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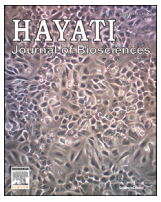

Original Research Article

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

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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

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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

Table 1. Primer sequences

Gene	Sequence (5′–3′)		References
	Forward	Reverse	
CD24	CCCACGCAGATTTATTCCAG	GACTTCCAGACGCCATTTG	Modur *et al.* 2016
CK18	ATCTTGGTGATGCCTTGGAC	CCTGCTTCTGCTGGCTTAAT	Makino *et al.* 2009
ITGB1	GTTACACGGCTGCTGGTCTT	CTACTGCTGACTTAGGGATC	Qiu L *et al.* 2012
ITGA6	CAAGATGGCTACCCAGATAT	CTGAATCTGAGAGGGAACCA	Qiu L *et al.* 2012
CD73	GACCTGGCTTTGTGACAGCAA	CTGACCCTGAGTAATCATGTCAGTCT	Designed by Ricky Fong
CD105	GACTGTCTTCACGCGCTTGA	GGAAGGCACCAAAGGTGATG	Designed by Ricky Fong
NANOG	CCAGTCCCAAAGGCAAACA	TCTTGACCGGGACCTTGTCT	Designed by Ricky Fong
OCT4	GATGTGGTCCGAGTGTGGTTCT	GTTGTGCATAGTCACTGCTCGAT	Designed by Ricky Fong
SOX2	CTAGAAACCCATTTATTCCCTGACA	GACAACTCCTGATACTTTTTTGAACAA	Designed by Ricky Fong
ESR1	GAGACATGAGAGCTGCCAAC	ACCCTCTTTGCCCAGTTGAT	Designed by Fitriya N. Dewi
GREB1	CACGACGATGGATTTCACCC	GATGAGGCAGAGGGTGAACT	Primer ID Hs.PT.5826216464 (Integrated DNA Technology)
TFF1	AGAGACATGTACAGTGGCCC	GGAGGGACGTCGATGGTATT	Gupta *et al.* 2003
S1004A	GAGGGTGACAAGTTCAAGCTC	GGAGGGCCCCAGCTGGCAGA	Designed by Fitriya N. Dewi
GAPDH	CGGATTTGGTCGTATTGG	TCAAAGGTGGAGGAGTGG	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

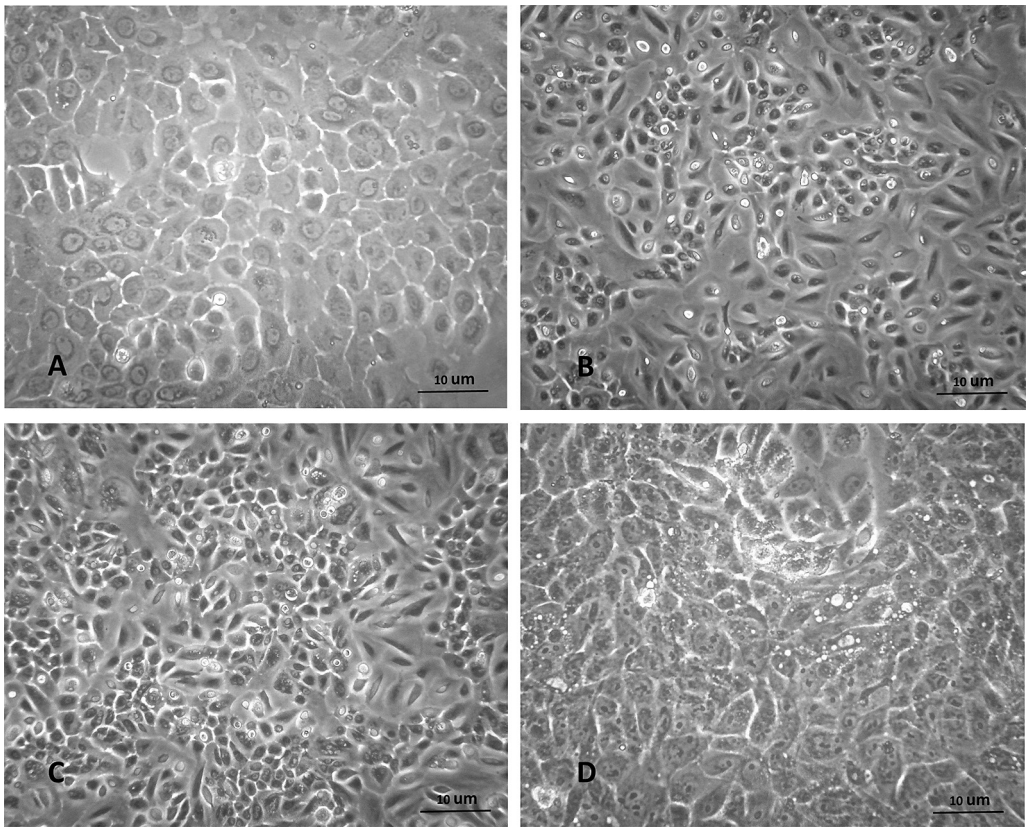

Figure 1. Cynomolgus monkey mammary gland culture predominated by epithelial-like morphology on first (A), second (B), and third (C) passages. Human mammary epithelial cell line MCF-12A is presented as morphological comparison (D).

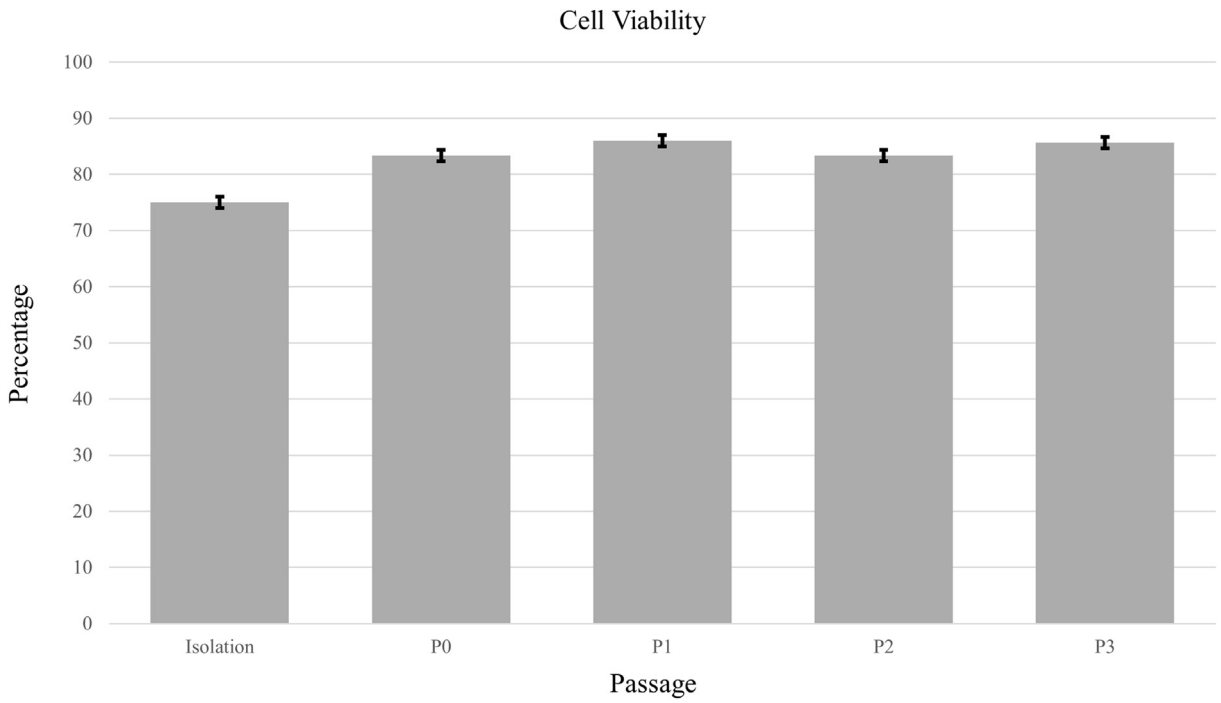

Figure 2. Viability of cells in the *Macaca fascicularis* mammary cell culture. Error bars = standard deviation.

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 makers 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

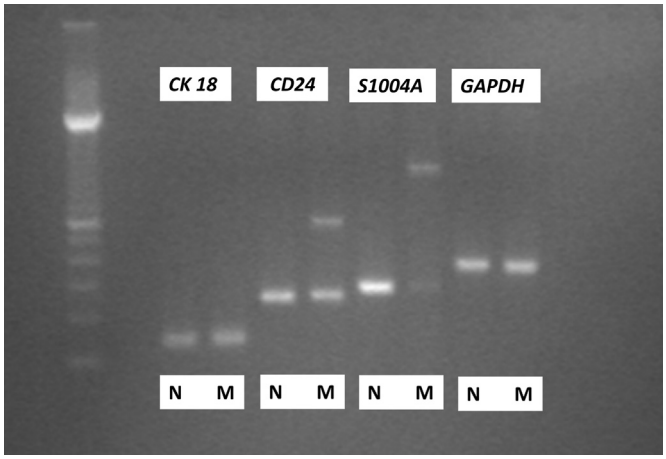

Figure 3. Expression of epithelial cell markers in *Macaca fascicularis* mammary cell culture (MfMC). Ladder 100 bp; *CK18* (137 bp), *CD24* (255 bp), *S1004A* (280), *GAPDH* (352 bp). N, MfMC; M, MCF-12A.

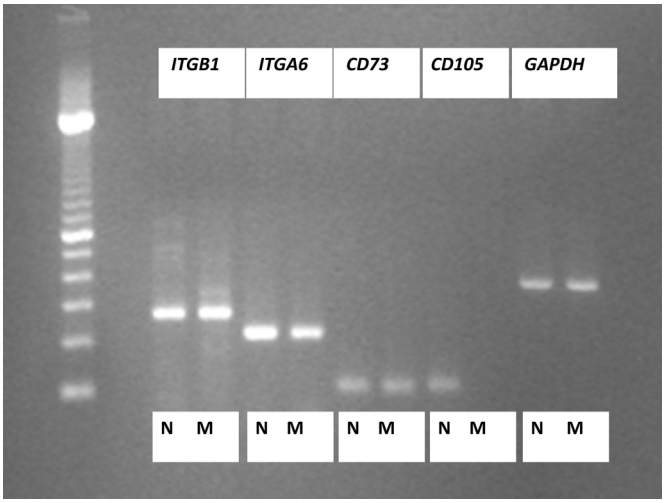

Figure 4. Expression of stem cell markers in *Macaca fascicularis* mammary cell culture (MfMC). Ladder l00 bp; *ITGB1* (264 bp), *ITGA6* (210 bp), *CD73* (101 bp), *CD105* (104 bp), *GAPDH* (352 bp). N, MfMC; M, MCF-12A.

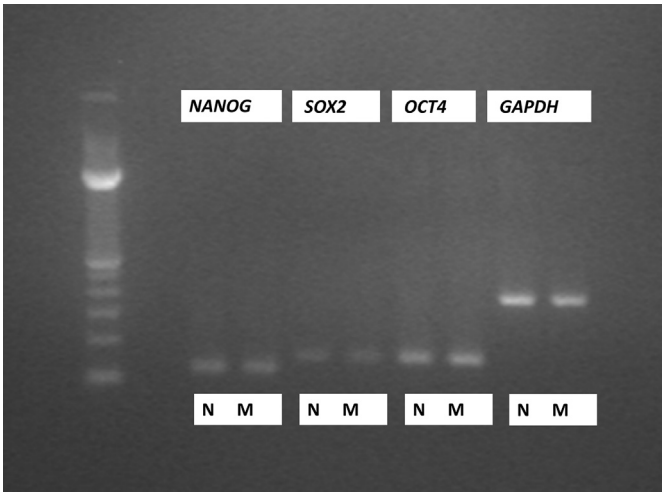

Figure 5. Expression of markers for pluripotency in *Macaca fascicularis* mammary cell culture (MfMC). Ladder l00 bp; *NANOG* (121 bp), *SOX2* (151 bp), *OCT4* (151 bp), *GAPDH* (352 bp). N, MfMC; M, MCF-12A.

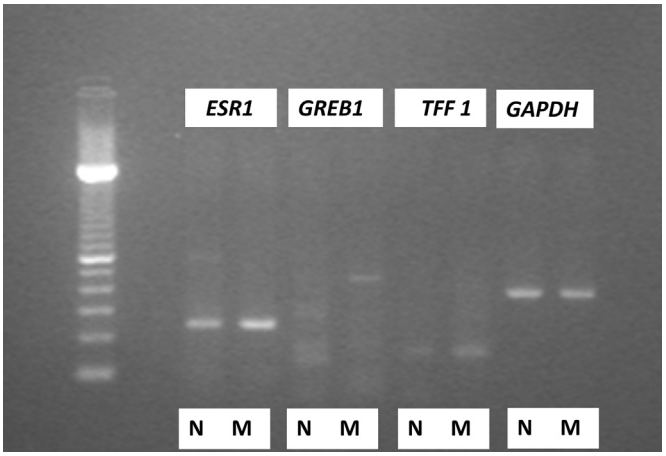

Figure 6. Expression of estrogen receptor and its activity markers in *Macaca fascicularis* mammary cell culture (MfMC). *ESR1* (241 bp), *GREB1* (163 bp), *TFF1* (155 bp), *GAPDH* (352 bp). N, MfMC; M, MCF12A.

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.

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