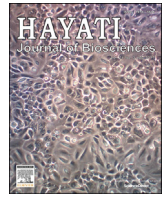


HOSTED BY



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 CellsSilmi 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 Sajuthi¹¹ 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 4 October 2017

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.

Copyright © 2017 Institut Pertanian Bogor. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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.

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% CO₂ 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% CO₂. 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

Table 1. Primer sequences

Gene	Sequence (5'–3')		References
	Forward	Reverse	
<i>CD24</i>	CCCACGCAGATTATTCCAG	GACTTCCAGACGCCATTG	Modur et al. 2016
<i>CK18</i>	ATCTTGGTGATGCCTTGGAC	CCTGCTTCTGCTGGCTTAAT	Makino et al. 2009
<i>ITGB1</i>	GTTACACGGCTGCTGGTCTT	CTACTGCTGACTTAGGGATC	Qiu L et al. 2012
<i>ITGA6</i>	CAAGATGGCTACCCAGATAT	CTGAATCTGAGAGGGAACCA	Qiu L et al. 2012
<i>CD73</i>	GACCTGGCTTTGTGACAGCAA	CTGACCCTGAGTAATCATGTCACTCT	Designed by Ricky Fong
<i>CD105</i>	GACTGTCTTCACGCGTTGA	GGAAGGCACCAAAGGTGATG	Designed by Ricky Fong
<i>NANOG</i>	CCAGTCCCAAAGGCAAACA	TCTTGACGGGACCTTGCT	Designed by Ricky Fong
<i>OCT4</i>	GATGTGGTCCGAGTGTGGTTCT	GTTGTGCATAGTCACTGCTCGAT	Designed by Ricky Fong
<i>SOX2</i>	CTAGAAACCCATTTATTCCTGACA	GACAACTCTGATACTTTTTGAACAA	Designed by Ricky Fong
<i>ESR1</i>	GAGACATGAGAGCTGCCAAC	ACCCTCTTGCCCACTTGAT	Designed by Fitriya N. Dewi
<i>GREB1</i>	CACGACGATGGATTTCACCC	GATGAGGCAGAGGGTGAACCT	Primer ID Hs.PT.5826216464 (Integrated DNA Technology)
<i>TFF1</i>	AGAGACATGTACAGTGGCCC	GGAGGGACGTCGATGGTATT	Gupta et al. 2003
<i>S100A4</i>	GAGGGTGACAAGTCAAGCTC	GGAGGGCCCCAGCTGGCAGA	Designed by Fitriya N. Dewi
<i>GAPDH</i>	CGGATTTGGTCTGATTGG	TCAAAGGTGAGGAGTGG	Tian et al. 2010

estrogenic treatment. The classic estrogen response marker *TFF1* was expressed more prominently in MCF-12A than in MfMC, whereas *GREB1* was not expressed (see [Figure 6](#)).

4. Discussion

In this study, we were able to maintain a primary culture of *M. fascicularis* mammary gland cells derived from the breast tissue collected via surgical biopsy. The cell population expressed the epithelial cell markers *CD24* and *CK18*, and a marker for fibroblast *S100A4*. Importantly, we were able to identify the presence of stem cells in the culture as shown by expression of various markers of

stemness. In addition, the expression of *ESR1* and *TFF1* was positive, indicating estrogen responsiveness of the model.

Mammary gland is an organized ductal network, wherein the ducts comprised an inner layer of luminal epithelial cells expressing cytokeratins such as CK18 and an outer layer of myoepithelial/basal cells expressing other cytokeratins and α -smooth muscle actin ([Joshi et al. 2012](#)). CK18 is also one of the established markers typically used to delineate the degree of differentiation of mammary epithelial cells, as it indicates mature luminal epithelial cells ([Eirew et al., 2008](#)). Another commonly used marker of epithelial cells is CD24, whereby the high and low expressions of CD24 typically correspond to luminal epithelial and myoepithelial/basal cells, respectively ([Sleeman et al. 2007](#)). MfMC grown in our study showed

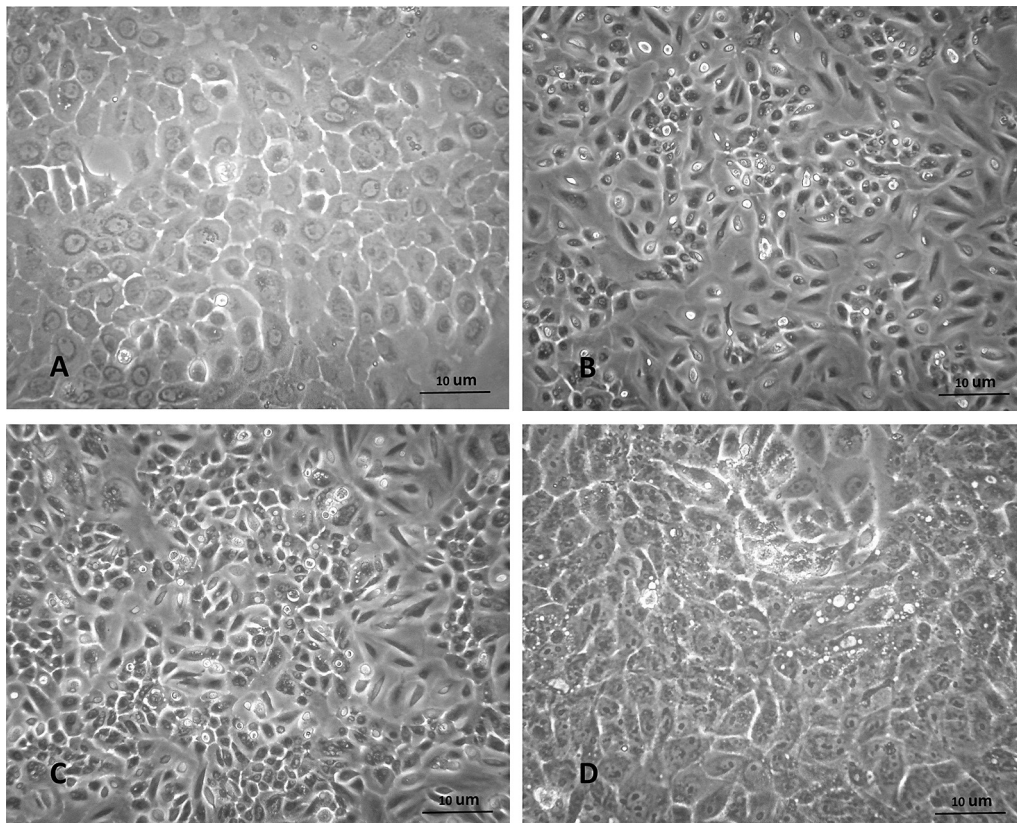


Figure 1. Cynomolgus monkey mammary gland culture predominated by epithelial-like morphology on first (A), second (B), and third (C) passages. Human mammary epithelial cell line MCF-12A is presented as morphological comparison (D).

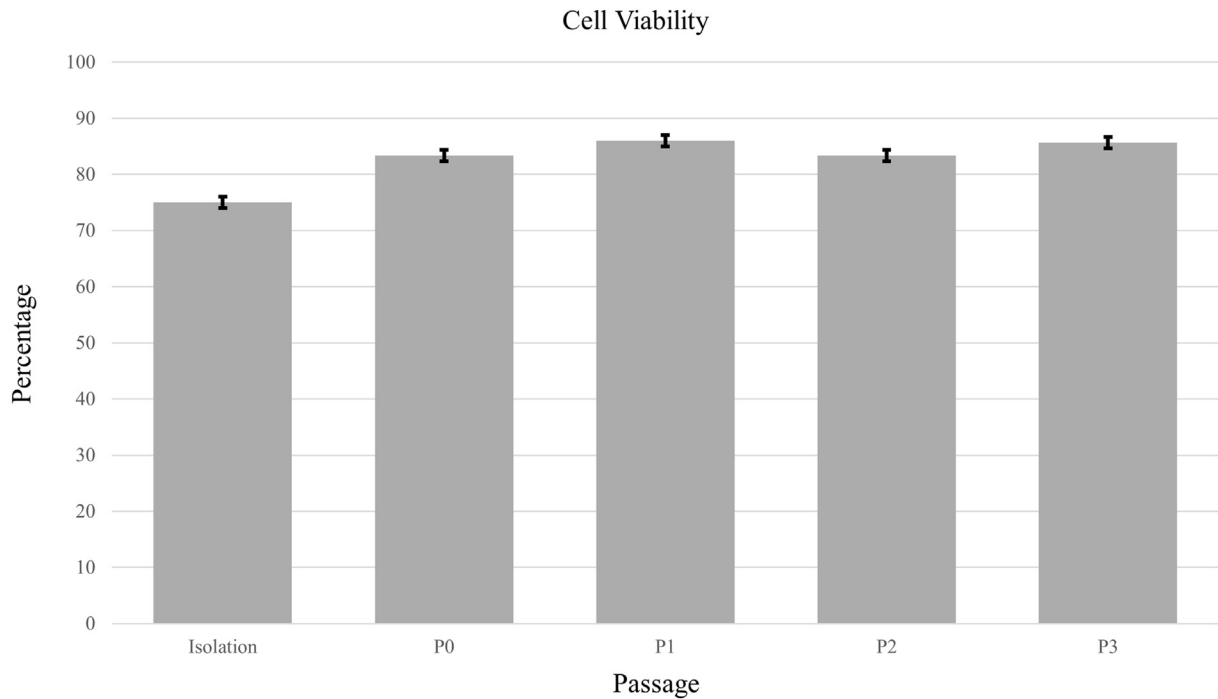


Figure 2. Viability of cells in the *Macaca fascicularis* mammary cell culture. Error bars = standard deviation.

expression of both CD24 and CK18 epithelial cell markers, which is consistent with the fact that the culture showed a predominant epithelial-like morphology. Importantly, this profile is comparable with the profile of human mammary epithelial cell line MCF-12A. Despite showing morphology of epithelial cells and being maintained in a medium selective for epithelial cells, the expression of a fibroblast marker was positive in MfMC, which may indicate the presence of stromal cell population in this model. This marker was also expressed in MCF-12A, although in lower intensity. Mammary gland is a heterogenous tissue, comprised epithelial cells and stromal components such as adipocytes, fibroblasts, endothelial cells, etc. (Richert *et al.* 2000). Further isolation will need to be performed to purify this model into an epithelial cell-specific cell culture system.

Mammary gland stem cells are important for normal mammary morphogenesis and tumor initiation. In the mouse model, the currently known cell surface markers to isolate MaSCs include CD24, CD29, and CD49f among others (Visvader 2009). The use of these markers has been a beneficial tool to isolate and enrich MaSCs. Moreover, cells positive for CD24 and CD29 were able to regenerate a functional mammary gland in murine model, which strongly supports the notion that these cells are part of a subpopulation that consists of mammary gland stem cells (Shackleton *et al.* 2006; Stingl *et al.* 2006). In humans, CD49f is among the known markers for MaSC with regenerative capacity, although the expression was also found in the epithelial luminal progenitor cells (Visvader 2009). Here, we found that alongside the epithelial

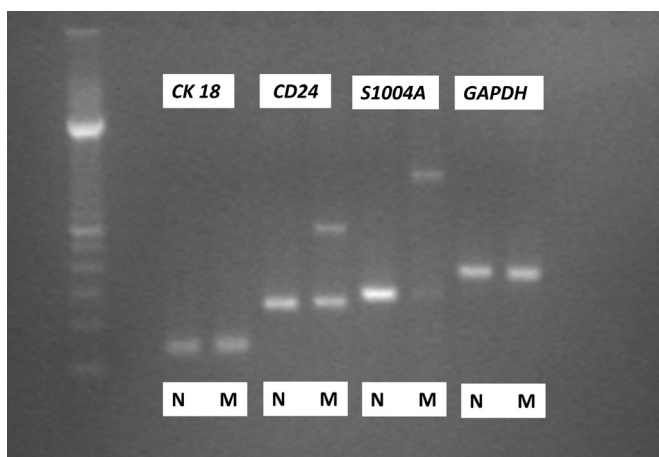


Figure 3. Expression of epithelial cell markers in *Macaca fascicularis* mammary cell culture (MfMC). Ladder 100 bp; CK18 (137 bp), CD24 (255 bp), S1004A (280), GAPDH (352 bp). N, MfMC; M, MCF-12A.

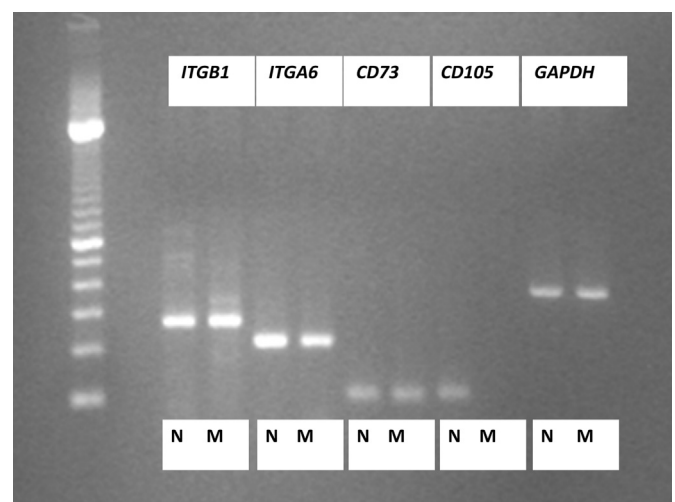


Figure 4. Expression of stem cell markers in *Macaca fascicularis* mammary cell culture (MfMC). Ladder 100 bp; ITGB1 (264 bp), ITGA6 (210 bp), CD73 (101 bp), CD105 (104 bp), GAPDH (352 bp). N, MfMC; M, MCF-12A.

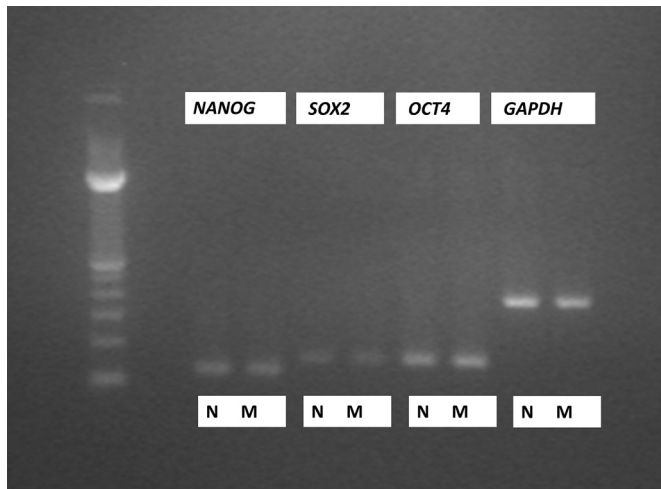


Figure 5. Expression of markers for pluripotency in *Macaca fascicularis* mammary cell culture (MfMC). Ladder 100 bp; *NANOG* (121 bp), *SOX2* (151 bp), *OCT4* (151 bp), *GAPDH* (352 bp). N, MfMC; M, MCF-12A.

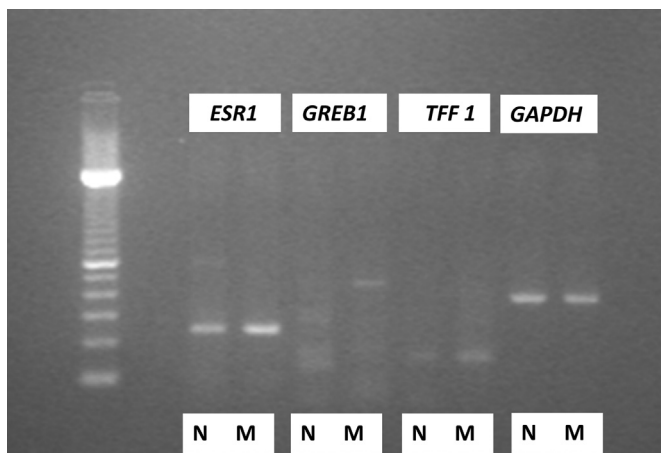


Figure 6. Expression of estrogen receptor and its activity markers in *Macaca fascicularis* mammary cell culture (MfMC). *ESR1* (241 bp), *GREB1* (163 bp), *TFF1* (155 bp), *GAPDH* (352 bp). N, MfMC; M, MCF12A.

markers CD24 and CK18, the MfMC cultures were also positive for CD29 and CD49f, suggesting that a population of MaSCs likely exists in this primary cell culture model.

The cell culture was derived from the breast tissue of nulliparous monkeys, as nulliparity is consistent with less differentiation of the mammary gland in monkeys and humans (Cline and Wood, 2008). We have previously reported a study in monkeys that showed terminal end bud (TEB) number was greater with less differentiation of the breast (Dewi et al. 2013). TEB has been thought to be the site where most MaSCs exist, as this TEB will give rise to epithelial cells (Visvader, 2009). It is likely that choosing the right developmental stage of the breast as tissue source is key in gaining a culture model with ample mammary gland stem cell population to be enriched further. Although our study indicates the likelihood of MaSC population to be present in the cell culture derived from nulliparous breast tissue, pubertal breast may also serve as a good source for MaSC culture because TEBs are most abundant in premenarchal breast (Dewi et al. 2013). Importantly, a subset of stem cell markers including CD29 were expressed at the highest level during puberty compared with other life stages in

monkeys (Stute et al. 2012). The use of monkey model may serve as a highly translational source to derive a culture model enriched for stem cells, especially from breast developmental stages that are ethically impossible to attain in humans.

Stem cells are characterized by their ability to self-renew, which makes them distinct from other cells. Stem cells are able to differentiate into other cells that are still in its lineage (Esmailpour and Huang, 2008). Other than adult MaSCs, in this study, we also evaluated the expression of mesenchymal stem cells (MSCs) using markers CD73 and CD105. These markers are typically expressed in bone marrow and matrix umbilical cord (Hass et al. 2011) and therefore perceived as gold-standard markers for MSCs. The expression of these markers in MfMC primary culture is likely due to the heterogeneity of the cell culture model. Despite using an epithelial cell-specific media, the MSCs were possibly derived from adipose tissue present as part of the mammary gland tissue. Although expression of mesenchymal markers has also been thought to be indicative of epithelial–mesenchymal transition naturally occurring in lactating breast and not of MSCs presence (Battula et al. 2012), it is unknown if such expression in the MfMC model is related to the multipotency of some cell population or other epithelial–mesenchymal transition-related activities.

Interestingly, pluripotent cells were reported to be present in disease-free breast tissue of parous and nulliparous women, whereby these cells are known to have remarkable lineage plasticity (Roy et al., 2013). These cells, known as endogenous pluripotent somatic cells or ePS cells expressed the markers OCT3/4, SOX2, and NANOG, were mortal and able to differentiate into functional three lineage derivatives. Another study reported that cells expressing the same pluripotency markers were present in breast milk, supporting the notion that pluripotent cells are present in normal breast (Hassiotou et al. 2012). The ePS cells are thought to be a different subset of population from the mammary-committed CD24⁻CD29⁺CD49f⁺ stem cells, although it was suggested that such MaSC is part of the cellular hierarchy that starts at the multilineage stem cell state (Hassiotou and Hartmann, 2014). Here, we showed that *OCT4*, *NANOG*, and *SOX2* were expressed clearly in MfMC culture comparable with that in MCF-12A. This finding suggests that such pluripotent cells were also present in the monkey breast-derived cell culture model. Further studies are needed to isolate ePS cells from MfMC and evaluate its pluripotency ability. Knowledge on plasticity of stem cells, progenitor cells, differentiated cells, and their interaction alongside the role of stroma for cell fate programming are important to bring together elements of biology and regulation within the mammary gland, which can be studied further using heterogenous culture models such as MfMC.

MfMC culture expressed ER and ER activity marker *TFF1*, in the level similar to that of MCF-12A. This profile indicates that the culture model is likely to be responsive to estrogen and estrogenic compounds. Mammary gland is a dynamic tissue responsive to the female reproductive hormones including estrogen. During certain developmental windows and reproductive cycles, the population of mammary gland stem cells are needed to expand and differentiate into new epithelium and therefore, they must be able to respond to the hormonal signs. As MaSCs are known to be negative for hormone receptors, they require niche components to relay the hormonal messages to cue MaSCs programming. This role may be served by ER⁺ and PR⁺ luminal epithelial cells as well as other niche elements like the stromal cells (Joshi et al. 2012). The positive expression of *ESR1* in this culture model can indicate the ability of niche cells to detect the level of circulating hormones, which are required for MaSCs growth. In addition, *TFF1* is typically used as a marker for ER activity. However, it is also commonly used to indicate the presence of oncogene in mammary gland and it may serve

as a beneficial information during the process of malignancy in breast cancer (Buache *et al.* 2011). Our findings highlight the potential use of MfMC culture model for future studies on estrogenic compound that may be important for breast cancer prevention, mainly those involving MaSC regulation.

The results indicate that stem cells are likely present in the primary cell culture model derived from the cynomolgus macaque breast. This finding suggests that MfMC may be a good source for population of MaSCs and potentially useful for further enrichment of stem cell population for future isolation purposes. Importantly, the culture model shows indication of estrogen responsiveness and mimics a heterogeneous breast tissue, which may serve as a promising tool for *in-vitro* works pertaining to carcinogenesis and cancer prevention, such as that involving estrogenic compounds and MaSC regulation.

Conflict of Interest Statement

None declared.

Acknowledgements

The authors would like to thank Dr Fitri Tafzi for providing MCF-12A cells, Dr Silvia A. Prabandari for the support on vaginal cytology, Ricky Fong for primers design, the staff of Research Animal Facility at Lodaya of PSSP-IPB, Microbiology and Immunology Laboratory and Biotechnology Laboratory of PSSP-IPB for their technical contributions. This study was financially supported by the Ministry of Research and Technology and Higher Education of the Republic of Indonesia.

References

- Battula VL, Evans KW, Hollier BG, Shi Y, Marini FC, Ayyanan A, Wang R, Briskin C, Guerra R. 2012. *NIH Public Access* 28. pp. 1435–45., 10.1002/stem.467. Epithelial-mesenchymal.
- Buache E, Etique N, Alpy F, Stoll I, Muckensturm M, Reina-San-Martin B, Chenard MP, Tomasetto C, Rio MC. 2011. Deficiency in trefoil factor 1 (TFF1) increases tumorigenicity of human breast cancer cells and mammary tumor development in TFF1-knockout mice. *Oncogene* 30:3261–73. <https://doi.org/10.1038/onc.2011.41>.
- Cline JM, Wood CE. 2008. The mammary glands of macaques. *Toxicol. Pathol.* 36: 134s–41s <https://doi.org/10.1177/0192623308327411>.
- Dewi FN, Wood CE, Lees CJ, Willson CJ, Register TC, Tooze JA, Franke AA, Cline JM. 2013. Dietary soy effects on mammary gland development during the pubertal transition in nonhuman primates. *Cancer Prev. Res.* 6:832–42 <https://doi.org/10.1158/1940-6207.CAPR-13-0128>.
- Dewi FN, Wood CE, Willson CJ, Register TC, Lees CJ, Howard TD, Huang Z, Murphy SK, Tooze JA, Chou JW, Miller LD, Cline JM. 2016. Effects of pubertal exposure to dietary soy on estrogen receptor activity in the breast of cynomolgus macaques. *Cancer Prev. Res.* 9:385–95 <https://doi.org/10.1158/1940-6207.CAPR-15-0165>.
- Dey D, Saxena M, Paranjape AN, Krishnan V, Giraddi R, Kumar MV, Mukherjee G, Rangarajan A. 2009. Phenotypic and functional characterization of human mammary stem/progenitor cells in long term culture. *PLoS One* 4. <https://doi.org/10.1371/journal.pone.0005329>.
- Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS. 2003a. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev.* 17:1253–70 <https://doi.org/10.1101/gad.1061803.potential>.
- Dontu G, Al-Hajj M, Abdallah WM, Clarke MF, Wicha MS. 2003b. Stem cells in normal breast development and breast cancer. *Cell Prolif.* 36(Suppl 1):59–72., 274 [pii].
- Eden JA. 2010. Breast cancer, stem cells and sex hormones. Part 2: The impact of the reproductive years and pregnancy. *Maturitas* 67:215–8. <https://doi.org/10.1016/j.maturitas.2010.07.012>.
- Eirew P, Stingl J, Raouf A, Turashvili G, Aparicio S, Emerman JT, Eaves CJ. 2008. A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability. *Nat. Med.* 14:1384–9 <https://doi.org/10.1038/nm.1791>.
- Esmailpour T, Huang T. 2008. Advancement in mammary stem cell research. *J. Cancer Mol.* 4:131–8.
- Gupta S, Hussain T, MacLennan GT, Fu P, Patel J, Mukhtar H. 2003. Differential expression of S100A2 and S100A4 during progression of human prostate adenocarcinoma. *J. Clin. Oncol.* 21:106–12 <https://doi.org/10.1200/JCO.2003.03.024>.
- Hass R, Kasper C, Böhm S, Jacobs R. 2011. Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue-derived MSC. *Cell Commun. Signal* 9:12. <https://doi.org/10.1186/1478-811X-9-12>.
- Hassiotou F, Beltran A, Chetwynd E, Stuebe AM, Twigger AJ, Metzger P, Trengove N, Lai CL, Filgueira L, Blancafort P, Hartmann PE. 2012. Breastmilk is a novel source of stem cells with multilineage differentiation potential. *Stem Cells* 30:2164–74. <https://doi.org/10.1002/stem.1188>.
- Hassiotou F, Hartmann PE. 2014. At the dawn of a new discovery: the potential of breast milk stem cells. *Adv. Nutr.* 5:770–8 <https://doi.org/10.3945/an.114.006924.770>.
- Joshi PA, Di Grappa MA, Khokha R. 2012. Active allies: hormones, stem cells and the niche in adult mammapoiesis. *Trends Endocrinol. Metab.* 23:299–309 <https://doi.org/10.1016/j.tem.2012.04.002>.
- LaMarca HL, Rosen JM. 2008. Minireview: hormones and mammary cell fate – what will I become when I grow up? *Endocrinology* 149:4317–21. <https://doi.org/10.1210/en.2008-0450>.
- Liu S, Dontu G, Wicha MS. 2005. Mammary stem cells, self-renewal pathways, and carcinogenesis. *Breast Cancer Res.* 7:86–95 <https://doi.org/10.1186/bcr1021>.
- Makino T, Yamasaki M, Takeno A, Shirakawa M, Miyata H, Takiguchi S, Nakajima K, Fujiwara Y, Nishida T, Matsuura N, Mori M, Doki Y. 2009. Cytokeratins 18 and 8 are poor prognostic markers in patients with squamous cell carcinoma of the oesophagus. *Br. J. Cancer* 101:1298–306. <https://doi.org/10.1038/sj.bjc.6605313>.
- Meier-abt F, Milani E, Roloff T, Brinkhaus H, Duss S, Meyer DS. 2013. Parity induces differentiation and reduces Wnt/Notch signaling ratio and proliferation potential of basal stem/progenitor cells isolated from mouse mammary epithelium parity induces differentiation and reduces Wnt/Notch signaling ratio and proliferation potential of basal stem/progenitor cells isolated from mouse mammary epithelium 36. *Breast Cancer Res.* 15:R36.
- Modur V, Joshi P, Nie D, Robbins KT, Khan AU, Rao K. 2016. CD24 expression may play a role as a predictive indicator and a modulator of cisplatin treatment response in head and neck squamous cellular carcinoma. *PLoS One* 11:1–21. <https://doi.org/10.1371/journal.pone.0156651>.
- Qiu L, Low HP, Chang CI, Strohsnitter WC, Anderson M, Edmiston K, Adami HO, Ekbohm A, Hall P, Lagiou P, Trichopoulos D, Hsieh CC. 2012. Novel measurements of mammary stem cells in human umbilical cord blood as prospective predictors of breast cancer susceptibility in later life. *Ann. Oncol.* 23:245–50 <https://doi.org/10.1093/annonc/mdr153>.
- Richert MM, Schwertfeger KL, Ryder JW, Anderson SM, Richert MM, Schwertfeger KL, Schwertfeger KL, Ryder JW, Anderson SM, Anderson SM. 2000. An atlas of mouse mammary gland development. *J. Mammary Gland Biol. Neoplasia* 5: 227–41. <https://doi.org/10.1023/A:1026499523505>.
- Roy S, Gascard P, Dumont N, Zhao J, Pan D, Petrie S, Margeta M, Tlsty TD. 2013. Rare somatic cells from human breast tissue exhibit extensive lineage plasticity. *Proc. Natl. Acad. Sci. U. S. A* 110:4598–603. <https://doi.org/10.1073/pnas.1218682110>.
- Russo J, Mailo D, Hu Y, Balogh G, Sheriff F, Russo IA. 2005. Breast differentiation and its implication in cancer prevention. *Clin Cancer Res* 11:931–6.
- Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. 2006. Generation of a functional mammary gland from a single stem cell. *Nature* 439:84–8. <https://doi.org/10.1038/nature04372>.
- Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ. 2007. Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. *J. Cell Biol.* 176:19–26 <https://doi.org/10.1083/jcb.200604065>.
- Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li H, Eaves CJ. 2006. Purification and unique properties of mammary epithelial stem cells. *Nature* 439:993–7. <https://doi.org/10.1038/nature04496>.
- Stute P, Wood CE, Kaplan JR, Cline JM. 2004. Cyclic changes in the mammary gland of cynomolgus macaques. *Fertil. Steril.* 82:1160–70 <https://doi.org/10.1016/j.fertnstert.2004.04.035>.
- Stute P, Sielker S, Wood CE, Register TC, Lees CJ, Dewi FN, Williams JK, Wagner JD, Stefanelli U, Cline JM. 2012. Life stage differences in mammary gland gene expression profile in nonhuman primates. *Breast Cancer Res. Treat.* 133:617–34.
- Tian H, Bharadwaj S, Liu Y, Ma H, Ma PX, Atala A, Zhang Y. 2010. Myogenic differentiation of human bone marrow mesenchymal stem cells on a 3D nano fibrous scaffold for bladder tissue engineering. *Biomaterials* 31:870–7. <https://doi.org/10.1016/j.biomaterials.2009.10.001>.
- Visvader JE. 2009. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev.* 23:2563–77 <https://doi.org/10.1101/gad.1849509>.
- Wiseman BS, Werb Z. 2002. Stromal effects on mammary gland development and breast cancer. *Science* 296:1046–9. <https://doi.org/10.1126/science.1067431>. **Stromal.**