

Subgroup of Reproductive Functions of Progesterone Mediated by Progesterone Receptor-B Isoform

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Progesterone regulates reproductive function through two intracellular receptors, progesterone receptor-A (PR-A) and progesterone receptor-B (PR-B), that arise from a single gene and function as transcriptional regulators of progesterone-responsive genes. Although *in vitro* studies show that PR isoforms can display different transcriptional regulatory activities, their physiological significance is unknown. By selective ablation of PR-A in mice, we show that the PR-B isoform modulates a subset of reproductive functions of progesterone by regulation of a subset of progesterone-responsive target genes. Thus, PR-A and PR-B are functionally distinct mediators of progesterone action *in vivo* and should provide suitable targets for generation of tissue-selective progestins.

The steroid hormone progesterone (P) functions in establishment and maintenance of pregnancy. The physiological effects of P are mediated by interaction with specific intracellular progesterone receptors (PRs) that are members of the nuclear receptor superfamily of transcription factors (1, 2). PRs are expressed as two protein isoforms, PR-A and PR-B, that are produced from a single gene by transcription at two distinct promoters and by translation initiation at two alternative AUG signals (3, 4). Mice lacking a functional PR gene display pleiotropic reproductive abnormalities including inability to ovulate, uterine hyperplasia and inflammation, severely limited mammary gland development, and impaired thymic function and sexual behavior (2).

The production of two PR isoforms from the PR gene is conserved in a number of vertebrate species (3, 5, 6), and the ratios of the individual isoforms vary in reproductive tissues as a consequence of developmental (6) and hormonal status (7) and during carcinogenesis (8). The PR-A and PR-B isoforms differ only in that PR-B contains an additional NH₂-terminal stretch of about 165 amino acids. This region encodes a transactivation function that is specific to PR-B and is required to specify target genes that can be activated by PR-B but not PR-A (9).

When expressed individually in cultured cells, PR-A and PR-B display different transactivation properties that are specific to both cell type and target gene promoter used (10).

PR-B functions as a strong activator of transcription of several PR-dependent promoters and in a variety of cell types in which PR-A is inactive. Under these conditions, agonist-bound PR-A can repress transcriptional activity of PR-B and other steroid receptors including estrogen receptor α (ER α) (11). Finally, when bound to some progestin antagonists, PR-B, but not PR-A, can be converted to a strongly active transcription factor by modulating intracellular phosphorylation pathways (12).

To determine whether the functional differences between the PR isoforms observed *in vitro* are reflected in a differential physiological capacity to mediate the diverse reproductive functions of P, we have selectively ablated expression of PR-A in PR-A knockout (PRAKO) mice.

Previous studies have shown that mutation of an internal ATG codon at which translation of PR-A is initiated (ATG_A) selectively abolishes expression of PR-A, but not of PR-B, when the full-length cDNA encoding PR is expressed in cultured cells (3, 13). Thus, we have used a CRE/loxP-based gene targeting strategy (14) to introduce a conservative amino acid substitution of the ATG_A codon (Met¹⁶⁶→Ala) in the murine PR (mPR) gene in embryonic stem (ES) cells (15). Male chimeras generated from three independent clones carrying the ATG_A mutation transferred the mutation to the next generation, and litters born from PRAKO^{+/-} intercrosses resulted in normal Mendelian inheritance of the mutation.

The absence of PR-A in PRAKO^{-/-} female mice was confirmed by protein immunoblot analysis of uterine extracts from estrogen (E)-treated animals (16, 17). A strong

immunoreactive band corresponding to the PR-A protein was detected in wild-type (WT) mice, but this band was absent from uterine extracts of PRAKO^{-/-} animals (Fig. 1). The PR-B protein was present in similar amounts in both WT and PRAKO^{-/-} mice and was typically detected as a doublet, presumably as a result of phosphorylation (18).

All animals appeared normal except that PRAKO^{-/-} females were infertile, a phenotype that was similar to that previously observed in PRKO mice in which both PR isoforms were ablated (2). To determine whether the infertility of the PRAKO^{-/-} mice was due to an inability to ovulate, we administered pregnant mare serum gonadotropin and human chorionic gonadotropin to 21-day-old mice to induce superovulation (2). Normal superovulation occurred in WT and PRAKO^{+/-} mice, with comparable numbers of oocytes produced in both cases (Table 1). PRAKO^{-/-} mice produced reduced numbers of oocytes, whereas PRKO mice produced no oocytes. Crosses between superovulated PRAKO^{-/-} females and WT males also failed to result in successful pregnancies despite the release of a small number of oocytes from PRAKO^{-/-} females. Uteri of these females failed to show decidualization of stromal cells in response to traunal stimulation (19), indicating that infertility was also associated with defective uterine implantation. Consistent with this finding, analysis of the expression of several implantation-specific uterine epithelial target genes indicated that PR-B regulated the expression of only a subset of these genes (Fig. 2, A to D). Expression of calcitonin (CT), histidine decarboxylase (HDC), and amphiregulin (AR) is increased in the WT uterine epithelium in response to P (in association with uterine receptivity) (20). Expression of lactoferrin (LF) is increased by E and decreased in response to P (21). Ovariectomized mice were administered a single dose of either vehicle (control), E, P, or E plus P, and uterine extracts were prepared for RNA and protein analysis (16). Ablation of PR-A resulted in a complete loss of regulation of CT, whereas the regulation of HDC was retained (Fig. 2, A and B). P-induced expression of AR

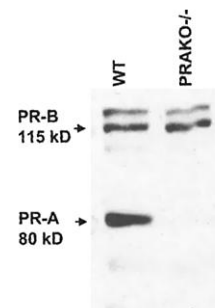


Fig. 1. Ablation of PR-A expression in PRAKO mice. Protein immunoblot analysis of PR-A and PR-B in WT and PRAKO^{-/-} uterine extracts.

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Fig. 2. Expression of P-responsive target genes in the uterine epithelium of PRAKO^{-/-} mice. Expression of (A) CT, (B) HDC, and (C) AR in uterine total RNA derived from WT, PRAKO^{-/-}, or PRKO animals. RNA levels were standardized to that of cyclophilin (CPH) and represented as an average fold increase in four experiments \pm SEM. (D) Protein immunoblot of uterine LF expression in WT, PRAKO^{-/-}, or PRKO mice. No hormone treatment (C), estrogen (E), or estrogen plus progesterone (EP). (E) PR immunohistochemistry of uterine sections obtained from ovariectomized untreated (control) and E-treated (E) WT, PRAKO^{-/-}, and PRKO animals. (Insets) Panels represent higher magnifications of areas indicated by the asterisk. Bars, 100 μ m (controls) and 50 μ m (plus E).

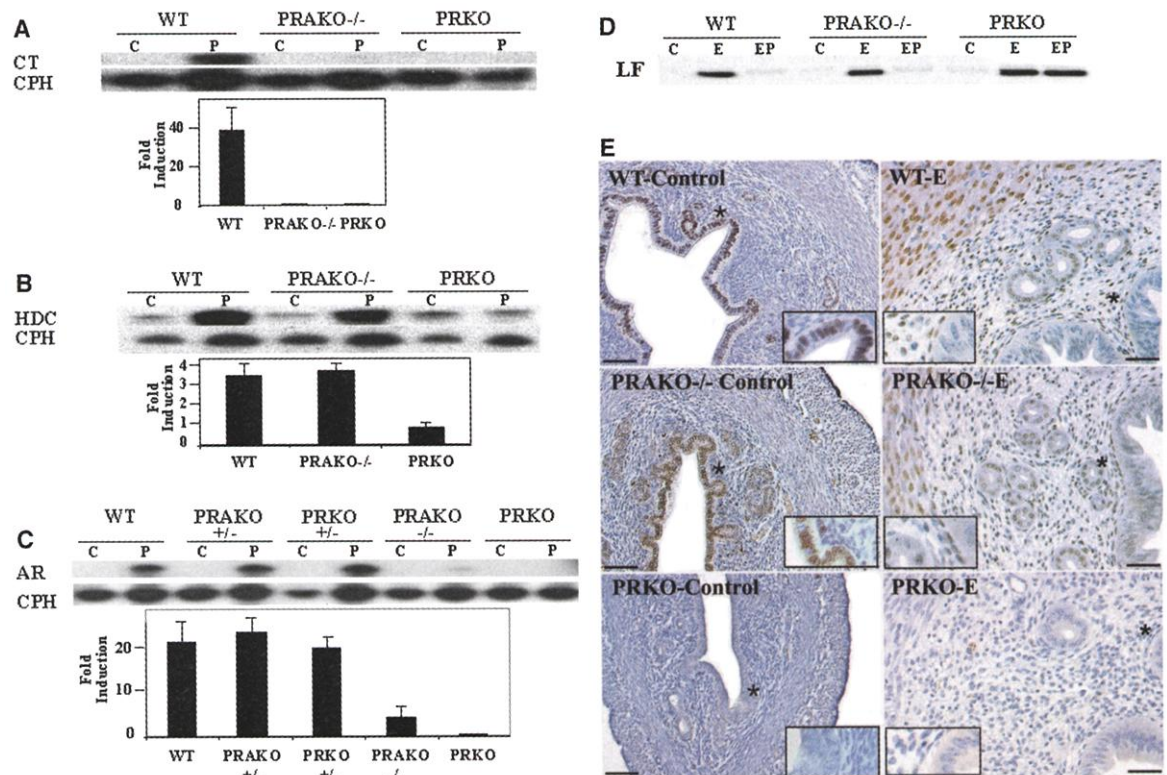


Table 1. Dependence of ovulation on PR-A.

Group	Oocytes*
WT	32 \pm 4
PRAKO ^{+/-}	33 \pm 3
PRAKO ^{-/-}	9 \pm 2
PRKO	0

*Average number of oocytes (\pm SEM) released per mouse after superovulation in WT, PRAKO^{+/-}, PRAKO^{-/-}, and PRKO mice; *n* = 8 per test group.

was also lost in PRAKO^{-/-} mice (Fig. 2C). PR-B alone completely inhibited the E-dependent induction of epithelial LF (Fig. 2D). These data indicate that defective implantation in PRAKO^{-/-} uteri is associated with loss of P-regulated expression of a subset of genes associated with receptivity. This differential target gene regulation by PR-B was not due to differences in spatiotemporal expression of PR-B relative to that of PR-A. Expression of PR-B in the uterine epithelium of untreated PRAKO^{-/-} mice was similar to that observed in WT uteri (Fig. 2E). E-treatment of PRAKO^{-/-} mice resulted in decreased expression of PR-B in the epithelium and increased expression in the stromal and myometrial compartments.

Progesterone is a potent antagonist of E-induced proliferation of the uterine epithelium (2). We examined mice administered daily with either vehicle, E alone, or E plus P for 4 days and injected with 5-bromo-2'-deoxyuridine (BrdU) for 2 hours (16). Histo-

logical analysis of uterine sections indicated that the epithelial morphology was similar in control WT, PRAKO^{-/-}, and PRKO mice (Fig. 3). Treatment of all groups with E resulted in hyperplasia of the luminal epithelium (LE) and the appearance of numerous scattered BrdU-positive proliferating cells. The addition of P with E resulted in an inhibition of uterine epithelial proliferation in WT animals. However, as previously observed in PRKO mice (2), this antiproliferative effect of P was absent in PRAKO^{-/-} mice. Indeed, treatment of PRAKO^{-/-} mice with E plus P resulted in a P-dependent increase in proliferation over that observed with E alone. This observation was confirmed by quantitative comparison of BrdU-labeled cells in E- and E plus P-treated WT, PRAKO^{-/-}, and PRKO uterine epithelium (15). These results indicate that selective ablation of PR-A results in a gain of P-dependent proliferative activity mediated through PR-B that is not observed in E- and P-treated WT or PRKO mice. This acquisition of a P-dependent proliferative response indicates that PR-A may diminish overall P as well as E responsiveness in the uterus. The finding that PR-B can contribute to, rather than inhibit, uterine epithelial cell proliferation may have important clinical implications for the hormonal management of uterine endometrial dysplasias. The relative expression of PR isoforms under these conditions will be an im-

portant determinant of the effectiveness of progestin therapy. Thus, generation of PR-A-selective progestin that can distinguish between different conformations of PR-A and PR-B as recently demonstrated in the case of ER (22) may be of significant clinical value.

To determine whether PR-B can elicit morphogenic responses of the mammary epithelium to P, we compared the morphology of mammary glands of ovariectomized WT, PRAKO^{-/-}, and PRKO mice treated with E and P for 3 weeks (23). Analysis of whole mounts of the thoracic mammary glands showed extensive hormone-dependent ductal branching that filled the fat pad and the appearance of multiple alveolar lobules in PRAKO^{-/-} mice (Fig. 4A). Further, the organized expression of E-cadherin outlining the mammary epithelial cells in PRAKO^{-/-} mice (Fig. 4B) indicated normal architecture and basal membrane integrity. Thus, PR-B is sufficient to elicit normal proliferation and differentiation of the mammary epithelium in response to P. Finally, E- and P-treated PRAKO^{-/-} mice also displayed normal thymic involution, a process we have previously shown to be PR dependent (24).

Our data indicate that PR-B mediates reproductive responses to P in a tissue-selective manner and that the PR-A and PR-B isoforms are functionally distinct mediators of progestin action in vivo. Thus, correct relative expression of the PR-A and

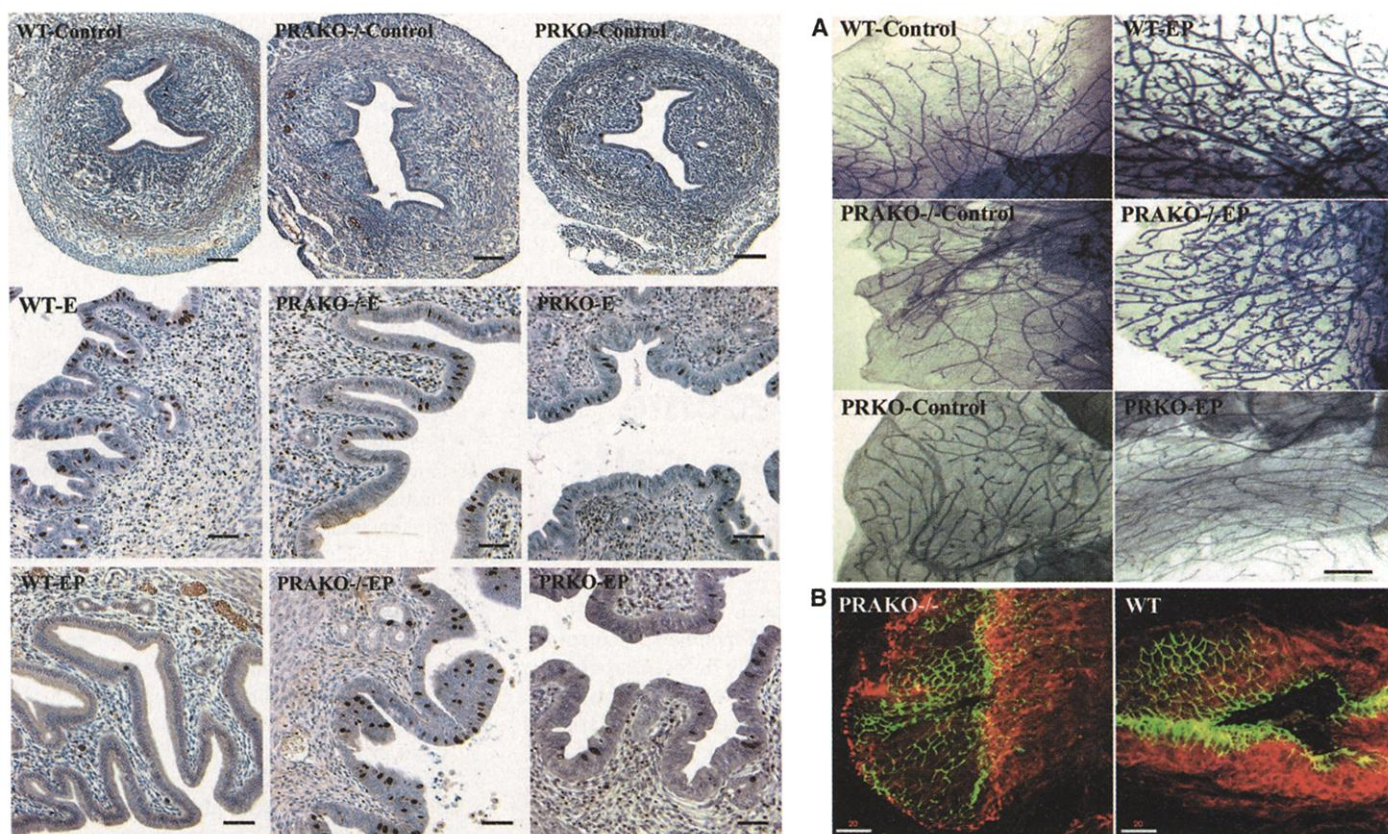


Fig. 3 (left). Abnormal proliferative responses to E and P treatment in PRAKO^{-/-} uteri. BrdU immunolabeling of uteri from ovariectomized WT, PRAKO^{-/-}, and PRKO mice treated with sesame oil (Control), E, or E plus P (EP). Bars, 200 μ m (controls) and 50 μ m (hormone-treated). **Fig. 4 (right).** Independence of mammary gland alveologenesis and tertiary ductal side branching from PR-A. (A) Thoracic

mammary gland whole mounts of untreated (Control) or E plus P (EP)-treated WT, PRAKO^{-/-}, and PRKO mice. Bar, 500 μ m. (B) E-cadherin immunofluorescence shows an organized expression pattern for E-cadherin in the mammary gland of both the WT and PRAKO^{-/-} mice (green); cytokeratin-14 in myoepithelial cells (red). Bars, 20 μ m.

PR-B isoforms is likely to be of critical importance to ensure appropriate reproductive tissue responses to P. Isoform-selective modulators of PR activity may allow tissue-selective modulation of progestin activity in hormonal therapy.

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- Ovariectomies were done on 6- to 8-week-old mice, and 14 days later animals were treated with hormone [E (1 ng/ μ l) or P (10 ng/ μ l) or both] dissolved in sesame oil. For protein immunoblot analysis of uterine PR expression and BrdU immunolabeling, ovariectomized mice were given daily subcutaneous (s.c.) injections of E (100 ng) alone or E (100 ng) and P (1 mg) for 4 days. Immunoblots were probed with polyclonal rabbit immunoglobulin G (IgG) to PR (1:1000 dilution; Santa Cruz Labs, Santa Cruz, CA) or polyclonal rabbit IgG to LF as described (21). Uterine sections (5 μ m) were probed for immunohistochemistry with rabbit polyclonal IgG to PR (1:100 dilution; DAKO, Denmark) as described (17). For BrdU immunolabeling, mice were administered intraperitoneal injections of BrdU (30 μ g per gram of body weight) 2 hours before being killed. Uteri were fixed in 4% paraformaldehyde and embedded in paraffin, and 5- μ m sections were prepared. BrdU uptake was detected with the Cell Proliferation kit (Amersham-Pharmacia Biotech, Buckinghamshire, UK). Image Tool software (UTHSCS, San Antonio, TX) was used to determine immunoreactive cells per unit area. For each animal, four independent sections each containing 1000 to 1500 cells were counted. For protein immunoblot analysis of LF, mice were given a single injection of E (100 ng) or E (100 ng) plus P (1 mg). For ribonuclease protection (RPA) analysis, uterine RNA was isolated 6 hours (AR) or 14 hours (CT and HDC) after a single dose of P (1 mg). Total RNA (10 μ g) was analyzed with ³²P-labeled uridine 5'-triphosphate-antisense RNA probes (MAXI-script; Ambion, Austin, TX) and the RPA assay kit (RIPA III; Ambion, Austin, TX). A 400-base pair (bp) CT clone was from EST bank (958362). Clones HDC [350-bp Bam HI-Eco RI fragment in pGEM7ZF (+)] and AR [475-bp Sac I-Dra II fragment in pBS-SKII(+)] were gifts from S. K. Dey (University of Kansas Medical Center, Kansas City, KS).
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P or no hormones were implanted s.c. on days 1 and 10, and tissues were collected 11 days later. Mammary glands were fixed in 10% formalin and stained with hematoxylin as described (2). For indirect immunofluorescence, frozen mammary tissue was sectioned at 50- μ m thickness, permeabilized with 5% Triton X-100, and fixed in 4% paraformaldehyde. Sections were incubated overnight at 4°C in rabbit polyclonal antiserum to cytokeratin-14 (1:500 dilution; a gift from D. R. Roop, Baylor College of Medicine) containing 1% bovine serum albumin and rat

monoclonal IgG to E-cadherin (1:500 dilution; Zymed, San Francisco, CA). Antibodies were visualized with Texas Red-goat antibodies to rabbit IgG (1:200 dilution; Molecular Probe, Eugene, OR) and fluorescein isothiocyanate-goat antibodies to rat IgG (1:400 dilution; PharMingen, San Diego, CA). Serial optical sectioning was performed with a confocal laser-scanning microscope (Multiprobe 2001, Molecular Dynamics). Three-dimensional images were reconstructed with Image Space Software (Molecular Dynamics).

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Oligodendrocyte Precursor Cells Reprogrammed to Become Multipotential CNS Stem Cells

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During animal development, cells become progressively more restricted in the cell types to which they can give rise. In the central nervous system (CNS), for example, multipotential stem cells produce various kinds of specified precursors that divide a limited number of times before they terminally differentiate into either neurons or glial cells. We show here that certain extracellular signals can induce oligodendrocyte precursor cells to revert to multipotential neural stem cells, which can self-renew and give rise to neurons and astrocytes, as well as to oligodendrocytes. Thus, these precursor cells have greater developmental potential than previously thought.

Oligodendrocyte precursor cells (OPCs) are arguably the best-characterized precursors in the mammalian CNS. They arise from multipotential cells in spatially restricted germinal zones and then migrate widely through the developing CNS (1–3). After a number of cell divisions, most OPCs terminally differentiate into postmitotic oligodendrocytes (4, 5), although some persist in the adult CNS (6, 7). Fetal calf serum (FCS) (8) and certain cytokines, including some bone morphogenic proteins (BMPs) (9), induce OPCs in culture to differentiate into type-2 astrocytes, although there is no convincing evidence that OPCs normally become astrocytes in vivo. Here we show that a 3-day exposure to these signals that induce type-2 astrocytes, followed by culture in basic fibroblast growth factor (bFGF), causes many purified OPCs to revert to a state that resembles that of multipotential CNS stem cells. The reverted cells can self-renew and give rise to neurons and type-1 astrocytes, as well as to oligodendrocytes. These unexpected findings suggest that extracellular signals can reverse glial cell specification and can convert specified precursors into multipotential stem cells.

We purified OPCs from postnatal day 6

(P6) rat optic nerve to greater than 99% purity by sequential immunopanning, as previously described (10). We cultured the cells in poly-D-lysine (PDL)-coated culture dishes in serum-free medium containing platelet-derived growth factor (PDGF) but no thyroid hormone (TH), to stimulate their proliferation and to inhibit their differentiation (11). After 5 days in culture, we tested their proliferative response to bFGF by removing the PDGF and adding bFGF: >99% of the cells stopped dividing and differentiated into oligodendrocytes within 5 days (not shown), just as they do when PDGF is removed in the absence of bFGF (11). This finding attests to the purity of the cultures and indicates that bFGF alone is a poor mitogen in these conditions. When, however, we first induced the OPCs to differentiate into type-2 astrocytes by adding 15% FCS for 3 days (Fig. 1B) and then removed the FCS and PDGF and cultured the cells in bFGF for a further 5 days, >40% of the cells could be labeled by a 6-hour pulse of bromodeoxyuridine (BrdU) (Fig. 1A). Thus, FCS-treated OPCs become responsive to the mitogenic effect of bFGF, a growth factor that is mitogenic for some CNS stem cells (12). Epidermal growth factor (EGF), which is also mitogenic for some CNS stem cells (13), did not stimulate BrdU incorporation under the same conditions (Fig. 1A). Using this protocol, we could keep the cells proliferating in bFGF, without serum, PDGF, or TH, for many weeks, during which time most of the cells reverted to a bipolar

morphology characteristic of perinatal OPCs (4) (Fig. 1C). When we cultured the cells in the same way, but in tissue culture dishes without the PDL coating, the cells produced floating neurosphere-like bodies after 10 days (Fig. 1D), just as CNS stem cells do under similar conditions (13, 14). Together, these findings raised the possibility that OPCs cultured sequentially in PDGF, FCS, and bFGF may acquire some of the properties of multipotential CNS stem cells.

To test this possibility, we assessed whether OPCs cultured in this way acquired the ability to produce neurons. We induced purified P6 OPCs to become type-2 astrocytes in 15% FCS and PDGF for 3 days and then cultured them in PDL-coated culture dishes in bFGF, without serum, PDGF, or TH, for 5 days or 1 month. We then stained the cells with neuron-specific monoclonal antibodies that recognize microtubule-associated protein 2 (MAP2), low molecular weight neurofilament protein (NF-L), or middle molecular weight neurofilament protein (NF-M), all of which are widely used as neuronal markers. We also stained the cells with the A2B5 monoclonal antibody (15) to identify OPCs (8), monoclonal anti-galactocerebroside (GC) antibody (16) to identify oligodendrocytes (17), and rabbit antibodies to glial fibrillary acidic protein (GFAP) to identify astrocytes (17, 18). As shown in Table 1, the proportion of cells that expressed neuron-specific markers greatly increased after 5 days in bFGF and increased two to three times further between 5 days and 1 month in bFGF, and most of these cells were A2B5-negative (A2B5⁻) (Fig. 1, E to G); at either time point, the proportion of A2B5-positive (A2B5⁺) OPCs was <40% and the proportions of GC⁺ oligodendrocytes and GFAP⁺ astrocytes were <1%. By contrast, when we stained the cells after 1 month in PDGF or after 3 days in 15% FCS, >80% were A2B5⁺, less than 5% were MAP2⁺, and none were NF-L⁺ or NF-M⁺ (Table 1). Thus, OPCs cultured sequentially in PDGF, FCS, and bFGF can give rise to neurons.

When we cultured cells treated in this way in bFGF for 2 months, many of them remained MAP2⁺, but <5% now expressed NF-L or NF-M (Fig. 2A). As it was shown previously that PDGF encourages CNS stem cells to develop into neurons (14, 19), we

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