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Chapter 1

Neural Induction

Karla Loureiro Almeida, José Abreu, and C.Y. Irene Yan

Contents

Abstract Neural induction, i.e. definition of the neural domain from the ectoderm, is a fundamental topic that has fascinated developmental biologists for years. The concept was first proposed by Spemman and Mangold after their classic experiment in the amphibian Xenopus laevis where transplantation of the embryo's dorsal blastopore lip induced a complete neural axis from the acceptor embryo's ectoderm. Since then, much effort has been applied into identifying the signals that bias the ectoderm into neural fate and the resulting picture clearly indicates that neural induction is a multi-step process that requires the interplay of various pathways. A major part of our current understanding of neural induction originates from the original amphibian model *Xenopus laevis*. Recently, the chick embryo has added another layer of complexity to the interpretation of the results obtained from the amphibian model. Here, we will focus on the landmark experiments that address the earliest step of neural induction in these two models. Specifically, we will discuss the Neural Default model that was generated from experiments in the amphibian embryo to explain the choice between epidermal and neural precursor fate and the modifications on this model based on conclusions derived from the chick embryo.

Keywords BMP signaling · Ectoderm · FGF · Neural induction · Smad

Abbreviations

BMP: bone morphogenetic protein
TGF-β: transforming growth factor β
FGF: fibroblast growth factor
MAPK: mtogen activated protein kinase

1.1 Introduction

The induction of neural tissue is a fundamental question that has fascinated developmental biologists since the classic experiment by Spemmann and Mangold. In 1924, based on their results from grafting experiments performed in amphibian embryos, the authors proposed for the first time the concept of neural induction. At the time, it was known that the blastopore lip initial involution site during gastrulation marked the dorsal region of the embryo, and that the future neural plate arose from the dorsal ectoderm – the ventral ectoderm forms mainly epidermal tissue. Spemann

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and Mangold transplanted the blastopore lip of donor embryos to the ventral region of host embryos in gastrula stage. The host embryos went on to develop a second, ventral neuraxis and anterior nervous system. More strikingly, the duplicate nervous system was fully composed of host tissue, whilst the transplant gave rise to a second notochord (dorsal mesoderm) underlying it. This result suggested strongly that the grafted tissue's "determinative influences on its surroundings" converted the surrounding ventral ectoderm into the second nervous system (Spemann and Mangold, 1924). The authors named the dorsal blastopore lip the Organizer, and hypothesized that during normal development this region determined the choice of a neural fate for the dorsal ectoderm. They also proposed that the effect of the Organizer on the responsive ectoderm necessarily would involve cell-to-cell communication.

In the ensuing years, much effort has been applied for identifying the exact signals that emanate from the Organizer and activate the signaling pathways that bias the ectoderm into neural fate in vertebrates. The resulting picture, derived from data obtained by various groups, indicates that neural induction is a multi-step process. The amphibian model, *Xenopus laevis,* has continued to be of major importance to our understanding of neural induction due to the ease of experimental readout of neural induction in ectoderm explants. In recent years, the chick embryo has added another layer of complexity to the interpretation of the results obtained from the amphibian model. In the following sections we will present the major results derived from both model systems and the model that is emerging from those results. For the purposes of this chapter, we will focus our discussion on the earliest step of neural induction, which is the choice between epidermal and neural precursor fate.

1.2 Neural Induction in the Xenopus Embryo – The Early Experiments

In the decade of 1990–2000, the search for the Organizer's neural inducing factors intensified and was mainly performed in the Xenopus embryo. Based on the characteristic of the Organizer, it was agreed that a *bona fide* candidate for direct neural inducer had to fulfill certain criteria: it should cause axis duplication in whole embryos, it should be expressed in the dorsal blastopore (Organizer) region and elimination of its activity should interfere with normal neural development. The experimental paradigm used to screen for candidate neural inducers was based on the fact that, by definition, induction involves a signaling source and a responsive target. Based on Spemmanś experiment, the endogenous source of neural inducers is the Organizer and the responsive tissue the ectoderm. Thus, ectoderm explants assays were used as an initial screen for candidates. Ectoderm explants (also known as animal caps) are cultured from a piece of ectoderm excised from the animal pole of late blastulas, the lower part of which constitutes the blastocoele roof (Fig. 1.1a). At this stage, the ectoderm is not yet committed to an epidermal or neural fate and responds to growth factors in the media or overexpression of relevant mRNAs by adopting different cell fates, which are verified through the expression of marker genes. When cultured as an intact tissue in saline solution, ectoderm explants express genes characteristic of epidermal tissue (Kintner and Melton, 1987). However, if the explant is co-cultured with a dorsal blastopore lip, neural markers are expressed instead (Kintner and Melton, 1987). Thus, a gene's neural-inducing activity is identified if there is upregulation of the expression of neural markers and decrease in the expression of epidermal genes. Importantly, because the Organizer is part of the dorsal mesoderm, genes that increased neural marker expression but also induced mesoderm markers, were not considered direct neural inducers, as their effect could be indirect, through additional factors secreted by the mesoderm.

The first molecule to fulfill all of the abovementioned criteria for direct neural induction was Noggin, a secreted polypeptide first identified by Smith and Harland (1992) in the Xenopus. Afterwards, Follistatin (Hemmati-Brivanlou et al., 1994) and Chordin (Sasai et al., 1994), were also isolated from Xenopus embryos on the basis of their neuralizing activity. All of these factors fulfilled the abovementioned conditions, including expression at the Organizer. At the time, these molecules were thought to act by directly stimulating neural fate, albeit through an as yet unidentified mechanism.

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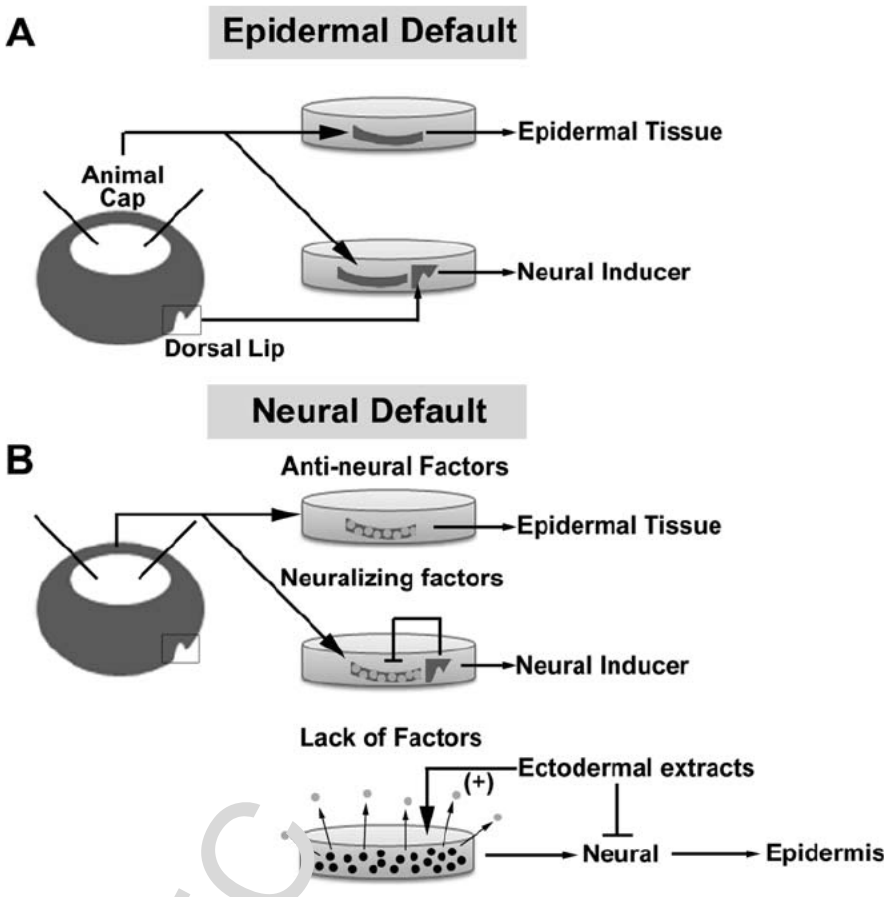

Fig. 1.1 Epidermal default model versus neural default Model. (**a**) In the "epidermal default model" the normal fate of an ectodermic tissue would be epidermal, unless this ectoderm is stimulated by external factors (such as those provided by addition of the dorsal blastopore lip). (**b**) In the "neural default model", the intact ectoderm secretes anti-neural/pro-epidermal factors. Induction of neural fate occurs either by co-culture with the dorsal blastopore lip, which secretes neuralizing factors or dissociation of the ectoderm. In the former, neuralizing factors counteract the effect of endogenous pro-epidermal factors. In the latter case, dissociation of the ectoderm dilutes these factors and generates neural fate. Addition of ectoderm extract restores epidermal fate

1.3 Neural Default Model

Insight on the mode of action of these molecules came from a second series of experiments that explored the effect of cell dissociation on ectoderm cell fate. When ectoderm explants are dissociated into individual cells and cultured as such for a set period of time, they express neural markers, instead of epidermal ones (Fig. 1.1b). Remarkably, this occurs in the absence of the dorsal blastopore lip and without the addition of exogenous factors (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989; Wilson et al., 1997). These data led to the hypothesis that neuralization is the default fate for ectodermal cells, and that the cell–cell interactions that occur in an intact ectodermic tissue somehow inhibit this developmental path, resulting in an epidermal fate (Fig. 1.1b). Once the tissue is dissociated, these "epidermal factors" are sufficiently diluted so as to allow development of neural fate (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989). Thus, it was proposed that the ectoderm has "neural default" fate, which is revealed in the absence of exogenous signaling (reviewed by Muñoz-Sanjuán and Brivanlou, 2002).

The addition of concentrated ectodermal supernatant to dissociated cell cultures prevented the expression of neural markers after ectodermal dissociation (Grunz and Tacke, 1990). Thereafter, candidate proteins for the role of "epidermal factor" were added onto dissociated cultures and tested for their ability to restore epidermal fate while suppressing neuralization. These screens identified Bone Morphogenetic Protein 4 (BMP4), a member of the Transforming Growth Factor beta (TGF-β) superfamily as a potent epidermal inducer. When BMP4 is added to a culture of cells dissociated from the ectoderm it induces the expression of epidermal markers (Wilson and Hemmati-Brivanlou, 1995). Moreover, the expression pattern of BMPs in

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the Xenopus gastrula is consistent with the role of "epidermal factor": BMP4 is found throughout the ectoderm prior to gastrulation but, afterwards it is excluded from the neural plate (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). Finally, inhibition of BMP signaling in ectodermal cells with dominant-negative receptors or antisense BMP4 RNA neuralizes ectodermal cells (Sasai et al., 1995). This last set of data was consistent with the model that inhibition of endogenous BMP signaling, through dilution, directs dissociated ectodermal cells towards neural fate.

1.4 BMP and the Neural Inducers

The discovery of the neuralization-suppressing effect of BMP4 suggested a new hypothesis for the mode of action of the direct neuralizers (Noggin, Chordin and Follistatin), that is through the inhibition of BMP4 action. Further experiments showed that, indeed, Noggin and Chordin directly bind to BMP4 protein and interfere with its ligation to its receptor (Zimmerman et al., 1996; Piccolo et al., 1996). Follistatin also binds to BMPs and, while still allowing ligation to its receptor, forms a trimeric complex that inhibits signaling (Nakamura et al., 1990; Fainsod et al., 1997; Iemura et al., 1998). Interestingly, molecular studies have shown that different from Noggin and Follistatin the inhibitory activity of Chordin on BMP resides in specific cysteine-rich (CR) domains and is phylogenetically conserved (Abreu et al., 2002).

The model that emerged was one in that the decision on the on neural or epidermal fate of the ectoderm depends on the level of BMP signaling. When BMP signaling is decreased, either through dilution in dissociated cultures or inhibition by neural inducers, ectoderm will progress towards a neural fate. Conversely, when BMP signaling prevails, the ectoderm will form epidermis.

This model is consistent with the conditions occurring during normal Xenopus development: On the ventral ectoderm of the gastrulating embryo, which is diametrically opposite to the Organizer and which develops into the epidermis, high levels of BMP are detected (Jones et al., 1996; Reém-Kalma et al., 1995). In contrast, the dorsal ectoderm, where neurulation occurs, is in close proximity to the Organizer, which is the source of BMP-inhibiting neural inducers. Accordingly, it has relatively low levels of BMP signaling. Likewise, this model explains the double-neural axis phenotype in Spemann and Mangolds´original Organizer graft experiment: The grafting of an additional Organizer in the ventral region provided a source of neural inducers that inhibited BMP signaling in that region, allowing the ventral ectodermal cells to follow their default neural fate.

1.5 Challenges to the Neural Default Model

The model of neural induction based on the simple inhibition of BMP signaling by its antagonists expressed at the Organizer has been challenged, however, by results which suggest that neural induction is a more complex process, involving additional factors. One of these might be Fibroblast Growth Factor (FGF; Kengaku and Okamoto, 1993). FGF treatment increases expression of neural markers and decreases that of epidermal markers, (Kengaku and Okamoto, 1993, 1995; Lamb and Harland, 1995; Uzgare et al., 1998). Furthermore, dominant-negative FGF receptor inhibits the neuralizing effects of ectoderm dissociation and of noggin overexpression in whole embryos (Hongo et al., 1999; Launay et al., 1996). Together, these data suggested that FGF might also be necessary to promote neural induction. This was just the beginning of a series of questions regarding the sufficiency of BMP inhibition in the neural induction model, which was primarily based on amphibian embryos. The strongest evidence against the neural default model of BMP inhibition, however, came from experiments conducted in chick embryos.

1.6 Neural Induction and the Avian Node

Unlike the Xenopus embryos, whose development is completely external, the avian embryo initiates its development in the oviduct (reviewed in Wittler and Kessel, 2004). The initial cleavage cycles that occur there generate a flat blastoderm disc overlying the yolk. When the egg is laid, the avian embryo is a translucent disc composed of an epithelial monolayer – the

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epiblast –, which is subdivided into a central *area pellucida* and an yolk-rich, extra-embryonic *area opaca*. The circumference where the pellucida and the opaca meet is known as the Marginal Zone. After a few hours, a half-moon-shaped thickened region appears at the marginal Zone. This structure is known as Kohler's sickle and is the morphological landmark for the posterior end of the embryo and the site for initiation of gastrulation. At the stage of its appearance, the epiblast cells migrate posteriorly in a bilaterally symmetric movement and anteriorly at the midline, forming the primitive streak through which epiblast cell ingress and form the definitive endoderm and mesoderm (Hatada and Stern, 1994; Voiculescu et al., 2007; Joubin and Stern, 1999). When sickle cells and the central epiblast cells meet at the anteriormost edge of the primitive streak, they form a thickened structure known as Hensen's Node, or simply the node (Fig. 2; Lawson and Schoenwolf, 2001; Bachvarova et al., 1998). As gastrulation continues, the primitive streak continues expanding anteriorly and bisects the embryo into left and right regions (Fig. 1.2).

The node is considered the avian homologue of the amphibian dorsal blastopore lip. Its neural inductive abilities and gene expression pattern are reminiscent of the Organizer: transplantation of the node to the extraembryonic area opaca induces a secondary neuraxis (Waddington, 1932; Storey et al., 1992), with minimal participation of donor node cells (Storey et al., 1992). Furthermore, the node expresses the avian homologues of Goosecoid (Izpisua-belmonte et al., 1993), Goosecoid-like gene (Gsx, Lemaire et al., 1997) and **C**hordin (Streit et al., 1998), which are found in the Xenopus Organizer.

1.7 Epiblast – The Responsive Tissue

The induction and patterning of the avian nervous system is a stepwise process that can be subdivided into the ability of the epiblast to respond to neuralizing signals (competence), the progressive stabilization of this response (specification) and the subsequent patterning of the neural region in its diverse axis. The initial experiments by Waddington (1932) showed that the avian blastula's epiblast layer is competent to respond to neuralizing signals derived from the node. Indeed, fate mapping experiments show that neural structures arise from a widespread region of the epiblast prior to gastrulation (Hatada and Stern, 1994 García-Martínez et al., 1993). Waddington's conclusions were further refined by Storey et al. who transplanted ectopic nodes to progressively older host embryos and determined that the epiblast can generate a full antero-posterior neural axis up to early gastrula stages (Storey et al., 1992; Streit et al., 1997). Thereafter, the epiblast cannot be induced to form anterior neural structures.

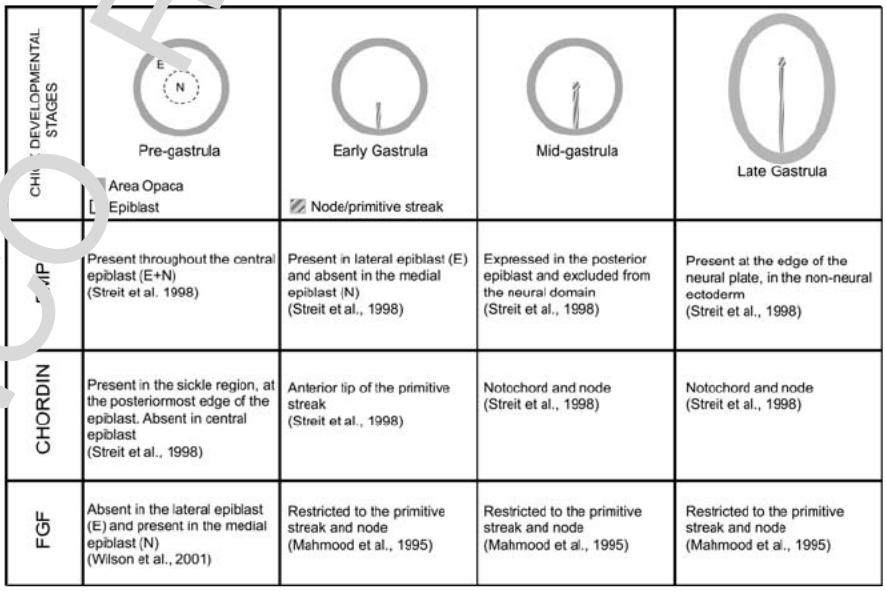

Fig. 1.2 The expression pattern of BMP, Chordin and FGF during different stages of early chick development. The first row represents a simplified dorsal view of the pre-gastrula and gastrulating embryo

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The precise stage at which the epiblast first demonstrates that it is competent to follow neural fate has been progressively pushed back as more molecular markers have become available. For instance, the early neural marker Sox3 and late marker Sox2 have been used as standard indicators of chick neural specification (Rex et al., 1997; Streit et al., 2000, 1997; Uchikawa, 2003). Sox3 is detected throughout the epiblast before neural induction in pre-gastrula embryos and becomes restricted to the future neuroectoderm as development progresses. Sox2 is first detected around the time when neural induction is believed to occur and its expression is limited to the neuroectoderm (Rex et al., 1997; Muhr et al., 1999).

Accordingly, immediately prior to gastrulation, the potential of different regions of the epiblast differ. Cultures of explants derived from central epiblast generated Sox2 and Sox3-positive cells whereas cultures derived from explants removed from regions closer to the marginal zone did not. Rather, these peripheral explants express genes indicative of epidermal fate (Fig. 2, Wilson et al., 2000). Thus, by following the expression of Sox3 and Sox2 in cultured epiblast explants, the earliest stage in which epiblast is compartmentalized into neural and epidermal domains was identified to be immediately prior to egg-laying (Wilson et al., 2000). At this stage, neural fate is restricted to the central epiblast and epidermal fate to the peripheral epiblast.

1.8 Inhibition of BMP in the Avian Context

The search for avian neural inducers that compartmentalize the epiblast into neural or epidermal fate was initially based on a parallelism between the inductive abilities of Hensen's Node and Spemann's Organizer. In support of this idea was the expression pattern of BMPs and its inhibitors in late Primitive Streak stages: Prior to egg-laying, BMP is present throughout the epiblast but, when neuro-epidermal compartmentalization occurs, it becomes excluded from the prospective neural tissue (Wilson et al., 2000; Streit et al., 1998, Watanabe and Le Douarin, 1996; Streit et al., 1998). Likewise, the TGF-beta inhibitors chordin and noggin, which are expressed anterior to Kohler's Sickle prior to gastrulation, are found at the anterior tip of the primitive streak in early gastrulas and are restricted to the notochord and the node in late gastrulas (Streit et al., 1998, Streit and Stern, 1999; Connolly et al., 1997). Altogether, these data suggested that in chick, similar to Xenopus, BMP and its inhibitors are present in complementary regions and that definition of a BMP-activity-free neural domain plays a crucial role in neural induction.

However, contrary to the results obtained in amphibian embryos, application of ectopic chordin onto early gastrula embryos cannot induce neural fate in non-neural ectoderm (Streit et al., 1998). Moreover, it would be expected, from the results in the frog model, that exposure to ectopic BMP would convert the presumptive neural domain into epidermal. Surprisingly, application of BMP onto early gastrulas' neural domains does not inhibit Sox3 or Sox2 expression (Streit et al., 1998). Inhibition of BMP signaling through overexpression of Smad6 or dominant negative BMP receptor is also not sufficient for neural induction (Linker and Stern, 2004). These results, together with the findings that central epiblast is specified as neural prior to egg-laying (see previous section), indicated that at early gastrula stages the neuro-ectodermal regions are already specified and that the search for the initial neuralizing step should include earlier developmental stages.

Thus, Wilson and collaborators investigated the identity of the signals that compartmentalized the central and peripheral epiblast into their respective neural and epidermal fates in pre-gastrula embryos. At this stage, the central epiblast is still susceptible to BMP and will respond to its presence by converting from neural to epidermal fate (Streit et al., 1998; Wilson et al. 2000). Thus, in early chick epiblasts, the *Xenopus* neural induction model holds true, in that BMP signaling confers an epidermal bias and that its absence is necessary for neural fate. The dynamics of BMP expression at this stage is consistent with its role as the endogenous epidermalizing signal – BMP is downregulated in central epiblast and maintained in peripheral epiblast (Streit et al., 1998; Wilson et al., 2000). This plasticity ends with the onset of gastrulation (HH4) (Fig. 2; Wilson et al., 2000). The neural domain's progressive resistance to BMP reflects the gradual commitment to neural fate that occurs during normal embryonic development.

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1.9 FGF Signaling and Neural Induction

The question that remains is: what is the identity of the endogenous factor(s) that inhibit BMP signaling in the pre-gastrula central epiblast? Contrary to expectations, BMP signaling cannot be directly antagonized by secreted BMP-inhibitors in pre-gastrula embryo. Although Chordin is expressed at the gastrula's node, neither Chordin, Noggin, Follistatin or Caronte were detected in central or peripheral epiblast in pre-gastrula embryos (Levin, 1998; Wilson et al., 2000). Moreover, these inhibitors cannot induce neural markers by themselves (Streit et al., 1998, 2000). In other words, an alternative signaling mechanism must maintain the central epiblast BMP-free for the initial step in neural induction to occur.

The answer came from a series of elegant experiments that provided strong evidence that FGF meets all the requirements for a role as an endogenous inhibitor of BMP in avian blastulas. Firstly, FGF3 is expressed in pre-gastrula central epiblast (Wilson et al., 2000, 2001). Furthermore, exogenously applied FGF can induce the expression of early neural markers (Streit et al., 2000). Blockade of endogenous FGF signaling inhibits expression of Sox3. Inhibition of FGF signaling blocks neuralization and induction of ectopic neural plate by a grafted organizer (Streit et al., 2000). Lastly, the FGF pathway is required for downregulation of BMP levels in the central epiblast, and absence of FGF signaling in the central epiblast can be compensated for by the addition of BMP inhibitors (Streit et al., 2000; Wilson et al., 2000, 2001). Together, these data suggest that FGF is a putative early neural inducer that acts by counteracting BMP signaling in the central epiblast.

These results agree with the previously mentioned effects of FGF on Xenopus embryos. However, at the time that those reports appeared, FGF was considered mainly a posteriorizing signal that acted secondarily on the neural domain generated by inhibition of BMP signaling. In light of the compelling data obtained from chick embryos, the role of FGF as a primary neuralizing signal was revisited in the amphibian embryo as well. This reassessment was done with *ex vivo* ectodermal explants and in vivo analysis of ventral ectoderm fate in whole embryos. The results derived from in vivo experiments differed somewhat from the classical *ex vivo* experiments. While overexpression of truncated TGF-beta receptor was sufficient to induce Sox2 expression in amphibian ectodermal explants (Wilson and Hemmati-Brivanlou, 1995), it did not induce a similar response in whole embryo ventral ectoderm (Linker and Stern, 2004; Delaune et al., 2004). In this experimental paradigm, ectopic expression of neural markers was achieved when there was concomitant inhibition of BMP and stimulation of FGF signaling (Linker and Stern, 2004). Moreover, in the absence of FGF signaling, the ectoderm cannot be neuralized by inhibition of BMP (Delaune et al., 2005). These results strongly suggest that, similar to the avian embryo, neuralization in the amphibian embryo requires interaction of the FGF and BMP pathways.

The interaction between both pathways has been mapped to Smad1, a downstream nuclear effector of the BMP pathway. Smad1 nuclear translocation and transcriptional activity are increased when it is phosphorylated at the carboxy-terminal upon activation of the BMP receptor serine/threonine kinase (Massagué and Chen, 2000). This activity is required for BMP-induced epidermal fate (Wilson et al., 1997; Nakayama et al., 1998). In contrast, when Smad1 is phosphorylated by MAPK in the central linker region, both nuclear translocation and transcription are inhibited (Kretzschmar et al., 1997).

FGF signals through receptor tyrosine kinases that ultimately activate MAPK, which in turn phosphorylates Smad1 (Pera et al., 2003). Underscoring the importance of the MAPK pathway during Xenopus neural development, MAPK activity is required for neural induction by FGF and cell dissociation in ectoderm explants (Uzgare et al., 1998; Kuroda et al., 2005). Thus, Smad1 integrates signals from the FGF and BMP pathway. Its activity results from the opposing effects between FGF-induced linker region phosphorylation versus the BMP-driven phosphorylation of the carboxy-region. Consistent with this idea, overexpression of a MAPK-kinase insensitive Smad1 inhibited neural development in whole embryos, whereas mutation of both MAPK and BMP-sensitive regions resulted in very mild phenotype (Pera et al., 2003). Thus, the final model that emerges places Smad1 in the centre of the choice between neural and epidermal fate. In the presence of high levels of BMP signaling, Smad1 is phosphorylated in the carboxy terminal, which activates its nuclear activity and culminates in epidermal fate. This epidermalizing effect can be counteracted by FGF, which phosphorylates the

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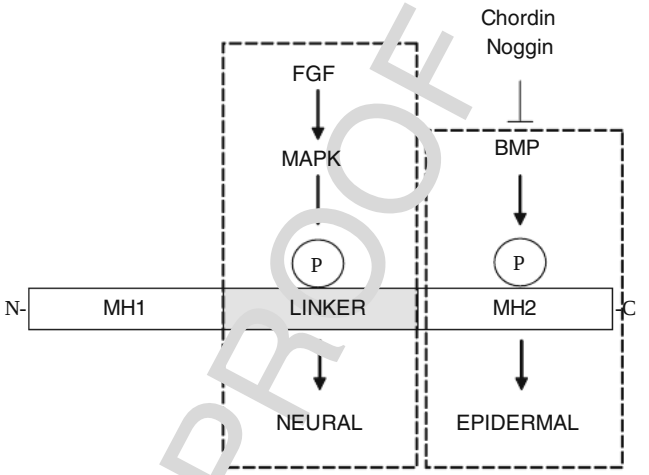

Fig. 1.3 Neural and epidermal fate are determined by Smad1 activity, which in turn is regulated by phosphorylation of its serine/threonine residues. FGF-induced phosphorylation of the linker region retains Smad1 in the cytoplasm and results in neural fate, whereas BMP-induced phosphorylation of the carboxy terminal promotes translocation of Smad1 to the nucleus and results in epidermal fate

Smad1 linker, inhibits its nuclear functions, resulting in adoption of neural fate (Fig. 1.3).

Although this model accounts for most of the results in the field, there are some points that must be considered: firstly, besides FGF there are other growth factors that can activate MAPK activity, which raises the possibility that additional secreted proteins can modulate neural induction (Linker and Stern, 2004). Second, MAPK has other target proteins, amongst them Smad2 and Smad3, components of another TGF-beta pathway. Therefore, it is possible that FGF modulates additional pathways for its neuralizing effect. Indeed, there is evidence that suppression of both Smad1 and Smad2 activity are necessary for neural induction in ventral ectoderm (Chang and Harland, 2007). Furthermore, the FGF pathway itself is modulated by other signals that are present during acquisition of neural competence. For instance, in the chick embryo, the Wnt pathway suppresses FGF signaling in the lateral epiblast (Wilson et al., 2001). Lastly, as mentioned above, cell fate induction occurs in a continuous and progressive fashion. Therefore, the response of a target tissue to neuralizing or epidermalizing signals depends on its differentiation state at the time of exposure. An example of this is neuralization through BMP inhibition in Xenopus embryos. The response to BMP inhibition is lost prior to the onset of gastrulation (Wawersik et al., 2005). Likewise, neural induction in Xenopus embryos is most sensitive to removal of FGF signaling during mid-blastula transition (Delaune et al., 2005). Although these results are still under discussion (de Almeida et al., 2008) and the exact period when each identified player is required for normal progression of neural development is still unclear, it is the general consensus that the plasticity of the ectoderm decreases with time due to stabilization of cell fate (Streit et al., 1998; Wawersik et al., 2005; Linker and Stern, 2004; reviewed in Stern, 2005).

In conclusion, since the molecular identification of direct neural inducers the development field has proposed and refined models for the signaling that underlies the choice between epidermal and neural fate from the ectoderm. Even though the current model does not account for all the complexity that occurs in this process, the speed with which new findings are collected and incorporated into the most recent hypothesis has increased, and a more comprehensive panorama should emerge in the next few years.

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