HAYATI Journal of Biosciences xxx (2017) 1–6

HOSTED BY

Contents lists available at ScienceDirect

HAYATI Journal of Biosciences

journal homepage: http://www.journals.elsevier.com/hayati-journal-of-biosciences

Original Research Article

Mammary Gland Cell Culture of *Macaca fascicularis* as a Reservoir for Stem Cells

Silmi Mariya,[1*] Fitriya Nur Annisa Dewi,[1] Irma Herawati Suparto,[1] Gregory K. Wilkerson,[2] J. Mark Cline,[3] Permanawati,[1] Diah Iskandriati,[1] I Nengah Budiarsa,[1] Dondin Sajuthi[1]

[1] *Primate Research Center, Bogor Agricultural University, Jalan Lodaya II/5 Bogor, 16151, Indonesia.*
[2] *Michale E. Keeling Center for Comparative Medicine and Research, MD Anderson Cancer Center, Bastrop, TX, USA.*
[3] *Wake Forest School of Medicine, Medical Center Blvd, Winston-Salem, NC, 27157, USA.*

ARTICLE INFO

Article history:
Received 21 July 2017
Received in revised form
5 September 2017
Accepted 8 September 2017
Available online xxx

KEYWORDS:
breast cancer,
mammary gland,
nonhuman primate,
stem cells

ABSTRACT

The mammary gland contains adult stem cells that are capable of self-renewal and are likely target for neoplastic transformation leading to breast cancer. In this study, we developed a cell culture derived from the mammary glands of cynomolgus monkeys (*Macaca fascicularis)* (MfMC) and furthermore identified the expression of markers for stemness and estrogen receptor-associated activities. We found that the primary culture can be successfully subcultured to at least 3 passages, primarily epithelial-like in morphology, the cultured cells remained heterogenous in phenotype as they expressed epithelial cell markers *CD24*, *CK18*, and marker for fibroblast *S1004A*. Importantly, the cell population also consistently expressed the markers of mammary stem cells (*ITGB1* or CD29 and *ITGA6* or CD49f), mesenchymal stem cells (*CD73* and *CD105*) and pluripotency (*NANOG, OCT4, SOX2*). In addition to this, the cells were also positive for Estrogen Receptor (ER), and ER-activated marker Trefoil Factor 1, suggesting an estrogen responsiveness of the culture model. These results indicate that our cell culture model is a reliable model for acquiring a population of cells with mammary stem cell properties and that these cultures may also serve as a reservoir from which more purified populations of stem cell populations can be isolated in the future.

1. Introduction

Multipotent stem cells, known as adult stem cells, are essential to the maintenance of most tissues in the body throughout life. These cells have the ability to undergo self-renewal to produce two stem cells or can divide in a fashion such that one cell remains a stem cell, whereas the other daughter cell undergoes further differentiation. Adult stem cells are normally only present in small numbers within most tissues after gestational development. Mammary gland development is unique, however, as full differentiation of this organ is only attained at adulthood through pregnancy and lactation (Liu *et al.* 2005). Consistent with this fact, nulliparous breasts are known to contain large numbers of undifferentiated stem cells (LaMarca and Rosen 2008; Stingl *et al.* 2006).

Adult mammary glands consist of the lobular and ductal structures composed of three cell lineages: alveolar epithelial cells that line the alveoli and synthesize milk proteins; ductal epithelial cells that line the lumen of the ducts; and myoepithelial cells that form the basal layer of both the ducts and alveoli. Alveolar, ductal, and myoepithelial components of the mammary gland initially originate from a common multipotent adult stem cell, the mammary stem cell (MaSC; Shackleton *et al.* 2006). During its normal developmental cycle, the mammary gland shows many characteristics similar to those previously associated with breast carcinogenesis and it has therefore been concluded that factors implicating normal mammary development are also important in breast carcinogenesis. In turn, it is likely that a better understanding of normal breast development may prove useful in elucidating how tumors originate and thrive (Wiseman and Werb, 2002).

Breast cancer is proposed to originate primarily from the populations of undifferentiated stem cells that reside within the lobular aspects of the breast. This hypothesis has been supported by numerous studies including those investigating the vulnerability of

* Corresponding author.
E-mail address: mariyasilmi@gmail.com (S. Mariya).
Peer review under responsibility of Institut Pertanian Bogor.

https://doi.org/10.1016/j.hjb.2017.09.002

mammary cells to chemical-induced carcinogenesis (Russo *et al.* 2005; Eden 2010). Based on the stem cell theory of carcinogenesis, the long-lived stem cells of the mammary gland have the potential to be exposed to larger numbers of mutagenic events over their lifetime than do the shorter-lived, more-differentiated cells of the gland. The switch from normal development to carcinogenesis can then either occur in the stem cells themselves as these cells acquire multiple deleterious mutations over time, or cancerous cells can arise from the progeny of these stem cells as they acquire additional mutations and the ability to self-renew. In light of this information, the purification and characterization of normal MaSCs is likely to be extremely useful for understanding normal mammary development, as well as carcinogenesis risk (Dontu *et al.* 2003b).

Difficulty in obtaining MaSCs for study of the adult MaSCs is due in large part to the small numbers of cells typically found within the mammary gland, limited availability of markers for the characterization of MaSCs, and limited techniques to maintain the MaSCs in an undifferentiated state. (Dontu *et al.* 2003a). MaSCs are likely to be more abundant at specific life stages, such as during puberty or in early adulthood before first pregnancy (i.e. nulliparity) when the breasts are less differentiated (Meier-abt *et al.* 2013). Therefore, choosing the right developmental stage is critical to ensure that enough stem cells are present in the breast tissue to allow for isolation and/or enrichment of these cells. As acquiring normal breast tissue from the developmental stages of greatest interest to stem cell researchers is restricted by ethical constraints in humans, the use of nonhuman primate (NHP) models is likely to be useful in by-passing such limitations. NHPs have similarities with humans in genomics, anatomy, and physiology. Importantly, the cynomolgus macaque (*Macaca fascicularis*) mammary gland has been demonstrated to have high similarity with human breast with regard to development, morphology, molecular profile, and carcinogenesis (Cline and Wood 2008; Dewi *et al.* 2013; Dewi *et al.* 2016). Therefore, the use of cynomolgus monkey-derived tissue and cells provides the benefit of studying breast development and breast cancer risk at specific reproductive ages, including that involving estrogenic exposure as well as stem cell regulation. Here, we developed a cell culture model derived from the mammary gland of cynomolgus monkeys or *M. fascicularis*, abbreviated as *M. fascicularis* mammary cell culture (MfMC). This highly translational cell culture model shall serve as a reservoir for MaSCs population, which will be potential for further enrichment.

2. Material and Methods

2.1. Animals

We conducted breast biopsy on adult nulliparous *M. fascicularis* ($n = 3$; age 5–6 years) to collect mammary gland tissues. All procedures involving animals were performed at Research Animal Facility-Lodaya, Primate Research Center at Bogor Agricultural University (PSSP-IPB), an AAALAC International-accredited facility, following ethics approval from PSSP-IPB Institutional Animal Care and Use Committee. Validation of menstrual cycle of monkeys at the time of biopsy was performed by vaginal cytology, following daily observation to identify menstrual bleeding pattern. This validation was performed to identify the cycle stage of the animal (i.e. luteal phase) because hormone profile during menstrual cycle influences the expression of ER in the breast tissue (Stute *et al.*, 2004; Stute *et al.*, 2012). Breast biopsy was performed on deeply-anesthetized animals; under aseptic condition, subcutaneous tissue (approximately 2 cm × 0.5 cm in size) that contains mammary glands were collected. On removal, the tissues were placed in transport media (Dulbecco's Modified Eagle's Medium, antibiotics, antifungal). Intensive peri- and postoperative care were performed, whereby animals were given analgesics and antibiotics, and closely observed throughout the week after biopsy.

2.2. Cell culture

Mammary tissues obtained from biopsy comprised adipose and glandular tissues. The texture was relatively hard and therefore difficult to dissociate. Digestion with the enzymes collagenase and hyaluronidase allowed for easier mincing and disaggregation. Cells dissociation were performed mechanically and enzymatically according to the method previously described (Dey *et al.* 2009) with slight modifications. The collected tissues were digested in 0.075% collagenase (Sigma Aldrich, USA) and 1 mg hyaluronidase (Sigma Aldrich, USA), and incubated in a humidified atmosphere at 37°C, 5% CO_2 for 16–18 hours. The tissues were minced and centrifugated at 500 *g* for 10 min. Supernatant was removed and resuspended in 10 mL phosphate-buffered saline twice. Cells were resuspended to ensure single cells suspension was formed. Hemocytometer was used to confirm the presence of single cells suspension; viable cells were calculated using trypan blue. Cell suspension was plated at appropriate density in selective medium for mammary epithelial cells (Lonza, USA) and incubated in a humidified atmosphere at 37°C, 5% CO_2. Subculture was performed when cell population reached 80% confluency. Human mammary epithelial cell culture (MCF-12A ATCC^R CRL 10782) was used as a comparison. Cells were maintained with the same condition as MfMC.

2.3. Reverse transcription-polymerase chain reaction

RNA was extracted from cells using RNeasy Kit (Qiagen, Germany), and reverse transcribed using *SuperScript* III *Reverse Transcriptase* (Invitrogen, USA), according to the manufacturer's instructions. Gene expression was evaluated using thermocycler polymerase chain reaction; primers used for polymerase chain reaction amplification are presented in Table 1.

3. Results

3.1. Cell morphology

Monkey breast-derived cells were cultured in selective medium specific for mammary epithelial cells enrichment. Although the morphology of MCF-12A in the same medium remained epithelial-like throughout different passages, the MfMC cell population grown showed predominant epithelial-like morphology alongside fibroblast-like and adipocyte-like morphologies (Figure 1). The MfMC culture was successfully subcultured up to three passages with cell viability of 74%–88% (Figure 2).

3.2. Markers validation

MfMC cell population expressed the mRNA of epithelial cell markers *CD24* and *CK18* on all passages (Figure 3). Despite the use of medium selective for epithelial cells, expression of a stromal cell marker *S100A4* was also positive. This finding, however, was somewhat consistent with that in MCF-12A, which is a human-derived mammary epithelial cell culture model. In MCF-12A, the stromal cell marker was also expressed, although in relatively lower level compared with that in MfMC. Importantly, various markers of stemness (Figure 4) and markers for pluripotency (Figure 5) were expressed in MfMC throughout all passages, suggesting the presence of stem cell population within the culture. This was also the case for MCF-12A.

3.3. Estrogen receptor markers

Markers for estrogen receptor (ER; *ESR1*) and ESR1-regulated activity were evaluated in MfMC and MCF-12A. *ESR1* was clearly expressed, indicating that the cells are likely to be responsive to

Please cite this article in press as: Mariya, S., et al., Mammary Gland Cell Culture of *Macaca fascicularis* as a Reservoir for Stem Cells, HAYATI J Biosci (2017), https://doi.org/10.1016/j.hjb.2017.09.002