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4   **Neural induction: Historical views and application to pluripotent stem cells**

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19   **Abstract**

20   Embryonic stem (ES) cells are a useful experimental material to recapitulate the differentiation

21   steps of early embryos, which are usually invisible and inaccessible from outside of the body, especially

22   in mammals. ES cells have greatly facilitated the analyses of gene expression profiles and cell

23   characteristics. In addition, understanding the mechanisms during neural differentiation is important for

24   clinical purposes, such as developing new therapeutic methods or regenerative medicine. As neurons

25   have very limited regenerative ability, neurodegenerative diseases are usually intractable, and patients

26   suffer from the disease throughout their lifetimes. The functional cells generated from ES cells *in vitro*

27   could replace degenerative areas by transplantation.

28   In this review, we will first demonstrate the historical views and widely accepted concepts

29   regarding the molecular mechanisms of neural induction and positional information to produce the

30   specific types of neurons in model animals. Next, we will describe how these concepts have recently

31   been applied to the research in the establishment of the methodology of neural differentiation from

32   mammalian ES cells. Finally, we will focus on examples of the applications of differentiation systems

33   to clinical purposes. Overall, the discussion will focus on how historical developmental studies are

34   applied to state-of-the-art stem cell research.

Neural induction in *Xenopus* and chick embryos

Neural induction, where uncommitted naïve cells acquire neural fate, is one of the events that occur early in vertebrate embryogenesis. As early mammalian embryos are too small and difficult to access, the molecular mechanisms for vertebrate neural induction were historically analysed using model animals that develop outside the mother's body, such as amphibians and chickens (Grunz, 1997; Munoz-Sanjuan and Brivanlou, 2002; Storey et al., 1992). The embryos of African clawed frog (*Xenopus laevis*) and chicks reach the gastrula stage in half a day after fertilisation, in which uncommitted cells begin to acquire the specific characteristics of three germ layers of ectoderm, mesoderm, and endoderm (Sasai et al., 2008). At this stage, the secreted factors that are collectively called "neural inducers" are produced in the specific area, called the organiser. The organiser differentiates into the dorsal mesoderm (notochord), and the neural inducers produced in the organiser act on the adjacent ectodermal cells and convert the neural progenitors into neurons (Stern, 2005). While the production and existence of neural inducing molecules in the organiser region were proven by classical experiments (e.g., transplantation experiments) (Spemann and Mangold, 2001), the responsive molecules remained elusive for many years. In the early 1990s, the development of techniques in molecular biology allowed the genes expressed in tiny areas in the embryos to be isolated. As a result, the secreted Noggin, Follistatin, and Chordin molecules, which assign a neural fate to the uncommitted cells, were isolated in *Xenopus* (Hemmati-Brivanlou et al., 1994; Sasai et al., 1994; Smith and Harland, 1992). These molecules are expressed in the organiser region and act on the uncommitted ectodermal cells to establish a neural fate. Moreover, while each of the inducers acts in a compensatory manner, the attenuation of all of them perturbs neural cells as well as the dorsal mesoderm development (Khokha et al., 2005), suggesting that these neural inducers are essential for neural cell fate decision (Fig. 1A).

Animal cap explant prepared from the animal pole area of *Xenopus* gastrula embryos, which is equivalent to the post-implantation epiblast of mouse embryos, is a good experimental system to recapitulate differentiation into specific types of cells (Green, 1999; Sive et al., 2007). Animal cap cells treated with Chordin differentiate into neural cells (Sasai et al., 1995), suggesting that neural differentiation can be regulated *in vitro*.

Subsequent mechanistic analyses revealed that neural inducers bind to a secreted growth factor BMP4 in the extracellular space and inhibit BMP4 binding to the BMP receptor (Hemmati-Brivanlou et al., 1994; Piccolo et al., 1996; Zimmerman et al., 1996). Consistently, overexpression of the dominant-negative mutant of the BMP receptor also induces neural cell fate in a cell-autonomous manner (Suzuki et al., 1994). The ectodermal cells exposed to the BMP signal differentiate into epidermis; therefore, naive ectodermal cells have a binary fate decision between the epidermis and neural tissue depending on the existence of the BMP signal.

This fact suggests that it is epidermal fate that must be actively induced by the BMP signal; otherwise the uncommitted cells are fated to neural as their default status (Hemmati-Brivanlou and

71 Melton, 1997; Munoz-Sanjuan and Brivanlou, 2002; Stern, 2005). This postulation, or the "neural

72 default model", turned out to be partly true as supported by an experiment in which dissociated animal

73 cap cells tended to differentiate into neural cells (Munoz-Sanjuan and Brivanlou, 2002). However, later

74 studies revealed that the mere signal blockade is not sufficient for the neural induction. For instance,

75 the blockade of the FGF signal inhibits the neural induction in *Xenopus* (Marchal et al., 2009; Pera et

76 al., 2003). Moreover, in chick embryos, the blockade of the BMP signal by Chordin and Noggin was

77 shown to be insufficient for neural induction (Streit et al., 1998), suggesting that additional inducing

78 signals are required, or more upstream factor(s) are involved in the neural induction.

79   Currently, the integration of the FGF signal in addition to the BMP blockade by the neural

80 inducer is thought to be required for vertebrate neural induction (Fig. 1A) (Linker and Stern, 2004;

81 Marchal et al., 2009; Pera et al., 2003), and more unidentified signals are also suggested for the stability

82 of the neural identity.

83

84 **Positional information**

85   In *Xenopus* and chicks, the anterior-posterior (A-P) and dorsal-ventral polarities in neural

86 tissues are thought to be generated after the cells attain a neural fate, and the neural plate is formed on

87 the dorsal side of the embryos. It has been suggested that the neural tissue originally induced by BMP

88 blocking factors possess the anterior neural fate and can be transformed by the transient input of

89 posteriorising signals (two-step model) (Sasai and De Robertis, 1997). The representative posteriorising

90 factors identified so far are FGF, Wnt, and retinoic acid (RA). In other words, the blockade of these

91 posteriorsing factors is required for the maintenance of the anterior fate (Rallu et al., 2002). For instance,

92 Dickkopf-1 (Dkk1) (Glinka et al., 1998) is expressed in the prechordal mesoderm, or head mesoderm,

93 and acts as an antagonist of Wnt. Dkk1 is required for head formation, as the injection of the neutral

94 antibody against Dkk1 causes microcephaly and cyclopia (Glinka et al., 1998). Lefty is a secreted

95 molecule antagonising Nodal (Juan and Hamada, 2001), and Cerberus (Piccolo et al., 1999; Silva et al.,

96 2003) inhibits multiple factors of Nodal, BMP and Wnt, and gives rise to the anterior cell fate.

97 Importantly, Nodal induces the expression of Lefty and Cerberus (Whitman, 2001), suggesting that

98 these molecules form a negative feedback loop. Likewise, *Shisa*, which encodes a protein localised to

99 the endoplasmic reticulum, is expressed in the head region, and specifically binds to the immature forms

100 of FGF receptor and Wnt receptor (Frizzled) to inhibit their maturations and trafficking to the cell

101 surface; thereby the anterior cells get insensitive to FGF and Wnt signals (Yamamoto et al., 2005). On

102 the other hand, activation of FGF and Wnt signals provides caudal identities (Brafman and Willert,

103 2017; Mulligan and Cheyette, 2012). Therefore, the gradients of FGF and Wnt correspond to the A-P

104 identity (McGrew et al., 1997; Yamaguchi, 2001). The combinatorial treatment of Chordin and

105 Wnt/FGF in explants provides posterior identities (Christen and Slack, 1997) in the nervous system,

106 and also produces neural crest cells (Fig. 2) (LaBonne and Bronner-Fraser, 1998; Sasai et al., 2001).

107   Retinoic acid also has a posteriorising activity; embryos treated with RA lose the anterior
108   structure (Strate et al., 2009). The activity, however, seems to be more localized compared to Wnt and
109   FGF and is mostly involved in the patterning within the hindbrain region and anterior spinal cord (Dupe
110   and Lumsden, 2001; Glover et al., 2006; Nordstrom et al., 2006; Sirbu et al., 2005). The hindbrain is
111   divided into eight regions of rhombomeres r1-r8, and the posterior parts (r4-r8) are the areas where RA
112   activity is high (Schilling et al., 2016). In contrast, the anterior rhombomeres (r1-r3) express the RA
113   hydroxylase Cyp26 and the RA activity is rather blocked (Schilling et al., 2016).

114   The dorsal-ventral polarity within the neural tissue is generated by the gradients of BMP and
115   Wnt (expressed at the dorsal side) and Sonic Hedgehog (Shh) (at the ventral side). The treatment with
116   different concentrations of Shh on the neural explant isolated from chick embryos provides different
117   neural subtypes, indicating the morphogen model is correct (Dessaud et al., 2010; Dessaud et al., 2007;
118   Marti et al., 1995; Wichterle et al., 2002; Yamada et al., 1993).

119   The aforementioned signal molecules, including RA (Schilling et al., 2012; Shimozono et al.,
120   2013), form gradients in neural tissues and determine the cell fate according to their concentrations.
121   Therefore, they are called morphogens, meaning "form-giving substances" (Ashe and Briscoe, 2006;
122   Wartlick et al., 2009) (Fig. 2).

123

124   **Conserved and varied mechanisms of neural induction in mammalian embryos**

125   In parallel with the studies in amphibian and avian, the functions of the mammalian orthologues
126   have been analysed, mainly utilizing gene knockout mice. In mouse, the compound mutant of *noggin*
127   and *chordin* genes demonstrates the perturbation of head development as well as the forebrain formation
128   (Bachiller et al., 2000), suggesting that the requirement of the blockade of the BMP signal for neural
129   fate decision is conserved among vertebrate species. Likewise, Wnt signalling is required for dorsal
130   neural specifications, including neural crest differentiation (Ikeya et al., 1997), and the mice with the
131   *Dkk1* gene knockout exhibits the defects in head formation (Mukhopadhyay et al., 2001). Shh was also
132   shown to be critical for ventral neural identities, as well as head formation (Chiang et al., 1996).

133   On the other hand, unexpectedly, some genes are not directly related to neural induction and
134   specification. For instance, while Cerberus is required for head formation in *Xenopus* (Silva et al., 2003),
135   this gene is not required for head formation in mice (Simpson et al., 1999). Likewise, the mice devoid
136   of the *Shisa* homologues do not demonstrate any phenotypes in the head formation (Furushima et al.,
137   2007). These apparent "discrepancies" could be explained either by the redundant functions of genes
138   and/ or by the difference in the steps during the neural development.

139   As for the neural specification, it has been suggested that there exist bipotent cells in the caudal
140   lateral epiblast that can differentiate into spinal cord and paraxial mesoderm (Tzouanacou et al., 2009).
141   This analysis suggests that the three germ layers of ectoderm, mesoderm, and endoderm are not
142   separated at the same time, but rather neuroectoderm and mesoderm are segregated later when

143 compared to other lineages (Tzouanacou et al., 2009). These cells are called neuromesodermal

144 progenitors (NMps). Importantly, neural cells that differentiate from NMps are mainly the trunk

145 (posterior) type (Tzouanacou et al., 2009). It was further hypothesized that the Wnt/FGF signals are

146 involved in the establishment and the fate decision of NMps (Henrique et al., 2015). This hypothesis

147 was determined to be accurate by the studies using ES cells, as discussed later. Recently the existence

148 of NMps has also been found in chick embryos, at the anterior edge of the primitive streak, suggesting

149 the conservation of the NMps in amniotes (Guillot et al., 2020).

150   The discovery of NMps has suggested that the neurons at the anterior (forebrain and hindbrain)

151 and the trunk (spinal cord) areas have different lineages; the A-P regionalisation occurs before the

152 neural induction, which highlights the difference from the conventional concept where the posterior

153 identities are produced by the transformation of the anterior cells. While the concept of NMps is

154 applicable to *Xenopus* is still unclear, the "transformation model" along the A-P decision must be

155 revised at least in mouse (Fig. 1B).

156

157 **Neural induction from mammalian ES cells**

158   Since mouse ES cells were established, studies to efficiently differentiate the cells into neural

159 and differentiated neurons (neuronal cells) have been conducted, and several protocols have been

160 established mainly by applying the mechanisms revealed by classical analyses.

161   The representative differentiation protocols and their characteristics are listed in the Tab.1. In

162 principle, these protocols were based on medium minimised with cytokines and growth factors that may

163 inhibit neural differentiation (Hemmati-Brivanlou et al., 1994; Sasai et al., 1994; Smith and Harland,

164 1992). Several combinations of media and supplements have been developed as differentiation media.

165 One representative medium contains Glasgow Minimum Essential Medium (GMEM) (Kawasaki et al.,

166 2000; Watanabe et al., 2005). GMEM has twice the concentration of amino acids and vitamins as the

167 other widely used cell culture medium Dulbecco's Modified Eagle Medium (DMEM). This medium

168 must be accompanied by supplements, such as pyruvate and knockout serum replacement (Abranches

169 et al., 2009; Kawasaki et al., 2000).

170   The ES cells cultured on these media start to express the pan-neural precursor markers Sox1

171 and Nestin as early as a few days after the start of the differentiation. Subsequently, the postmitotic

172 marker expressed at mature stages, TuJ, is found around one week. When cells are cultured in the

173 presence of the feeder cells PA6, the cells further differentiate into the midbrain dopaminergic neurons

174 in two weeks (Kawasaki et al., 2000), while the effect of PA6 is still unknown. On the other hand, the

175 cells in a floating culture tend to differentiate into the telencephalic precursors (Watanabe et al., 2005).

176 In this protocol, treatment with Lefty and Dkk1 (Glinka et al., 1998; Juan and Hamada, 2001) increases

177 the positive cells for the telencephalic marker BF1, suggesting that the principles revealed in the

178   *Xenopus* experimental system, in which the blockade of Wnt provides the anterior cell fate, are
179   conserved in the neural differentiation in mouse.

180   Another medium widely used is one composed of a 1:1 ratio of Neurobasal and DMEM/F-12
181   media supplemented with N2 and B27 (Ying et al., 2003b). These cells can further differentiate into
182   tyrosine hydroxylase-positive dopaminergic neurons through treatment with FGF8 and Shh.

183   In these cases, although the neural inducing factors are yet to be identified, BMP treatment
184   blocks neural induction through the upregulation of the transcription factor Ids, suggesting that the
185   blockade of BMP signal is required for the neural induction (Kawasaki et al., 2000; Tropepe et al.,
186   2001; Ying et al., 2003a; Zhang et al., 2010). Therefore, the mechanisms for neural induction in mouse
187   ES cells are conserved with those in other vertebrates. As predicted from the studies in *Xenopus* (Launay
188   et al., 1996) and chicks (Streit et al., 1998), the FGF signal is required for neural induction from ES
189   cells; treatment of the cells with FGF inhibitors or the overexpression of the dominant-negative FGF
190   receptor inhibited the induction (Ying et al., 2003b), suggesting that ES cells express FGF or
191   autonomously activate the FGF signal. A later investigation has revealed that ERK1/2 signalling
192   stimulated by the FGF signal is required for the transition of the pluripotent state to lineage commitment
193   (Kunath et al., 2007).

194   The "neural default model" was attempted in ES cell differentiation. ES cells seeded in a
195   medium in which all components were defined chemicals differentiate into the rostral hypothalamus
196   (Wataya et al., 2008), suggesting that the "neural default mode" is partly conserved among vertebrates
197   in that the cells tend to convert into neural cells.

198   By adding additional signalling molecules such as BMP and Shh, further directed
199   differentiation was achieved. For instance, the multiple types of BMPs confer dorsal interneuron
200   identities (Andrews et al., 2017). In contrast to the BMP signal by which the dorsal neurons are assigned,
201   the treatment of graded concentrations of Shh enabled the ES cells to differentiate into varied ventral
202   subtypes (Kutejova et al., 2016; Mizuseki et al., 2003; Wichterle et al., 2002; Yatsuzuka et al., 2019).

203   During neural differentiation, cells first enter the epiblast stage, positive for FGF5 (Abranches
204   et al., 2009), and gradually differentiate into neuroectodermal cells. The transcription factor Zfp521 is
205   required for the conversion from the epiblast state into the neuroectoderm (Kamiya et al., 2011). In the
206   absence of Zfp521, the cells remain in the epiblast step and cannot progress into the neuroectodermal
207   stage (Kamiya et al., 2011).

208   For differentiation into the spinal cord level, the Wnt signal must be added at the epiblast stage
209   (Gouti et al., 2014) to allow the cells to acquire the posterior state. This treatment produces NMps, and
210   further treatment with RA confers the cells to the neural cell fate (Garriock et al., 2015; Gouti et al.,
211   2014). Moreover, treatment with Wnt (or CHIR99021, which mimics the effect of Wnt by blocking
212   GSK3β) has been shown to segregate anterior and posterior epiblasts, and this segregation is dependent
213   on the presence of the Wnt signal. Moreover, the Wnt signal provides distinct chromatin accessibility

214   on the cells, and this determines the anterior/posterior cell fates (Metzis et al., 2018). This study

215   demonstrated that the epiblast cells are already regionalised; the cells have acquired the A-P identity

216   before neural induction. Further specification within the spinal cord level can be achieved by treatment

217   with RA and different concentrations of Shh (or its agonist SAG) (Sagner et al., 2018).

218

219   **Requirement of the timely signals for the neural differentiation**

220   One important feature in the neural differentiation is that the inducing signals have to be applied

221   at the right time point. For instance, neural inducers cannot produce neural cells even when their

222   expression is forced in mature tissues, suggesting that the cells have to be able to respond to the neural

223   inducers. The ability of the cells to respond to specific inducing signals is called "competence"

224   (Waddington, 1940), which represents an important concept in developmental biology in parallel with

225   the concept of "induction", conferred by the inducing signals from the organisers.

226   Also, the neural progenitor cells at different differentiation steps respond to the same signal

227   molecules in a different manner. For instance, BMP inhibits the neural differentiation when it is applied

228   at the step of the neural induction, but has a dorsalising effect within the nervous system when applied

229   after the cells are fated to neural (Mizuseki et al., 2003; Tozer et al., 2013). Also, while Wnt signals are

230   critical for the posteriorisation of the neural plate at the early steps of the neural differentiation, the

231   same signals applied later acts on the fine patterning within the forebrain without exerting the

232   posteriorizing activity (Green et al., 2020; Kirkeby et al., 2012; Polevoy et al., 2019; Rifes et al., 2020).

233   As another example, the neural progenitor cells applied with the same signal molecule of Shh

234   at different time points respond in a different manner. The neural progenitor cells at an early time point

235   exposed to a high concentration (at the saturated level) of Shh differentiate into the floor plate cells,

236   whereas the cells at a later time point tend to differentiate into the ventral interneuron progenitor cells

237   when exposed to the same level of Shh (Kiecker et al., 2016; Sasai et al., 2014).

238   Although the molecular mechanisms have not been fully elucidated, the competence seems to

239   be determined by a specific chromatin state of the cells. As the differentiation systems from the ES cells

240   enable to isolate the specific differentiation steps of the cells, the molecular characterisation of the

241   competence is warranted in the future investigations.

242

243   **Clinical applications**

244   One possible application of ES cells is in clinical purposes. Once differentiated, neurons cannot

245   regenerate by themselves; therefore, the only fundamental therapeutic method for neurodegenerative

246   diseases is to replace cells in the body with cells differentiated *in vitro* at the site where functions are

247   lost. Below are some examples of the neurodegenerative diseases that studies on ES cells aim to cure.

248   For the establishment of the stem-cell based therapeutic methods, primate (monkey) ES and iPS

249   (induced pluripotent) cells have been used in addition to mouse ES cells, because the differentiated

250　neurons can be experimentally applied to the disease model individuals and the effects can be examined

251　in the conditions which are closer to those of humans.

252

253　**Parkinson's disease**

254　　Parkinson's disease, which is named after the clinician who discovered its symptoms, is a long-

255　term neurodegenerative disease affecting overall movement (Armstrong and Okun, 2020; Kalia and

256　Lang, 2015; Poewe et al., 2017). The symptoms include shaking, stiffness, and difficulty with walking,

257　balance, and coordination (Aging). The degeneration of dopaminergic neurons, which are located at the

258　ventral-most region of the midbrain, is a significant cause of the outset of the disease (Arenas et al.,

259　2015). The degeneration usually occurs in conjunction with ageing, and the symptoms start at over 60

260　years old. The prevalence is higher in men than in women, and more than 10 million people suffer from

261　this disease worldwide (Foundation). There is no fundamental cure for Parkinson's disease, although

262　oral medication of L-dopa is often taken (disease).

263　　Dopaminergic neurons can be generated from ES cells in several ways (Arenas et al., 2015). In

264　mouse ES cells, as described above, when the ES cells are seeded on PA6, feeder cells tend to

265　differentiate into tyrosine hydroxylase-positive dopaminergic neurons (Kawasaki et al., 2000). The

266　other protocol contains the treatment with Shh and FGF8 in Nurr1 (Nuclear receptor related 1/NR4A2)-

267　overexpressed ES cells, and the differentiated neurons are shown to be successfully incorporated into

268　Parkinson's disease model mice (Kim et al., 2002). Mechanistically, the transcription factor LIM

269　Homeobox Transcription Factor 1 Alpha (Lmx1a) is essential for the induction of dopaminergic

270　neurons. (Andersson et al., 2006). Lmx1a, in cooperation with Lmx1b, regulates proliferation,

271　specification, and differentiation of midbrain dopaminergic progenitors (Yan et al., 2011). Furthermore,

272　Lmx1a/b also regulate the mitochondrial functions of the midbrain dopaminergic neurons (Doucet-

273　Beaupre et al., 2016), suggesting that Lmx1a/b are essential not only for induction but for maintenance

274　or cell survival.

275　　The generation of the dopaminergic neurons and treatment in higher mammals are now feasible

276　as well (Kawasaki et al., 2002; Kikuchi et al., 2017).

277

278　**Retinopathy**

279　　In humans, approximately 80% of all external information delivered to the brain is processed

280　by the visual system (Haupt and Huber, 2008). Therefore, vision loss severely impacts the quality of

281　life and daily functioning.

282　　Pigment epithelium cells (RPE) and photoreceptor cells are located in the retina, which is

283　formed at the back in the eyeball (Wright et al., 2010). Among the multiple types of cells in the retina,

284　photoreceptor cells are the first cells that perceive light and colour stimuli. Moreover, RPE and

285　photoreceptor cells run a redox cycle among RA, retinal and retinol, which is called the visual cycle, in

286 conjugation with each other and convert the visual inputs from the periphery to electrical signals to

287 relay them to other cells (Tsin et al., 2018). RPE also phagocytoses the outer segment discs of

288 photoreceptor cells and activates the mTORC1 signal (Yu et al., 2018), thereby encouraging the renewal

289 of photoreceptor cells. Thus, the photoreceptor cells and RPE are critical components of the retina, as

290 well as other eye regions.

291 Age-related macular degeneration and retinitis pigmentosa are two major eye diseases caused

292 by the malfunction or degeneration of photoreceptor and RPE cells (Wright et al., 2010). The prevalence

293 of these diseases is 1 in 3,000 to 4,000 people worldwide, with frequent cases occurring due to an

294 inherited trait.

295 The symptoms start with nyctalopia (night blindness) and deficits in the visual field with

296 gradual vision loss, resulting in complete vision loss in some cases (degeneration). There are no

297 standardised clinical treatments, and therefore these diseases are assumed to be "designated intractable

298 diseases" in Japan (Kanatani et al., 2017).

299 Therefore, the generation of RPE and photoreceptor cells *in vitro* is an important sight research

300 objective for transplantation to replace non-functional regions. Photoreceptor cells were successfully

301 differentiated from mouse ES cells by modifying the serum-free floating culture of embryoid body-like

302 aggregates (SFEB). In this method, considering that primordial retina arises from the ventral region of

303 forebrain (Ikeda et al., 2005), ES cells were treated with DKK1, LeftyA, serum, and Activin to induce

304 the cells positive for rhodopsin and recoverin. The differentiated photoreceptor cells were transplanted

305 into the mouse retina and were confirmed to be incorporated there.

306 Impressively, the whole retinal structure, which is called an organoid, was successfully

307 generated in mouse (Eiraku et al., 2011) and human (Nakano et al., 2012). Further studies are required

308 to apply these ES cell-derived tissues for clinical purposes. For instance, investigations of the functional

309 validation, efficient growth and reproducible generation of the tissues are of importance. However, the

310 method is definitely powerful for future regenerative medicine.

311 Pigment epithelium cells (Osakada et al., 2009b) and photoreceptor cells (Osakada et al.,

312 2009a) can be generated from human ES cells as well, and the transplantations of RPE cells into patients

313 with age-related macular degeneration are ongoing (Liu et al., 2018; Qiu, 2019).

314 The explorations of both the two-dimensional (2D) differentiation (generation of the specific

315 retinal components) and three-dimensional (3D) differentiation (making the whole retinal tissues)

316 methods will be useful for clinical purposes, as the 2D differentiation will be more advantageous for

317 transplantation to the disease areas, while the 3D differentiation will be useful for recapitulating the

318 progression of the retinopathy and developing novel therapeutic methods.

319

320 **Motor neuron disease**

321   ALS, Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disorder in which motion

322 is gradually weakened by the loss of both upper and lower motor neurons. In addition to the sporadic

323 form, more than 30 genes have been recognised as causative genes for the inherited form. Among them,

324 the mutations in *c9orf72*, encoding a guanine nucleotide exchange factor (Balendra and Isaacs, 2018),

325 *SOD-1* (*superoxide dismutase-1*) and *TDP-43* (*TAR DNA-binding protein-43*) genes have been found

326 in many cases (van Es et al., 2017). SMA, spinal muscular atrophy (SMA) is another lethal motor

327 neuron disease characterised by the loss of somatic motor neurons and innervation to voluntary skeletal

328 muscles, leading to death by respiratory failure. One of the causal genes of this disease is *survival motor*

329 *neuron gene 1* (*SMN1*); in 95% cases, the patients with this disease have lost the *SMN1* gene. Moreover,

330 its homologue *SMN2* exists in human, and a single mutation at the splicing site perturbs the proper

331 splicing and the non-functional protein is produced. Although some medicines have been developed,

332 fundamental therapeutic strategies are still awaited (Ebert and Svendsen, 2010).

333   Transplantation of the motor neurons generated from the ES cells is a promising therapeutic

334 method to cure these diseases. Motor neurons can be generated from ES cells by the sequential treatment

335 with RA (posteriorisation) and Shh (ventralisation) (Wichterle et al., 2002; Wichterle and Peljto, 2008).

336 Further analysis revealed that the motor neuron in this method is lateral motor column, and can be

337 grafted into chick spinal cord and was confirmed to settle in appropriate columnar domains (Peljto et

338 al., 2010). Motor neuron can also be generated from human ES cells (Shin et al., 2005) and iPS cells

339 (Dimos et al., 2008; Qu et al., 2014; Sances et al., 2016). Further, the motor neuron was differentiated

340 from the human ES cells with the SOD1 mutation and the alterations of cell morphology and reduction

341 of cell survival was recapitulated (Karumbayaram et al., 2009). This study is an excellent example of

342 using the ES cell-derived motor neuron for disease modelling, with which the mechanisms of the disease

343 or the effects of candidate therapeutics can be investigated.

344

345 **Perspectives**

346   By applying the principles revealed by classical model animals such as *Xenopus* and chick, it

347 is now feasible to recapitulate the developmental process *in vitro* by ES and induced pluripotent stem

348 cells. Now novel principles can also be elucidated using the ES/induced pluripotent stem cell

349 differentiation systems.

350   For future perspectives, more detailed analyses in neural differentiation from stem cells will be

351 possible. For instance, it would be feasible to describe gene expression profiles using single-cell

352 expression analysis and compare the expression profiles with those from embryos. This kind of analysis

353 will identify new genes essential for specific stages of differentiation and reveal new gene regulatory

354 networks.

355   It is noteworthy that the recently established CRISPR/Cas9 method (Andrey and Spielmann,

356 2017; Zhang et al., 2017) has enabled targeted modifications on the genome. Using this method, it is

357   now possible to create many genetically modified ES cells, including gene knockouts and reporter lines

358   systematically, and to analyse their effects during the differentiation (Nakatake et al., 2020). This

359   method can be significantly advantageous to conventional analytical methods using gene knockout

360   embryos in terms of time and resources. Moreover, as *in vitro* neural differentiation from ES cells occurs

361   without being affected by the other developing organs, the direct effects of gene deficiencies in neural

362   differentiation can be analysed. This research will be conducive to establish the protocol in which

363   specific functional neurons can be created more efficiently.

364   However, whether ES cells are the only way to obtain specific purified neurons efficiently *in*

365   *vitro* must be reconsidered. As discussed above, the ES cells have to undergo several regulatory steps

366   (Fig. 1), and many subtle techniques are needed to obtain a single type of neurons with a high purity.

367   One idea is to use neural progenitor cells. If neural progenitor cells could be stably maintained,

368   the steps required for differentiation into postmitotic neurons are only those for the terminal

369   differentiation, and the efficiency will be expected to be significantly improved. Such cells can be

370   isolated from the postnatal brain (Lupatov et al., 2017; Palm et al., 2015), and it has been shown that

371   these cells can be maintained and can differentiate into astroglia (Palm et al., 2015) and dendritic cells

372   (Lupatov et al., 2017). Further analyses will elucidate the characteristics of this type of stem cells.

373   In addition, strategies for *in vivo* reprogramming are now being explored. These strategies

374   include the delivery of reprogramming factors into the injured or degenerated areas by DNA injection

375   or adeno-associated virus, attempting to let the live cells acquire the target cell fate (Ofenbauer and

376   Tursun, 2019). In the cases of neuron, the Müller cells in the retina injected with β-catenin to encourage

377   the glial proliferation followed by the injection of three transcription factors of Otx2, Crx and Nrl

378   directly reprogrammes the Müller glial cells to the photoreceptor fate (Yao et al., 2018). Once this

379   method is established, it will be useful as the transplantation of the cells can be bypassed.

380   Almost thirty years have passed since neural inducers were isolated in frogs, and now the

381   generation of whole organs is conceivable (Kim et al., 2020); the combination of classical knowledge

382   and cutting-edge research methods will be warranted for the development of molecular mechanisms for

383   neural differentiation and stem cell techniques.

384

390

391   **Competing Interest**

392   The authors declare that no competing interests exist.

393 **Figures**

394 **Fig.1 The outline of the neural differentiation and representative signal molecules involved in**

395 **each differentiation step.** (A) Conventional/historical and (B) revised models for the neural induction

396 and specification.

397

398

399 **Fig. 2 A simplified schematic showing the signal molecules providing the positional information**

400 **in the embryo.** A: anterior, P: posterior, D: dorsal, V: ventral, FB: forebrain, MB: midbrain, HB:

401 hindbrain, SC: spinal cord. A-P polarity is formed at the late gastrulation to the neural plate stages

402 (around e5.0-6.0 in the mouse and HH stages 3-5 in the chick), and the D-V patterning is formed later

403 after the neural tube is formed (around e8.0-9.0 in the mouse and HH stages 10-12 in the chick).

404

405 **Table**

406 **Tab. 1 Representative protocols for neural differentiation.**

407

408 **Additional Reference in the Tab. 1**

409 (Bain et al., 1995; Chanoumidou et al., 2018; Eiraku and Sasai, 2011; Forouzanfar et al., 2015; Rao et

410 al., 2020)

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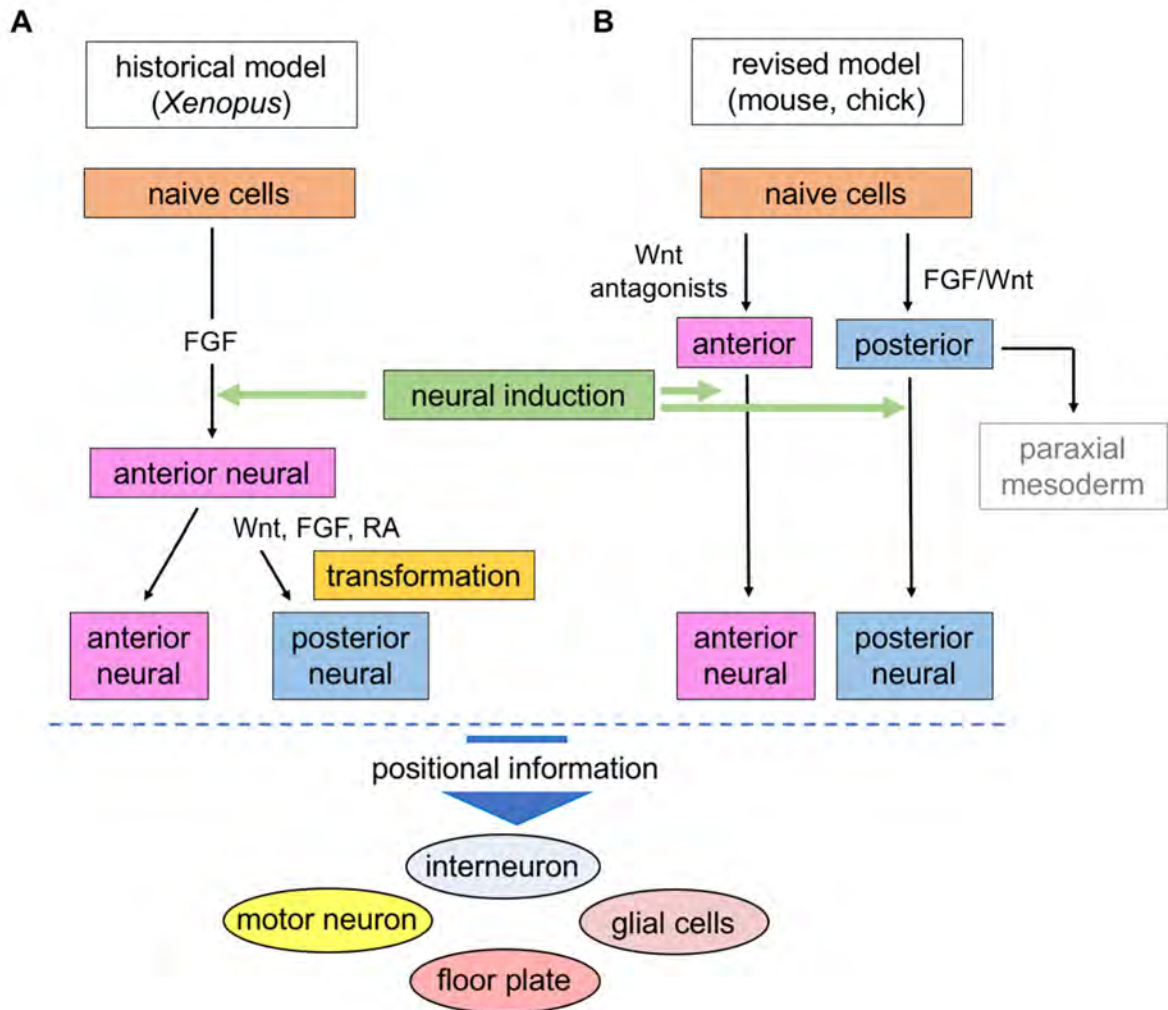729   epithelium. Sci Signal 11.
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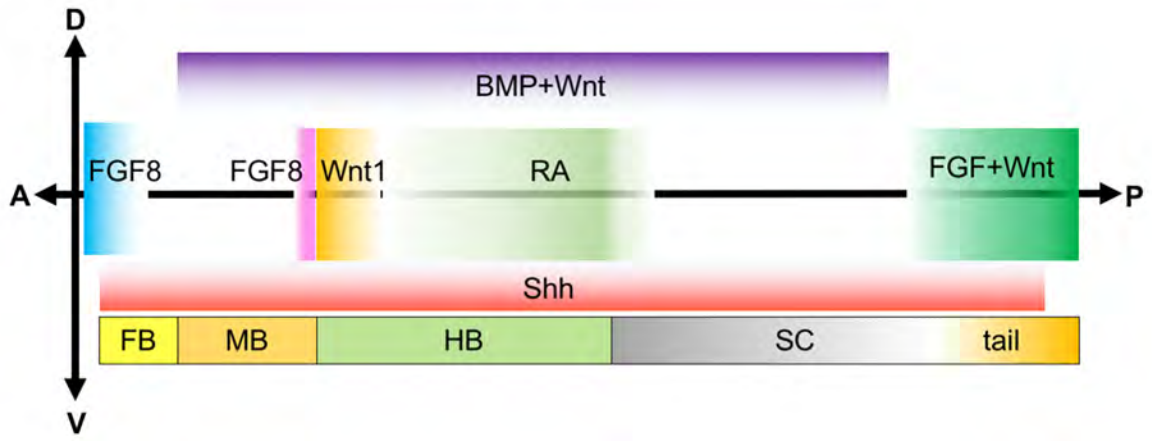736   binds and inactivates bone morphogenetic protein 4. Cell 86, 599-606.
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Protocol	Statements	References
Pre-application of knowledge on development biology		
Retinoic acid induction	- ES cells cultured in non-adherent surface, forming embryoid bodies (EBs). - 8 days culture: 4 days without RA followed by 4 days in the presence of RA (4-/4+). - Difficulty in analyzing and controlling the differentiation due to EBs containing different types of cells (mesodermal & endodermal cells).	Bain et al. (1995)
DMEM/F12 + ITSFn medium DMEM/F12 + modified N3 medium (containing bFGF and laminin)	- ES cells cultured as suspension for 4 days. - EBs then cultured with DMEM/F12 with bFGF and insulin, transferrin, selenium chloride and fibronectin (ITSFn medium). - Neuroepithelium precursors (Nestin+) appear after expansion in mN3 medium in the presence of bFGF and further differentiation (dopaminergic neurons and glial cells) occurs by withdrawal of bFGF.	Okabe et al. (1996); Lee et al. (2000)
Application of knowledge on development biology		
Stromal cell-derived inducing activity (SDIA)/coculture with PA6 (stromal cells derived from skull bone marrow) cells	- PA6 coculture for 8 days in GMEM/10% KSR and 6 days in GMEM/N2 (removal of FCS). - Generation of neural precursor cells (Nestin+ and TuJ+) and mesencephalic dopaminergic neurons (TH+).	Kawasaki et al. (2000)
Adherent monolayer culture with serum-free N2B27 medium (1:1 DMEM/F12 medium + modified N2 and Neurobasal medium + B27)	- Sox1-GFP and Tau-GFP ES cells. - Generation of neuroepithelial cells with rosette morphology (Nestin+), and extended neuronal processes (GABA+). - Replating of cells with addition of FGF8 and Shh resulted in generation of neurons (TH+).	Ying et al. (2003)
SFEB culture with GMEM + 5% KSR	- SFEB culture (8 days) and adherent culture (2 days) generated TuJ1+ neurons and Nestin+ neural precursors. - Combination treatment of Wnt and Nodal antagonists (Dkk1 and LeftyA) further enhanced neural differentiation. - Telencephalic differentiation occurs in the presence of Dkk1 or/and LeftyA (first 5 days of SFEB culture). - Regional specification of telencephalic precursor cells in respond to dorsoventral patterning signal (Wnt3a and Shh).	Watanabe et al. (2005)

SFEB culture with growth factor-free chemically defined medium (gfCDM)	- Spontaneous differentiation into rostral hypothalamic progenitor-like cells (Rax+/Six3+/Vax1+). - Generation of dorsal hypothalamic progenitors (Pax6+/Nkx2.1-) and vasopressinergic neurons (Otp+/Brn2+) without Shh. - Generation of ventral hypothalamic progenitors (Pax6-/Nkx2.1+), glutamatergic neurons (SF1+) and dopaminergic neurons (TH+/Nkx2.1+) with Shh.	Wataya et al. (2008)
SFEB culture with GMEM + 10% KSR, followed by DMEM/F12	- Generation of telencephalic neuroepithelium (Bf1+) - Cell aggregates contain several rosettes (Pax6+/Tbr1+) recapitulate early embryonic corticogenesis	Eiraku et al. (2012)
Post application of knowledge on developmental biology		
Fndc5 overexpression	- Addition of RA and Fndc5 overexpression was induced by Doxycycline lead to formation of neural precursor cells and improved differentiation into neuronal cells and astrocytes.	Forouzanfar et al. (2015)
GRG5 overexpression	- GRG5 promotes neural fate specification of ES cells through suppression of Wnt and Bmp signalling. - GRG5 overexpression in neural stem cells enhances self-renewal ability through Notch and Stat3 signalling.	Chanoumidou et al. (2018)
E2A overexpression	- Neural lineage commitment is perturbed in E2A knockout ES cells. - E2A activates neural lineage associated genes (Sox1 and Foxd4) and suppresses Nodal signalling.	Rao et al. (2020)