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CHARACTERIZATION OF MESENCHYMAL STEM CELLS ISOLATED FROM FELINE AND CANINE ADIPOSE TISSUE

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Abstract

Multipotent mesenchymal stem cells (MMSCs) are a promising tool of regenerative medicine for treatment of various small animal diseases. MMSCs have a high proliferative activity, multipotent properties, low immunogenicity, as well as the ability to migrate to the damaged tissue and promote its healing and regeneration. Currently, the methods of regenerative medicine are actively developing to solve problems that are difficult to cope with alternative treatments. However, data on the use of these cells in the clinic are ahead of the study of the properties of these cells in culture. This paper is our first report on isolation cells with phenotype similar to mammalian multipotent mesenchymal stem cells from feline and canine adipose tissue. The aim of the presented research was to study the cells properties with a phenotype similar to MMSC isolated from feline and canine adipose tissue (AT) in vitro. Isolation of cells was achieved by mechanical and enzymatic treatments of the AT. For enzymatic dissociation, the tissues were treated with a 0.01 % collagenase type II solution based on DMEM-LG (PanEco, Russia) with low glucose (1 g/l) at 37 °C for 60 min. The comparative analysis of properties of the derived cellular populations is carried out. Cells isolated from feline and canine adipose tissue had similar morphological characteristics and were represented by two cellular types: small round cells and larger narrow spindle like fibroblast. They had a strong adhesion to cultural plastic and high colonies formation ability, 88.3±0.10 % for feline MMSCs and 88.0±0.15 % for canine MMSCs. The generation time of feline MMSCs was 34.6±0.02 h, while in canine MMSCs it was 50.0±0.01 h. Mitotic index of feline and canine MMSCs was 3.4 ‰ and 2.7 ‰, respectively. The ability of the MMSCs to induced osteo-, chondro- and adipogenic differentiation in vitro was demonstrated using StemPro® Osteogenesis Differentiation Kit, StemPro® Chondrogenesis Differentiation Kit и StemPro® Adipogenesis Differentiation Kit (Gibco, USA), respectively. Adipogenic differentiation accompanied by the appearance of rounded cells with lipid vesicles in the cytoplasm that were identified with the specific dye Oil red O. Specific staining of feline and canine MMSCs for endogenous alkaline phosphatase was positive on day 14 of culture in the induction medium. MMSCs stained by von Kossa revealed extracellular matrix formation on day 21 after induction. Alcian blue staining of cells cultured in chondrogenic medium for 21 days visualized formation of round structures with isogenic groups similar to the lacunae of hyaline cartilage. Thus, it was shown that cells isolated from feline and canine adipose tissue exhibit in culture the properties of MMSC. The derived cell cultures were propagated and deposited to Kovalenko VIEV Specialized Collection of somatic cell cultures of farm and commercial animals.

Keywords: multipotent mesenchymal stem cells, adipose tissue, culture, induced differentiation in vitro, feline, canine

Development of methods of stem cell derivation from animal tissues without a health damage, as well as their culture and storage provided wide use of these cells in various scientific branches. As laboratory models, the cell cultures used in cell biology, genetics, toxicology, virology, medicine and biotechnologies for a long time. In terms of veterinary virology, they are applicable to viral reproduction studies, di-

agnostic testings and production of various antiviral products. To this end, diploid cell cultures derived from animal organs/tissues and their fetuses are often used. These cell cultures have several disadvantages (including lack of standardization and short-term culture (up to 50 cytogenerations)). Continuous immortalized (immortal) cell lines are the most promising in this regard. However, their application is restricted with loss of tissue specificity resulted from long-term culture that leads to viral attenuation. Moreover, continuous cells isolated from farm animal tissues are typically contaminated with viruses. In light of this, multipotent mesenchymal stem cells (MMSCs) derived from animals can be considered as a new cell model that has several advantages over diploid and continuous cell cultures.

Mammalian MMSCs are promising tool to solve many veterinary, medical and biotechnological issues. Human MMSCs were detected in bone marrow (BM), adipose tissue (AT), skeletal muscles, placenta, umbilical blood and other tissues [1]. MMSCs have unique properties. They can maintain genome stability during self-renewal in vitro (> 50 cytogenerations) for a long time, and form bone, chondral and adipose cells in vitro during induced differentiation [2]. Previously, we isolated MMSCs from equine umbilical blood [3], as well as from bovine BM and AT [4]. MMSCs extracted from equine umbilical blood are suitable for equine infectious anemia virus study [3]. Small domestic animal MMSC cultures should be obtained, in particular, due to their promising application to manufacturing biotechnology of carnivore antiviral products (including vaccines to combat canine, mink, feline and fox parvoviral enteritis, distemper and, of course, rabies).

Nowadays, a great focus is placed on human MMSC biology in vitro studies. At the same time, the cells are regarded as a biomedical cell product (Federal Law no. 180-FZ 'On Biomedical Cell Products' dated 23.06.2016). It is generally believed that, in terms of cell therapy, MMSCs act as immunosuppressors [5]. According to reported data, similar studies involve small domestic animals, i. e., cats [6-9] and dogs [10, 11]. MMSCs represent promising cell material to treat osseous and chondral disorders (including osteoarthritis and herniated disks in cats and dogs) [12-14]. In this regard, we need a great number of cells obtained with their conversion to culture. The optimum culture conditions to provide efficient increase in cell mass are of primary concern. It was noted that current practical MMSC application, unfortunately, anticipates their studies in culture. In this view, these cell properties should be assessed beyond tissue/organ they were derived from. Preclinical studies of biomedical cell products are conducted in laboratory animals (i.e., mice or rats). In turn, this often complicates interpretation of a study findings. In this light, cats and dogs can be more suitable models, and their MMSC isolation can solve an issue related to pre-clinical study control. Isolation and study of MMSC properties in culture as per appropriate human cell standards enable development of large banks and national collections of certified stem cell cultures.

In the paper we derived canine and feline adipose cells demonstrating all the primary features of multipotent mesenchymal stem cells. Also, we obtained their cultures. Chemically induced cells differentiate into adipocytes, chondrocytes, and osteocytes. It was found that feline MMSC cultures have higher proliferative activity.

Our purpose was to isolate cells with a phenotype similar to multipotent mesenchymal stem cells from feline and canine adipose tissue in vitro, and to study their properties in culture.

Techniques. AT was collected in 8-month-cats (n = 3) and 1-year-dogs (n = 3) experienced ovariectomy as per the Declaration of Helsinki (World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, 1964-2013) in the veterinary clinic of Kovalenko All-Russian Research Institute of Experimental Veterinary. These samples

were shipped to a laboratory for < 30 min. Cells were isolated as per the previously described method (16). Finished inner AT sample (2-4 g) was carefully washed with PBS (PanEco, Russia) without Ca^{2+} and Mg^{2+} ions. Then, it was pulverized and subjected to enzymatic treatment with 0.01% collagenase type II solution based on DMEM LG (PanEco, Russia) with low glucose (1 g/l) at 37 °C for 60 min. Collagenase action was neutralized with equal volume of DMEM LG medium with 10% fetal bovine serum (FBS), and centrifuged at 1000 g for 10 min. Cells were washed twice in DMEM LG medium with antibiotics (final streptomycin and penicillin concentrations are 50 μg/ml and 50 U/ml, respectively). Further, it was precipitated with centrifugation at 800 g for 10 min.

After final precipitation we added DMEM LG to cell precipitate. It was resuspended carefully and filtered through cell grids (SPL Life Sciences Co., Ltd., Korea) on a step-by-step basis. At the first stage stromal vascular fraction (SVF) cells were collected with 70 μm grids. To select stem cells, we filtered SVF through 10 μm filters.

Counted in a Goryaev chamber (1.2×10^6), cells were placed in a culture flask with 25 cm^2 growth surface area. DMEM LG with 10% FBS (HyClone, Perbio Scientific, Belgium) and antibiotics (final streptomycin and penicillin concentrations are 50 μg/ml and 50 U/ml, respectively) was a primary MMSC growth culture medium. In 24 h the medium was replaced by a fresh one; adhesive cells were caused to grow in a CO_2-incubator (5% CO_2, 37 °C).

Duration of tested MMSCs' cell cycle was assessed in virtue of doubling time. G_0-pool was not considered. Growth rate of cellular populations was evaluated in virtue of cell number alteration until a monolayer was formed. Mean doubling time was calculated as per the formula as follows: $t_d = t/\log_2 (N_t/N_0)$, where t_d — doubling time, t — a period between an initial and a final cell countings, N_0 and N_t — initial and final cell count, respectively [17]. Mitotic index of each cellular population was calculated during the logarithmic phase. It is expressed as a ratio of generation number and total cell count multiplied by 1000 (‰).

Native and Romanowsky-Giemsa-stained cell morphology was evaluated visually (Axio Observer D.1 inverted phase-contrast microscope, Carl Zeiss, Germany, magnification ×100, ×200, ×630; AxioVision Rel. 4.8 software, Carl Zeiss, Germany).

Clone formation efficiency was assessed during cell plating (1.5×10^3) in culture flasks (25 cm^2). It was expressed as ratio of total cell count and number of clones formed on day 10 of culture.

Feline and canine ability to inductive differentiation into adipocytes, chondrocytes, and osteocytes in vitro was studied with StemPro® Osteogenesis Differentiation Kit, StemPro® Chondrogenesis Differentiation Kit and StemPro® Adipogenesis Differentiation Kit (Gibco, USA). In this regard, MMSCs were plated in 12-well plates (SPL Life Sciences Co., Ltd., Korea) (1×10^5 cells/well) at 2-3 passages. When cells reached 70-80% of a monolayer, we removed working medium and added induction media as per the manufacturer's guidelines. Induction media were replaced every 4 days for 21 days. Feline and canine MMSC differentiation was evaluated on day 14 and day 21. Cells were fixed with ice-cold methanol (–20 °C) for 10 min. Then, they were stained with specific colorants. All the colorants were produced by Sigma-Aldrich (USA). Cell alkaline phosphatase (AP) was assayed with Alkaline Phosphatase Kit (Sigma-Aldrich, USA). To detect phosphates and carbonates associated with the osteogenic differentiation of MMSCs, cells were stained by von Kossa method according to the reagent manufacturer's guidelines. During silvering fixed cells were treated with 2% aqueous

$AgNO_3$ solution for 10-15 min. Then, they were placed under direct lamp light (60 W) for 1 hour. Cells were washed with distilled water and treated with 2.5% aqueous sodium thiosulphate solution for 1 min. They were washed with tap water and put under a microscope (Axio Observer D.1, Carl Zeiss, Germany, magnification ×100, ×200, ×630). To assess chondrogenic MMSC differentiation, we used Alcian blue stain (LabPoint, Russia) staining mucopolysaccharides produced by chondral cells in extracellular matrix. Adipogenic differentiation efficiency was evaluated with Oil Red O staining to detect lipid inclusions in cell cytoplasm. Nuclei were counterstained with hematoxylin.

To perform statistical processing, we calculated arithmetic mean (*M*) and its standard error (±SEM). Difference significance was evaluated with Student's *t*-test ($p < 0.05$).

Results. MMSCs derived from canine and feline AT demonstrated strong adhesive properties. In 24 h after the isolation they were detected adherent to a culture flask bottom. This meets one of the minimum obligatory criteria for culture mammalian MMSCs [2]. Cell number increased on day 4 of culture. Cells derived from feline and canine AT had similar morphological properties (Fig. 1, A, B). Final cellular populations represented two following cell types: small round cells (⌀=10±0.2 μm, arrowed in the Figure) and larger (⌀=20±0.5 μm) narrow spindle ones with fibroblast-like morphological properties. A single cytogeneration took 34.6±0.02 h and 50±0.01 h in three feline and canine MMSC cultures, respectively. Feline and canine MMSC mitotic indices were 3.4‰ and 2.7‰, respectively (see the Table). Feline MMSCs formed a monolayer on day 12 (Fig. 1, C) of culture when 1×10^4 cells/cm^2 were plated; canine MMSCs formed a monolayer on day 15 under the same conditions (Fig. 1, D). According to the tabulated data, MMSCs derived from canine and feline AT showed high clone formation efficiency. Also, this verifies their affiliation with MMSCs.

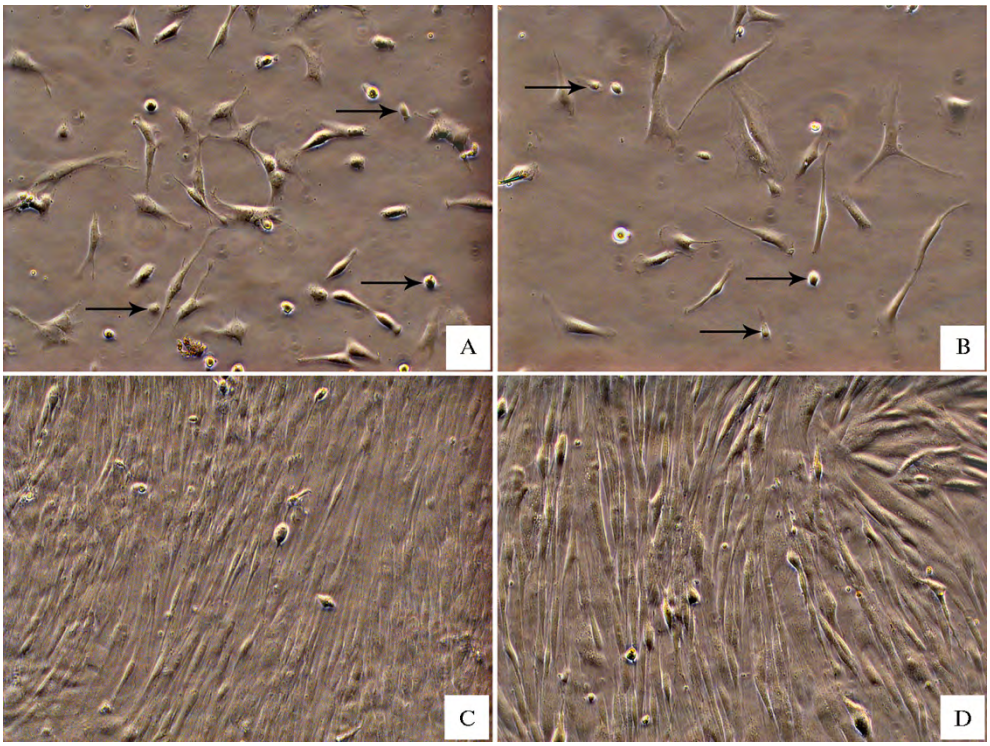

Fig. 1. Multipotent mesenchymal stem cell (MMSC) phenotype cells derived from small domestic animal adipose tissue on day 4 after isolation (A — feline MMSCs, B — canine MMSCs)**; on day 12 (mon-**

olayer) after isolation (C — feline MMSCs) **and on day 15 (monolayer) after isolation** (D — canine MMSCs**)**. Small round cells are arrowed. Native preparation, phase-contrast microscopy (microscope Axio Observer D.1, Carl Zeiss, Germany; magnification ×200).

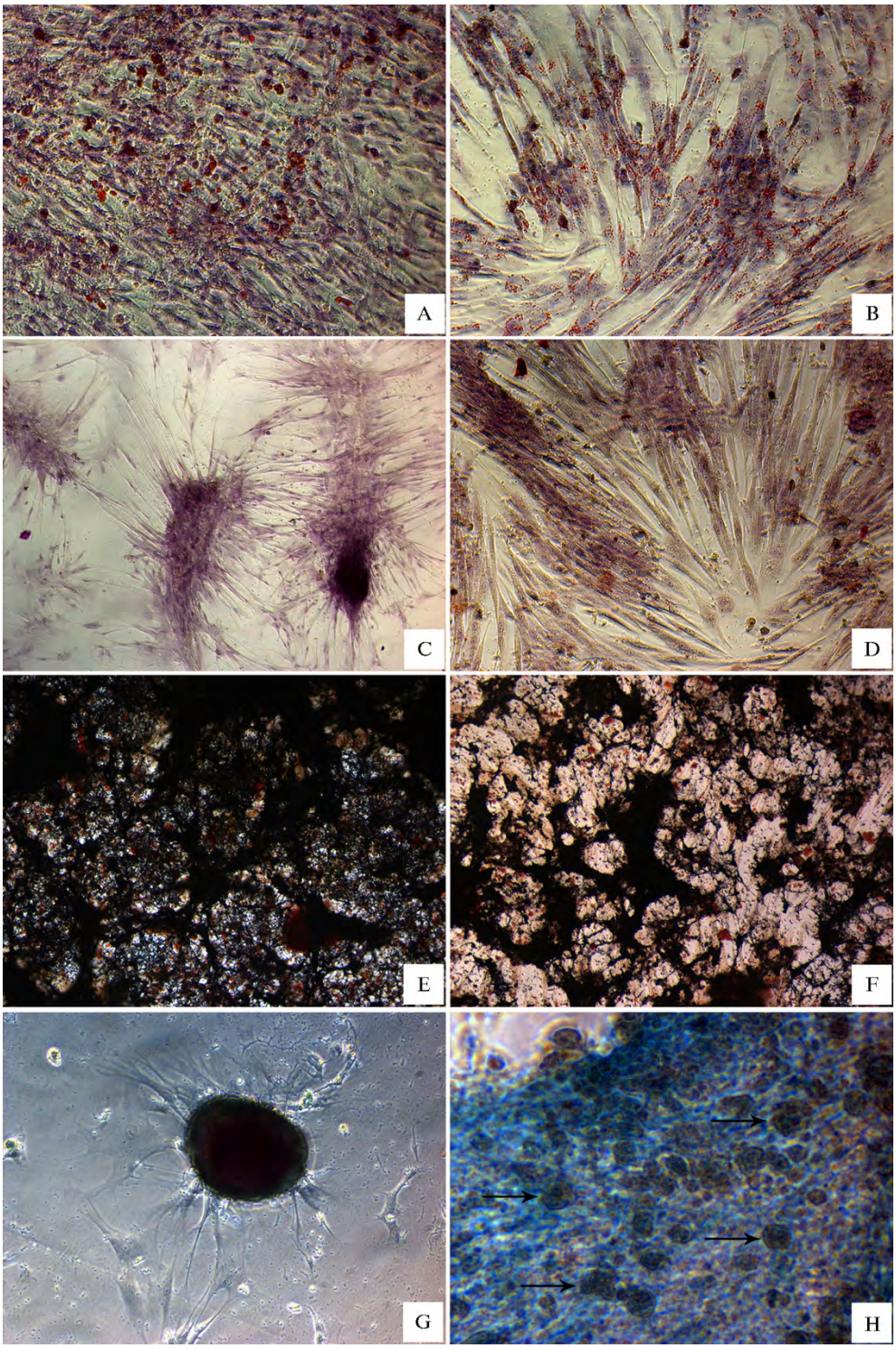

Fig. 2. Ability of induced multipotent mesenchymal stem cells (MMSCs) **derived from small domestic animals' adipose tissue to form adipose, osseous and chondral tissues:** A, B — feline and canine MMSCs, respectively, on day 21 of culture (Oil Red O staining, adipogenic medium); C, D — feline and canine MMSCs on day 14 of culture (alkaline phosphatase staining), E, F — feline and canine MMSCs on day

21 (von Kossa silvering) (osteogenic medium); G, H — feline and canine MMSCs on day 21 (Alcian blue staining, chondrogenic medium). Isogenic groups whose morphological properties are similar to hyaline cartilage lacunae are arrowed. Phase-contrast microscopy (microscope Axio Observer D.1, Carl Zeiss, Germany), magnification - ×200 (A, B, D, G), ×100 (C, E, F) and ×630 (H)..

Properties of multipotent mesenchymal stem cell (MMSC) **culture in vitro derived from feline and canine adipose tissue** (M±SEM, n = 3)

Cell culture	Sample	Metaphases/cell count	MI, ‰	Cytogeneration time, h	Clone formation efficiency, %
Feline MMSCs	1	34/1000	3.4	36.0±0.02	89.0±0.22
	2	33/1000	3.3	33.0±0.04	89.0±0.01
	3	36/1000	3.6	35.0±0.04	87.0±0.07
	Average	34.3/1000	3.4	34.6±0.02	88.3±0.10
Canine MMSCs	1	28/1000	2.8	50.0±0.01	88.0±0.40
	2	24/1000	2.4	52.0±0.02	87.0±0.01
	3	30/1000	3.0	48.0±0.06	89.0±0.05
	Mean	27.3/1000	2.7	50.0±0.01	88.0±0.15

Note. MI is mitotic index. Means (M) and their error (±SEM) of three independent tests are presented

Previously it was shown that MMSCs derived from feline [18-21] and canine [22, 23] AT are stained with fluorochrome-labeled antibodies (AB) to CD29 (β-1 integrin), CD44 (hyaluronic acid receptor), CD90 (Thy-1), CD105 (endoglin) and CD166 (ALCAM) antigens (AG), and are not stained with AB to AG markers of hematopoietic cells CD34 (sialomucin), CD45 (LCA, leukocyte common AG) and CD73 (5′-terminal nucleotidase)). In this case, we evaluated the cell affiliation with MMSCs according to their functional properties (i.e., in vitro induced adipose, osseous and chondral cell formation). Cells derived from feline and canine AT demonstrated morphological changes even in 14 days after induction of medium containing adipogenic growth factors. Adipocyte differentiation was accompanied by formation of round cells with lipid vesicles in cytoplasm which were detected with a specific Oil Red O staining (Fig. 2, A, B).

It is a known fact that a direct relationship is seen between AP cell level and osteogenic differentiation [16]. Feline and canine MMSC staining for specific activity of endogenous AP demonstrated positive results even on day 14 of culture in the induction medium (Fig. 2, C, D). In 21 days after the induction (von Kossa silvering) we detected extracellular matrix in vitro as indicated by black coloration of phosphates and carbonates (Fig. 2, E, F). This evidences an ability of isolated MMSCs (i.e., both types) to osteogenic differentiation in vitro.

Round structures stained with Alcian blue were observed after 21-day cell culture in the chondrogenic medium (Fig. 2, G). Mature chondroblasts start to produce chondral intercellular matrix containing mucopolysaccharides [24] that turn blue with the stain. Although cells could be hardly differentiated morphologically in preparations, isogenic groups whose morphological properties are similar to hyaline cartilage lacunae were visualized (Fig. 2, 3). Thus, MMSCs derived from feline and canine inner AT can form cells similar to osseous, adipose and chondral ones after induced differentiation in vitro. Our findings comply with other reported data [25-27]. Owing to maintained functional abilities in culture, we can assume that feline and canine MMSCs can be a promising material to treat various osseous and chondral disorders in small domestic animals. Our results improve our knowledge concerning cell behavior in culture. So, we can take a step closer to further understanding of processes occurring in progenitor cells in vitro.

Therefore, we obtained multipotent mesenchymal stem cell (MMSC) cultures isolated from feline and canine adipose tissue (AT). These isolated cells exhibit all the primary MMSC properties such as strong adhesion to plastic surface, high clone-forming ability (88.3±0.10% for feline MMSCs vs. 88.0±0.15%

for canine MMSCs), as well as differentiation into adipocytes, chondrocytes, and osteocytes after chemical induction in vitro. Comparative analysis of feline and canine MMSCs revealed insignificant differences. Despite similar morphological properties of cell cultures, a single MMSC cytogeneration took 34.6±0.02 h and 50.0±0.01 h in cats and dogs, respectively; mitotic index is 3.4‰ and 2.7‰, respectively, indicating higher proliferative activity of feline MMSC cultures. The derived cell cultures were propagated and deposited to Kovalenko VIEV Specialized Collection of continuous somatic cell cultures of farm and commercial animals.

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