Research Article

Somatic cell conservation of the Punganur cattle: An Indian heritage breed

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ABSTRACT

ICAR-NBAGR is the nodal agency for conserving indigenous livestock genetic resources as embedded in the Sustainable Developmental Goal of the United Nations specifically 2.5.1.a. Indigenous Punganur cattle is one of the shortest cattle breeds of the world that has <10,000 true to the breed animals (19th Livestock Census). Breed Watch list 2022- ICAR-NBAGR, Karnal enlists Punganur as a vulnerable breed and hence it was considered on priority for conservation. Ear skin tissue samples of both sexes were used for generating the somatic cells by *in vitro* explant and cell culture. Initially, tissue fragments were cultured in Dulbecco's Modified Eagle Medium supplemented with 20% FBS and 2% antibiotic–antimycotic solution under a controlled atmosphere (37 °C, 5% CO_2). Fibroblast cells having fusiform shape and centrally positioned oval nuclei were isolated and multiplied in culture till the 4th passage. The cell lines demonstrated stable growth characteristics and maintained normal morphological features. At least 30 cryovials per animal ($1x10^6$ cells/ vial) were cryopreserved in a liquid nitrogen container at -196 °C for posterity. The growth potential of cryopreserved cells was found to be comparable to that of the cells before freezing. Advances in the cloning of animals using somatic cells for potential revival of a breed and future breakthroughs are likely to enhance the use of these established cell lines in diverse fields, such as disease modeling and treatment, tissue engineering, cellular reprogramming, and the production of extracellular matrix. Therefore, the stored biological material is valuable both for *ex-situ* conservation of the genetic resources and for future research avenues.

Key words: Cattle, Conservation, Punganur, Somatic cells

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INTRODUCTION

Punganur, a registered cattle breed of India (INDIA_CATTLE_0100_PUNGANUR_03022) is native to the Chittoor district of Andhra Pradesh in Southern India. Its breeding tract is confined to the Vayalpad, Madanapalle, and Palamaner mandals of the district. The breed derives its name from a village in the Chittoor district, Punganur (Mouli *et al.,* 2022). The Jamindars of Punganur being Diwans in the erstwhile Princely state of Mysore liked the small cows and improved this breed. The breed is famous for its short size, low maintenance and comparatively good milk yield. The animals have high disease resistance, heat tolerance and very good adaptability to tropical climatic conditions (Ekambaram *et al.,* 2015).

Punganur breed is integral to the socio-cultural fabric of the region. Tirumala Tirupati Devasthanams maintains a farm for Punganur cows since its milk is used for 'Ksheera abhishekam'. The ghee prepared from the milk of these cows is used in the 'archana' (offering) of Lord Venkateswara. The ghee is also used for making the very famous Tirupathi temple 'laddu'. Unfortunately, the unique Punganur cattle is one among the 38 indigenous Indian livestock and poultry germplasm which are categorized to be "at risk" as per the latest ICAR-NBAGR Breed Watch List, 2022. Punganur cattle are enlisted under the vulnerable category (https://nbagr.icar.gov.in). Extensive indiscriminate crossbreeding in the breeding tract is the main reason behind the current ominous scenario.

Animal genetic resource conservation has been an international priority for decades and has gained further momentum with the United Nations Sustainable Development Goals (SDGs) where target 2.5 advocates the preservation of genetic diversity in domesticated animals (Kumar *et al.,* 2024). Cattle breeds have evolved with combinations of mutation, genetic drift, differential selection pressure of the local environment and endemic diseases, available feed and fodder along with human selection (Sharma *et al.,* 2020). Therefore, each breed encompasses unique genetic combinations which need to be conserved for future generations. Accordingly, immediate efforts are required to conserve the unique Punganur cattle breed (*Bos indicus*). Cryopreservation is an integral component of farm animal conservation strategies (Dua *et al.,* 2021). Advances in cryobiology allow the cryopreservation of any type of cells, however, in farm animals, the

somatic cell cryobanks (SCBs) are in vogue as breeds can be revived by somatic cell nuclear transfer (SCNT) methods and cells can also provide biomaterials for a wide variety of scientific and academic research (Groeneveld *et al.,* 2016). We initiated the somatic cell cryobanking of Punganur cattle intending to conserve them for posterity and also to make cryopreserved cells available to the scientific communities for research endeavors.

MATERIALS AND METHODS

Tissue sample collection

True to the breed type animals were selected from its breeding tract. The animals had a concave and broad forehead and white, grey, or light to dark brown coat colour. The skin, muzzle, eyelids and hooves were black. Crescent-shaped horns were stumpy in males and longer in females and the tail touched the ground (Fig.1). Sampling was done from two male and two female Punganur cattle, sourced from the Punganur cattle herd being maintained at Livestock Research Station, Palamaner of the Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh. The marginal ear region of each animal was washed with water and sterilized with 70% alcohol to facilitate aseptic tissue sample collection. Ear marginal tissue biopsy (0.5-1.0 cm^2), one per animal was collected using an ear notcher following the ethical standards of the institute. The tissue samples were transported to the laboratory facility at ICAR-NBAGR, Karnal in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% Fetal bovine serum (FBS) and 2% antibiotic solution. Transportation was done within a timeframe of 24 to 36 hours at 4°C.

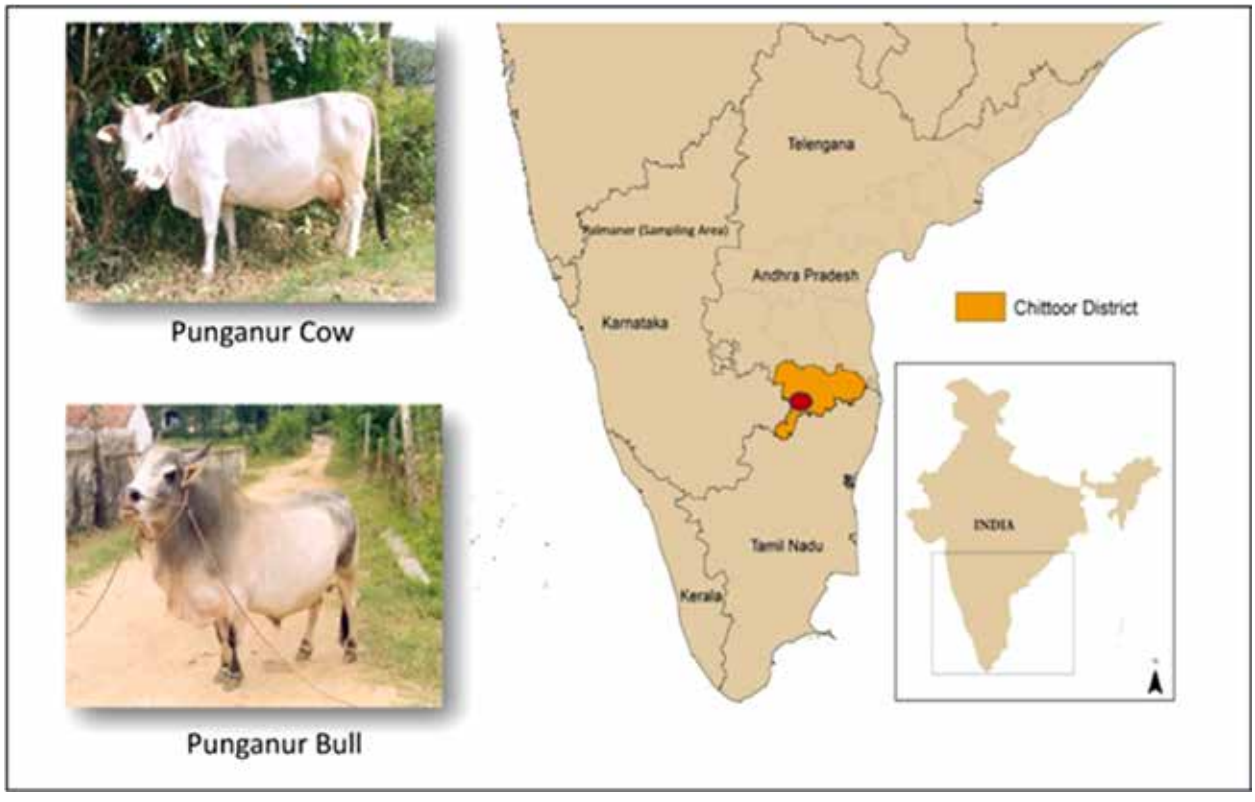

Fig. 1: *Representative animals of the Punganur breed, its distribution area (marked in yellow) in Andhra Pradesh, India, and sampling location (marked in red).*

Explant culture

All reagents and culture media utilized in this protocol were procured from Sigma-Aldrich (St. Louis, MO, USA) and Gibco-BRL (Carlsbad, CA, USA). The samples in the laboratory underwent a thorough washing s utilizing 70% alcohol and Dulbecco's Phosphate-Buffered Saline (DPBS) with a Ca^{+2} and Mg^{+2}, to ensure decontamination while maintaining osmolarity. Washed tissues were processed and sliced into smaller pieces measuring 1.0 to 2.0 mm^3 which were seeded evenly across 5 to 6 cell culture plates (60mm) having 500µl culture media (DMEM with 20% FBS and 2% antibiotic solution). The plates were incubated at 37°C in a 5% CO_2 incubator for 10-12 h. After incubation, 2 ml of fresh culture media was added to each petri plate and kept for culturing at 37°C in a 5% CO_2 incubator. Cultures were maintained until 70-80% confluence of cell outgrowths. Subsequently, each plate was treated with 0.25% trypsin-EDTA solution for 1-2 min for the preferential dissociation of fibroblast-like cells. The cell suspension

was centrifuged in a 15 ml tube (4,500 rpm, 10 min) and the pellet was resuspended in 1 ml of culture media.

Establishment of the culture and cellular evaluations

The method was adopted from the previously described protocols of Sharma *et al.,* 2018a and 2018b. Cells were cultured using DMEM with 10% FBS and 1% antibiotics solution (100 IU/mL penicillin and 100 µg/mL streptomycin) in T-25 tissue culture flasks under an optimum atmosphere (37°C; 5% CO_2). The medium was changed twice a week to maintain proper proliferation and growth of cells. Morphological characteristics including cell shape, and nuclear and cytoplasmic extensions were examined under an inverted microscope. After trypsinization, the cell suspension was centrifuged (250 g, 20 min) and the cell pellet was split into new culture flasks having culture media. The standard cell passaging was performed to expand somatic cells for cryobanking.

Cell viability analysis

Harvested cells were counted using a hemocytometer and viability was checked by trypan blue assay (Freshney, 2000). Suspended cells (10 µl) in the centrifuge tube were diluted with 10 µl of 0.4% trypan blue dye. Viable (colorless) and dead cells (stained blue) were counted. The viability of cells was determined by the relative cell count of live cells to the total number of cells.

Cell proliferation assay

The proliferative rate of established cell culture was elucidated by seeding 8×10^4 cells per 25 cm^2 flask. A duplicate culture for 9 days was set up. Average cell counts were recorded after cell trypsinization every 24 h to draw the cell growth curve and calculate the population doubling time (PDT). PDT was estimated as PDT = T ln 2 / ln (Xe/Xb) where PDT is the time required to double the culture (h), T is the incubation time, ln is the Napierian logarithm, Xe is the number of cells at the end of the incubation period, and Xb is the number of cells at the beginning of the incubation period.

Cryopreservation, recovery, and cryobanking of cells

Fourth Passage cells in the exponential phase of growth (70-80% confluency), were harvested with 0.25% trypsin-EDTA. The cell suspension ($1x10^6$ viable cells) was aliquoted into 2 ml cryogenic vials having 1 ml culture media with 10% FBS and 10% DMSO as the freezing medium. Labeled cryovials (animal identity, passage number, and date) were utilized for the storage. These were placed in a 1ºC cooler at -80 ºC overnight and then transferred to liquid nitrogen (-196 °C) for long-term storage. To recover cells, cryovials were quickly thawed at 37ºC, contents were transferred into a flask containing DMEM/Ham's F-12 media with 10% FBS and cultured at 37ºC with 5% CO_2 in a humidified incubator.

RESULTS AND DISCUSSION

India has been blessed with a large and rich biodiversity of indigenous bovine population (*Bos indicus*) which represents an enormously important bovine gene pool due to their adaptability to diverse agro-ecological and climatic conditions (Sharma *et al.,* 2015). They are considered to have superior heat tolerance, immunity, and ability to sustain on restricted feed and fodder than the exotic (*Bos taurus*) (Sharma *et al.,* 2023). The breed watch list recently published by ICAR-NBAGR (https://nbagr.icar.gov.in) highlighted the need to take immediate steps for the conservation of 9 of 53 registered cattle breeds due to their endangered/ vulnerable status. Changing developmental, economic and social aspects are adversely affecting the numbers and genetic diversity of these breeds (Sharma *et al.,* 2023). Conserving the indigenous cattle germplasm is essential for harnessing hybrid vigor, preserving specific genes and gene combinations, and also as an insurance against climate and social changes (K *et al.,* 2024).

It is a well-established fact that the cryopreservation of animal cells is a superb tool for the long-term preservation of animal genetic resources (Saadeldin *et al.,* 2019). Successful cryopreservation of somatic cells has been reported in farm animals across the species such as cattle, sheep, goat, pig, horse, and buffalo (Dua *et al.,* 2021). The fibroblast cell lines and their cryopreservation are accepted as a complementary strategy for the conservation of genetic resources of valued and endangered animals due to their usage as nuclear donors in the SCNT procedure (Pathak *et al.,* 2023). Conservation of the indigenous livestock biodiversity is the mandated activity of the ICAR-National Bureau of Animal Genetic Resources (NBAGR), Karnal. Semen and embryos of some indigenous breeds are cryopreserved for posterity in the Genebank of Bureau. We initiated somatic cell banking under the Consortium Research Project on Agrobiodiversity (sponsored by the ICAR) and were the first to cryopreserve the *Camelus bactrianus* fibroblasts in the world (Sharma *et al.,* 2017). The present research effort targeted the somatic cell conservation of Punganur a dwarf cattle breed of southern India that is one among the nine 'At risk' breeds.

The important milestones in establishing skin-derived fibroblast-like cells are depicted in Fig. 2. Skin explant displayed early outgrowth of the monolayer of cells on days 4-6 of explant seeding (Fig. 2C).

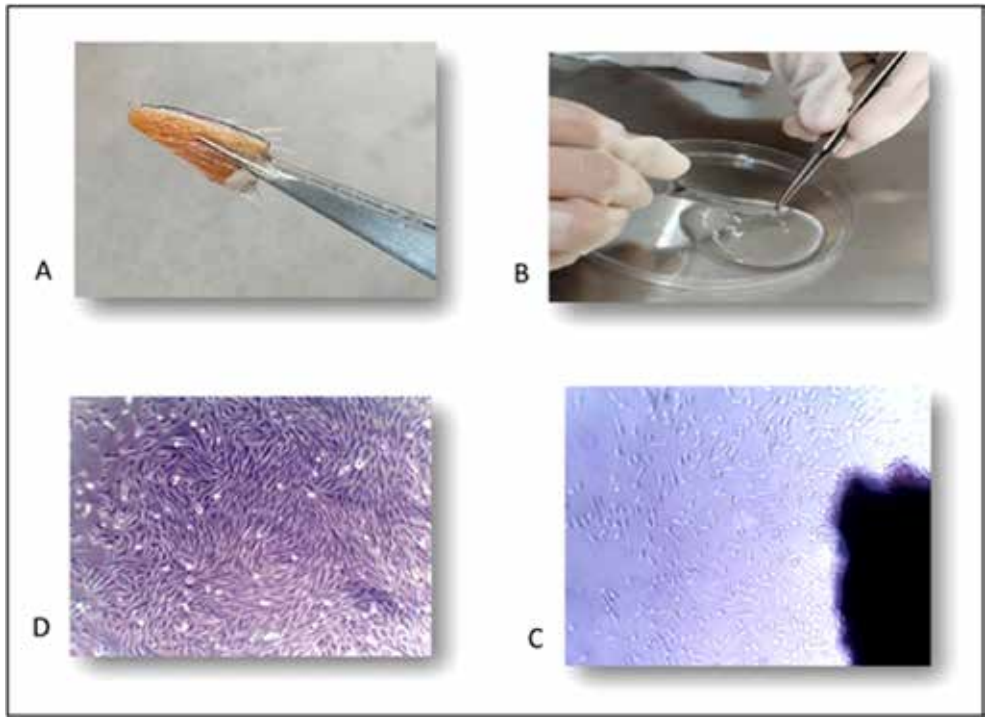

Fig. 2: *Culture of Punganur cattle skin-derived fibroblast-like cells (A) Ear marginal tissue piece (0.5-1 cm) (B) Chopping of ear marginal tissue piece after removing hair, epidermis and fat layer (C) An outgrowth of somatic cells from seeded explants after 4-8 days (D) Confluent fibroblast-like cells.*

Interestingly, cells appeared only in the attached explants and not in the floating explants. The majority of literature reports a period of 7-10 days for the appearance of cells during the primary culture (Selokar *et al.,* 2018) hence the method adopted was efficient in the generation of primary cells. Cells were mostly of fibroblast type. Mixed types of cells emerged from some of the explants where epithelial cells appeared as a sheet of uniform cuboidal cells near the tissue explants while fibroblast-type cells were far apart from the tissue. The culture medium was changed every three days till the Petri plates were more than 60-70% confluent (Fig 2D). At this stage, cells were harvested by trypsinization. The two types of cells behaved differentially on incubation with trypsin-EDTA. Fibroblasts were observed as round cells after 1.5-2 min of trypsinization while epithelial cells remained as sheets and dissociated at a later stage of incubation (>5 min). Thus it was possible to differentially harvest the fibroblast-like cells for secondary culture. It is documented in the literature that the fibroblasts attach and trypsinize more quickly than the epithelial cells (Liu *et al.,* 2014).

Fibroblasts with spindle morphology were isolated preferentially at around 80% confluency at secondary passages based on their sensitivity to trypsin. Moreover, fibroblasts progressively outdo their epithelial counterparts by sequential passaging due to their better growth capacity (Wang *et al.,* 2012). These properties enabled the development of a pure Punganur fibroblast culture after three to four passages. Passage 2 onwards the somatic cells had fibroblast-like morphology and culture characteristics. The cells displayed a classic fusiform morphology with oval nuclei positioned in the center, radiating fibroblast-like structures, and migratory patterns resembling flames. The secondary cultures grew faster than primary cultures in achieving 70–80% confluency, which may be related to their adaptability to the cultural environment. The culture was checked for any microbial contamination on every 2nd day. The absence of turbidity in culture media indicated no microbial multiplication during the cultivation of cells. Furthermore, neither fungi nor bacteria were identified by observing the flasks under the microscope.

Subculture and cell multiplication were continued up to the fourth passage. The cells of 4th passage were harvested and cryopreserved in liquid nitrogen. Freezing of the cells was done using a slow freezing method, and at least 30 cryovials of each animal were prepared for the cryobanking. The informative datasheets incorporating animal details, sex, photograph, population status, place of tissue biopsy collection, date and time of sample collection, number of cryovials stored per animal, passage number at freezing, and date of freezing were made and maintained as electronic and printed inventory records.

The recovery of cryopreserved cells was done using the standard thawing and culture method. Typically, more than 70% of thawed cells attached to culture dishes and proliferated within 48 h and reached confluency after 5-6 days. The thawed and resurrected fibroblasts at different stages of culture are shown in Fig 3.

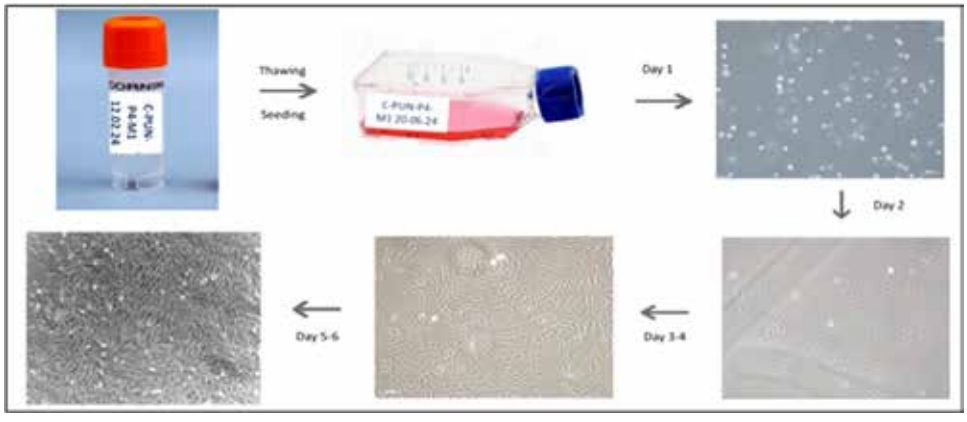

Fig. 3: *The representative images depicting various steps involved in the revival of frozen cells after three months of cryopreservation in liquid nitrogen (-196°C).*

The establishment of fibroblast cell lines that have a high rate of proliferation, lifespan, and genetic stability is crucial for the success of future applications (Sharma *et al.,* 2017). Thus, cells before and after thawing were compared for growth characteristics and *in-vitro* dynamics including their population-doubling time (Fig. 4.).

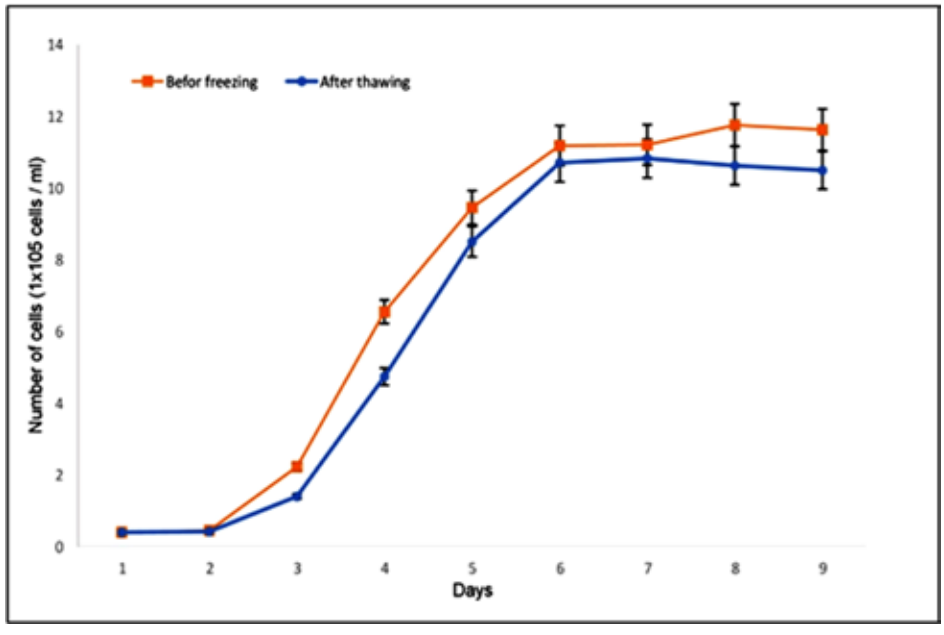

Fig. 4: *The growth curves depicting the proliferation of cells in culture before and after storage in liquid nitrogen. The values are mean ±SD of three independent observations.*

The growing cells followed normal S-shaped growth dynamics and the population doubling time of 24.54 to 31 hrs before and after freezing. It confirmed that the followed protocol ensured the ideal growth of cells during culture and also ensured cell survival during freezing and recovery.

The present endeavor added Punganur cattle to the somatic cell repository. Dwarf cattle breeds play a very important role in the context of sustainability because of their limited feed requirements. Their smaller body size, good grazing habits, adaptability to local conditions, resilience to diseases, and less feed requirement are beneficial to marginal or landless dairy farmers (Manjunath *et al.,* 2020). Hence it is essential to take immediate steps for the conservation of declining dwarf cattle breeds in India such as Punganur. The preserved cells are a storehouse of the viable diploid genome of Punganur cattle and can provide useful biomaterials for a wide variety of scientific studies (Groeneveld *et al.,* 2016). These include but are not limited to regenerating the animals by somatic cell nuclear transfer, tissue

engineering, induced pluripotent stem cell applications, production of transgenic or genome-edited animals, and wound healing research. The successful generation of Punganur cattle somatic cells and their conservation give impetus to the cryobanking of other Indigenous cattle breeds and livestock in need of conservation. The achievements also contribute to India's commitment to the United Nations Sustainable Development Goals (SDG).

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