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Unlimited expansion of intestinal stem cells from a wide range of ages

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Abstract

The recent technical advance in cloning and culturing ground-state intestinal stem cells (ISC) provides us an opportunity of accurate assessment of age-related impact on the function of highly proliferative intestinal stem cells. Our ability of indefinitely and robustly expanding single-stem-cell derived pedigrees *in vitro* allows us to study intestinal stem cells at the clonal level. Interestingly, comparable number of ISC clones was yielded from 1mm endoscopic biopsy of all donors despite the age. They were passaged *in vitro* as pedigrees and expanded to 1 billion cells in approximately sixty days without changes in stemness demonstrated by clonogenicity and multipotency. Therefore, our study shows that ISCs from a wide range of ages can be cloned and expanded to unlimited number *in vitro* with similar efficiency and stability. These patient-derived ISCs harbor intrinsic immortality and are ideal for autologous transplantation, supporting the promise of adult-stem-cell based personalized medicine.

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Conflicts of interest statement

W.X., F.M., M.D. and M.V have filed a patent related to the technology used in the present work and Drs. Xian and McKeon have financial interests in MultiClonal Therapeutics Inc.

Introduction

Autologous transplantation using wild-type or transgenic epidermal stem cells have been proven to be extremely successful in patients with severe burns, chronic wounds and junctional epidermolysis bullosa [1–3]. Conceivably, adult stem cells derived from other regenerative tissues such as intestine can be used to restore the intestinal epithelial functions following autologous transplantation in patients with severe forms of short bowel syndrome (SBS) [4], or those with congenital disorders [5] or those with inflammatory bowel disease (IBD) [6,7].

However, caution needs to be taken when these patient-derived adult stem cells being used for autologous transplantation. Although there is an intriguing amount of evidence suggesting that the stem cells residing in the intestinal tissues of aged people are still quite capable, it is unclear whether their stem cell behavior is similar to those taken from a younger individual. Thus, whether old stem cells are inherently dysfunctional is a question of considerable relevance to the practical development of stem cell therapies based on autologous transplantation for people at all ages.

Cloning, screening and expanding ISCs had been challenging due to our inability of cloning stem cells from columnar epithelial tissue and maintaining their immaturity during *in vitro* expansion. Consequently, ISCs had to be carried forward as regenerative, differentiating “organoids” with very low percentage of clonogenic cells, which limits the kinetics of their propagation as well as their utility for exploring the elemental stem cell [8–10].

Recently, a new technology was developed to support cloning the ground state ISCs in their highly immature, clonogenic state. These cultured ground-state ISCs demonstrated remarkable stability in their genome and epigenetic commitment programs, maintained clonogenicity and unlimited replicative expansion, suggesting their tremendous potential for personalized regenerative medicine [11,12].

In this study, we used the ground state stem cell cloning technology to study intestinal stem cells derived from donors in a wide range of ages. We found the behavior of ISCs removed from the old cellular environments was identical to those taken from a younger individual. They can all be passaged robustly and stably *in vitro,* suggesting the intrinsic immortality of ISCs is age independent.

Results

Ground state ISCs derived from patients with a wide range of ages

In order to understand whether ground-state intestinal stem cells (ISC^{GS}) can be successfully cloned and cultured from patients at all ages, we chose ten patients between age 10 to 20, ten patients between age 30 to 50 and ten patients between age 50 to 80 (Supplementary Table 1). The 1mm biopsies from the intestinal epithelium of these patients were enzymatic digested and seeded in a system including 3T3J2 feeder and specialized medium [11]. We detected approximately 100 colonies that can be derived from each of all thirty patients. Starting from one ISC^{GS} colony, a billion ISC^{GS} cells can be generated from all thirty

patients independent of age in approximately sixty to eighty days (Figure 1A). The ISC^{GS} derived from all ages displayed indistinguishable morphology (Figure 1B). In order to test their multipotent differentiation ability, pedigree lines of ISC^{GS} of 16,42-and 76-years old patients were differentiated in air-liquid interface (ALI) cultures for 10 days. All the ISC^{GS} formed a highly uniform, 3D serpentine pattern (Figure 1C). Histological sections of these differentiated ISC^{GS} revealed a columnar epithelium of villus-like structures (Figure 1D, E) marked by goblet (Mucin 2+), endocrine (chromogranin A+), and Paneth cells (defensin alpha 6+) (Figure 1, Supplementary Table 2), indicating that the progeny of a single ISC^{GS} from a wide range of patients (10yr-76yr) can give rise to all epithelial lineages typically found in the intestine.

Long-term culturing of ground state ISCs

We next wanted to study the self-renewal ability of ISCs derived from these thirty patients (10yr-76yr). We first showed that ISC^{GS} from all patients included in this study are highly clonogenic. Approximately 70% clonogenicity was observed across the patients (Figure 2A). Due to this high clonogenicity, single cell derived colony can be rapidly expanded to single-cell derived pedigree (Figure 2B). We next examined the functional stability of the ISC^{GS} pedigree in culture. We compared the ISC^{GS} at early passage (P1) and late passage (P10). Each passage includes culturing *in vitro* for ten days with approximately 17 cell divisions. Despite passaging number, the stem cells were able to differentiate into intestine-like structures in ALI culture system and expressed differentiation markers such as goblet cell marker (Muc2+) (Figure 2C). Furthermore, these ISC^{GS} remained high clonogenic ability (>60%) despite long-term culturing (Figure 2D). This result is consistent with what we observed in human fetal ISC^{GS} during *in vitro* expansion [13]. Thus, the stable and robust culturing of ISC^{GS} is age independent and these cells provide reliable stem cell source for personalized regenerative medicine.

Discussion

Stem cell based autologous transplantation may improve outcomes of patients with a wide range of disorders of the gastrointestinal tract, characterized by an impaired mucosal barrier function, including IBD, necrotizing enterocolitis, fistulas, NSAID-induced damage, or gastroduodenal bleeding [5,14]. There are critical and unanswered questions relevant to the older patients such as whether the ISC^{GS} derived from these aged individuals are capable of being expanded faithfully and rapidly to sufficient numbers to functionally regenerate the intestinal epithelium.

The current direction in regenerative medicine is to use patient's own stem cells for autologous transplantation. If aged stem cells were inherently dysfunctional, that would greatly limit the ability to use this type of therapies for older people. However, if old stem cells are still maintaining intact stemness, in another words if the intrinsic immortality of ISC^{GS} is age independent, then this approach to regenerative medicine for age-related disease could be very promising. We showed here that ISC^{GS} can be cloned from a wide range of patients aged from 10 to 76. We did not detect any age-related loss of self-renewal or differentiation ability. In approximately 60 to 80 days, a single ISC^{GS} can be expanded to

about 1 billion cells among all 30 patients included in this study, suggesting they may serve as ideal stem cell source for autologous transplantation tailored for patients with intestinal disorders.

In 1980s, Howard Green and colleagues demonstrated the first example of cell therapy using cultured adult stem cells. They showed that human epidermis could be grown in the laboratory and transplanted onto burnt patients to reconstitute a functional epidermis [15,16]. Since then, this procedure has been shown repeatedly life-saving for patients with severe burns [2]. Furthermore, long-term effectiveness and safety of using genetically modified epidermal stem cells to correct the severe skin blistering disease epidermolysis bullosa has been shown clinically [3]. The successful clinical usage of epidermal stem cells has demonstrated a close correlation with the number of long-lived stem cells used in the procedure that can extensively self-renew *in vitro* and *in vivo* [1,3]. It remains unclear whether autologous transplantation of cultured intestinal cells can achieve the same success in clinical settings. Although it has been claimed that successful transplantation of organoids including a small fraction of intestinal stem cells can be achieved in murine models of experimental colitis, which shows that these organoids adhere to and become an integrated part of the epithelium [8] it is likely that extremely limited number of stem cells in the organoid structures cannot support the long term intestinal epithelium regeneration in human.

In comparison to less than 1% presence of intestinal stem cells in the organoids structures, the ground state ISC culture comprises over 60–70% of ISC^{GS}. Based on the previous lessons that we learned through clinical usage of cultured epidermal stem cells, it is conceivable that usage of ISC^{GS} will significantly improve the efficacy and success of the transplantation. Certainly, another challenging problem is that the influence of the aged or diseased tissue environment must be solved to build effective regenerative therapies for the patient. Nevertheless, with the knowledge that stem cells from elderly patients have similar capabilities to growth and differentiation as those from younger patients and our ability of culturing them *in vitro* robustly, we foresee a promising future of adult stem cell-based regeneration approaches for patients suffering intestinal disorders.

Methods

Cell cloning from endoscopic biopsies

Patients were recruited for this study under informed consent consistent with institutional review board protocols. All biopsies were derived from patient's endoscopy.

1mm endoscopic biopsies were collected into RPMI media (Gibco) with 2% fetal bovine serum and subsequently digested in 2mg/ml collagenase A (Roche) at 37°C for 1.5 hour. Cells were washed by centrifugation in RPMI, digested with 0.5% trypsin (Gibco) 10 min, passed through a 40um Nylon mesh (Falcon), and seeded onto a feeder layer of lethally irradiated 3T3-J2 cells in StemECHO expansion medium and StemECHO enhancer (MultiClonal Therapeutics, West Hartford, CT). The culture medium was changed every two days. Cells were digested by 0.25% trypsin and passaged every seven to 10 days.

Stem cell differentiation

Air-liquid interface (ALI) culture of ISC^{GS} was performed as described [11,12]. Briefly, cells were cultured on Transwell plates (Corning) in StemECHO expansion medium media. At confluence, the medium on the inserts was removed by careful pipetting and the cultures continued for an additional 10–20 days prior to harvesting. The StemECHO differentiation medium (MCT) was changed every two days. After 2 weeks, the differentiated structures were harvested.

Histology and immunostaining

Histology and immunofluorescence were performed using standard techniques. For immunofluorescence and immunohistochemistry, 4% paraformaldehyde fixed, paraffin-embedded tissue sections were subjected to antigen retrieval in citrate buffer (pH 6.0, Sigma-Aldrich, USA) at 95°C for 20 min, and a blocking procedure was performed with 5% bovine serum albumin (BSA, Sigma-Aldrich, USA) and 0.01% Triton X-100 (Sigma-Aldrich, USA) in phosphate-buffered saline (PBS; Gibco, USA) at room temperature for 1 h. Primary antibodies used in this study and staining condition were listed in Supplementary Table 2. All images for section slides were captured by using LSM 510 confocal microscope (Zeiss) with LSM software. Bright field cell culture images were obtained on an Eclipse TS100 microscope (Nikon) with Digital Sight DSFi1camera (Nikon) and NIS-Elements F3.0 software (Nikon).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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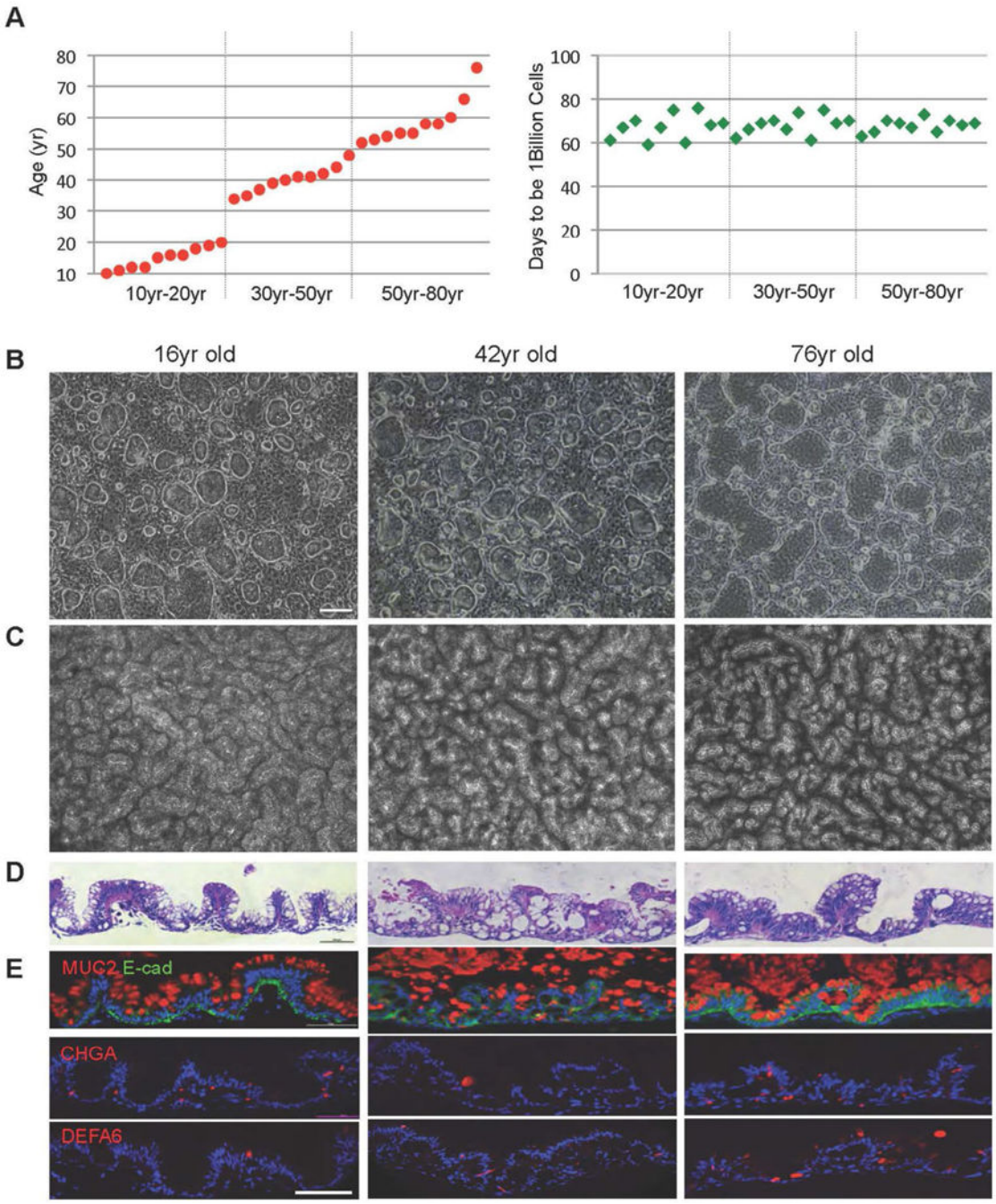

Figure 1.

Cloning ISC^{GS} from patients of wide-range ages

A. *left,* Histogram depicting ages of the patients included in this study. *right,* Histogram depicting days needed for single ISC^{GS} from each patient dividing to 1 billion ISCGS. B. Phase contrast images of typical colonies derived from biopsies of 16yr, 42yr and 76yr old patients. Scale bar, 100μm. C. Surface view of ALI cultures. D. Histological sections through ALI cultures via hematoxylin-eosin staining. E. Immunofluorescence on sections of

ALI cultures with indicated antibodies to secretory cell markers Mucin 2, Chromogranin A and Defensin A6. Scale bar, 100μm.

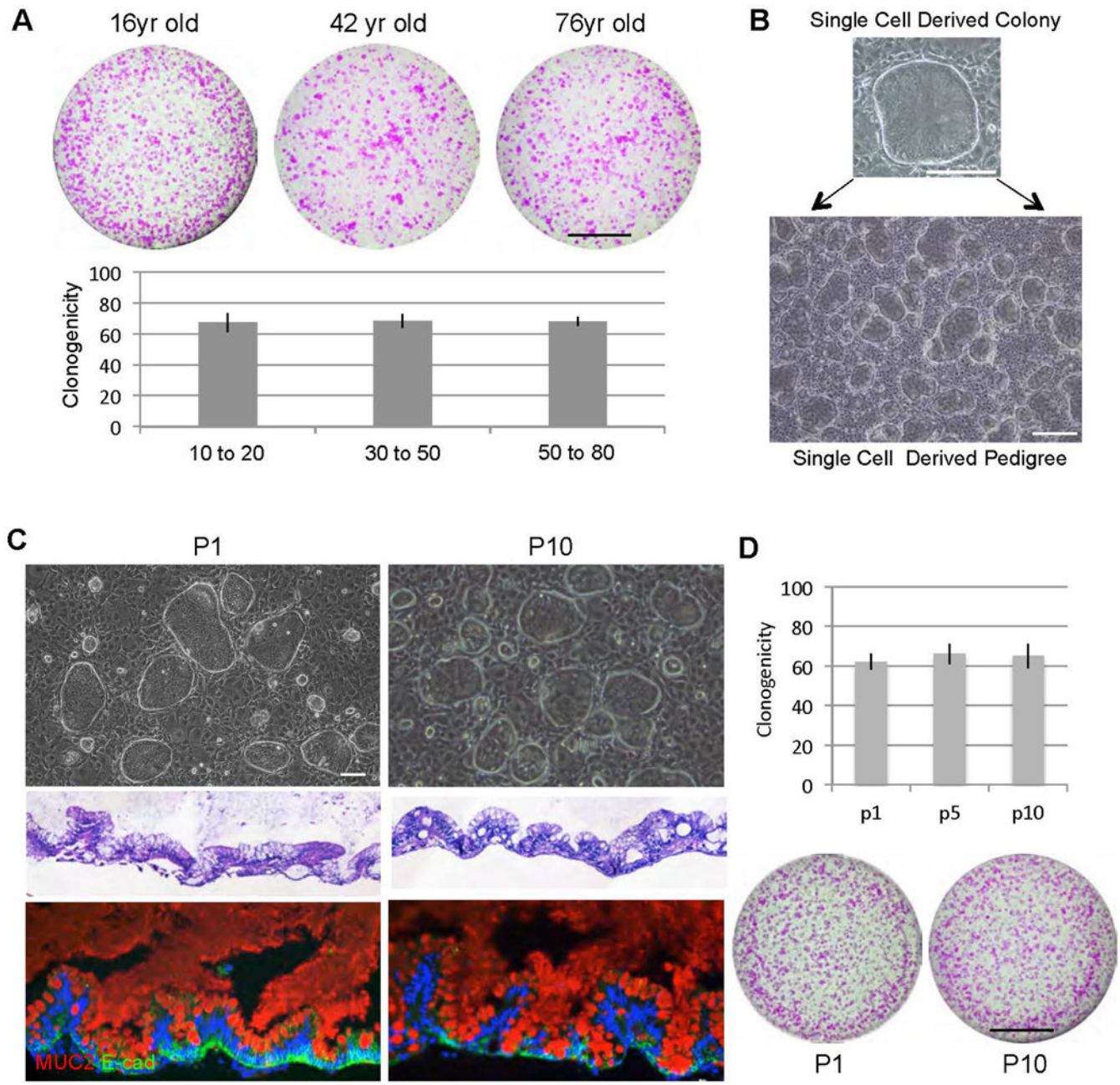

Figure 2.

Long-term stability of ISC^{GS}

A. *upper*, Clonogenicity assay revealing Rhodamine red-stained colonies grown 20days following seeding 1000 ISC^{GS} cells. Scale bar, 10mm. *lower*, Quantification of clonogenicity of ground state stem cells from endoscopic biopsy at indicated age range. Error bars, s.d. B. Individual colonies are sampled from the pool and grown as separate lines in isolation. C. *upper*, Phase contrast images of typical clones at early and late passage of wild-type ISC^{GS}. *middle*, Histological sections through ALI cultures via hematoxylin-eosin staining. *lower,* Immunofluorescence on sections of ALI cultures with indicated antibodies to secretory cell markers Mucin 2. D. Clonogenicity assay of early and late passages of

ISC^{GS} revealing nearly unchanged number of Rhodamine red-stained colonies grown 10 days following seeding 2,000 passaged ISC^{GS}.