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ORIGINAL PAPER

Role of BMP, FGF, Calcium Signaling, and Zic Proteins in Vertebrate Neuroectodermal Differentiation

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Abstract More than a decade has passed since Zic family zinc finger proteins were discovered to be transcription factors controlling neuroectodermal differentiation (neural induction) in *Xenopus laevis* embryos. Although BMP-signal blocking has been shown to be a major upregulator of Zic genes in neuroectodermal differentiation, recent studies have revealed that FGF signaling and intracellular calcium elevation are also involved in regulating the expression of Zic genes. Different regulatory mechanisms have been found for the Zic1 and Zic3 genes, raising the possibility that functional synergism between them partly accounts for the integration of BMP-signal blocking and FGF signaling in neuroectodermal differentiation. Furthermore, mammalian Zic1 and Zic3 have been found to be neural-cell-fate-inducing and pluripotency-maintaining factors, respectively, leading us to the intriguing question of whether the mechanism underlying amphibian neuroectodermal differentiation is applicable to mammals. Comprehensive understanding of the Zic family genes is therefore essential for the study of the neuroectodermal differentiation and stem cell biology.

Keywords Neural induction · Zic · Calcium signaling · Default model · FGF signaling · Stem cell

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Neuroectodermal Differentiation

In the course of vertebrate development, a part of the dorsal ectoderm (neuroectoderm, NE) differentiates into the neural plate and the neural plate border region (neuroectodermal differentiation, NED). NED is the earliest event in vertebrate neural development and occurs during gastrulation, in which the three germ layers (ectoderm, mesoderm, and endoderm) are formed through highly coordinated cell movement. In the frog *Xenopus laevis*, gastrulation is initiated by formation of a slit-like blastopore in the future dorsal side, followed by involution of the marginal zone cells, and convergence of cells at the blastopore [1] (Fig. 1). The part of the dorsal equatorial region that includes the dorsal lip of the blastopore is called the organizer (Spemann's organizer). The organizer itself differentiates into dorsal mesoderm-derived tissues or organs such as notochord; however, more critically for neural development, the organizer emanates diffusible factors called neural inducers. The neural inducers act on the naive ectoderm and induce its differentiation [2, 3] (Fig. 1). Therefore, NED is a core process of neural induction.

BMP-Signal Blocking and the Default Model

Several important discoveries that have helped us understand the molecular mechanism underlying NED have been described in the last two decades; however, the most important discoveries may be the molecular identification of neural inducers (including chordin and noggin) and the elucidation of their effect on NED [2, 3]. In Xenopus embryos, NED starts at the blastula stage based on the expression and activities of neural inducers and requires the combined activities of two distinct signaling centers:

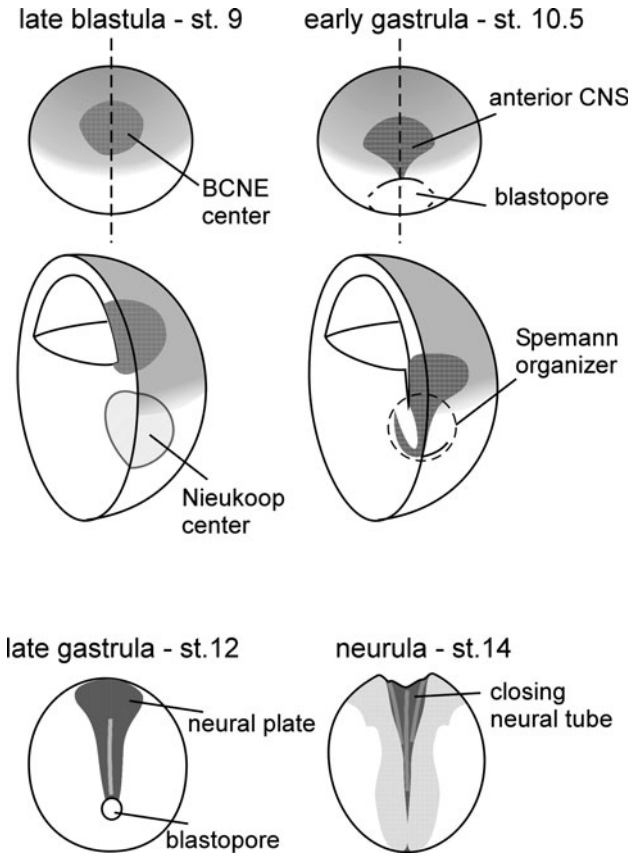

Fig. 1 Gastrulation and NED in Xenopus embryos. *Dorsal views* of Xenopus embryos at late blastula stage (St. 9), early gastrula stage (St. 10.5), late gastrula (St. 12), and neurula (St. 14). The position of NE, blastopore, Spemann's organizer, BCNE, and Nieuwkoop center are shown in the *right* hemisections of the late blastula and early gastrula stage embryos. The *dorsal views* and staging are based on [59], and the hemisection diagrams are based on [3]

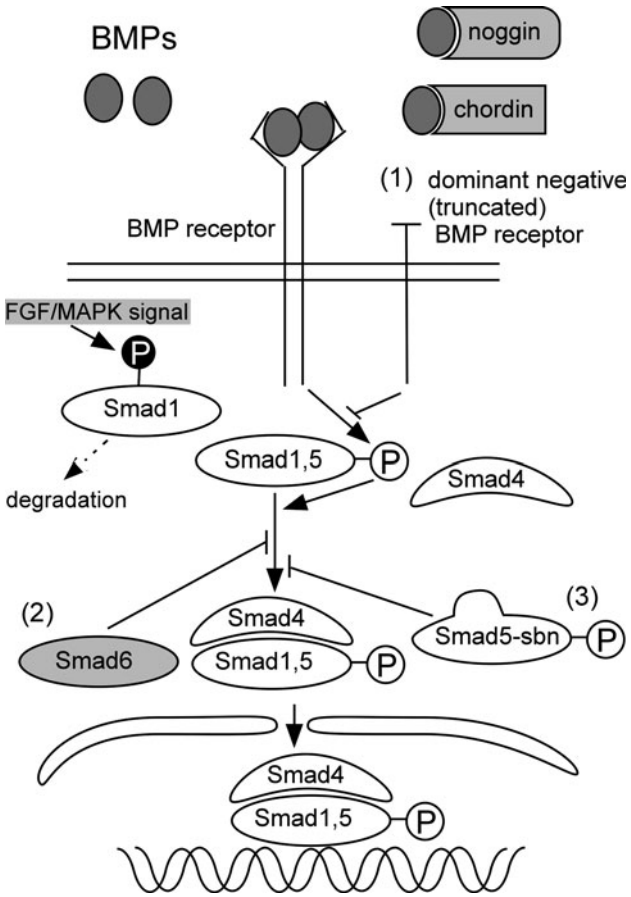

Fig. 2 BMP signaling and its blocking. In the course of gastrulation, natural BMP-signal blocking occurs in BCNE through the physical interaction between BMP2/BMP4 and noggin/chordin. Three ways of experimentally blocking BMP signal in Xenopus embryos are shown: (1) dominant-negative type BMP receptor; (2) Smad6 (inhibitory Smad); and (3) Smad5-sbn (somitabun), which is thought to be deficient in binding to Smad4 and to form an inactive heteromeric complex with intact Smad5 and Smad1, resulting in efficient inhibition of the BMP signaling pathway. Phosphorylation of Smad1 by FGF/MAPK signaling (*P* on *black background*) occurs at a different amino acid position from that of BMP receptor-mediated phosphorylation (*P* on *white background*), and inhibits the transcriptional activity of Smad1. Natural inhibitory factors for *BMP* signaling are *highlighted with gray*

the Blastula chordin- and noggin-expressing center (BCNE), which contains the prospective neuroectoderm and Spemann's organizer precursor cells, and the Nieuwkoop center, which secretes nodal-related factors (potent mesoderm inducers) and cerberus (a wnt/nodal/BMP-antagonist) and forms the anterior endomesodermal cells that underlie the ectoderm in the head region [4]. Chordin, noggin, and cerberus cooperate in the formation of the central nervous system (CNS) [4]. Cerberus and other wnt-antagonists have critical roles in head induction [5], but this will not be discussed here.

Noggin and chordin commonly antagonize the activities of BMP2 and BMP4 (secreted factors belonging to the TGFβ family), which instruct the ectoderm to differentiate into epidermis [2]. The blocking of the BMP signals intrinsically contained in the ectoderm is sufficient to induce NED under certain conditions. Therefore, the NED mechanism based on the neural inducers is called the "default model" [2, 6]. The word implies that the "default" fate of the ectoderm is the neural tissue and that the epidermal cell fate is added by the BMPs. The BMPs bind to a single membrane-spanning protein receptor (a heterodimer of the type I and II BMP receptor subunits), and the binding results in the phosphorylation of the carboxy termini of regulatory Smad proteins (Smad1 and Smad5), which form a transcription factor complex with the common Smad (Smad4) and regulate their target genes [7] (Fig. 2).

Many transcription factors are influenced by BMP-signal blocking. These include Zic1, Zic2, Zic3, and SoxD [8–12], all of which are upregulated in the NE region and can promote NED. The expression of Zic family genes is upregulated upon blockade of BMP signaling by dominant-negative

BMP receptors or noggin mRNA injection [8, 11]. Therefore, Zic genes are thought to link the neural inducers and NED [13].

FGF Signaling is Essential for NED

While the importance of the default model is widely recognized, this model is not sufficient to explain NED. In Xenopus development, overexpression of a dominant-negative FGF receptor inhibits the activation of NED by chordin [14] or noggin [15]. Furthermore, it has been proposed that FGFs can enhance NED [16–18]. Therefore, FGFs have been thought to include potential neural inducing factors, together with the neural inducers from the organizer. While BMP-signal blocking is clearly required for neural induction, whether it is sufficient for NED remains controversial.

To clarify this point, cell-autonomous blocking of BMP signaling has been carried out by injecting cell-autonomous BMP-signal inhibitors (dominant-negative BMP receptor, Smad6 [inhibitory Smad], or Smad5-somitabun [Smad5-sbn; a mutant Smad5 that lacks the interaction with Smad4]) (Fig. 2) into blastomeres that generate ventral epidermal cells [19–21]. Injection of the BMP-signal inhibitors mRNA alone did not induce neural tissue in the ventral side, but injection of BMP-signal inhibitors mRNA together with a low amount of FGF4 mRNA caused ectopic ventral NED [19, 20]. Although it was possible that FGF4 promoted NED by stimulating the formation of neural-inducer-producing tissues, this did not seem to be the case because dorsal mesodermal markers were absent in the regions of ectopic NED [21]. Thus, the case for a requirement of FGF4-mediated signaling has been consolidated in Xenopus embryos.

The requirement for FGF signaling in NED is strongly supported by findings in chick neural development. In chick embryos, the expression patterns of BMPs and their antagonists do not fit the default model. Furthermore, ectopic expression of BMP antagonists does not induce neural markers, and introduction of a source of BMP by grafting does not inhibit NED [reviewed in 22]. Studies in zebrafish have shown that both BMP-signal blocking and FGF activity can directly cause NED [23, 24]. In various vertebrate species, FGF signaling is accepted as a critical signaling pathway involved in NED.

Calcium Signaling and NED

Besides the well-known signaling pathways of the paracrine growth factors (BMPs and FGFs), accumulating evidence indicates the involvement of calcium signaling in NED [25]. The addition of noggin to the naive ectoderm from amphibian embryos triggers an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) [26]. In Xenopus, the increase in $[Ca^{2+}]_i$ lasts 10–20 min and represents approximately 15% of the resting $[Ca^{2+}]_i$ [25, 27]. The increase is inhibited by an antagonist of the dihydropyridine (DHP)-sensitive Ca^{2+} channel (DSCC, L-type Ca^{2+} channel). Treatment with a DSCC agonist causes NED even in the presence of BMP [26], whereas DSCC antagonists inhibit NED. Furthermore, drugs that induce Ca^{2+} release from internal Ca^{2+} stores (caffeine and theophylline) are potent neural inducers [26]. These results indicate that $[Ca^{2+}]_i$ increases can facilitate NED irrespective of the Ca^{2+} sources. When $[Ca^{2+}]_i$ was analyzed in intact gastrulating embryos using a Ca^{2+} imaging technique, a higher $[Ca^{2+}]_i$ was observed in the anterior dorsal part of the ectoderm [28]. As gastrulation proceeded, the $[Ca^{2+}]_i$ increased and reached a peak level by mid-gastrulation, just prior to NED [28]. The investigators later proposed that the $[Ca^{2+}]_i$ increase might be localized in the BCNE [29]. A transient $[Ca^{2+}]_i$ increase is therefore the first directly visualized event linked to neural induction [29].

In isolated ectodermal explants, expression of the neural-cell-fate-inducing gene Xlpou2 (Xenopus homologue of Pou3f4) is observed soon ($\sim$30 min) after the increase in $[Ca^{2+}]_i$, and the DSCC antagonist blocks expression of Xlpou2 in response to noggin [29]. These results, together with findings on another neural-cell-fate-inducing gene, Zic3 (see below), demonstrate the direct NED-facilitating actions of an increase in $[Ca^{2+}]_i$.

However, the involvement of the $[Ca^{2+}]_i$ increase in NED may not be limited to the direct action on the ectoderm. Palma et al. [30] found misexpression of DSCC caused NED in embryos, but not in ectodermal (animal cap) explants. The NED in these embryos was shown to be caused by ectopic dorsal mesoderm expressing cerberus and chordin in the ventral side [30], raising the possibility that Ca^{2+} influx can facilitate the formation of dorsal mesoderm. It seems likely that the role of increased $[Ca^{2+}]_i$ in NED is bimodal in that it acts directly in the ectoderm and indirectly through the dorsalization of the mesoderm.

Targets Downstream of BMP-Signal Blocking, FGF4, and Calcium Signaling in NED

The emergence of the three signals discussed above (BMP-signal blocking, FGF4, and $[Ca^{2+}]_i$ increases) led us to the important question of how these signals are conveyed to the actual executers of NED (Fig. 3). Several transcription factors are thought to be downstream targets of BMP-signal blocking [22]. Here, we focus on the Zic family of transcription factors, which have been analyzed as downstream

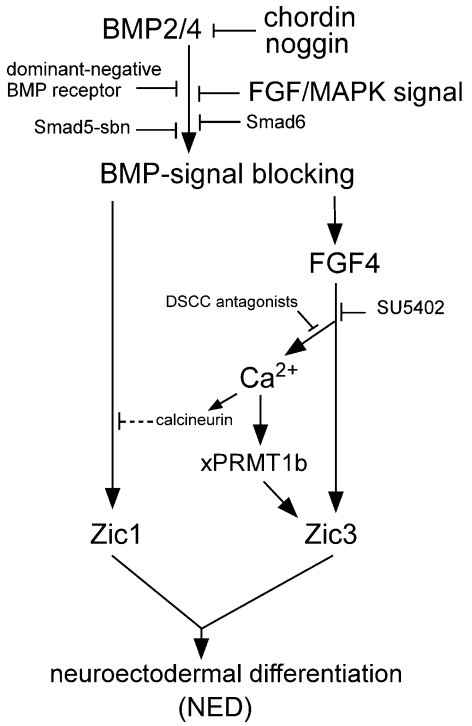

Fig. 3 NED signaling pathways and Zic1/3 genes. BMP-signal blocking, FGF, and calcium signaling regulate the expression of the NED-enhancing transcription factors Zic1 and Zic3

targets of all three NED-inducing signals. After blocking of BMP signaling in Xenopus embryos (either by mRNA injection of truncated BMP receptor, noggin, or chordin), the Zic1 and Zic3 genes are induced in NE [8, 11]. Overexpression of Zic1 and Zic3 results in the expansion of neuroectoderm, following the activation of bHLH-type transcription factors, including ascl3, neurogenin1, or neurod1 [8, 11]. A noggin-responsive sequence was identified in the promoter region of the Zic1 gene [31]. The sequence may provide us with a clue to understanding the processes downstream of BMP-signal blocking.

Zic3 expression is also regulated by $[Ca^{2+}]_i$ [28, 32]. The pattern of $[Ca^{2+}]_i$ accumulation in ectoderm-mesoderm planar explants (Keller explants) correlates with the mRNA expression of Zic3 [32]. When the internal Ca^{2+} transients are blocked with DSCC antagonists, the level of Zic3 expression is dramatically reduced both in explants [32] and embryos [28] (Fig. 3). The arginine *N*-methyltransferase gene, xPRMT1b, which is one of the early $[Ca^{2+}]_i$-dependent genes involved in NED, can induce Zic3 expression, whereas an oligonucleotide against xPRMT1b inhibits caffeine-induced Zic3 expression in isolated ectodermal explants [27]. This suggests that xPRMT1b is a direct link between a transient $[Ca^{2+}]_i$ increase and downstream genes involved in NED [27].

Marchal et al. [21] addressed the differential activation of target genes downstream of FGF4 and BMP-signal blocking in Xenopus embryos [21]. They screened a large number of candidate genes and found that the genes encoding Zic1 and Zic3 are activated by noggin overexpression and suppressed by an FGF signaling inhibitor (SU5402). Interestingly, the extent of SU5402-mediated inhibition on the noggin-overexpressing embryos differs between Zic1 and Zic3 genes—Zic1 expression is maintained whereas Zic3 expression is totally suppressed. In agreement with this, when embryos injected with Smad5-sbn are then treated with SU5402, expression of Zic1, but not Zic3, is maintained. They also found that Zic3, but not Zic1 expression, is upregulated in the presence of a low dose of cycloheximide, an inhibitor of translation, indicating that Zic3 is one of the first cycloheximide-resistant neural targets of FGF signaling in Xenopus embryos. Furthermore, they observed that Zic1 expression is activated by noggin in the presence of cycloheximide, whereas Zic3 is not. Therefore, BMP-signal blocking and FGF signaling are responsible for the initiation of Zic1 and Zic3 expression, respectively.

The findings by the Kodjabachian [21] and Moreau [27, 28, 32] groups provide us with intriguing clues as to the relationship among BMP-signal blocking, FGF signaling, and calcium signaling in NED. Because Zic1 and Zic3 are structurally related and are paralogs with overlapping function and expression, and they respond differentially to the three NED-inducing signals, it seems likely that the actions of Zic1 and Zic3 co-ordinate the final merging of the three NED-inducing signals (Fig. 3).

It is known that the pathways involving the three signals share contact points and interactions (crosstalk) besides the regulation of Zic genes. For instance, Smad1 transcriptional activity is inhibited by the FGF-signal-mediated phosphorylation of its linker region (distinct target phosphorylation residue from that of BMP receptor-mediated one) [33], and BMP-antagonist-mediated signals and FGF signals are integrated at the level of Smad1 [3] (Figs. 2, 3). The integration at the Smad1 level is independent of Zic1 and Zic3 coordination because Zic3 is induced by FGF4 even in the presence of an FGF-signal-insensitive Smad1 mutant [21]. Furthermore, the sensitivity of Zic1 expression to BMP-signal blocking remains in the presence of an FGF-signaling inhibitor [21]. The two integration systems (Smad1 level and Zic1/3 coordination) may function concurrently although the significance of each system and their relationship should be addressed further. Integration points between FGF and calcium signaling also exist. FGF signaling activates Ca^{2+} channels in isolated ectodermal explants [34]. Noggin induces elevation of $[Ca^{2+}]_i$, and this effect is blocked by SU5402, indicating that the noggin-mediated activation of calcium signaling requires the FGF signal [35]. In addition, FGF4-induced $[Ca^{2+}]_i$ increases are inhibited both by SU5402 and DSCC antagonists [35]

(Fig. 3). Another possible mechanism for the integration of the NED-inducing signals is Ca^{2+}/calmodulin-dependent phosphatase 2B (calcineurin) inhibition of the phosphorylation of Smad1, which results in BMP-signal blocking [25] (Fig. 3).

Interactions Among the Zic Genes

Having reviewed the three most potent NED signals, BMP-signal blocking, FGF, and Ca^{2+}, we will now discuss another basis for their signaling integration. Marchal et al. [21] injected Zic1 and Zic3 function-suppressing morpholino oligonucleotides into Xenopus embryos and found that the combined injection of Zic1 and Zic3 morpholinos suppresses the proper expression of the neural plate marker, Sox2. Consistent with this result, the combination of mouse Zic1 and Zic3 null mutations results in severely impaired forebrain development, which is not obvious in either of the single mutants [36]. Taken together, these results suggest that functional integration of the pathways downstream of the three NED signals can occur at the level of synergism between Zic1 and Zic3. Both Zic1 and Zic3 can bind the same target sequences [37] reflecting the high conservation of the zinc finger domain sequences, and their overexpression causes both the expansion of neural plate and enhanced neural crest tissue generation [8, 9].

There are similarities in the expression of Zic genes during gastrulation between mouse, Xenopus, and zebrafish. In mouse, Zic3 expression can be seen in the epiblast layer of E6.0 prestreak stage embryos before gastrulation [38]. At the early gastrulation stage (E6.75–E7.0), Zic3 expression is found in NE and underlying mesoderm [38–40]. In contrast, Zic1 expression in NE is first detected at E7.25 [39]. In Xenopus, the first moderate expression of Zic1 and Zic3 can be seen in the dorsal marginal zone before gastrulation; however, during gastrulation the expression of Zic3 is much higher than that of Zic1 in the involuting mesoderm/prospective neuroectoderm) [41] (Fujimi et al., unpublished). In zebrafish early gastrula, Zic3 is expressed in posterior NE, whereas Zic1 is not expressed in this tissue [42]. Zic1 expression starts in the anteriormost domain of NE at mid-gastrula. Therefore, in these three species, Zic3 is expressed in both mesoderm and NE at the early gastrulation stage, whereas Zic1 is preferentially expressed in prospective NE.

The expression patterns and the loss-of-function phenotypes in Xenopus embryos indicate that Zic1 and Zic3 share a critical role in NED. Together with the differential gene expression activated by BMP-signal blocking and FGF signaling, the interaction between Zic1 and Zic3 can be regarded as a site of integration of the two NED signals. Further study of the molecular mechanism of FGF- and Ca^{2+} signaling-dependent Zic3 gene expression regulation in early embryos should provide a better understanding of NED.

Implications from Stem Cell Biology

Zic genes are versatile tool-kit genes that are used in many eumetazoan developmental contexts [43–47]. They are implicated in human congenital anomalies and are markers for brain tumors (medulloblastoma and meningioma) [48–50]. Recent studies have revealed that Zic genes play important roles in the regulation of mammalian embryonic development by controlling the differentiation status of stem cells. For example, Zic3 is required for the maintenance of pluripotency in mouse and human embryonic stem (ES) cells [51]. Interestingly, RNA interference–mediated suppression of Zic3 in ES cells induces expression of several markers of the endodermal lineage. Furthermore, expression of Nanog, a repressor of extraembryonic endoderm specification in ES cells, is reduced in Zic3-suppressed cells [51], and the Nanog promoter is directly upregulated by Zic3 [52]. Thus, Zic3 has been hypothesized to maintain the pluripotency of ES cells by preventing endodermal differentiation [51]. The proposed role of Zic3 in preventing endodermal fate in ES cells seems rational considering that Zic3 possesses NED-enhancing and mesodermal-development (MED)-controlling abilities. Mouse ES cells possess the cell properties of the inner cell mass, which is a developmentally earlier stage than that in which NED occurs. Therefore, Zic3 might act early as the regulator of meso-ectodermal cell fate competence, and later as the NED/MED controlling factor.

Another intriguing finding is that Zic1 has been identified as a neuronal cell-fate inducing gene in mouse fibroblasts [53]; in a screen for genes that induce neural cell fate, five genes (Pou3f2, Pou3f4, Myt1l, Zic1, and Olig2) were found to substantially potentiate the neuron-inducing activities of Ascl1. Zic3 and other Zic genes were not among the genes available to be screened.

Thus, it is likely that NED mechanisms related to Zic1 and Zic3 are highly conserved between amphibians and mammals during embryonic development. The involvement of Zic family genes in stem cell regulation might not be limited to Zic3 because the Zic2 protein is detected in the inner cell mass of blastocysts [54], and Zic2 and Zic5 have been reported as potential downstream target genes of transcription factors essential for pluripotency maintenance and self-renewal (Pou5f1, Sox2 and Nanog) [55]. Furthermore, functional redundancy of Zic2 and Zic3 are indicated by the Zic2/Zic3 compound mutant mice phenotypes [40]. We consider that the biological characterization of Zic family genes would contribute greatly to our ability to control the differentiation of embryonic and

neural stem cells. In particular, both FGF signaling [56, 57] and Ca^{2+} influx [58] can enhance NED of mouse ES cells. It is clear that the role of Zic genes as downstream targets of the BMP-blocking/FGF/Ca^{2+} signaling in NED should be explored further.

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