



RESEARCH ARTICLE

Visualization of Bonghan Microcells by Electron and Atomic Force Microscopy

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Abstract

Objectives: The origin of adult stem cells remains an open question. If they derive from embryos, it is difficult to determine the mechanism which interrupts their differentiation during tissue formation. In the 1960s, the Bonghan microcell was suggested as one possible, yet to be described, route of stem cell production, such that they have the potential to proliferate to produce normal cells.

Materials and Methods: In this study, Bonghan microcells were isolated from Bonghan tissues on rat organ surfaces, and their detailed morphology examined by electron and atomic force microscopy.

Results: The ultrastructure observed distinguished them from apoptotic bodies and other microorganisms, and their unique, possible proliferation feature, as protruding threads, was imaged by atomic force microscopy.

Conclusions: The unique threadlike structure of the Bonghan microcell is consistent with Prof. Kim's observation in the first step of making a cell. Understanding of the functions of this threadlike structure may give a clue to understand the origin or the differentiation cue of adult stem cells.

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1. Introduction

The most interesting part of Bonghan theory may be the report of micro-sized Bonghan microcells (BH-MC) flowing through Bonghan ducts [1,2]. BH-MCs were reported to have the pluripotent ability to divide and differentiate into many kinds of cells in the same way that adult stem cells are able to.

Their ability to form a specific cell was tested *in vivo* as well as *in vitro* in the 1960s, about the same time that adult stem cell research began [3]. Though not described in modern medical terminology, BH-MC formation and proliferation is well worth investigating as a possible origin of adult stem cells.

Whether adult stem cells are "leftover" embryonic stem cells or arise in some other manner has

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yet to be determined [4], and the origin of cancer stem cells has become a subject of great interest in oncology and stem cell research [5]. Buikis et al suggested a very interesting phenomenon, called “sporosis”, as a possible origin of cancer stem cells [6]. Sporosis is a cytological mechanism for microcell formation which is distinguished from apoptosis and necrosis at the cellular level. They observed that microcells in cancer tissues were metabolically active, intensively accumulated nucleoproteins in their nuclei and cytoplasm, and transformed into young undifferentiated cells via rapid mitosis. This indicated that stem cells could arise from microcells and that the successive propagation of undifferentiated cells via mitosis could lead to cell clone expansion and subsequent senescence.

The differences between BH-MCs and cancer tissue microcells are that BH-MCs are generated through normal physiology while Buikis’s microcells were from pathological (tumor) tissues. The origin of the BH-MCs is the micronucleation of some cells in organs and tissues, and these BH-MCs, conversely, proliferate in certain medium or injured tissues. Microcells contain micronuclei consisting of one or a few chromosomes, together with a small amount of cytoplasm enclosed by a membrane [7,8], and have insufficient genetic information for a cell. In this light, these two observations suggest the presence of an unknown mechanism by which microcells can develop into undifferentiated cells. Prof. Kim’s suggested unique transformation process for converting BH-MCs to a cell may provide a clue to this heretofore unknown process [1,2].

As Prof. Kim did not record a specific method for the isolation and identification of BH-MCs, his results have been difficult to repeat. Recently, several researchers have confirmed the existence of the Bonghan system inside blood or lymphatic vessels [9,10], on the surfaces of organs [11,12], and in brain ventricles [13]. They have also successfully isolated DNA-containing BH-MCs from Bonghan systems on organ surfaces using a differential centrifugation method [14]. Those BH-MCs were spherical, 1–2 μm in diameter, and their DNA was fragmented. Transmission electron (TEM) and atomic force microscopy (AFM) have been used to investigate their specific morphology [15]. Some of their properties could be examined right after isolation [16,17], however, their cultivation has not been successful yet.

The goal of the present work was to observe budding BH-MCs, as the first step of their proliferation, from Bonghan systems on the surfaces of rat organs. We were able to successfully image BH-MCs, as protruding threadlike structures, using TEM, scanning electron microscopy (SEM), and AFM.

2. Material and Methods

2.1. Animal preparation and surgical procedure

Sprague-Dawley rats aged 8–10 weeks and obtained from Central Lab Animal, Inc. (Seoul, Korea) were housed in a 23°C temperature-controlled environment with 60% relative humidity, a 12 hour light-dark cycle, and fed food and water *ad libitum*. Procedures involving animals and their care conformed to institutional guidelines and in full compliance with current laws and policies [18]. Rats were anesthetized with intraperitoneal urethane (1.5g/kg), and, under general anesthesia, the abdominal wall dissected along the linea alba. Threadlike structures and corpuscles on organ surfaces were observed under a stereomicroscope (SZX12, Olympus, Japan) and were sampled using small surgical instruments.

2.2. Separation of Bonghan microcells (BH-MCs)

Corpuscles and threadlike structures from rats were disrupted by agitation in a glass homogenizer in 2 mL of 0.15M phosphate-buffered saline (PBS), without Ca^{2+} and Mg^{2+} , at pH 7.4 (Invitrogen, USA) for 1 minute. The BH-MCs were separated from the resulting homogenate of tissue debris, BH-MCs, and whole cells, including erythrocytes, by differential centrifugation consisting of two successive centrifugations at increasing rotor speeds of a fixed angled rotor (A1.5S-30) in a Combi 514R centrifuge (Hanil Science Industrial, Korea) at 4°C. First, the homogenate was transferred into microtubes (2 mL) and centrifuged at 200g for 10 minutes, sedimenting the tissue debris and whole cells, and following this the supernatant, containing BH-MCs, was poured into a fresh microtube and centrifuged at 20,000g for 30 minutes. The supernatant was discarded, leaving behind a pellet of ~200 μL .

2.3. DNA staining

The pellet containing BH-MCs was resuspended and dropped on ready-made CELLTAK (BD sciences, USA)-coated cover glass. CELLTAK mixed with 2M sodium carbonate (4/1, v/v) had been incubated on a cover glass at 37°C for 20 minutes, washed with distilled water, washed twice with PBS, pH 7.4 (Invitrogen, USA), stained with 0.1% Acridine orange (A6014, Sigma) for 15 minutes at room temperature, and washed overnight with the previous buffer. Stained samples were mounted with anti-fade reagent (Prolong[®] Antifade kit) and viewed by confocal microscopy (LSM510, Zeiss, Germany)

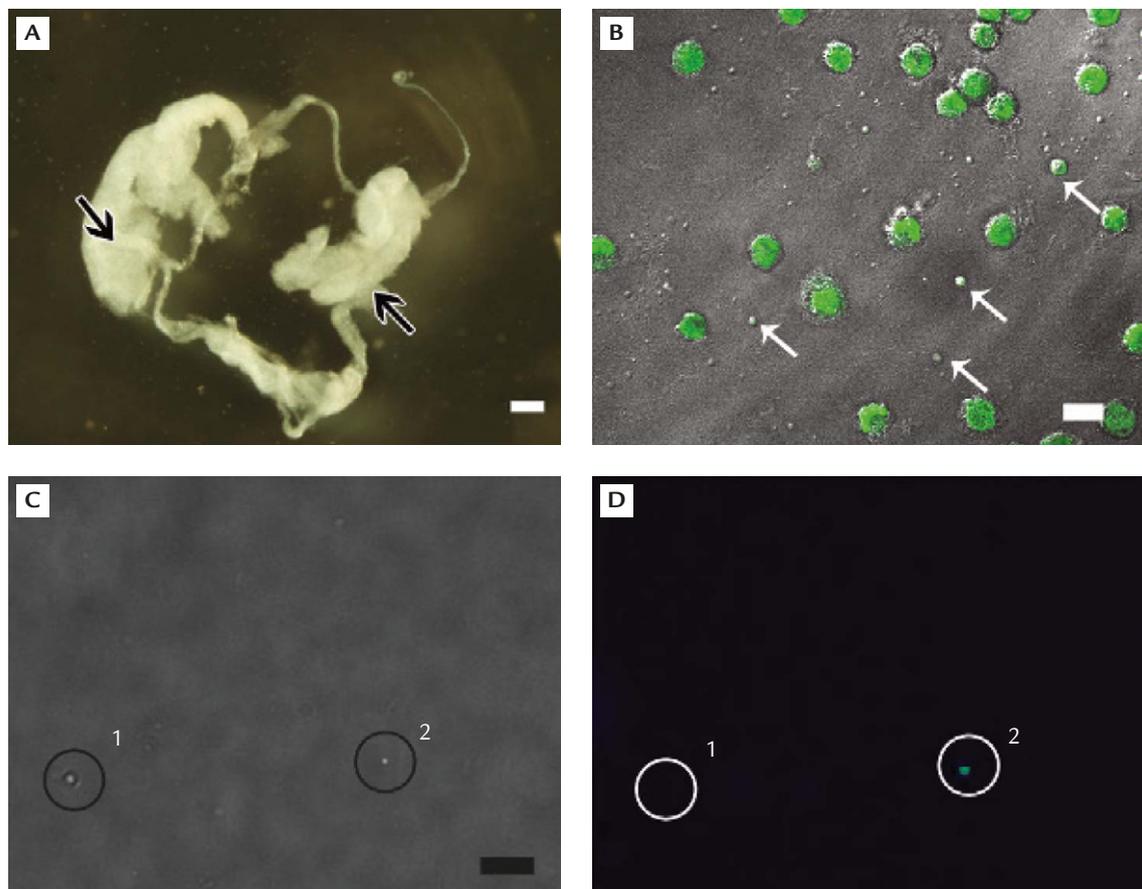


Figure 1 (A) Bonghan ducts on the surface of rat small intestine. Connections of ducts and corpuscles indicated by arrows; scale bar, 200 μm . (B) Macro and microcells naturally flowing out from Acridine orange stained cut; various sizes of microcells (arrows) stained among leukocytes. (C, D) Bonghan microcells (BH-MCs), 1–2 μm in size, separated by differential centrifugation, DNA-containing BH-MCs selected; two microcells, one on right stained with Acridine orange. Scale bars, 10 μm for Figure B, D.

with a 560 high pass filter and a 505–550 band pass filter.

2.4. Electron microscopy (EM)

A pellet containing BH-MCs was resuspended and dropped on a CELLTAK (BD sciences, USA) coated carbohydrate membrane filter (Millipore, pore size 0.2 μm) [19]. After fluorescence observation, interesting areas of the filter were excised for selective investigation of DNA-containing BH-MCs. For TEM, BH-MCs on an excised filter were fixed with modified Karnovsky's fixative (without calcium chloride and with 2.5% glutaraldehyde) at 4°C for 2 hours [20], washed in 0.1M sodium cacodylate buffer at pH 7.2, postfixed in 1% OsO_4 (Sigma, USA) in 0.1M sodium cacodylate buffer at pH 7.2 for 2 hours, stained in 0.5% uranyl acetate (Sigma, USA), dehydrated with a series of ethanol and propylene oxide washings (Sigma, USA), and embedded in Poly/Bed 812 resin (Polyscience Inc., USA). Ultrathin sections were collected onto 300-mesh copper grids, contrasted with 2% uranyl acetate and Reynolds'

lead citrate, and then examined with a JEM1010 transmission electron microscope (JEOL, Japan) at an accelerating voltage of 80kV. For SEM, fixed BH-MCs were dehydrated with an ethanol series, lyophilized in a vacuum chamber, and examined using a JSM5410LV (JEOL, Japan).

2.5. AFM

A pellet containing BH-MCs was resuspended and dropped on a CELLTAK-coated coverglass. Though it adhered to cell-tak coated substrates, their round shape and approximate 1 μm height difference made it difficult to image in a live state. To dry the BH-MCs, it was fixed with Karnovsky's fixatives, dehydrated by a series of ethanol, and lyophilized in a SEM chamber (FEI Phillips XL30 sFEG) for 12 hours. Slow evaporation of liquid nitrogen dried the sample with minimal damage, while the fixation procedure also helped maintain the morphological structure. The sample was then imaged in air in tapping mode using an atomic microscope (Dimension 3,100, DI) with a typical nitrite tapping cantilever ($k=0.16\text{N/m}$).

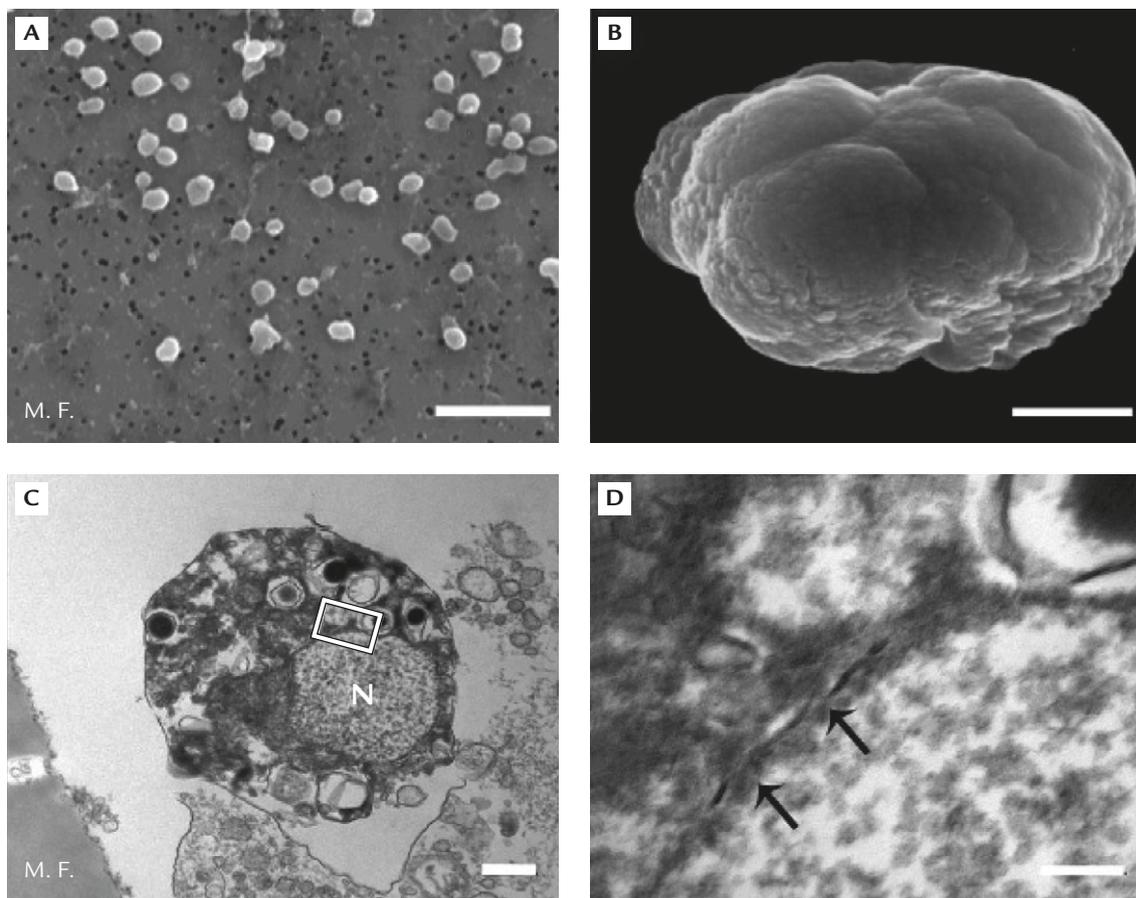


Figure 2 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of Bonghan micro-cells (BH-MCs) on polycarbonate membrane filter. (A) SEM image showing round BH-MCs, $\sim 1\ \mu\text{m}$ diameter; scale bar, $5\ \mu\text{m}$. (B) High magnification showing bumpy surface structure; scale bar, $200\ \text{nm}$. (C) DNA-containing BH-MC selected by staining; TEM of ultrathin section; largest part assumed to be micronucleus “N” based on Acridine orange staining; several $250\ \text{nm}$ sized granules and many smaller granules inside membrane; all granules surrounded by lipid membrane and with electron dense contents; scale bar, $500\ \text{nm}$. (D) Magnification of (C) rectangle showing the border of the micronucleus “N”; the membrane is not a double bilayer structure as in a nuclear membrane; scale bar, $100\ \text{nm}$.

3. Results and Discussion

Bonghan ducts can be found on the abdominal organ surfaces of rats and parts of these ducts are distinctively afloat from the mesenteric membranes. The unique properties of these ducts have been investigated by several researchers [21,22]. When a Bonghan duct was cut at its connection to the membrane of small intestines, it appeared to consist of thin ducts $10\text{--}100\ \mu\text{m}$ in diameter and of several corpuscles $500\ \mu\text{m}$ thick and $1000\ \mu\text{m}$ long (Figure 1A). During incubation at 37°C for 20 minutes, cells and micro-sized bodies emerged naturally (Figure 1B). DNA staining revealed that these cells were mostly leukocytes and that some of the bodies contained DNA as well. After differential centrifugation, only $1\text{--}2\ \mu\text{m}$ -sized bodies were separated, but all bodies were not stained by a DNA specific dye (Figure 1C, D). Because the separation method was not a density gradient centrifugation, the pellet

contained other particles, such as microparticles generated from activated cells or organelles from necrotic cells, produced during processing. Though mitochondria containing DNA were among those bodies, their small DNA content ($\sim 10^4$ bps) was essentially negligible in fluorescence imaging compared with the chromosome-sized DNA content of BH-MCs ($\sim 10^8$ bps) [1]. Thus it was assumed here that the stained bodies were BH-MCs based on their sizes, shapes, and DNA content.

For further examination, such as by EM, separated BH-MCs were passed through a $0.2\ \mu\text{m}$ -pore-sized CELLTAK-coated polycarbonate membrane filter, retaining BH-MCs larger than the pore size and allowing them to adhere to the coating sufficiently strongly for staining *in situ* and observation; DNA-containing bodies were selected for further investigation. SEM imaging showed that they were almost spherical or oval shaped and $\sim 1\ \mu\text{m}$ in diameter (Figure 2A) and magnified SEM showed a bumpy

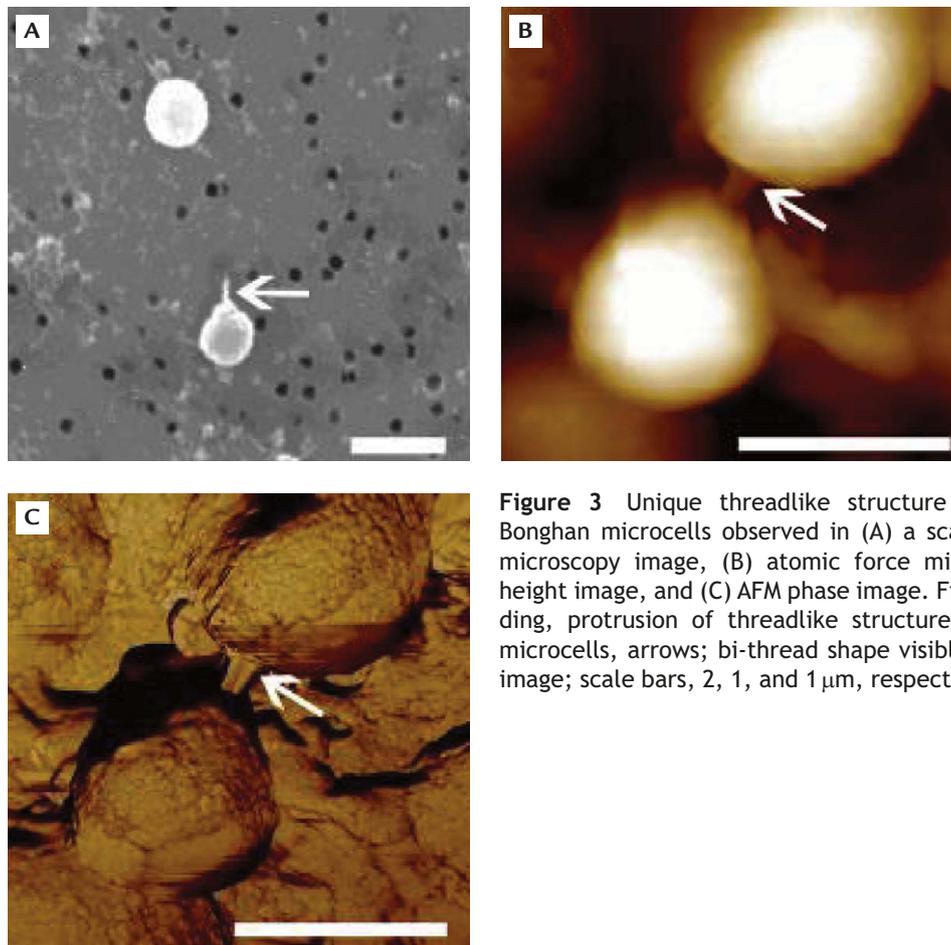


Figure 3 Unique threadlike structure of lyophilized Bonghan microcells observed in (A) a scanning electron microscopy image, (B) atomic force microscopy (AFM) height image, and (C) AFM phase image. First step of budding, protrusion of threadlike structure from Bonghan microcells, arrows; bi-thread shape visible in AFM phase image; scale bars, 2, 1, and 1 μm , respectively.

surface structure (Figure 2B). Adhered BHMCs were embedded in resin and cut by using ultramicrotome. The interested area was trimmed after semithin section observation and ultrasectioned into 80 nm thickness. TEM imaging showed a 2.6 μm diameter BH-MC with an inner ultrastructure of a 1.5 μm sized central region and many small 50–500 nm sized granules in the peripheral region (Figure 2C). All structures were enclosed by a lipid membrane to show their viability. Following Prof. Kim's depiction, we conjectured here that the biggest central region contained the DNA which had made sufficiently strong fluorescence to be detected under a fluorescent microscope. The low electron density of the central area can be explained by a fragmented DNA status as studied before [14]. But at higher magnification, the central region showed to be bounded by a single membrane (Figure 2D). The single membrane and the low electron density inquires further molecular analysis. However, we can confirm that this inner structure is different from that of apoptotic bodies which have highly condensed inner structures [23].

While most of the BH-MCs had round shapes (Figure 2A), one of the BH-MCs had a unique protrusion, appearing as a swollen part (Figure 3A); this

observation was to be expected in light of Prof. Kim's description of BH-MC budding [1]. According to his observations, initially the BH-MC protrudes a thread and produces a daughter microcell from that thread, such that with proliferation, it forms a bundle of BH-MCs, which then fuse to make a nucleus-like structure. Finally, the structure is enclosed by a membrane to form a cell. About 10% of the BH-MCs observed in SEM images showed such protrusions, having a threadlike structure 100 nm in diameter and 400 nm in length. More detailed images from AFM revealed threadlike structures with a unique bi-thread shape, most clearly visible in the phase AFM image (Figure 3B, C, arrows). As the width of these threads was 50 nm, we assumed that the thread in the SEM image consisted of two threads (Figure 3A). This observation may represent the first step of the doubling in Prof. Kim's description and, although he did not address the bi-thread shape in his model, the present technological advances have made it possible to visualize the shape in detail.

The argument has been made that these structures are microorganisms that replicate by yeast-like budding or by making fungal-like hyphae [24], but the TEM image didn't confirm these possibilities.

As opposed to other microorganisms, the BH-MCs observed here did not have cell walls (Figure 2C) and the bumpy surfaces observed further supported the lack of a cell wall (Figures 2B, 3C). The present results are the first observations of cells without cell walls budding through a threadlike structure. There has been no information about how this threadlike structure ignites budding. This thread might play a role in transferring small molecules through, or in providing a polarized tension for further processes. Further study on their unique structure can provide exciting information about the mechanism of budding. Further study on BH-MC's unique structure can provide exciting information about microcell budding.

The ultrastructure of the BH-MC was investigated using EM and AFM and the results showed differences between these structures and organelles, microorganisms, and apoptotic bodies. We observed a unique threadlike structure, similar to descriptions by Prof. Kim of BH-MCs in the first step of proliferation. In conclusion this structure supported the hypothesis that these microcells could proliferate to form an undifferentiated macro cell, in a manner similar to that whereby cancer microcells become cancer stem cells.

Acknowledgments

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