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Much is known about how cell proliferation is controlled at the single cell level, but much less about the control of cell numbers in developing populations. Cell number might be determined by an intracellular division limiter or, alternatively, by the availability of mitogens or other factors outside the cell. We investigated the relative importance of intracellular and extracellular controls for one well-defined population of neural precursor cells, namely the glial progenitors that give rise to oligodendrocytes in the mouse spinal cord.

We found by cumulative BrdU labeling in vivo that the progenitor cell division cycle slows down markedly as their numbers increase during embryogenesis. When cultured in saturating PDGF, the main mitogen for these cells, their cell cycle accelerated and was independent of their prior rate of division in vivo. This shows that mitogens are limiting in vivo, and suggests that division normally slows down because the PDGF concentration declines. In PDGF-transgenic mice, cell number was proportional to the PDGF supply and apparently unsaturable; at ten times the normal rate of supply, cell number was still increasing but the animals were no longer viable.

Progenitor cell proliferation in the embryo is limited by environmental factors, not a cell-intrinsic mechanism. The linear relationship between PDGF supply and final cell number strongly suggests that cells deplete the mitogenic activity in their environment at a rate

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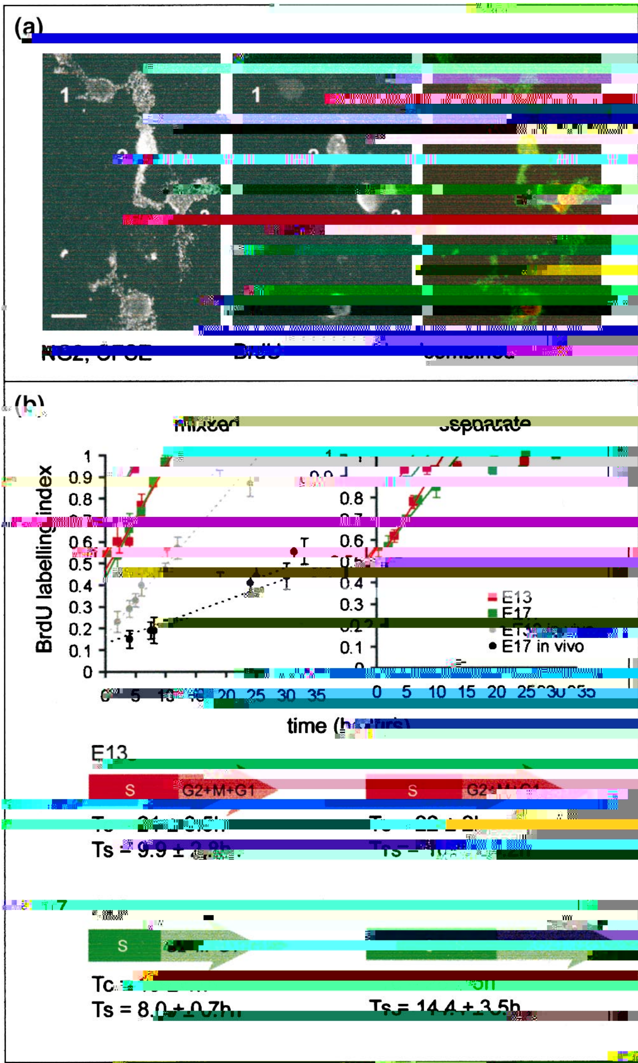

Cumulative BrdU labeling of oligodendrocyte progenitors in vitro. ( ) Dissociated E13 spinal cord cells were prelabeled with the fluorescent dye CFSE (see Materials and methods) before they were mixed with unlabeled E17 cells and cultured overnight. BrdU was then added for various times before fixing and immunolabeling with anti-NG2 and anti-BrdU. The CFSE and fluorescein signals are both green, but can be distinguished because CFSE is in the cytoplasm whereas NG2 is on the cell surface. Numbered cells: (1) an (NG2^{1}, BrdU^{2}, CFSE^{2}) E17 progenitor, (2) an (NG2^{1}, BrdU^{1}, CFSE^{1}) E13 progenitor, and (3) an (NG2^{1}, BrdU^{1}, CFSE^{2}) E17 progenitor. The scale bar represents 10 mm. ( ) The BrdU labeling index was plotted against time for E13 and E17 cells (see key) in mixed-age cultures (left graph) and separate cultures (right graph). Tc and Ts were calculated from the lines of best fit and are shown graphically. The in vivo labeling data from Figure 1 are also shown for comparison. Points that appear to be on the plateau were not included when calculating the line of best fit. There is no significant difference between the cell cycles of E13 and E17 cells, unlike the situation in vivo. Note also that the division rate is speeded up in vitro compared to in vivo for both E13 and E17 cells.

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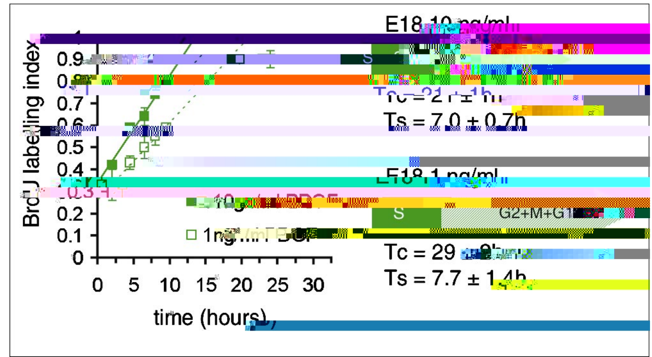

PDGF concentration controls progenitor cell cycle time in vitro. Spinal cords from E18 embryos were dissected and cultured overnight in 10 ng/ml or 1 ng/ml PDGF-AA. BrdU was added and the cumulative labeling index was determined. The oligodendrocyte progenitor cell cycle was significantly longer at 1 ng/ml than at 10 ng/ml PDGF. Variation was largely in the G2-M-G1 phases of the cycle.

PDGF, than they did in vivo prior to dissociation (Figure 2). We conclude from the latter observation that PDGF is limiting for cell division in vivo at both E13 and E17. There might also be an inhibitory activity in vivo that is lost when the cells are placed in culture. The reason the cell cycle slows down in vivo between E13 and E17 is not because the cells' inherent ability to respond to mitogens decreases, but because there is less mitogenic activity in the extracellular environment. That is, the PDGF concentration falls and/or a putative inhibitory activity rises.

To test the principle that a falling PDGF concentration might slow the progenitor cell cycle in vivo, we measured the cell cycle time of E18 progenitor cells cultured in either 10 ng/ml or 1 ng/ml PDGF. In both saturating and subsaturating PDGF concentrations, the progenitors behaved as uniform populations of cycling cells; the proportion of BrdU-labeled cells increased linearly with time until almost all of the cells were labeled (Figure 3). The cell division rate depended on PDGF concentration, however. In 10 ng/ml PDGF, the cell cycle time was 21 6 1 hr, whereas in 1 ng/ml PDGF it was 29 6 2 hr (Figure 3). Even the latter time is much shorter than is observed in vivo at E17 (100 6 17 hr), suggesting that the effective PDGF concentration might fall significantly below 1 ng/ml in vivo. In any case, this experiment demonstrates that the falling cell division rate in vivo could potentially be caused by a dwindling PDGF concentration. Note that the length of S phase was not affected by the PDGF concentration in vitro, consistent with previous studies on cultured fibroblasts. A similar dependence of cell cycle time on PDGF concentration was also observed for E14 progenitors (data not shown).

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Cell-intrinsic mechanisms do not appear to be responsible for limiting progenitor cell number during normal embryonic development (see previous two paragraphs). It might be expected that there would be some inbuilt limit on proliferation as a fail-safe device, however, in case mitogenic activity in the environment were deregulated somehow during development or neoplasia. We imagined that a cell-intrinsic mechanism would kick in to halt cell division if we were to induce extreme hyperproliferation of progenitor cells by elevating PDGF expression beyond some critical point in vivo. We tested thisty

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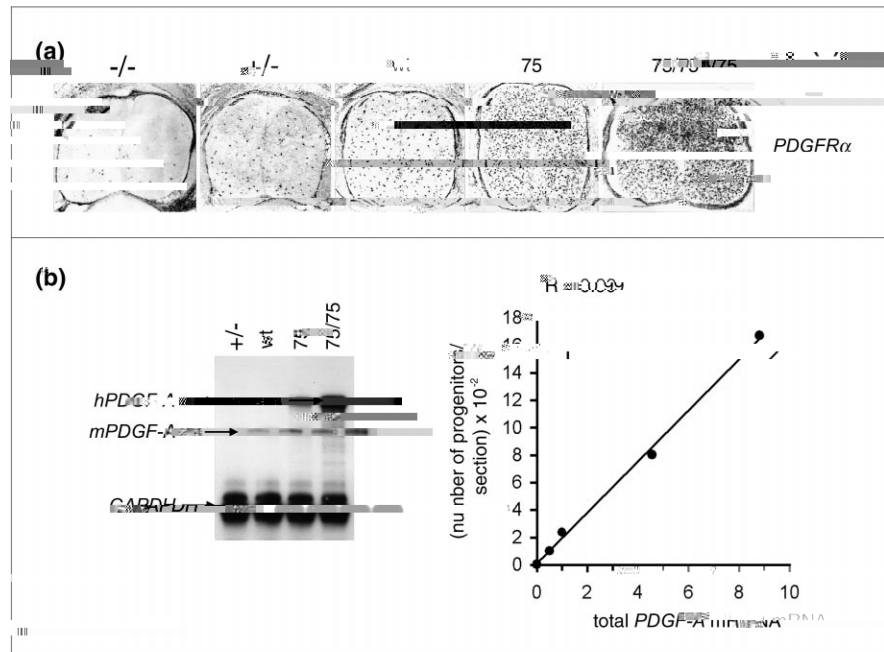
Progenitor cell number is proportional to the PDGF supply. The linear relationship between PDGF-A and final cell number also holds when PDGF is underexpressed in PDGF-A knockouts. ( ) Spinal cord sections of E15 mouse embryos hybridized in situ to a PDGFRa probe: homozygous PDGF-A knockout (2/2), heterozygous PDGF-A knockout (1/2), wild type (wt), hemizygous NSE-PDGF-A (75), and homozygous NSE-PDGF-A (75/75). Progenitor cell numbers rise with increasing levels of PDGF-A expression. The scale bar represents 100 mm. ( ) Total PDGF-A mRNA level was estimated by RNase protection and plotted against the number of PDGFRa progenitors counted by eye in photographic enlargements of the in situ micrographs. The amount of functional PDGF-A mRNA was assumed to be zero in (2/2) embryos and half of normal in (1/2) embryos, since our RNase protection assay could not distinguish wild-type from nonfunctional, hybrid PDGF-A-[illegible] transcripts in the knockouts. The dose response is linear (correlation coefficient R 5 0.99). Taken together with Figure 5, we conclude that progenitor cell number is directly proportional to PDGF supply over a range of expression levels from near zero (PDGF-A null) to more than ten times normal (75,82/75,82 transgenic).



lular environment. This follows from our observation that the cell division rates of E13 and E17 progenitors were indistinguishable in vitro in saturating PDGF, even though their division rates in vivo were very different. Slowing of the cell cycle is accompanied by a decline in the rate of increase of progenitor cell number, so that the cell number reaches a plateau before birth and remains stable for some time after birth. It seems likely that cell cycle dynamics directly influence the final cell number and therefore that the cell number is controlled by the environment, not by a cell-intrinsic counting mechanism. This does not mean that the intracellular state of cells stays constant during embryogenesis, but that any intrinsic changes that might limit the proliferation rate are probably a consequence of the changing mitogenic environment outside the cell and remain reversible, at least for a few days (between E13 and E17).

The progenitor cell population in the embryonic cord can be expanded seemingly without limit, simply by increasing the rate of supply of PDGF. This strongly supports our conclusion that the only effective constraint on progenitor cell number in the embryonic cord is the extracellular mitogen supply, and raises the question of whether progenitor cells would proliferate indefinitely in vivo if an inexhaustible supply of PDGF could be provided. We could not approach this condition because higher levels of PDGF caused spinal deformity and killed the animals. It is striking that cell number is directly proportional to *PDGF* transcript levels (which we take to be an indicator of the rate of PDGF synthesis and secretion) over a wide range of *PDGF* expression. This is perhaps counterintuitive, being that mitogen dose–response curves in vitro are typically sigmoidal in shape, rising from background to maximal (plateau) response over a limited range of concentrations, about a 10-fold range for oligodendrocyte progenitors and PDGF [12]. In vitro dose responses, however, usually record [illegible] versus mitogen [illegible], whereas our in vivo dose response reflects [illegible] versus [illegible] of mitogen. We do not know what the effective PDGF concentration is in vivo except that it is limiting (subsaturating).

It should be possible to estimate the effective concentration of PDGF from the measured cell cycle time in vivo, given that cell cycle time seems to be predictably related to the PDGF concentration in vitro (Figure 3). At E13, cell cycle time in vivo was comparable to that measured in vitro in the presence of 1 ng/ml PDGF-AA (~30 hr). Cell cycle time at E17 in vivo was ~100 hr, however, suggesting that the effective PDGF concentration is much less than 1 ng/ml by that time. We have not recapitulated a 100-hour cycle in vitro, because in very low concentrations of PDGF the cells tend to drop out of division and differentiate, as they do in the complete absence of PDGF. It would be interesting to see whether the combination of a low concentration of PDGF together with fibroblast growth factor, which is known to inhibit

oligodendrocyte differentiation in vitro [13], would be able to keep progenitors dividing slowly in vitro. Omitting thyroid hormone from the medium might also help [14].

It should be noted that overexpressing PDGF does not affect the final number of postmitotic, differentiated oligodendrocytes that accumulates after birth [3], despite the increased number of progenitor cells. This is because numbers of progenitors and oligodendrocytes are controlled separately, progenitors by the mitogenic effect of PDGF and oligodendrocytes by axon-derived survival factors [15]. Our previous experiments showed that the rate of oligodendrocyte production is elevated in *N E-PDGF-A* mice but that the excess oligodendrocytes are removed by programmed cell death, leaving a normal number of mature, myelinating cells [3].

We previously estimated the average progenitor cell cycle time at steady state (E17) to be about 24 hr [3]. To explain how the cells could continue to divide once a day while their number remained constant, we had to invoke large-scale death and removal of newly formed cells, around 200 cells per 10-mm section per day. The fact that we could not detect large numbers of dying oligodendrocyte lineage cells in the prenatal spinal cord was not perceived to be a problem, as dead cells are usually cleared rapidly. Our revised cell cycle time of ~100 hr at steady state, however, means that the scale of oligodendrocyte death in the prenatal spinal cord can now be revised downward to around 50 cells/section/day.

We do not understand why our previous estimates of cell cycle time (Tc) were 4-fold shorter than they should have been. The measured labeling index (L) after a single BrdU pulse at E17 was similar then and now (~20%). We previously assumed an invariant S phase (Ts) of 4 hr; given that it now seems that the S phase is closer to 12 hr at E17 (Figure 1), this goes a long way toward explaining the discrepancy at E17 (since Tc 5 Ts/L). It does not explain, however, the discrepancy at E13, when Ts ≈ 5 hr (Figure 1). We tried without success to replicate our previous results using both our present and former experimental paradigms. We conclude that, whatever the source of error before, our present data are more reliable and should take precedence.

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A simple explanation that fits our observations (though not the only explanation; see Potential role of antiproliferative factors, below) is that the proliferating cells limit their own proliferation by consuming the available PDGF. As the cells multiply, the rate at which they consume PDGF (by receptor binding and internalization) increases in proportion to cell number until the rate of consumption matches the fixed rate of supply. At this point, increased competition for PDGF prevents further proliferation. This simple model explains the linear relationship between the rate of PDGF supply and the final cell number (Figures 5 and 6). It also explains why the cell cycle slows down as the cell number rises (Figure 1), because the shifting balance between PDGF supply and demand should cause the PDGF concentration to decrease with time. Finally, it explains why the ultimate rate of cell division is the same in normal mice and transgenic PDGF overexpressers (Figure 4), as the PDGF concentration should be determined solely by the relative rates of PDGF supply and consumption, which, according to the model, are always equal at steady state.

We tried to detect a decline in PDGF concentration in vivo by Western blotting. We were unable, however, to detect PDGF-A in embryonic spinal cord tissue (E12 to birth) from either wild-type or homozygous *N E-PDGF-A* mice, using any of several antibodies against PDGF-A or an antibody against the Myc tag on the carboxy terminus of transgene-derived human PDGF-A (data not shown). We were also unable to detect PDGF-A by immunohistochemistry in spinal cord sections. In contrast, we were easily able to detect the product of another *PDGF* transgene encoding a nonsecreted form of PDGF-A that has an endoplasmic reticulum retention signal (KDEL) appended to its carboxy terminus, either by immunohistochemistry [3] or Western blot (data not shown). It is likely that PDGF is normally secreted rapidly after synthesis and is difficult to detect once it leaves the cell. We have in the past been able to detect PDGF immunoreactivity in situ, but only inside cells, not in the extracellular space [16].

We also tried to detect PDGF activity indirectly by visualizing activated PDGFRa on Western blots of newborn spinal cord, using antibodies against both the receptor and phosphotyrosine. Although we could easily detect PDGFRa protein, we could not detect autophosphorylation. We could readily detect phosphorylated receptors on NIH 3T3 cells that were cultured in the presence of 10 ng/ml PDGF-AA. We tentatively conclude that the level of receptor phosphorylation in oligodendrocyte progenitors in vivo is low relative to PDGF-stimulated 3T3 cells, again consistent with our conclusion that the effective PDGF concentration in vivo is very low.

It has been proposed before that dividing cells should limit their own proliferation by consuming mitogens, on the basis of mathematical modeling [17]. Moreover, Holley and Kiernan [18] showed that the final population density attained by fibroblasts in culture is proportional to the initial serum concentration, and interpreted this in terms of depletion of serum growth factors by the expanding cell population. This explanation is similar to, and sets an early precedent for, the in vivo mitogen depletion model we propose here.

Mitogen depletion could potentially explain other puzzling examples of cell number control. One example is

is TGFβ1 [24]. Thus, it is not unlikely that they produce autocrine inhibitors of cell proliferation in vivo too, al-

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bearing cells were also NG-2 immunoreactive, and more than 95% of NG-2-positive cells were PDGFRα positive.

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1. Conlon I, Raff M: . *C* 1999, **6** 235-244.
2. Slack JM: h . *C B* 1997, **7** 467-469.
3. Calver AR, Hall AC, Yu WP, Walsh FS, Heath JK, Betsholtz C, *et al.*: h . *N* 1998, **20** 869-882.
4. Takahashi T, Nowakowski RS, Caviness VS Jr: h h h h . *J N* 1995, **15** 6046-6056.
5. Pringle NP, Richardson WD: α- h h h h . *D* 1993, **117** 525-533.
6. Hall A, Giese NA, Richardson WD: h α- . *D* 1996, **122** 4085-4094.
7. Nishiyama A, Lin XH, Giese N, Heldin CH, Stallcup WB: - 2 (,)-333776 0 (6) 5.4377 . ( ) 1 h 46 h
23. Louis JC, Muir D, Varon S: h - -2 (-2) . *G* 1992, **6** 30-38.
24. McKinnon RD, Piras G, Ida JA Jr, Dubois-Dalcq M: -β . *J C B* 1993, **121** 1397-1407.
25. Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, *et al.*: h β 1 h. *N A A* 1993, 0 770-774.
26. Paulsson Y, Karlsson C, Heldin CH, Westermark B: - h h -β 1 h h - - h α- . *J C* 1993, **157** 97-103.
27. Huang JS, Huang SS, Deuel TF: - h h α 2- . *N A A* 1984, **81** 342-346.
28. Schweitzer R, Howes R, Smith R, Shilo BZ, Freeman M: h ***Drosophila*** h . *N* 1995, **376** 699-702.
29. Temple S, Raff MC: h . *C* 1986, **44** 773-779.
30. Durand B, Fero ML, Roberts JM, Raff MC: **27** 1 h - h h . *C B* 1998, **8** 431-440.
31. Raff MC, Lillien LE, Richardson WD, Burne JF, Noble MD: -