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Parthenogenetic stem cells in postnatal mouse chimeras

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Summary

The ability of parthenogenetic (pg) cells to contribute to proliferating stem cell populations of postnatal aggregation chimeras was investigated. Using DNA in situ analysis, pg participation was observed in highly regenerative epithelia of various regions of the gastrointestinal tract, e.g., stomach, duodenum and colon, in the epithelia of tongue and uterus and in the epidermis. Pg cells also contributed to the epithelium of the urinary bladder, which is characterized by a relatively slow cellular turnover.

Using a sensitive proliferation marker to determine division rate of pg and normal (wt) cells in tissues of a 24-day-old chimera, no significant differences between pg and fertilized cells were observed. However, in colon and uterus of a pg ↔ wt chimera aged 101 days, a significant loss of proliferative capacity of pg cells was found. In the colon, this loss of proliferative potential was accompanied by an altered morphology of pg crypts. In general, they were situated at the periphery of the epithelium and lacked access to the lumen, with consequent cystic enlargement and flattened epithelium. No obvious morphological changes were observed in the pg-derived areas of the uterine epithelium of this chimera.

Our results provide evidence that pg cells can persist as proliferating stem cells in various tissues of early postnatal chimeras. They suggest that pg-derived stem cells may cease to proliferate in restricted areas of the gastrointestinal tract and in the uterine epithelium of pg ↔ wt chimeras of advanced age. However, no indications of such a loss of proliferative potential of pg cells could be observed in other areas of the digestive tract, e.g., in the stomach and duodenum, or in oral epithelium and in the epidermis.

These findings argue for a high degree of specificity of selection against pg cells in postnatal life. They also suggest that an impaired proliferative capacity is not a general feature of pg cells. Hence, it is possible that, to explain the impaired growth of of pg ↔ wt chimeras and the overall stringent selection against pg cells in such chimeras during fetal development, additional and/or alternative causes apart from a decreased proliferative potential of pg cells have to be considered.

Apart from their contribution to the endoderm-derived epithelia of stomach, gut and uterus, to the mesoderm-derived epithelium of uterus and to ectoderm-derived oral epithelium and epidermis, pg-derived cells differentiated into the mesoderm-derived mesenchymal cells of the stromal layers that lie between the epithelial cells and smooth muscle of stomach, gut, uterus and urinary bladder. Contribution to smooth muscle was also observed.

Thus our results show that pg cells are able to differentiate into various cell types of different embryonic origin in tissues of composite structure.

Key words: parthenogenesis, chimeras, stem cells, proliferation, mouse.

Introduction

In mammals, both parental genomes are required for normal prenatal development (Barton et al., 1984; Mann and Lovell-Badge, 1984; McGrath and Solter, 1984; Surani et al., 1984). This is presumably due to the non-equivalence of imprinted genes active in embryonic growth and differentiation, whose expression depends on their parental origin. Experimentally produced uniparental mouse embryos die at midgestation. Interestingly, androgenetic and parthenogenetic/gynogenetic embryos develop opposite phenotypes (Surani et al., 1986). It is possible to rescue uniparental embryos by integrating them in aggregation or injection chimeras with normal mouse embryos (Stevens et al., 1977; Surani et al., 1977; Mann et al., 1990; Barton et al., 1991; Mann and Stewart, 1991).

Previous investigations have shown that chimeras composed of parthenogenetic (pg)/gynogenetic and fertilized cell lineages exhibit a highly specific and consistent phenotype. Parthenogenetic ↔ fertilized chimeras (pg ↔ wt chimeras) with even a relatively small contribution of pg cells are considerably smaller than their normal littermates (Stevens et al., 1977; Stevens, 1978; Anderegg and Markert, 1986; Fundele et al., 1989, 1990; Nagy et al., 1989). This effect was consistently observed, even when strain combinations were used where the pg embryos were derived from a mouse strain whose individuals are larger and heavier than those of the strain contributing the fertilized embryos (Fundele et al., 1991). Another characteristic feature of pg ↔ wt chimeras is a stringent selection against the pg cell lineage which occurs during fetal development (Nagy et al., 1987; Surani et al., 1988; Clarke et al., 1988; Thomson and Solter, 1988; Fundele et al., 1990).

In adult PFCs, pg cells are consistently absent from skeletal muscle (Fundele et al., 1989; Nagy et al., 1989) but are able to contribute to most other tissues, although not regularly and only at low levels. However, pg cells frequently persist in brain, cardiac muscle and regions of the gastrointestinal tract and, in these tissues, they may form a considerable proportion of the total cell population (Fundele et al., 1989, 1991).

The mechanisms that cause the selection against pg cells during fetal development have so far not been identified. This phenotype can be explained either by a decreased proliferative capacity of pg cells or by increased rate of pg cell death or by various combinations of these two mechanisms (Surani et al., 1990a, b). It is also feasible that different selection mechanisms might apply in different tissues of the developing organism.

If one assumes that an impaired proliferative potential is a general feature of pg cells and the main cause for the selection against them, then their persistence in highly proliferative stem cell populations of postnatal chimeras would be extremely unlikely.

Therefore, to address this question, various tissues from pg ↔ wt chimeras containing epithelia with continuous cellular turnover were analysed by in situ hybridisation. The tissues selected for this study were epidermis (Potten, 1983), epithelium of the tongue (Hume, 1983), three different areas of the gastrointestinal tract, e.g., stomach, duodenum and colon (Lipkin, 1987; Potten and Loeffler, 1990) and the mesodermal epithelium of the uterus (Boutin et al., 1992).

Materials and methods

Animals

Outbred albino CFLP and NMRI mice, C57BL/6 × CBA hybrids (F_1) and strain 83 mice were used. Strain 83 mice are homozygous for a high copy transgenic insertion of a plasmid containing a mouse β-globin gene (Lo, 1986). This transgenic marker was used in the past for cell lineage studies in chimeric mice (Lo et al., 1987; Clarke et al., 1988; Thomson and Solter, 1988), because it allows a classification of single cells in a mosaic tissue. Using GPI-1 allozymes as markers such questions could not be addressed. CFLP mice were originally obtained from Bantin and Kingman, Colchester, UK, NMRI from the Zentralinstitut für Versuchstierzucht, Hannover, FRG. C57BL/6 females and CBA/J males were obtained from both suppliers. The transgenic 83 mouse strain was originally established by C. Lo and given to us with the kind permission of C. Lo.

Embryos and chimeras

Superovulation of females, the collection, activation and handling of eggs and embryos, and embryo aggregation were carried out according to standard procedures described in detail (Surani et al., 1988). For the production of diploid pg ↔ wild type (wt) chimeras, activated day-3 4- to 8-cell embryos derived from (F_1 × strain 83) hybrid females were aggregated with day-2 2-cell fertilized CFLP or NMRI × F_1 embryos. (F_1 × strain 83) hybrids are heterozygous for the transgenic insert and pg embryos derived from them are either homozygous or wild type. Control chimeras were produced by aggregating day 3 embryos derived from crosses between (F_1 × strain 83) females and strain 83 males with day-3 CFLP embryos. Aggregated embryos were transferred to recipient females on day 3 of pseudopregnancy, the day of the vaginal plug being counted as day 1 of pregnancy.

Analysis of chimeras

Postnatal pg (F_1 × strain 83) ↔ CFLP × CFLP chimeras were identified by patchy eye and coat pigmentation as pg (F_1 × strain 83) are either agouti or black. To identify pg ↔ wt chimeras whose pg component carried the transgene, tail biopsies were performed and DNA was isolated by standard extraction procedures (Allen et al., 1987). Equal amounts of DNA were loaded onto a 1% agarose gel and electrophoresed (Maniatis et al., 1982). The DNA was transferred to a Nylon membrane (Gene Screen plus, NEN) which was probed with radioactively labelled pMßG∂2 to detect the presence of the transgene. The signal intensity provided a first estimate of the contribution of pg cells to the embryo. Phenotypic identification of pg (F_1 × strain 83) ↔ NMRI × F_1 was possible when pg-derived hair was black, as NMRI × F_1 hybrids are agouti. Hence, tail biopsies were performed on all mice derived from pg (F_1 × strain 83) aggregations. DNA was made accessible by boiling and proteinase K digestion of homogenized tissue. Polymerase chain reaction (PCR; Saiki et al., 1988) was performed using primers situated in the pBR322 plasmid sequences of the pMβG∂2 transgene resulting in a 369 bp reaction product.

With one exception, chimeras whose pg component carried the transgenic insert were killed by CO_2 inhalation. Tissues were fixed overnight at 4°C in Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid), equilibrated against 70% ethanol, dehydrated and embedded in paraffin. Tissues were sectioned at 7 µm and floated on glass slides coated with 3-aminopropyltriethoxysilane (Sigma; Maddox and Jenkins, 1987)

In situ hybridization (ISH) and immunohistochemistry (IHC)

Probe pMßG∂2, a kind gift from Dr. J. Rossant, Toronto, was labeled with digoxigenin-dUTP according to the manufacturer´s recommendations (Boehringer Mannheim). Sections of chimeric tissues were digested for 10 minutes at 42°C with proteinase K at a concentration of 50 µg ml^{-1} in 100 mM Tris-HCl, pH 8.0, 50 mM EDTA. Nuclear DNA on tissue sections was denatured either in 70 mM NaOH for exactly 3 minutes or in 85% formamide/15% 20 × SSC for 20 minutes at 60°C. Prehybridization was performed as suggested in the Boehringer application manual. Hybridization was carried out for 24 hours at 60°C. The hybridization mixture contained approximately 5 ng labelled probe, 5 µg salmon sperm DNA, 10 µg heparin in 10% dextrane sulfate and

5 × SSC per slide in a volume of 50 µl. Cover slips were attached to the slides with rubber cement. After hybridization, slides were washed twice in 2 × SSC, 0.1% Triton X-100 and once in 0.1 × SSC, 0.1% Triton X-100 for 5 minutes at room temperature (RT), followed by a 10 minutes wash at 60°C in 0.1 × SSC, 0.1% Triton X-100. After a final wash in 0.1 × SSC, 0.1% Triton X-100, 5% bovine serum albumin (BSA) for 5 minutes at RT, sections were incubated in alkaline phosphatase-conjugated anti-digoxigenin antibody (AB) diluted according to the supplier's recommendations in Tris-buffered saline (TBS) for 1 hour. Staining was carried out according to the manufacturer's recommendations using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-nitroblue-tetrazolium chloride (NBT) as substrates.

Two pg (F_1 × strain 83) ↔ F_1 × NMRI chimeras only were injected intraperitoneally with 50 mg bromodesoxyuridine (BrdU; Sigma) per kg of bodyweight between 30 minutes (chimera 1711) and 4 hours (chimera 0202) prior to death. BrdU is incorporated into newly synthesized DNA and by applying a monoclonal AB against BrdU (Dako) may be used as a cell proliferation marker (Gratzner, 1982). After ISH, sections were denatured a second time in 70 mM NaOH for 3 minutes at RT. Indirect IHC was performed according to standard conditions with AB dilutions in the range recommended by the supplier. An alkaline phosphatase-conjugated anti-mouse AB (Dako) was used as second AB. Staining was carried out using hexazotized New Fuchsin as dye. Levamisol was added to a final concentration of 1 mM. Sections were counterstained with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI). When a more detailed morphological analysis was found to be necessary, serial sections adjacent to those that had undergone ISH were stained with hematoxylin and eosin (HE) without prior ISH. Microscopical analysis of in situ labeled sections was done with a Zeiss Axiophot microscope and pictures were taken with Kodak Ektachrome 100 film.

Quantitation of ISH and IHC

For the quantitation of pg contribution, slides were selected randomly. Sections from at least two different areas of any given tissue were evaluated. In epidermis, epithelium of the tongue and uterus, labeled and unlabeled cells were counted. In epithelia of stomach, duodenum and colon, the number of pg- and wt-derived glands and crypts was compared, as intestinal crypts and gastric glands are populated by a single stem cell. For the quantitation of cell proliferation in stomach and gut, the number of cells labeled by anti-BrdU IHC versus unlabeled cells in pg and wt glands and crypts was compared. Tissue sections from at least four different planes of section were analyzed. Wt crypts lying adjacent to pg-derived crypts were used for controls. For analysis of tongue and epidermis, continuous areas with adjacent patches of pg and wt cells were counted. Statistical significance of proliferation differences between pg and wt cell populations was calculated using a chi^2 contingency table.

Results

Chimeras

The pg ↔ wt and wt ↔ wt chimeras analyzed in the present study were produced in three experiments (Table 1). Of the eight chimeras obtained in experiment 1 (carried out at the AFRC-IAPGR, Babraham), four carried the transgene, based on Southern blot analysis of tail DNA. One of these chimeras (No. 194) died overnight at 11.5 days of age. The other three pg ↔ wt chimeras (Nos. 215, 216, 217) obtained in experiment 1 were all killed at the age of 21 days.

Pg ↔ wt chimera 194 was considerably retarded when compared with its chimeric litter-mates. It had a bodyweight of 2.27 g after death compared with 6.74, 7.56 and 7.52 g of three other pg ↔ wt chimeras. Postmortem dehydration probably contributed to this extreme weight reduction. All non-chimeric litter mates that had lost the pg component were killed to avoid competition with the smaller chimeric animals. A comparison between pg ↔ wt chimeras and wt mice derived from the same litter was therefore not possible.

The 22 pg (F_1 × strain 83) ↔ NMRI × F_1 aggregations obtained in experiment 2 (carried out at the Institut für Biologie III, Freiburg) were transferred to three recipient females. One of these recipients was used for another experiment. The two remaining females which had received 14 aggregations, gave birth to 11 pups. Seven large newborns were killed on day 1 without further analysis. At the age of 12 days, the four remaining mice were analyzed by PCR for the presence of the transgene. A PCR reaction product migrating in the expected position was obtained from all of these mice. The smallest mouse (No. 1711) was killed at 24 days. It had a bodyweight of 11.2 g, compared with 12.2 (No. 0202), 16.3 and 18.6 g of the other chimeras. Another chimera was killed at the age of 101 days (No. 0202). This was the only overt coat colour chimera produced in this experiment, with black pg-derived hairs in a mainly agouti coat.

Distribution and proliferation of pg cells

After ISH, signals were found on 91% (511 out of 559 nuclei counted) of nuclei in the colonic epithelium of a control (F_1 × strain 83) hybrid mouse hemizygous for the transgene, thus showing the efficiency of the technique (Fig. 1A). Representative results of ISH on sections of pg ↔ wt and wt ↔ wt chimeras are shown in Figs 1 and 2. A summary of the quantitative contribution of pg cells to epithelia of several organs of pg ↔ wt and wt ↔ wt chimeras is provided in Table 2.

Serial sections of dorsal skin from two different areas of the back of pg ↔ wt chimeras 1711 and 0202 were analyzed (Fig. 1D). From the frequency of epidermal cells labeled by ISH, a pg contribution of approximately 5 to 10% and 60 to 70% to epidermis of chimeras 0202 and 1711, respectively, could be estimated. Since the patches that were analyzed were comparatively small compared with the total area of epidermis, a sampling error in this estimate cannot be excluded. Hence, the overall pg contribution to epidermis of pg ↔ wt chimeras 1711 and 0202 could have been considerably higher or lower than determined. Two proliferating cell populations were found in the skin of chimeras 1711 and 0202, e.g., cells situated in the hair follicles and epidermal cells. Only cells lying in the epidermis proper were counted. Adjacent patches of of pg and wt cells were analyzed (Table 3). In both 1711 and 0202, the pg cells seemed to have a slight proliferative advantage; in chimera 0202 this is marginally significant at the 5% level. Dorsal skin was not dissected from the other pg ↔ wt chimeras or from any wt ↔ wt chimeras.

The other ectoderm-derived epithelium that was analyzed for the present study was the epithelium of the tongue (Fig.

1E). A considerable participation of pg cells in the differentiation of this oral epithelium was observed in some pg ↔ wt chimeras (Table 2). No significant decrease of proliferative potential of pg cells compared with wt cells was seen in the tongue epithelia of 1711 and 0202 (Table 3).

Three different areas of the gastrointestinal tract were analyzed. Stomach areas exhibiting a glandular epithelium were selected for analysis, as the gastric glands are monoclonal, thus facilitating the evaluation of pg contribution (Fig. 2A,B). For the quantitation of proliferating cells in the small intestine, only cells situated in the crypts were counted, as dividing cells are spatially restricted to crypts. In stomach and colon, all cells situated in a gastric gland or crypt, respectively, were counted. Contribution of pg cells to epithelia of the gastrointestinal tract varied between 0% (colon 217) and 80% (duodenum 194). In most cases, however, contribution was between 5 and 20%, reflecting the strong selection against pg cells in pg ↔ wt chimeras. In the gut regions of two wt ↔ wt chimeras that were analyzed, a comparable consistent reduction of fertilized (F_1 × strain 83) × strain 83 derived cells was not observed (Table 2; Fig. 1B).

Pg ↔ wt chimera 194 was found to be exceptional. Pg-derived cells had populated extensive areas of the gastrointestinal tract of this chimera (Table 2; Fig. 1F). In one cross-section through the duodenum, 43 out of 54 crypts (80%) had been populated by pg stem cells. Chimera 194 was extremely retarded, as assessed by its reduced bodyweight. The possibility was taken into account that the small size and ensuing death of this chimera was caused by a functional disturbance of its numerous gastric and intestinal pg cells. However, no obvious malformations of various regions of the small intestine (duodenum, ileum, jejunum) with high pg participation could be discerned by HE staining of 7 µm paraffin sections. Shedding of the epithelium into the lumen, a morphological change that occurred mainly in distal parts of the small intestine, affected both pg- and wt-derived patches (not shown). These alterations can probably be regarded as normal post-mortem disintegration that occurred in the period (< 17 hours) between death and fixation. Shedding of large areas of the intestinal epithelium was not observed in sections of other pg ↔ wt and wt ↔ wt chimeras. Another trait specific to chimera 194 was the occurrence of cells in the lumen of distal regions of the small intestine. By HE staining some of these cells could be positively identified as neutrophilic granulocytes. This finding indicates that inflammatory processes had taken place in these gut regions during the life of chimera 194.

No obvious morphological alterations of pg-derived gastrointestinal structures like villi, crypts and gastric glands of the pg ↔ wt chimeras killed at the age of approximately three weeks were observed (chimeras 215, 216, 217, 1711; Fig. 1C, 2B). No significant reduction in BrdU integration could be detected in the epithelia of stomach and colon of chimera 1711; however, somewhat less pg than wt nuclei were labeled by anti-BrdU IHC in duodenal crypts of this chimera (P < 5%; Table 3).

Proliferation of pg stem cells was normal in stomach and duodenum of pg ↔ wt chimera 0202 (Table 3) and morphology of pg-derived gastric glands seemed to be normal. In contrast, some pg crypts in the duodenum of 0202 seemed to exhibit a somewhat abnormal morphology (Fig. 1G). In the colon of chimera 0202, a significant reduction of pg proliferation was observed. Only 4.6% of cells situated in pg crypts had integrated BrdU in detectable amounts during the four hours between injection and death, compared with 12.9% of controls. In addition, pg-derived crypts showed a distinctly abnormal phenotype and localization (Fig. 2C,D,E,F). Of 28 pg crypts observed in 18 sections

Fig. 1. (A) Section through the colon of a non-chimeric (F_1 × strain 83) mouse hemizygous for the transgenic insert, to show the efficiency of the in situ hybridization technique (bar = 50 µm). (B) Section through colon of control chimeras 218. Unlabeled crypts are derived from a CFLP × CFLP embryo; labeled crypts are derived from a (F_1 × strain 83) × strain 83 embryo. The monoclonal origin of intestinal crypts is nicely visible; scattered labeled cells are probably mesenchymal. Considerable labeling is observed on this section, showing that wt (F_1 × strain 83) × strain 83 cells are not at a competitive disadvantage compared with CFLP × CFLP cells (bar = 50 µm). (C) In situ analysis of colon of pg ↔ wt chimera 215. Presence of pg cells in the epithelium is easiliy visible. Differentiation of pg cells into goblet cells can be detected without cell specific markers, by morphological criteria alone (bar = 50 µm). (D) A cross section through dorsal skin of pg ↔ wt chimera 1711 with a patch of pg-derived cells containing the transgenic insert is shown. Nuclei that have incorporated BrdU are stained red by anti-BrdU IHC and New Fuchsin staining. Not all nuclei in this patch are marked by ISH for the transgene. Some signals are not in the focal plane, in other nuclei the chromosomes carrying the transgenic insert were not on the 7 µm section (bar = 25 µm). (E) Section through tongue of chimera 0202. A patch of pg cells with adjacent patches of wt cells are shown. The columnar structure of the epithelium of the tongue is obvious. This section is counterstained with DAPI (bar = 25 µm). (F) Section through duodenum of chimera 194. Most crypts and villi on this section are of pg origin. The patches of mostly unlabeled cells are mesenchymal cells which form the core of the intestinal villi (counterstain is DAPI; bar = 50 µm). (G) Pg and wt crypts in duodenum of chimera 0202 are shown. A considerable proportion of the nuclei belonging to cells situated in the crypts are labeled by anti-BrdU IHC. One pg crypt (arrow) seems to exhibit a somewhat abnormal morphology (bar = 25 µm). (H) A colonic crypt (arrow) of chimera 1711 is shown, which exhibits both labeled (pg) and unlabeled (wt) cells, indicating that more than one stem cell is active in this crypt (bar = 50 µm).

Table 1. *Results of parthenogenetic ↔ fertilized and fertilized <-> fertilized aggregations*

	Activated	Aggregations (%)	Transferred (%)	Born (%)	Chimeras (%)
1 pg(F_1×83) ↔ CFLP×CFLP	250	126 (50)	44 (35)	16 (36)	8 (50)
2 pg(F_1×83) ↔ NMRI×F_1	96	42 (42)	22 (55)	11 (79)	4 (36)
3 (F_1×83)×83 ↔ CFLP×CFLP	-	77	55 (71)	28 (51)	15 (54)

Table 2. *Frequency of transgenic nuclei in tissues of parthenogenetic ↔ fertilized (pg ↔ wt) and fertilized ↔ fertilized (wt ↔ wt) chimeras*

	pg <-> wt						wt <-> wt	
	194	215	216	217	1711	0202	214	218
Epidermis	n.d.	n.d.	n.d.	n.d.	60-70%	5-10%	n.d.	n.d.
Tongue[a]	n.d.	344/1535 (22.4%)	444/1738 (25.5%)	n.d.	300/1628 (18.4%)	413/1727 (23.9%)	n.d.	n.d.
Stomach[b]	n.d.	35/360 (9.7%)	35/308 (17.5%)	13/291 (4.5%)	44/195 (21.0%)	19/276 (6.9%)	n.d.	n.d.
Duodenum[c]	150/219 (68.5%)	20/391 (5.1%)	24/381 (6.3%)	29/457 (6.3%)	58/336 (17.3%)	7/515 (1.4%)	24/108 (22.2%)	79/108 (73.1%)
Colon[c]	n.d.	54/416 (12.5%)	5/400 (1.3%)	0/604 (0%)	12/436 (8.8%)	9/554 (1.6%)	76/116 (65.5%)	79/108 (81.7%)
Uterus[a]	n.d.	n.d.	n.d.	n.d.	n.d.	50/25200 (0.2%)	n.d.	n.d.
Bladder[a]	309/911 (33.9%)	n.d.	58/884 (6.6%)	6/1210 (0.5%)	104/709 (14.7%)	102/2091 (4.9%)	n.d.	n.d.

Values are given as number of nuclei[a], gastric glands[b], or intestinal crypts[c] labeled by in situ hybridization/total number of labeled and unlabeled nuclei[a], gastric glands[b] or intestinal crypts[c]. Percentage values are given in parentheses.

Table 3. *Uptake of bromodeoxyuridine (BrdU) in nuclei of parthenogenetic (pg) and fertilized (wt) cells of different tissues of pg ↔ wt chimeras*

	Epidermis		Tongue		Stomach		Duodenum		Colon		Uterus	
	wt	pg	wt	pg	wt	pg	wt	pg	wt	pg	wt	pg
1711	48/629 (7.6%)	53/521 (10.2%)	47/1628 (2.9%)	9/300 (3.0%)	163/2568 (6.3%)	147/1871 (7.9%)	114/363 (31.4%)	119/495 (24.0%) $P<5\%$ $\chi^2=5.74$	64/842 (7.6%)	25/265 (9.4%)	-	-
0202	8/427 (1.9%)	9/192 (4.7%) $P<5\%$ $\chi^2=3.93$	29/1727 (1.7%)	6/413 (1.5%)	74/702 (10.5%)	61/758 (8.1%)	208/491 (42.4%)	171/402 (42.5%)	246/1908 (12.9%)	30/647 (4.6%) $P<0.05\%$ $\chi^2=34.2$	434/1200 (36.2%)	31/139 (22.3%) $P<0.5\%$ $\chi^2=10.56$

Values are given as BrdU-labeled nuclei/total number of nuclei. Percentage values are shown in parentheses; *P* values are given where differences in proliferation ratios of pg and wt cells were found to be significant (χ^2 values)

from different areas of the colon, only one had access to the lumen. The other pg crypts were located on the periphery of the gut. They showed cystic enlargement and flattening of the epithelium, probably due to continuing secretion from the goblet cells and increasing inner pressure (Fig. 2E,F).

The monoclonal origin of gastric glands and duodenal and colonic crypts was regularly visualized by the ISH technique. However, crypts were observed which posessed both labeled and unlabeled cell populations (Fig. 1H). This indicates that at least two stem cells, one wt- and one pg-derived, remained active in these crypts.

Contribution of pg cells to the uterine epithelium of chimera 0202 was extremely low (Table 2; Fig. 2G,H). In serial cross-sections through ten different parts of the uterus, a single patch of cells showing hybridization signals was detected. This patch contained approximately 50 cells in its largest spatial extension and approximately 3600 epithelial cells were counted per uterine cross-section, giving a contribution of 0.14%. Compared with wt epithelial cells, pg-derived cells exhibited a significant reduction of cellular proliferation with 36.2% of wt and 22.3% of pg cells marked by anti-BrdU IHC.

In some chimeras, pg cells formed a considerable subpopulation of the epithelium of the urinary bladder (Table 2; not shown). However, in both pg ↔ wt chimeras 1711 and 0202, no urothelial cells had incorporated BrdU (not shown), indicating a slow cellular turnover of this tissue.

Discussion

Previous studies have shown that pg-derived cells can persist in numerous tissues in adult chimeric mice (Fundele et al., 1989, 1990; Nagy et al., 1989). However, contribution of pg cells is usually low. It was therefore assumed that, during prenatal development, pg cells may undergo a slowing down or even cessation of their cellular division rate (Surani et al., 1990a, b; Fundele et al., 1990). To test this hypothesis, we have investigated the distribution of pg cells in pg ↔ wt adult chimera tissues that contain highly proliferating cells. The tissues analyzed were parts of the gas-

trointestinal tract, e.g., stomach, duodenum and colon, and the epidermis, oral epithelium and uterus.

An interesting finding not directly related to the main topic of the present study was the identification of crypts with two distinct cell populations. In adult mice, crypts are monoclonal as shown by histological analysis of aggregation chimeras and electrophoretic analysis of single crypts from female mice heterozygous at an X-chromosome-linked gene (Ponder et al., 1985). It was shown that, in duodenum of postnatal day-2 chimeras, 50% of crypts situated at patch boundaries are of mixed genotype (Schmidt et al., 1988). This number decreases steadily and by postnatal day 14 a large majority of crypts is monoclonal (Schmidt et al., 1988). It is therefore probable that the crypts of mixed origin that were observed in pg ↔ wt chimera 1711 were rare remnants that would have lost their pg or wt stem cell later in life. No mixed crypts were however observed in colon of two wt ↔ wt chimeras.

The observed distribution of pg cells, as visualized by ISH, in tissues of pg ↔ wt chimeras aged approximately three weeks allows several conclusions.

First, pg cells are able to develop into various differentiated cell types. These include smooth muscle, mesenchyme and various cell types found in endoderm- and ectoderm-derived epithelia. Since no differentiation-specific cellular markers were used in this study, no direct evidence exists that a pg subpopulation had reached a comparable degree of maturation as an adjacent wt cell population. However, in stomach, duodenum and colon, all the different cell types of the epithelium are derived from one stem cell, as intestinal crypts are monoclonal (Ponder et al., 1985; Schmidt et al., 1985, 1988). Cell migration from the crypt is accompanied by differentiation. As the morphology of pg-derived gastric glands and intestinal crypts and villi was apparently normal in pg ↔ wt chimeras aged approximately three weeks, our results indicate that pg cells can functionally differentiate into all mature cell types found in the epithelia of duodenum and colon and in the glandular epithelium of the stomach. Comparable conclusions may be inferred for the epithelium of the tongue which also shows a spatial patterning with monoclonally derived patches (Hume, 1983).

Second, normal proliferative potential of pg stem cells can be inferred. Most epithelial cells of the gastrointestinal tract, on whose proliferation profiles a considerable amount of work has been carried out, have extremely short life-spans ranging between three days in duodenum and stomach (Cheng and Leblond, 1974; Eastwood, 1977; Lipkin, 1987) and six days in colon (Chang and Leblond, 1971; for reviews see Potten and Hendry, 1983; Gordon, 1989; Potten and Loeffler, 1990). Exceptions are the Paneth cells and the enteroendocrine cells of the colon which have life-spans of approximately four and three weeks, respectively. To sustain this cellular turnover, an adequate supply of cells has to be maintained by the stem cells. It seems likely that a reduced division rate of pg stem cells would result in an abnormal morphology of pg crypts and villi if the life-span of mature epithelial cells remained constant. However, no obvious morphological abnormalities were observed in pg-derived structures of the digestive tract. The same applies for the other tissues that were analyzed. The argument that pg stem cells of epidermis, oral epithelium and the gastrointestinal tract proliferate normally, is also supported by the results of the BrdU labeling experiments.

Hence, it came as a surprise that, in two tissues of one chimera of approximately three months of age, pg cells exhibited a severe decrease in BrdU uptake. The significance of this finding is not clear as only one chimera of this age was analyzed. In the colon, the slowing down of proliferation was accompanied by morphological alterations of pg crypts. Interestingly, no comparable changes were observed in other regions of the gastrointestinal tract, but we cannot exclude the possibility that degeneration of pg crypts might have started later in other areas of the gut of this chimera. It is, however, worth noting that a better survival of pg cells in duodenum compared with colon of pg ↔ wt chimeras was described before (Fundele et al., 1990). The molecular basis for the selective or early loss of pg stem cells from the colon is not clear at all. An involvement of the imprinted genes *Igf2* (DeChiara et al., 1990; 1991; Ferguson-Smith et al., 1991) and *H19* (Bartolomei et al., 1991) can be excluded as these genes are not expressed in intestine of adult rodents (Beck et al., 1988; Stylianopoulou et al., 1988; DeChiara et al., 1991; Poirier et al., 1991). The restriction of the observed phenotype makes the involvement of an ubiquitous cell cycle control gene unlikely, too. This is also supported by the fact that normal proliferation of pg cells was observed in all other tissues of this chimera, with the exception of the uterine epithelium.

In summary, our results provide, for the first time, evidence that pg cells do not exhibit a **general** proliferative deficiency. This means that specific imprinted genes may be responsible for the observed phenotypes of pg ↔ wt chimeras, such as their small size or the consistent loss of pg cells from specific tissues such as skeletal muscle (Fundele et al., 1989, 1990; Nagy et al., 1989) or cartilage (Bender, R., Christ, B. and Fundele, R., unpublished data). This is supported by the striking phenotype of androgenetic (ag) ↔ wt chimeras (Mann et al., 1990; Barton et al., 1991; Mann and Stewart, 1991) which seems to be caused by an increased proliferative capacity of ag cells. However, this beneficial effect of a uniparental ag genotype seems to be restricted to a specific cell type, e.g., chondrocytes. In addition, the proliferation-inducing effect shows a spatial and temporal specificity. Furthermore, our findings in the colon of chimera 0202 indicate that genomic imprints are stably transmitted into postnatal life. It should be interesting to investigate whether the persistence of genomic imprints may cause aberrant behaviour of uniparental cells much later in adulthood and in senescence.

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Fig. 2. (A, B) Gastric glands of chimeras 1711 (B) and 0202 (A) are shown. Monoclonal origin of gastric glands and localization of proliferating cells can be discerned (A: bar = 25 µm; B: bar = 50 µm). (C, D) Aberrant morphology of pg crypts in colon of pg ↔ wt chimera 0202 is shown. Pg crypts do not have access to the lumen of the gut. No proliferating cells are found in any of the three crypts. In contrast, wt unlabeled crypts contain dividing cells marked by anti-BrdU IHC (bars = 25 µm). (E, F) Adjacent sections through colon of chimera 0202 are shown. In E, two pg crypts are marked by ISH for the transgene and proliferating cells are demonstrated by anti-BrdU IHC; the section is counterstained with DAPI. F is stained with HE, without prior ISH or IHC. Flattened epithelium is found only in the pg crypts (bars = 50 µm). (G) Section through uterus of pg ↔ wt chimera 0202. No pg cells were observed in epithelium of this cross-section. In contrast, considerable participation of pg cells can be detected in the stroma of the uterus (bar = 50 µm). (H) A patch of pg cells in the uterine epithelium of chimera 0202 is shown.