement for TGFβ inhibition. The SFEB protocol described above has also been adapted for hESCs and like in mESCs, it has been shown to recapitulate major spatial and temporal milestones during cortical development and generate forebrain precursors.[140] While this protocol utilizes inhibition of Activin/Nodal-SMAD2/3 and WNT signaling in hESCs but not BMP-SMAD1/5/8 inhibition, addition of exogenous BMP did inhibit neural induction, which again points towards endogenous BMP inhibition.[156] Use of WNT inhibitors in this system probably serves to prevent posteriorizing signals and non-neural differentiation.[157,158]

Downstream Mechanisms of Default Neural Induction in Mouse and Human ESCs

The ability to generate purified populations of neuralized ESCs *in vitro* combined with use of gain-of-function and loss-of-function approaches has permitted scrutiny of the mechanisms operating downstream of TGFβ inhibition by which pluripotent cells undergo neural conversion. Inhibition of Activin/Nodal-SMAD2/3 downregulates NANOG and promotes expression of ZEB2, a SMAD-binding protein.[151] In pluripotent cells, ZEB2 limits the mesoderm-inducing effects of Activin/Nodal signaling and is repressed directly by NANOG and OCT4. Once upregulated, ZEB2 promotes neuroectodermal differentiation of EpiSCs and hESCs. In addition, Activin/Nodal-SMAD2/3 and BMP-SMAD1/5/8 inhibition also promotes expression of a COUP-TFII (NR2F2), which is among the earliest TFs expressed during neural differentiation of hESCs.[155,159] In pluripotent hESCs, OCT4 and the OCT4-induced microRNA mir-302 regulate expression of NR2F2 by transcriptional and post-transcription mechanisms, respectively, whereas in the differentiating neuroectoderm, NR2F2 directly represses OCT4 expression and promotes expression of other neural-specific markers.

BMP-SMAD1/5/8 inhibition also contributes to neuroectodermal differentiation through several other mechanisms. First, it promotes the specificity of neural induction by inhibiting induction of non-neural germ layers such as trophectoderm, mesoderm, and non-neural ectoderm.[155] Indeed, inhibition of BMP signaling together with downregulation of OCT4 is a prerequisite for neuroectodermal specification in hESCs.[160] Second, inhibition of BMP signaling may serve to stabilize the neural fate by maintaining the expression of shared pluripotency and neural genes such as SOX2.[155] Third, absence of BMP signaling promotes the expression of cell-intrinsic neural determinants, such as the zinc finger TF ZNF521, which is necessary and sufficient for neural induction in hESCs as well as mEpiSCs.[156] Lastly, BMP inhibition may also promote acquisition of anterior neural fate, as neural induction protocols which involve inhibition of Activin/Nodal-SMAD2/3 in the absence of BMP inhibitors appear to adopt a more posterior neural identity in both mEpiSCs and hESCs.[149,150]

As mentioned above, FGF signaling maintains pluripotency in mEpiSCs and hESCs. It is thought that the FGF-MEK-ERK branch directly regulates NANOG expression in hESCs, but not mEpiSCs.[136,161] Hence, one way FGF inhibition may contribute to neural induction is by facilitating downregulation of pluripotency TFs, thereby permitting expression of the default neural program. In addition, during early differentiation, FGF-MEK-ERK signaling has been shown to directly repress expression of the neural determinant TF PAX6 in hESCs as well as EpiSCs.[136,155] Furthermore, inhibition of FGF signaling promotes rapid induction of the forebrain- and midbrain-enriched homeobox TF OTX2 in hESCs. OTX2 in turn directly binds to the PAX6 promoter and enhances its expression in hESCs.[155] Thus, like BMP-SMAD1/5/8 inhibition, FGF-MEK-ERK inhibition may promote neural induction through several mechanisms.

CONCLUSIONS AND PERSPECTIVES

The default model provides a molecular explanation for the rich observations made in early embryology experiments, as revealed in the embryo by potent local inhibition of global inhibitors of neural fate. This double negative still appears to be the most persuasive explanation for observations from *in vivo* and *in vitro* assays of neural specification from fly to human. However, many open questions remain. To what extent does a default mechanism or inhibition of an inhibitor repeat itself during nervous system development? For example, what is the default positional identity within the nervous system? What determines the timing of double inhibitory events? When does it end? How is the dynamic aspect of signaling and signal inhibition regulated at the network level? How is TGFβ inhibition integrated in the hierarchical network of signaling that occurs during neural induction to establish positional identity—and therefore cellular diversity—in the CNS? From an evolutionary point of view why should the nervous system be the default cellular identity? What advantage did this confer at the root of metazoan taxonomy? Future work in comparative developmental biology and evolution in diverse systems will begin to furnish responses to some of these questions.

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FURTHER READING

NIH resource for stem cell research: http://stemcells.nih.gov/info/basics/

Wikipedia article on neural development: http://en.wikipedia.org/wiki/Neural_development

Wikipedia article on TGFβ signaling: http://en.wikipedia.org/wiki/Transforming_growth_factor_beta

Wikipedia article on BMP signaling: http://en.wikipedia.org/wiki/Bone_morphogenetic_protein

Brivanlou Lab website: http://xenopus.rockefeller.edu/CV.html

