Intrinsic and extrinsic factors regulating vertebrate neurogenesis

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Recent studies of the factors regulating neurogenesis in vertebrates reveal three emerging themes. First, the number of cellular stages involved in this process may be greater than has previously been appreciated. Second, homologues of genes that regulate neurogenesis in invertebrates appear to play analogous roles in development of vertebrate nervous systems. Third, extrinsic factors can act to regulate neuron number during neurogenesis by controlling survival and differentiation, and not simply proliferation, of neural progenitor cells.

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Introduction

In this review, I synthesize recent information on the molecular factors that regulate neurogenesis in vertebrates. I will focus on a few classes of molecules, both intrinsic and extrinsic to neuronal precursor cells, that regulate their proliferation, differentiation, and survival. The studies that will be discussed make use of several different vertebrate systems, including rodents, birds, and *Xenopus*, and employ varied techniques, such as tissue culture, genetic analysis, and experimental embryology. One area of vertebrate neurogenesis that, for reasons of space, is not dealt with here is the interesting story that is emerging about the relationship between neurogenesis and song learning in songbirds. The reader is referred to a recent review by Doupe [1] for an introduction to this topic.

General features of vertebrate neurogenesis

The cells that make up vertebrate nervous systems ultimately descend from the ectodermally derived neuroepithelial cells of the neural tube and the neurogenic placodes [2]. In the peripheral nervous system, mesenchymal neural precursors (neural crest cells) emerge from these epithelial structures, and migrate to often distant targets, continuing to proliferate as they migrate [3]. In the central nervous system, most cell proliferation remains confined to an expanding neuroepithelium, which later comes to be called a germinal or ventricular zone as a growing mantle of postmitotic cells forms around it. Some exceptions to this rule occur in locations such as the cerebellum, where the precursors of granule neurons leave the ventricular zone and migrate to the pial surface of the developing brain to form a highly proliferative external granule layer [4].

Before the control of vertebrate neurogenesis can be fully understood, certain basic questions must be answered about how neurogenesis proceeds, such as what sorts of lineages give rise to the cells of the nervous system, and how many phenotypically distinct cellular stages precursors must pass through on their way to becoming fully differentiated neurons. The first question has been approached largely through lineage mapping and cell transplantation studies that involve the marking of precursors with non-transmissable viruses, dyes, or enzymes. The results of these studies indicate that, in many cases, a rather large degree of multipotentiality exists among neural precursors, sometimes even very late in their development [5–13], although this may not be the rule throughout the nervous system [14,15•,16•]. These results pertain to the issue of the existence or absence of lineage restrictions during vertebrate neurogenesis. Still lacking, however, are details about the 'form' of neural lineages, such as when symmetric versus asymmetric divisions occur, and whether self-renewing stem cells are present.

Are there neuronal stem cells?

The stem cell question is a particularly thorny one, because, unlike regenerating tissues such as liver, muscle or blood, most vertebrate nervous systems do not replace lost neurons. Thus, it is possible that most neuronal lineages operate without stem cells, simply producing

Abbreviations

E—embryonic day; **FGF**—fibroblast growth factor; **GGF**—glial growth factor; **HLH**—helix–loop–helix; **INP**—immediate neuronal precursor; **NGF**—nerve growth factor; **NT**—neurotrophin.

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a given number of post-mitotic progeny and leaving no cells behind that can repeat the process. On the other hand, neural stem cells may exist only early in embryonic development (some recent evidence points to the existence of embryonic neural stem cells in neural crest and cerebral cortex; cf. [17,18•]), or they may persist into adulthood, but simply remain quiescent [19•]. Recent studies that directly or indirectly demonstrate the existence of neuronal precursors in parts of the adult mammalian brain support the idea that persistent stem cells may exist in the mammalian central nervous system [19•,20,21•,22••]. However, it is not yet clear in all of these cases that the strict definitions of a stem cell — self-renewal, essentially unlimited proliferative potential, and the ability to reconstitute all of the differentiated products of a lineage — have so far been met.

Nonetheless, other reasons exist for maintaining that stem cells participate in at least some neuronal lineages. For example, it has been known for many years that the olfactory epithelium of all vertebrates undergoes continuous neuron turnover and renewal throughout life, virtually guaranteeing the existence of a stem cell in this lineage (reviewed in [23]). Similar continuous addition of neurons to the hippocampus and olfactory bulb of rodents has also long been observed [24–27].

Cellular stages in vertebrate neurogenesis

Probably the least well understood aspect of vertebrate neurogenesis concerns the identification of the phenotypic stages that precursor cells pass through on the way to becoming neurons or glia. In other tissues, the existence of such stages has been deduced from progressive changes in morphology or growth factor requirements (e.g. the hematopoietic system), changes in the expression of cell-surface antigens (e.g. lymphocytes), or changes in relative cell positions (e.g. epidermis and gastrointestinal epithelium) — reviewed in [28,29].

In vertebrate nervous systems, direct evidence for analogous stages has been hard to come by. Most neuroepithelial cells are morphologically indistinguishable from one another, as are most neural crest cells. Few antigens exist that are specifically expressed on neural precursors, and fewer still that are candidates for markers of particular stages in neural precursor development. Perhaps most significantly, evidence that the fates of many neural precursors may not be determined until very late in their proliferative life (in some cases, as late as the last cell division; cf. [13]) has raised the possibility that many neural precursors are 'blank slates', upon which information about cell fate is written quite late by local microenvironments. In that case, perhaps there is no need for distinct cellular stages in neurogenesis. Stem cells could simply give rise directly to relatively unprogrammed, generic precursors that then receive all of their instructions from their environment.

Although it is clear that this issue is far from resolved, it is important to point out that the stages that exist in proliferative lineages in other tissues do not exist solely for the purpose of restricting the fates of terminally differentiated progeny. Precursors occupying positions intermediate between stem cells and end-stage progenitors may themselves need to respond to systematically changing sets of environmental cues that control their proliferation and survival. Precursors may need to go through distinct phenotypic stages simply to assure that, ultimately, correct numbers of cells are made at the right times and in the right places. Thus, neural precursors may go through distinct stages that are not necessarily linked to changes in developmental potential. Some observations that suggest that this may indeed be the case are among those discussed below.

Intrinsic factors regulating vertebrate neurogenesis: insights from *Drosophila melanogaster*

In *Drosophila*, functions of two classes of genes — the neurogenic genes and the proneural genes — are required for proper specification of neuronal precursors (for recent reviews, see [30–34]). Briefly, the proneural genes are required for the intrinsic determination of neuroblasts (neuronal precursor cells) in the developing *Drosophila* nervous system. Their expression in undifferentiated ectodermal cells results in clusters of these cells (proneural clusters) being competent to become neuronal precursors. Among the proneural genes are the four genes of the *achaete–scute* complex (*achaete*, *scute*, *lethal of scute*, and *asense*), the gene *ventral nervous system condensation defective* (*vnd*), and *atonal*, the most recently identified [35,36]. Genetic experiments indicate that different proneural genes appear to specify nonoverlapping subsets of neuronal precursors: for example, deletions of the *achaete–scute* complex result in a failure of precursors for external sense organs, such as mechanosensory bristles, to form, whereas flies lacking *atonal* function lack chordotonal organs and a specific class of photoreceptors [35–37].

Proneural genes seem to determine whether a cell can become a neuronal precursor, but neurogenic genes seem to determine whether a cell will become a neuronal precursor. The segregation of neuroblasts from the proneural clusters of neural-competent cells appears to involve a process of lateral inhibition, in which neuroblasts that have been determined prevent their neighboring cells from also adopting a neural fate. This lateral inhibition process requires the function of neurogenic genes such as *Notch* [38]. Loss-of-function mutations of the neurogenic genes cause ectodermal cells that should become epidermis instead to adopt a neural fate and become neuroblasts. The function of the neurogenic genes can thus be thought of as repressing neural development in neurally competent ectodermal

cells, thereby allowing most ectodermal cells to develop as epidermis [30,32,33].

Notch and *achaete-scute* homologues exist in vertebrates

Drosophila proneural genes that have been sequenced show strong similarities in structure and function: they are all transcription factors that contain a helix-loop-helix (HLH) domain, a protein domain that is also found in vertebrate myogenic determination genes, such as *MyoD* [33–35,39,40]. Sequence conservation within HLH domains served as the basis for identifying vertebrate homologues of the *Drosophila achaete–scute* genes. Homologues of *achaete–scute* have been isolated in rat and mouse (*MASH-1* and *MASH-2*, for *Mammalian Achaete–Scute Homologue-1* and *-2*; [41,42•]), *Xenopus* (*XASH-1*, *XASH-3a* and *XASH-3b*; [43,44•,45••]), chicken (*CASH-1*; [46•]), and man (*HASH-1*; [47]; J Rothstein, personal communication).

Unlike proneural genes, the neurogenic genes of *Drosophila* do not share a common molecular structure. However, vertebrate homologues of the *Drosophila* neurogenic gene *Notch*, which encodes a large (~300 kDa), structurally complex transmembrane protein (see [48] and references therein), have been identified and studied in *Xenopus* (*Xotch*; [49]), rat (*Notch-1* and *-2*; [50,51]), mouse (*Notch-1*, *-2*, and *-3*; [52–55]), and man (*TAN-1*; [56]).

The roles of vertebrate *achaete–scute* homologues in neurogenesis

Localization studies of Mash-1 protein and mRNA in mouse and rat embryos indicate that this gene is transiently expressed during neural development, and expression is confined to subpopulations of neuronal precursor cells in the central and peripheral nervous systems [42•,57]. (*MASH-2*, unlike *MASH-1*, is not expressed in a neural-specific pattern; instead, *MASH-2* is strongly expressed by cells of the extraembryonic trophoblast lineage and appears to be required for the generation of those cells and the subsequent proper development of the placenta [58,59].) Like *MASH-1*, *CASH-1* and the three *Xenopus achaete–scute* homologues (*XASH-1*, *XASH-3a*, and *XASH-3b*) show transient expression that is restricted to zones of the developing nervous system that contain proliferating neuronal precursors [43,44•,45••,46•].

The broad similarity of such patterns of expression to those of *achaete–scute* genes during *Drosophila* nervous system development (e.g. [60]; reviewed in [33,34]) suggests that vertebrate *achaete–scute* homologues may subserve functions analogous to those of the proneural genes in *Drosophila*. More direct support for the view that vertebrate *achaete–scute* homologues play a crucial role in the determination of particular subsets of neuronal precursor cells has come from several recent studies (described below).

Experiments in which the *MASH-1* gene was 'knocked out' by homologous recombination in mice have shown that Mash-1 function is necessary, at some time in development, for the production of the majority of olfactory receptor neurons of the olfactory epithelium, and many autonomic and enteric neurons as well [61••]. *In vitro* studies of neurons associated with developing intestine indicate that it is specifically the serotonergic neurons of the enteric nervous system that fail to develop in *MASH-1*$^{-/-}$ mice (E Blaugrund *et al.*, Soc Neurosci Abstr 1994, 20:654).

Unlike the enteric nervous system, in the olfactory epithelium there is only one major neuronal lineage — that of the olfactory receptor neuron. Experiments to identify the site of *MASH-1* gene action in the olfactory epithelium suggest that expression of the Mash-1 protein demarcates a specific stage of neuronal precursor differentiation, rather than a distinct lineage: *MASH-1* is expressed transiently in proliferating neuronal precursor cells during olfactory epithelium neurogenesis *in vitro* [62,63••]. Similarly, *in vivo*, cells expressing *MASH-1* are present in low numbers in normal adult mouse olfactory epithelium, but their numbers increase dramatically and transiently when neurogenesis is induced by surgical removal of the olfactory bulb, the synaptic target tissue of olfactory receptor neurons (MK Gordon, RA Davis, AL Calof, unpublished data). Cells expressing *MASH-1* are immunologically and morphologically distinct from both terminally differentiated olfactory receptor neurons and the keratin-expressing basal cells that have been postulated to be stem cells of the olfactory epithelium. Their ^{3}H-thymidine incorporation kinetics *in vivo* and *in vitro* indicate that they are rapidly dividing. The transient appearance of cells expressing *MASH-1* during olfactory epithelium neurogenesis *in vitro* does not result from apoptosis; rather, it appears to result from their rapidly giving rise to a later stage of neuronal precursor, the immediate neuronal precursor of olfactory receptor neurons ([64,65]; AL Calof, JL Guevara, K Hannon, BB Olwin, MK DeHamer, Soc Neurosci Abstr 1994, 20:1275). Together with the results from the *MASH-1* gene 'knockout' experiments [61••], these studies suggest a model for the function of *MASH-1* in olfactory epithelium neurogenesis that is analogous to that of proneural genes in *Drosophila*: gene function is required transiently during the initial selection of neuronal precursor cells, but is then turned off in the differentiated precursors that are committed to specific neuronal fates [34].

Another approach that has been taken to determine whether vertebrate proneural homologues function like their *Drosophila* counterparts is to test whether ectopic expression of these genes can cause additional cells to

become neuronal precursors (as has been shown to occur in *Drosophila* [31,37]). Such experiments have not always met with success in vertebrate systems [58]. Recently, however, Turner and Weintraub [45••] examined the consequences of mosaic *XASH-3a/b* overexpression in *Xenopus* embryos by injecting synthetic RNA into one blastomere of two-cell embryos. As the first cleavage in *Xenopus* embryos defines the plane of symmetry of the animal, the injected RNA remains restricted to one side of the developing embryo, with the uninjected side serving as a control. Expression of *XASH-3a/b* in these animals resulted in a dramatic increase in the size of the nervous system on the injected side. In addition, the non-neural ectoderm adjacent to the expanded nervous system in injected embryos appeared to be reduced in size. Interestingly, treatment of embryos at midgastrulation with hydroxyurea and aphidicolin to block cell division still resulted in embryos with increased numbers of neuronal cells on the injected side. This suggests that cell division is not necessary for the expansion of nervous system resulting from ectopic *XASH-3a/b* expression, but rather that *XASH-3a/b* expression converts cells from an epidermal to a neural fate. Although cell division is not required for *XASH-3a/b* function, DNA binding and presumably transcriptional activation of downstream genes are: expression of a form of *XASH-3a/b* in which the DNA-binding basic region was disrupted, but the HLH dimerization domain remained intact, had no effect in injected embryos [45••]. Altogether, this study provides the strongest evidence thus far that a vertebrate *achaete–scute* homologue can function to direct neuronal determination in cells that express it.

The roles of vertebrate *Notch* homologues in neurogenesis

If vertebrate homologues of *Notch* behave like their *Drosophila* counterpart, then a loss of vertebrate *Notch* function would be expected to stimulate neurogenesis, whereas an increase in *Notch* function would be expected to inhibit neurogenesis. Genetic tests in vertebrates of the first of these predictions has proved difficult for technical reasons. Like *Drosophila Notch*, vertebrate *Notch* homologues are widely expressed in early embryos [49–51,54,55]. Probably as a result, mice homozygous for targeted deletions of the mouse *Notch-1* gene die before 11.5 days of gestation. At this stage, many phenotypic markers of the nervous system have not yet appeared, making a quantitative assessment of neurogenesis extremely difficult in these animals [66].

In contrast, testing the predicted outcome of increased *Notch* function has proved more tractable. A clue as to how to accomplish this was provided by the discovery of the human *Notch* homologue *TAN-1*, which lacks most of the extracellular domain and is associated with neoplastic transformation of T cells [56]. In *Drosophila*, deletions of the *Notch* extracellular domain result in gain-of-function alleles, which inhibit neurogenesis but allow epidermal development to proceed normally (e.g. see [67]). These studies suggest that *TAN-1*, as well as other forms of vertebrate *Notch* homologues that are engineered to contain large deletions of their extracellular domains, could be used to increase *Notch* function in vertebrate cells or embryos. Consistent with this view, when Nye *et al.* [68•] introduced a construct encoding just the intracellular domain of murine *Notch-1* (*mNotchIC*) into mouse P19 embryonal carcinoma cells, they observed a suppression of neurogenesis in cells expressing the transgene. Austin and colleagues (CP Austin *et al.*, Soc Neurosci Abstr 1994, 20:1275) have recently reported studies that suggest a similar function for *Notch* in the neural retina: embryonic day 4 (E4) chick retinal cells undergo rapid differentiation into ganglion cells when dissociated and cultured at low density; differentiation is inhibited in high-density cultures. Treatment of high-density cultures with antisense oligonucleotides directed against the *Notch* sequence cause an increase in ganglion cell differentiation, as predicted for *Notch* loss-of-function. Conversely, retrovirus-mediated transduction of *TAN-1* into retina caused a reduction in the number of ganglion cells present *in vivo* and inhibited the overproduction of ganglion cells seen *in vitro*.

In contrast to these experiments in which cell lines, primary cultures, or developing retinas were studied, somewhat different results have been obtained when widespread overexpression of *Notch* extracellular deletion constructs was attempted in intact *Xenopus* embryos. In this case, expression of a *Xotch* extracellular deletion construct actually caused an increase in both neural tissue and muscle in injected embryos [69•]. It was proposed that cells expressing the construct were delayed in early steps of differentiation, and thereby remained competent to respond to later neural and myogenic inductive signals. In support of this idea, animal caps isolated from blastula-stage injected embryos were shown to form more neural tissue than control caps when cultured in the presence of a neural inducer, indicating that cells expressing the *Xotch* extracellular deletion construct have an enhanced response to neural-inducing signals.

Assuming vertebrate *Notch* homologues do play roles analogous to those of *Drosophila Notch*, how might they exert their effects on neurogenesis? In *Drosophila*, activation of *Notch* is achieved through the binding of the Notch extracellular domain to a cell-surface counter-receptor Delta. Genetic experiments indicate that activation of Notch leads to inhibition of expression of proneural genes. The mechanism underlying this inhibition is not clear but in flies may involve the release, by the cytoplasmic domain of activated Notch, of the protein product of the *Suppressor of Hairless* gene, which then translocates to the nucleus where it could affect the actions of transcription factors such as the proneural genes [70]. Interestingly, Kopan and colleagues [71•] have recently found that the mNotchIC protein can function

as a repressor of the transcriptional activation function of Xash-3a, as well as the myogenic basic HLH proteins Myf-5 and MyoD. Thus, all of the fundamental elements of the pathway linking neurogenic and proneural gene function may be conserved in vertebrates.

Epigenetic control of neurogenesis: the role of growth factors

Gene products intrinsic to cells that have the potential to become neural precursors clearly play an important role in establishing the numbers and types of neural lineages in vertebrates. Nonetheless, extrinsic factors, such as secreted polypeptide growth factors, clearly play crucial roles in vertebrate neurogenesis. Indeed, recent studies indicate that extrinsic factors may act at every stage in the development of neuronal progenitor cells.

For example, Shah and colleagues [72••] have shown that glial growth factor 2 (GGF2; [73]), first isolated as a Schwann cell mitogen made by neurons [74], can act on early neural crest cells to direct their commitment to a glial, rather than a neuronal, pathway of differentiation. When grown at clonal density under appropriate conditions, neural crest cells derived from rat embryos are multipotent and give rise to colonies containing both neurons and glia [17]. However, when cloned neural crest cells were grown in GGF2, the majority (90%) of colonies that developed did not contain any neurons [72••]. This effect was not due to selection against the survival of neurons or neuron precursors: daily observation of expanding clones for 15 days after plating revealed that neurons never appeared in cultures grown in GGF2, nor did fewer neural crest cells survive to give rise to colonies in GGF2-treated cultures than in control cultures. Interestingly, *MASH-1*, which normally is transiently expressed by undifferentiated cells in ~84% of expanding neural crest cell colonies before neuronal differentiation, was not expressed by cells in GGF2-treated colonies, even though GGF2 was not toxic to pre-existing cells expressing *MASH-1* in control experiments. Taken together, these results indicate that exposure to extrinsic factors such as GGF2 can lead to restrictions in the potential of multipotent neural progenitor cells.

In contrast, the role of fibroblast growth factors (FGFs) in vertebrate neurogenesis may not be to determine the fates of early neural progenitors, but rather to regulate the number of cell divisions that precursor cells undergo. When embryonic mouse olfactory epithelium is cultured without growth factors, cells referred to as immediate neuronal precursors (INPs) divide once and generate two daughter cells, which undergo terminal differentiation to become olfactory receptor neurons [64,65]. Interestingly, several members of the FGF family (FGF1, FGF2, FGF4, and FGF7) can be shown to prolong neurogenesis in olfactory epithelium cultures. By labeling INPs through successive S phases with bromodeoxyuridine and ^{3}H-thymidine, DeHamer and colleagues [63••] demonstrated that FGFs enable INPs to undergo multiple rounds of division before differentiating into olfactory receptor neurons. By adding FGF at later and later times to olfactory epithelium cultures and then identifying the point at which FGF was no longer able to act, they determined that FGF must be present by early G1 phase of the INP cell cycle in order to exert its effects. Interestingly, this is the phase in which commitment to terminal differentiation would be expected to occur, suggesting the action of FGFs on INPs might be analogous to their action on myoblasts: repression of terminal differentiation in G1, thus allowing progression of cells through additional cell cycles [75].

FGFs were also shown to have a second action in cultured olfactory epithelium: they permit the extended proliferation of a rare cell, possibly a stem cell, that acts as progenitor to INPs [63••]. The effects of FGFs on neurogenesis are probably widespread, as recent reports indicate that FGFs stimulate proliferation of progenitor cells from a number of regions of the embryonic nervous system, including E10 telencephalon [76,77], cerebral cortex [78], corpus striatum [79,80], hippocampus [81], and retina [82].

In addition to regulating the determination and proliferation of neural precursors, polypeptide growth factors apparently can also regulate the survival of neuronal precursor cells at particular stages of precursor differentiation. The clearest examples of this phenomenon, so far, come from *in vitro* studies of the actions of neurotrophins on the precursors that give rise to sympathetic neurons (sympathetic neuroblasts). Nerve growth factor (NGF), the prototypical neurotrophin, has long been known to be a survival factor for post-mitotic sympathetic neurons (for a recent review, see [83]). However, during the genesis of sympathetic neurons, sympathetic neuroblasts express trkC, the receptor for the neurotrophin NT-3, before they express trkA, the receptor for NGF [84•,85•]. Although NT-3 does stimulate proliferation of sympathetic neuroblasts to some extent, it also promotes survival of these cells when they are cultured as an isolated cell fraction, whereas FGF2, a stronger proliferation-stimulating factor, does not enhance survival above control levels [84•]. This separation of NT-3's effects on proliferation and survival has been observed independently by DiCicco-Bloom and colleagues [85•], who found that NT-3 increased the number of sympathetic neuroblasts present at 24 hours in culture even when cells were blocked from entering S phase by treatment with aphidicolin, an inhibitor of DNA polymerase.

What could be the purpose for regulation by neurotrophins of the survival of proliferating neuronal precursor cells? Certainly this mechanism could provide a second level of control in regulating the size of neuronal precursor pools. Specifically, extrinsic factors could act to alter the number of precursors by controlling not only the number of precursor cell divisions, but also whether the progeny of these divisions survive to reach the next stage of their development.

Interestingly, however, in the case of sympathetic neuroblasts, neurotrophins can also act in another way to drive precursors toward the next stage of their development: culturing sympathetic neuroblasts in NT-3 not only promotes the survival of sympathetic neuroblasts, it also (at higher concentrations) induces expression of trkA and NGF responsiveness [86••]. The induction of trkA appears to be a consequence of the mitotic arrest of sympathetic neuroblasts that is brought about by high concentrations of NT-3. Indeed, both effects could be produced by treating cultures either with antimitotic agents (e.g. aphidicolin and mitomycin C) or with ciliary neurotrophic factor, a cytokine previously shown to inhibit proliferation of chick sympathetic neuroblasts [87]. NT-3 may also play a role in stimulating terminal neuronal differentiation by precursors in other areas of the nervous system. For example, a recent report indicates that NT-3 promotes differentiation into motoneurons of early (neural tube) progenitors cultured from quail [88].

Conclusions

Recent studies of precursors in several different neuronal lineages suggest that vertebrate neurogenesis is regulated by both intrinsic and extrinsic factors at every stage of neuronal precursor development, from initial commitment of multipotent progenitors to induction of the specific gene expression that is characteristic of terminal neuronal differentiation. The experiments that have led to this view have, in the process, also begun to reveal distinct cellular stages through which neurogenesis in several systems proceeds. Based on analogies (and homologies) with *Drosophila* neurogenesis, and on the diversity of responses of vertebrate neural precursors to extrinsic signals, it seems likely that developing vertebrate nervous systems are complex mosaics of distinct precursor populations, distinguishable in terms of patterns of gene expression, as well as in terms of developmental potency.

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