

KAv-1 is Better Suited to Chick Fibroblast Culture than DMEM or 199 Media

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Cultured cells are a useful resource for poultry scientists, since these cells allow scientists to evaluate biological responses to conditions such as infectious diseases *in vitro* while mimicking the whole-body response in birds. However avian cell culture requires an optimized basal medium, and there are currently relatively few options for this basal medium (medium 199 and KAv-1). This means that there is still room for the development of an optimal basal medium for avian cell culture. Here we compare KAv-1 medium, Dulbecco's modified Eagle medium (DMEM) and medium 199 during the culture of chick fibroblasts and determine that KAv-1 remains the optimal medium for these assays. Our results show that DNA damage is reduced in fibroblasts cultured in the KAv-1 medium, when compared to both DMEM and Medium 199 and that these cells also display improved growth dynamics in KAv-1 medium when compared to both DMEM and medium 199. To the best of our knowledge, this is the first study to describe a comparative analysis of culture media for avian cells, which would provide useful information for poultry scientists.

Key words: cell culture, cell growth, cellular senescence, chick fibroblasts, DNA damage, growth medium

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Introduction

Cell culture is a useful tool in poultry science as the cost of cell culture is significantly lower than that of whole-animal experiments. For example, cultured avian cells have been used in the evaluation of avian influenza sensitivity, which is one of the most lethal diseases in poultry (Hagiwara *et al.*, 2020). Furthermore, based on the concept of the three Rs (i.e., replacement, reduction, and refinement), we must consider replacing *in vivo* studies with cell culture to improve animal welfare. Thus, we believe that cell culture studies

will continue to make a significant contribution to the development of poultry science.

Efficient chicken fibroblast culture relies on the use of an optimized basal cell culture medium. Various types of mammalian cell culture media, including Dulbecco's modified Eagle medium (DMEM), Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12), Roswell Park Memorial Institute-1640 (RPMI-1640), and minimal essential media with alpha modifications (alpha-MEM medium) have been developed for *in vitro* assays, and have been widely applied by our groups and others in mammalian cell culture (Fukuda *et al.*, 2012; Katayama *et al.*, 2015; Gouko *et al.*, 2018; Katayama *et al.*, 2019a; Tani *et al.*, 2019; Orimoto *et al.*, 2020). However, unlike in mammalian cell culture, the number of culture media designated for use in avian cell culture remains quite limited and includes media such as medium 199 and KAv-1 (Morgan *et al.*, 1950; Kuwana *et al.*, 1996; Katayama *et al.*, 2019b). This means that there is still room for investigations into the types of media best suited to avian cell culture. In mammalian studies, the escape from cellular senescence is one of the critical factors in maintaining active cell growth (Haff and Swim, 1956; Hayflick, 1965). Based on these studies, we hypothesized that cell culture conditions that allow good cell growth are more advantageous for reducing cellular stress

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and cellular senescence, even after sequential passage.

Here we evaluate three types of media for cell culture of chicken fibroblasts: KAv-1 medium, DMEM, and medium 199. DMEM is the most common medium in mammalian cell culture while both medium 199 and KAv-1 were developed for avian cell culture. Medium 199 was developed for the culture of chick embryo fibroblasts, and KAv-1 was optimized for chicken primordial germ cell (PGC) culture (Morgan *et al.*, 1950, Kuwana *et al.*, 1996). We also investigated the relationship between chicken fibroblast growth and cellular senescence.

Materials and Methods

Chick Fibroblasts

We obtained fibroblasts from chicken embryos (*Gallus gallus domesticus*, white Leghorn) produced from fertilized eggs purchased from a commercial poultry farm (Goto Furanjyo, Inc., Kakamigahara City, Gifu, Japan). We incubated the fertilized eggs for seven days at 38°C and allowed the development of the embryo to continue over the course of this period to increase the number of fibroblasts available in each egg. As this study relied only on embryonic chickens we did not need to submit the animal experimental protocol for approval, based on the ethical standards of the National Institute of Environmental Studies (NIES, Tsukuba, Japan).

Cell Culture

Chicken fibroblasts were obtained from primary chicken embryonic tissue culture. Tissue was placed on a collagen-coated dish and cultured with Dulbecco's modified Eagle's medium (DMEM + GlutaMax; catalog no. 10566016; Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; catalog no. SH30396.03; cytiva, Marlborough, MA, USA) and 1% antibiotic-antimycotic mixed stock solution (catalog no. 161-23181; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan).

To culture the chick fibroblasts, we used four types of media: KAv-1 medium, DMEM, MEM, and medium 199. KAv-1 is an MEM-based medium with 5% fetal bovine serum (FBS) and 5% chick serum and has been described in detail in a previous publication (Kuwana *et al.*, 1996). Both DMEM and MEM (catalog no. 11900024; Thermo Fisher Scientific Inc., Waltham, MA, USA) were supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Medium 199 (catalog no. 11150059; Thermo Fisher Scientific Inc., Waltham, MA, USA) was supplemented with 10% FBS and 1% antibiotic-antimycotic solution. In addition, two types of serum were used in this study: FBS (catalog no. SH30396.03, Cytiva, MA, USA), and chick serum (catalog no. 16110-082, Thermo Fisher Scientific Inc., Waltham, MA, USA). Fibroblasts were incubated at 37°C in 5% CO₂ on gelatin-coated 6-well cell culture plates.

Cell Growth Analysis

We used sequential passage of the chick fibroblast cultures to evaluate the dynamics of long-term culture using each of the basal medias (Fig. 1). When one of the fibroblast cultures reached confluence, we treated the flask with trypsin and counted the number of cells and determined the live cell

ratio using a CountessTM cell counter (Thermo Fisher Scientific Inc., Waltham, MA, USA). We have described this protocol in detail in a previous report (Fukuda *et al.*, 2012, Qin *et al.*, 2012). Briefly, the fibroblasts were seeded in 6-well plates at a density of 1×10^5 cells per well. When the cells reached confluence, confluent fibroblasts were trypsinized, and the number of cells per dish was counted using a cell counter. Population doubling (PD), which is used as a measure of cell growth rate, was calculated using the following formula: $PD = 1/2 \log_2 (A/B)$, where A is the number of harvested cells and B is the number of plated cells.

In addition to sequential passage, we evaluated short-term cell growth and live cell ratios over five to six days (Fig. 2A, 2B, 5B, 5D, and S1A to C) of culture using a similar method. Live cell ratios were determined by trypan blue staining.

Growth Factors and Inhibitors

Growth factors and inhibitors were used to evaluate the cellular characteristics of the chick fibroblasts. We used between 5 and 20 ng/mL basic fibroblast growth factor (bFGF; catalog no. 064-04541; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan), 25 and 100 μ M Wnt1 (catalog no. 231-02251; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan), and 50 and 200 ng/mL R-spondin-1 (catalog no. 181-02801; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) as growth factors. In addition, we used between 0.5 and 2 μ M of the fibroblast growth factor receptor (FGFR) inhibitor PD173074 (catalog no. 160-26831; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan), and between 5 and 20 μ M of the Wnt inhibitor XAV939 (catalog no. 247-00951; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) as inhibitors.

Quantitative PCR (qPCR)

Total RNA was isolated from chick fibroblasts using the EZ1 RNA Tissue Mini Kit (catalog no. 959034; QIAGEN, Hilden, Germany). Complementary DNA (cDNA) was synthesized using a PrimeScriptTM reverse transcription (RT) reagent kit (catalog no. RR047A; Takara Bio Inc., Otsu, Japan) and was then used to analyze the expression levels of various growth factor genes. Polymerase chain reaction (PCR) was performed according to the manufacturer's protocol (KOD SYBR qPCR Mix; catalog no. QKD-201; TOYOBO; Osaka, Japan) and was used to analyze the expression of *FGF2*, *FGF3*, *FGF5*, *FGF10*, *Wnt3a*, and *Wnt5a*. In brief, the real-time PCR was performed in a reaction volume of 12.5 μ L containing $2 \times$ KOD SYBR qPCR Mix, 10 ng of cDNA, and 0.3 μ M of each primer. We normalized the expression of the target genes against that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The primers used in these assays are listed in Table S1.

Cell Cycle Analysis

We used the MuseTM Cell Cycle Assay Kit (Merck Millipore Corporation, Darmstadt, Germany) and the MuseTM Cell Analyzer (Merck Millipore Corporation, Darmstadt, Germany) to evaluate the cell cycle in our chicken fibroblast samples. All analyses were completed in accordance with the manufacturer's instructions.

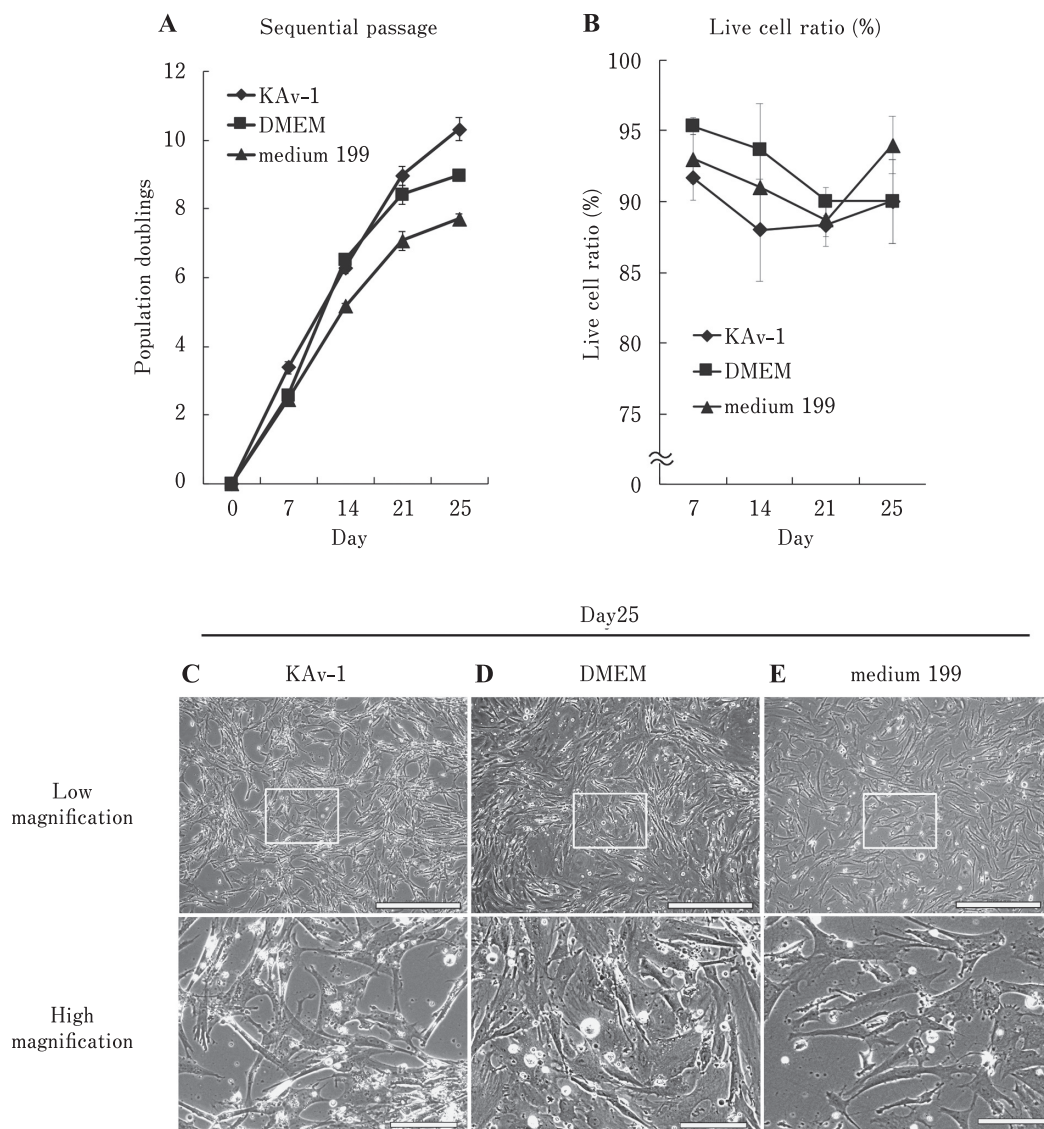


Fig. 1. Long-term culture of chick fibroblasts in KAv-1, Dulbecco's Modified Eagle Medium (DMEM), and medium 199.

A: Sequential passage of chicken fibroblasts in KAv-1, DMEM, and medium 199. The diamonds represent KAv-1, the squares represent DMEM, and the triangles represent medium 199. Error bars represent standard deviation (SD).

B: Live cell ratio of chicken fibroblasts in KAv-1, DMEM, and medium 199. The diamonds represent KAv-1, the squares represent DMEM, and the triangles represent medium 199. Error bars represent SD.

C: Cellular morphology of chicken fibroblasts grown in KAv-1 medium for 25 days. The upper and lower boxes represent the low and high magnifications, respectively. Scale bar=500 μ m (upper box) or 100 μ m (lower box).

D: Cellular morphology of chicken fibroblasts in DMEM on day 25. The upper and lower boxes represent low and high magnifications, respectively. Scale bar represents a length of 500 μ m (upper box) and 100 μ m (lower box).

E: Cellular morphology of chicken fibroblasts grown in medium 199 for 25 days. The upper and lower boxes represent low and high magnifications, respectively. Scale bar=500 μ m (upper box) and 100 μ m (lower box).

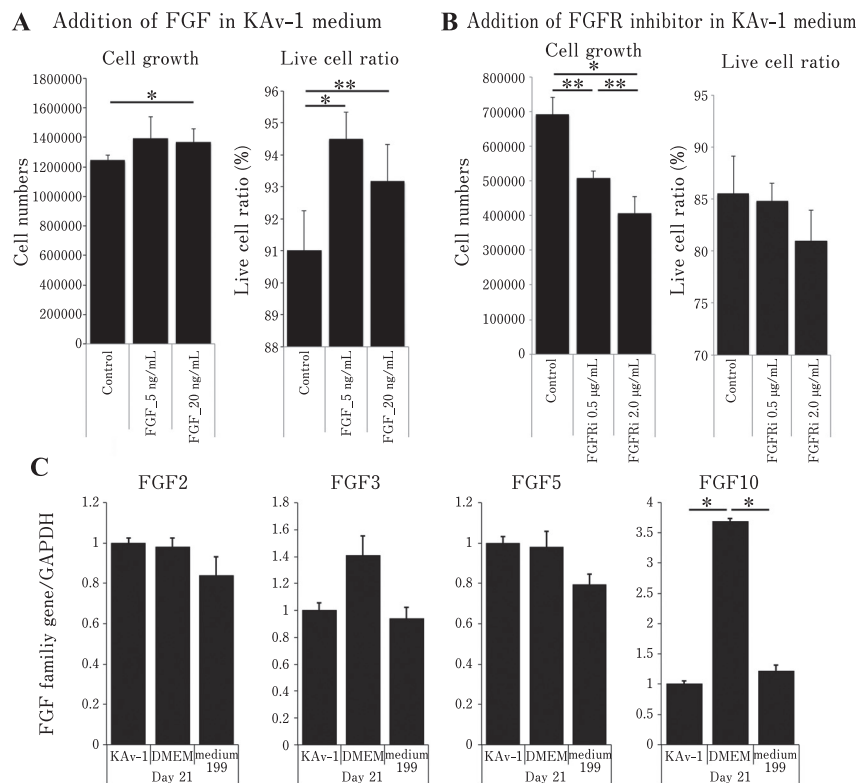


Fig. 2. Control of FGF signaling and the expression of several FGF genes in chick fibroblasts.

A: Addition of basic FGF (bFGF) to KAv-1 medium. The left graph represents chick fibroblast numbers on day 6, and the right graph represents the live cell ratio of the chick fibroblasts at day 6. The bars in the graph represent control (without bFGF), 5 ng/mL bFGF, and 20 ng/mL bFGF. Error bars represent SD ($n=6$). *: $p < 0.05$, **: $p < 0.01$.

B: Addition of FGF receptor (FGFR) inhibitor to KAv-1 medium. The left graph represents chick fibroblast numbers on day 5, and the right graph represents the live cell ratio of chick fibroblasts at day 5. The bars in the graph represent control (FGFR inhibitor minus), addition of 0.5 µg/mL of FGFR inhibitor PD173074 to the KAv-1 medium, and addition of 2 µg/mL of FGFR inhibitor PD173074 to the KAv-1 medium. Error bars represent SD ($n=6$). *: $p < 0.05$, **: $p < 0.01$.

C: Expression of FGF family genes in chicken fibroblasts. From left to right, the graphs represent *FGF2*, *FGF3*, *FGF5*, and *FGF10* gene expression levels in chicken fibroblasts. The expression levels of target genes were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Error bars represent SE ($n=6$). *: $p < 0.05$.

SA-β-Gal Staining

Senescence-associated beta-galactosidase (SA-β-Gal) staining was used to determine the extent of cellular senescence in the chicken fibroblast samples (Dimri *et al.*, 1995). Cell fixation and staining were performed using a senescence detection kit (catalog no. 0500-3115; BioVision Inc., Milpitas, CA, USA).

Gamma H2A.X Staining

Chick fibroblasts were stained with mouse anti-phospho-Histone H2A.X (Ser139) antibodies (1:20 dilution; catalog no. sc-519348; Santa Cruz Biotechnology Inc., Dallas, TX, USA) and an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (dilution, 1:200; catalog no. A11001; Thermo Fisher Scientific Inc., Waltham, MA, USA) before being counterstained with 4',6-diamidino-2-phenylindole (DAPI)

(dilution, 1:200; catalog no. 340-07971; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) and Alexa Fluor 568 Phalloidin (F-actin; dilution, 1:200; catalog no. A12380; Thermo Fisher Scientific Inc., Waltham, MA, USA). The green fluorescence is γ-H2A.X, red fluorescence is F-actin, and blue fluorescence is 4', 6-diamidino-2-phenylindole (DAPI).

Statistical Analysis

We used the Steel-Dwass test to evaluate all the non-parametric multiple comparison analyses described in Fig. 2, 3C, 4 B, 4D, 5 B, and S1. The data summarized in Fig. 5 D was evaluated using the Mann-Whitney U test. All statistical differences are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

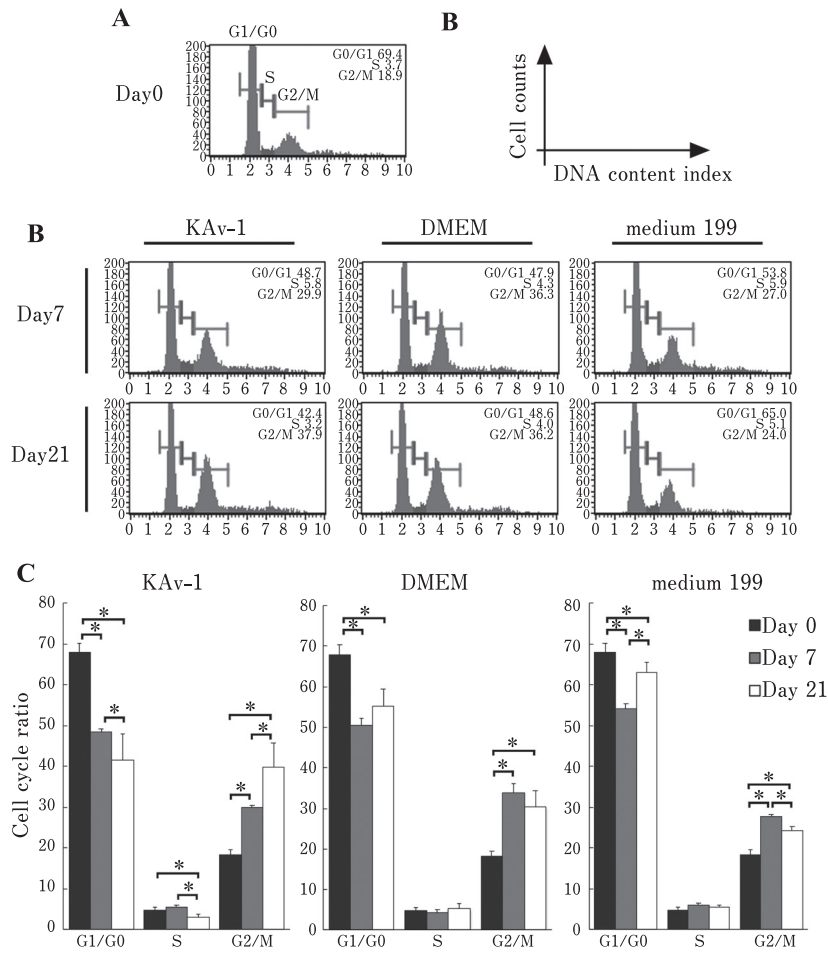


Fig. 3. Cell cycle analysis of chick fibroblasts grown in different media.

A: Histogram of chicken fibroblasts on day 0. The longitudinal axis represents cell counts. The horizontal axis represents DNA content index.

B: Histogram of chick fibroblasts on days 7 and 21 in KAv-1, Dulbecco's modified Eagle medium (DMEM), and medium 199. The upper and lower boxes represent days 7 and 21, respectively. The longitudinal axis represents cell counts. The horizontal axis represents DNA content index.

C: Comparison of the cell cycle in chicken fibroblasts grown in KAv-1, DMEM, and medium 199. The left bars represent the cell cycle ratio at day 0, the middle bars represent the cell cycle ratio on day 7, and the right bars represent the cell cycle ratio at day 21. Error bars represent SD ($n=6$). *: $p<0.05$.

Results

Growth of Chick Fibroblasts in Three Different Media

We evaluated the long-term culture of chicken fibroblasts in three types of media, namely, KAv-1 medium, DMEM, and medium 199. We used sequential passage to simulate long-term culture environments and although the live cell ratio was similar in all three media (88% to 95%), the fibroblasts exhibited the most active growth in the KAv-1 medium after 25 days in culture (PD was 10.316, 8.970, and 7.706 for KAv-1 medium, DMEM, and medium 199, respectively) (Fig. 1A, B). The KAv-1 medium also maintained the fibroblast morphology the best over the 25 days of culture, when compared to DMEM and medium 199 (Fig.

1C to E). Therefore, we concluded that KAv-1 medium is the best medium for the long-term culture of chick fibroblasts.

KAv-1 Medium did not Increase the Expression of the Endogenous FGF Genes in the Chick Fibroblasts at Day 21

In order to evaluate why the chick fibroblasts were able to actively proliferate in KAv-1 medium, we went on to examine the FGF signaling in these cells as these proteins are known to stimulate growth in mammalian fibroblasts. We observed that the addition of bFGF to the medium stimulated chick fibroblast growth (Fig. 2A) and that their growth was inhibited in a dose dependent manner when treated with the FGFR inhibitor (Fig. 2B). Our results suggest that that FGF signaling promotes chick cell growth. Therefore, we hy-

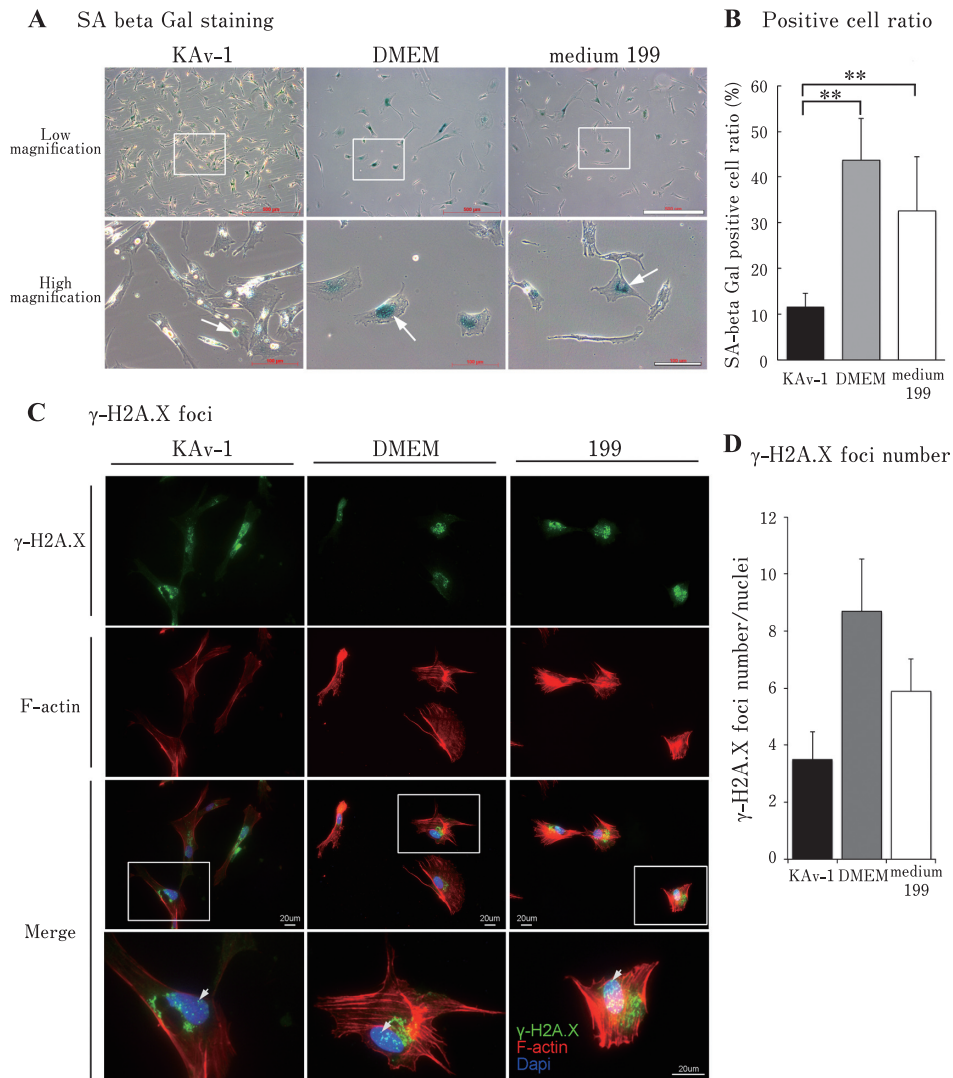


Fig. 4. Detection of cellular senescence and DNA damage in chick fibroblasts on day 25.

A: Senescence-associated beta-galactosidase (SA- β -Gal) staining in chicken fibroblasts grown in KAv-1, Dulbecco's modified Eagle medium (DMEM) and medium 199 at day 25. The upper and lower boxes represent low (5 \times) and high (10 \times) magnifications, respectively. The arrows indicate SA- β -Gal positive cells. Scale bar = 500 μ m (upper box) and 100 μ m (lower box), respectively.

B: Ratio of SA- β -Gal-positive chicken fibroblasts at day 25. The black bar represents chick fibroblasts growing in KAv-1 at day 25, the gray bar represents chick fibroblasts growing in DMEM on day 25, and the white bar represents chick fibroblasts growing in medium 199 at day 25. Error bars represent SD ($n=10$). **: $p < 0.01$

C: Detection of anti- γ -H2A.X positive foci in the nuclei of chick fibroblasts growing in KAv-1, DMEM, and medium 199 on day 25. The upper panel represents chicken fibroblasts with an anti- γ -H2A.X antibody. The middle panel shows chicken fibroblasts with an anti-F-actin probe. The lower panels represent the merged images of anti- γ -H2A.X (green fluorescence), F-actin (red fluorescence), and 4',6-diamidino-2-phenylindole (DAPI) (blue fluorescence) in chick fibroblasts. Scale bar = 20 μ m.

D: Number of anti- γ -H2A.X positive foci in chick fibroblast nuclei at day 25. The black bar represents chick fibroblasts growing in KAv-1 at day 25, the gray bar represents chick fibroblasts growing in DMEM on day 25, and the white bar represents chick fibroblasts growing in medium 199 on day 25. The error bars represent SD (KAv-1 medium: $n=9$, DMEM and medium 199: $n=10$).

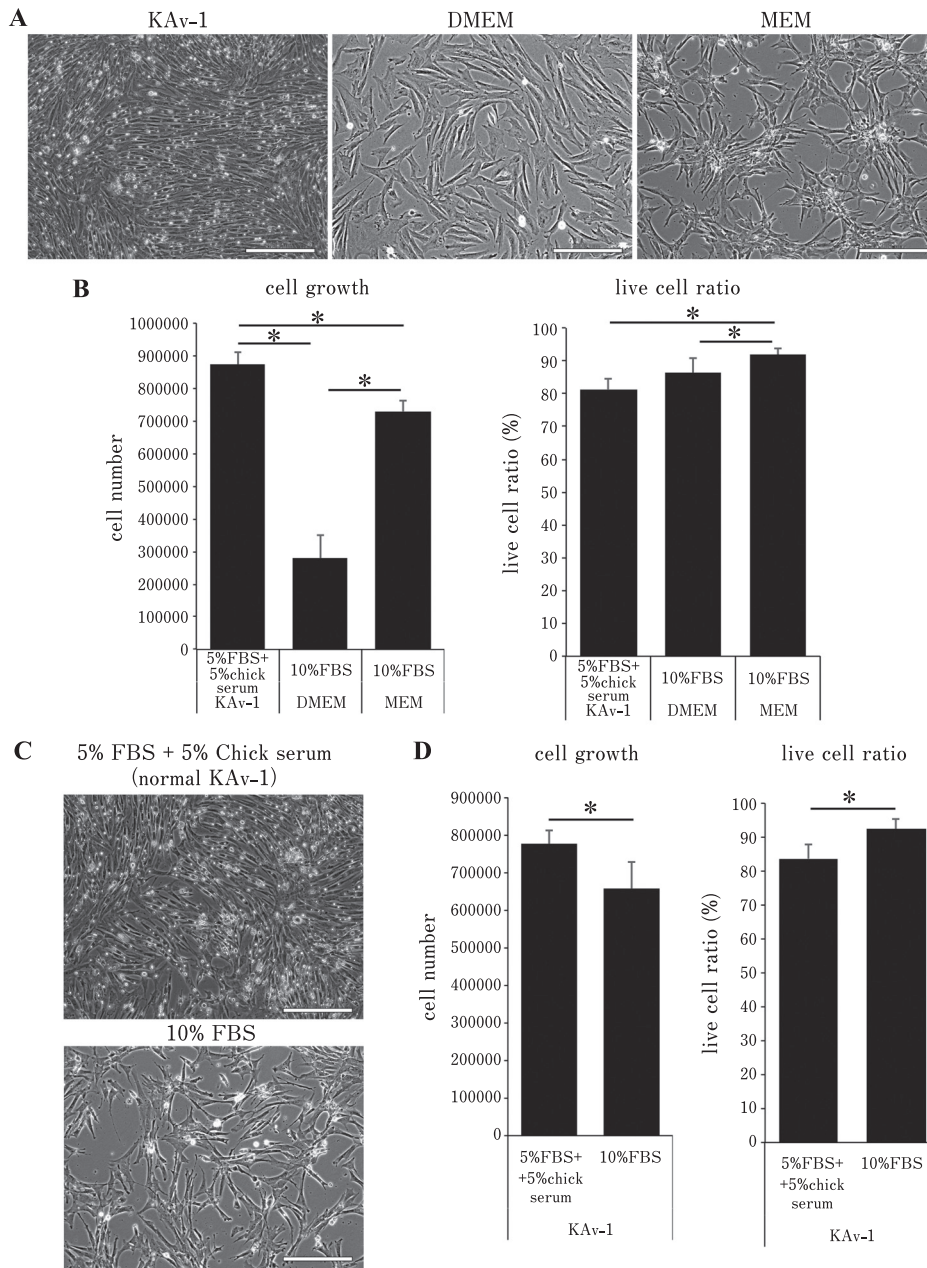


Fig. 5. Cell growth analysis in three media (KAv-1, DMEM and MEM) and comparison of cell growth following supplementation with 10% FBS and 5% chick serum with 5% FBS media.

A: Chicken fibroblast morphologies when cultivated in KAv-1, DMEM, and MEM for 3 days. The left panel shows chick fibroblasts in KAv-1 at day 3, middle chick fibroblasts in DMEM at day 3, and right chick fibroblasts in MEM medium at day 3. Scale bar=200 μ m.

B: The cell number and live cell ratio of chicken fibroblasts in all three media. The left image represents cell growth in each medium at day 3. The right image represents the live cell ratio for the chick fibroblasts in each medium at day 3. Left bars represent KAv-1 medium, middle bars represent DMEM, and right bars represent MEM. *: $p < 0.05$.

C: Morphology of chick fibroblasts in 10% FBS and 5% chick serum and 5% FBS on day 3. The upper portion represents chick fibroblasts in 5% chick serum and 5% FBS on day 3, and the lower portion depicts chick fibroblasts in 10% FBS at day 3. Scale bar=200 μ m.

D: The cell number and live cell ratio of chick fibroblasts grown in medium supplemented with 10% FBS medium, 5% chick serum and 5% FBS medium at day 3. The left image shows the growth of chick fibroblasts in 10% FBS, 5% chick serum and 5% FBS media on day 3. The right image shows the live cell ratio of chick fibroblasts in 10% FBS, 5% chick serum and 5% FBS media on day 3. Left bars represent KAv-1 medium, middle bars represent DMEM, and right bars represent MEM. *: $p < 0.05$.

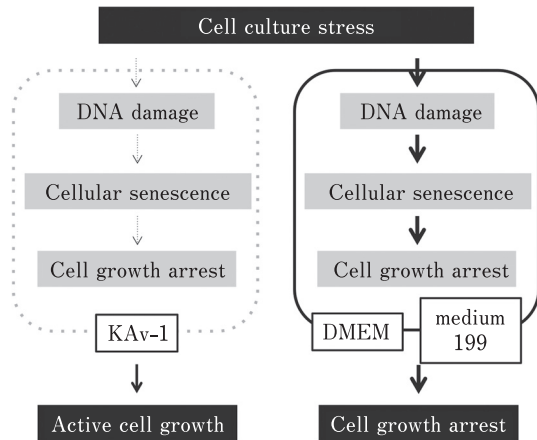


Fig. 6. **Summary of this study.**

KAv-1 medium was shown to be the optimal medium for chick fibroblast culture when compared with both DMEM and 199 medium.

DMEM - Dulbecco's Modified Eagle Medium

199 - Medium 199

hypothesized that KAv-1 medium induces high-levels of expression in the *FGF* genes in chicken fibroblasts. However, we did not observe a significant increase in *FGF* expression in these cells when compared to the fibroblasts grown in the other media (DMEM and medium 199) at day 21 (Fig. 2C). We concluded that the KAv-1 medium did not increase endogenous *FGF* expression and we hypothesized that the increased chick fibroblast growth observed in KAv-1 medium is mediated by other mechanisms, such as reduction of cell culture stress.

G2/M Phase Ratio Increases in Chick Fibroblasts at Late Passage

To analyze the condition of the chick fibroblasts during sequential passage, we went on to evaluate the cell cycle ratios in these cultures on days 0, 7, and 21. In all three media, the G1/G0 cell ratio at day 21 was lower than that of day 0. In contrast the G2/M ratio significantly increased on day 21 (Fig. 3A-C). Therefore, we can conclude that chick fibroblasts proceed towards cell cycle arrest at the G2/M phase, as a consequence of sequential passage.

Both Cellular Senescence and DNA Damage was Reduced in KAv-1 Medium at Late Passage

Based on the results of the cell cycle analysis, we hypothesized that KAv-1 medium is able to more efficiently reduce cell culture than DMEM and medium 199. First, we analyzed the ratio of SA- β -Gal positive cells, as an evaluation of cellular senescence in the chick fibroblasts growing in each of the three media at day 25. This analysis revealed that chick fibroblasts growing in KAv-1 medium had a significantly lower SA- β -Gal positive cell ratio than fibroblasts grown in DMEM and medium 199 after 25 days (Fig. 4A, B). Given this, we concluded that KAv-1 medium allows greater protection of fibroblasts from cellular senescence than DMEM and medium 199 during long-term fibroblast

culture.

Next, we evaluated whether KAv-1 medium could reduce the accumulation of DNA damage in chick fibroblasts at day 25. To detect the accumulation of DNA damage, we stained the chick fibroblasts with anti- γ -H2A.X antibodies and counted the number of γ -H2A.X positive foci in the nuclei of the cells. Although we did not observe any significant differences in the number of chick fibroblasts growing in the KAv-1 medium, we did note a lower number of γ -H2A.X positive foci in these cells when compared to those grown in DMEM or medium 199 (Fig. 4C, D). Based on the number of SA- β -Gal positive cells and anti-phospho-Histone-H2A.X positive foci, we could conclude that KAv-1 medium is an optimal choice for long-term chicken fibroblast culture.

Comparison of Chick Cell Growth in Each Media

KAv-1 is the optimal medium for chicken fibroblast culture when comparing KAv-1, DMEM, and medium 199. However, there is still room for investigation as to why KAv-1 medium is optimal for chick fibroblasts. To evaluate the effect of the basal medium, KAv-1, we first compared the cell growth of chick fibroblasts in KAv-1, DMEM, and MEM. Growth analysis showed that cells grown in KAv-1 demonstrated the most active proliferation with MEM in second place and DMEM in last place (Fig. 5A, B). Therefore, we can assume that although MEM is a more optimal medium than DMEM, KAv-1 medium is still more advantageous for chick fibroblast culture than either of the others. Thus, in addition to the reduction of cell culture damage mediated by MEM (which is the basal medium of KAv-1), other factors in KAv-1 promote the active growth of chick fibroblasts.

Next, we evaluated the effect of chick serum on the growth of chick fibroblasts, since, in contrast to other media, KAv-1 contains 5% chick serum and 5% FBS. Although the live cell ratios in the 5% FBS/ 5% chick serum medium was lower than that of the 10% FBS medium, chick fibroblasts were more active in the 5% FBS/ 5% chick serum medium, when compared with 10% FBS medium (Fig. 5C, D). Therefore, 5% chick serum is one of the critical factors mediating increased cellular growth in KAv-1. Taken together, these results suggest that the unique formulation of KAv-1 medium is the reason that it performs better in the culture of chick fibroblasts.

Discussion

Avian cell culture is more difficult than mouse and human cell culture. Several culture media have been developed for the culture of mouse and human cells. However, there is still room for the investigation and production of optimal basal media in avian cell culture. In this study, we demonstrated that KAv-1 medium is the best basal medium for the culture of chicken fibroblasts and to the best of our knowledge, this is the first study to perform a comparative analysis of avian culture media. Therefore, our results would provide useful information for poultry scientists starting cell culture.

We first hypothesized that KAv-1 medium might enhance the endogenous expression of *FGF* growth factors in chick

fibroblasts. Although we saw some response to increased *FGF* expression in late passage, there was no significant increase in *FGF* expression in fibroblasts cultured in the KAv-1 medium. *Wnt* proteins are also an important growth factor in mammalian fibroblasts (Davidson and Niehrs, 2010). Therefore, we analyzed *Wnt*-related signaling in chick fibroblasts at late passages in KAv-1 medium and although chick fibroblast growth was reduced in medium supplemented with *Wnt* inhibitor (XAV939), we did not observe any enhanced growth in the medium containing *Wnt* and R-spondin-1 (which activates *Wnt* signaling) (Fig. S1A to C). Furthermore, although the expression of *Wnt3a* was highest in the chick fibroblasts growing in KAv-1 medium, the expression of *Wnt5a* was not increased in these cells. Based on our results, we concluded that the acceleration in the chick fibroblast growth in KAv-1 medium may be related to other mechanisms, such as reduction of cell culture stress. Given this we went on to demonstrate that cell culture stress in chick fibroblasts was minimized in KAv-1 medium, when compared to DMEM and medium 199, with both DNA damage and cellular senescence shown to decrease under these culture conditions (Fig. 6). Given this we believe that KAv-1 medium is the most suitable medium for chicken fibroblast culture when compared to DMEM and medium 199.

Unpublished data suggests that KAv-1 medium may be the best medium for culturing fibroblasts from whooper swans as well. The whooper swan is well known as the host of the avian influenza virus (Sakoda *et al.*, 2010, Bui *et al.*, 2013, Bi *et al.*, 2015, Chen *et al.*, 2016, Li *et al.*, 2018). Avian influenza is one of the most lethal infectious diseases in poultry. Therefore, fibroblasts derived from whooper swans may have potential in the *in vitro* evaluation of avian influenza infection. A number of new avian influenza viruses are reported by various research groups every year (Yamayoshi *et al.*, 2014, Gamoh *et al.*, 2016). Therefore, developing *in vitro* evaluation systems for predicting the toxicity of new types of avian influenza using cultured chick fibroblasts and whooper swan-derived fibroblasts may have lasting impact on our ability to control these infections. In addition, there is widespread consensus that *in vitro* cell culture using avian fibroblasts could promote the progress of poultry science.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Author Contributions

MK was involved in the study design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing.

MO provided experimental material, and final approval of the manuscript.

TF provided experimental material, and final approval of

the manuscript.

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