Cell starvation increases uptake of extracellular Thymosin β4 and its complexes with calcium

Marco Piludu[a], Giuseppina Pichiri[b], Pierpaolo Coni[b], Monica Piras[b], Terenzio Congiu[b], Gavino Faa[b], and Joanna Izabela Lachowicz[*,b]

[a] *Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy*

[b] *Department of Medical Sciences and Public Health, University of Cagliari, Cagliari, Italy*

Keywords: Thymosin β4; calcium; cell starvation; tumour metastasis; immunostaining

Abstract

Cell metastasis is the main cause of cancer mortality. Inhibiting early events during cell metastasis and invasion could significantly improve cancer prognosis, but the initial mechanisms of cell transition and migration are barely known. Calcium regulates cell migration, whilst Thymosin β4 is a G-actin and iron binding peptide associated with tumor metastasis and ferroptosis. Under normal cell growth conditions, intracellular free calcium ions and Thymosin β4 concentrations are strictly regulated, and are not influenced by extracellular supplementation. However, cell starvation decreases intracellular Thymosin β4 and increases extracellular peptide uptake above the normal range. Unexpectedly, cell starvation significantly increases internalization of extracellular Ca^{II}/Thymosin β4 complexes. Elucidating the role of Ca^{II}/Thymosin β4 in the early events of metastasis will likely be important in the future to develop therapies targeting metastasis.

Introduction

Tissue invasion and metastasis are one of the six early features of cancer[1]. Metastatic dissemination of cells escaping the primary tumour is the main cause of cancer mortality[2]. Plasticity in cancer cells allows them to adapt to their demanding environment and maximize proliferation, employing strategies to tolerate stressful conditions such as high levels of reactive oxygen species[3] (ROS). Nevertheless, prolonged stress due to nutrient limitation, chemotherapy, severe hypoxia and ROS can initiate migration of starving cells[4].

Metastasis is a highly ineffective process. Most metastatic cells which enter the blood or lymphatic system do not survive[5, 6]. Recent studies have shown that translation reprogramming supresses anoikis and increases resistance to oxidative stress, possibly playing a key role in enhancing cell survival in the bloodstream and metastatic colonization of visceral organs[7].

Human Thymosin β4 (Tβ4) (Figure 1) is a G-actin binding peptide[8], with numerous functions in the human body including blood clotting, tissue regeneration[9], angiogenesis[10] and tumour metastasis[11, 12]. Tβ4 mRNA is expressed in different tissues, with the highest expression in those tissues with elevated concentrations of calcium, iron, copper and zinc ions[13]. Of note, Tβ4 expression is enhanced in tumour tissue[13]. The exact biochemistry of Tβ4 is unknown, and the binding of the G-actin protein cannot itself explain the multi-activity of Tβ4. Recent studies showed that Tβ4 is an iron chelator, involved in iron homeostasis and iron-related oxidative stress, thus inhibiting ferroptosis in cancer cells[14].

The specific Tβ4 functions are localized in distinct amino-acid-specific sequence of the peptide. The region comprising 1-4 amino acids (Ac-SDKP) peptide has antiinflammation, angiogenesis, and antifibrotic functions; 1-15 peptide protects against cytotoxicity of some agents and has antiapoptotic activity; while 17-22 peptide with actin-binding motif induces exocytosis and angiogenesis, and stimulates dermal wound repair and hair growth[15]. Of note, all this process are strictly correlated with calcium homeostasis, but the calcium/Tβ4 interactions has never been studied.

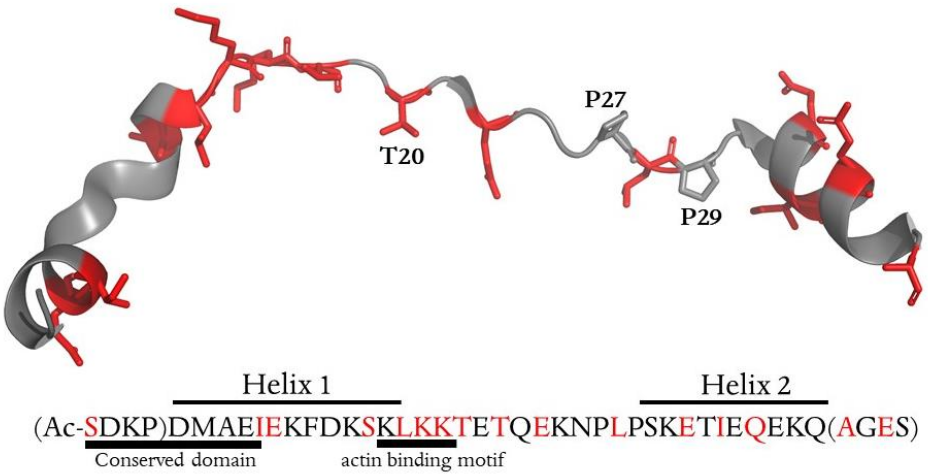

Figure 1. X-ray structure of Tβ4 extrapolated from the complex with actin (PDB: 4PL7). Residues potentially involved in calcium binding (based on preliminary NMR data) are marked in red. Amino acids in parentheses were not defined by the X-ray structure.

Calcium is a ubiquitous second messenger and regulator of cell migration[16]. Increased intracellular calcium concentration (as a result of Ca^{II} entry from extracellular space and/or liberation from intracellular sources) can initiate cell migration, but the sources of calcium and the mechanism by which it modulates this process in cancer cells are only now beginning to be understood[16]. Calcium signalling pathways play an important role in stem cell transition [17, 18], tissue development [19] and healing [20, 21] processes. However, the influence of different cell starvation conditions in those events is less known, respect to its role in the cancer metastasis.

Here, we discuss for the first time the biological importance of calcium complex with Tβ4 and its potential role in cell starvation. We observed that cell starvation increased uptake of extracellular Tβ4 and Tβ4 complex with calcium ions. The augmented intracellular concentration of both Tβ4 and calcium ions could be an initial signal for cell migration under starvation conditions, and targeting this pathway could be a future therapeutic strategy.

Results

Given the high expression level of Tβ4 and calcium dyshomeostasis in cancer cell lines[22-24], we used in experiments the non-cancerous human embryonic kidney cells (293T). Among different cell starvation conditions, serum deprivation was chosen as the best model stress, and an inducer of cell adaptation and survival mechanisms[25]. The use of non-cancerous cells gave a unique and rewarding possibility to observe processes, which could occur at early stages of malignancy, rather than enhancing already altered biochemical pathways in the tumor cells.

To track intracellular Tβ4, we used immunostaining with primary anti-Tβ4 antibodies and secondary fluorescent antibodies. Fluorescent Fura-2 was selected for intracellular calcium labelling, in accordance with previous studies[26]. Fura-2 forms stable green fluorescent complexes with free calcium ions. The dissociation constant (K_d) of Fura-2 for Ca^{II} was determined to be 285 nM at 37°C, utilizing the value of K'_{CaEGTA} adjusted by the method of Harrison and Bers[27]. This strategy permitted us to label both free calcium ions in combination with red fluorescence labelling of Tβ4 with secondary antibodies.

The endogenous intracellular Tβ4 ($[T\beta4]_i$) concentration in 293T cells was decreased in cells growing under starvation conditions (Figure 3B) respect to the ($[T\beta4]_i$) concentration in the cells growing under normal conditions (Figure 2B). Addition of calcium ions into the growth medium did not change intracellular Tβ4 concentration under normal conditions (Figure 2F), nor under starvation (Figure 3F). However, supplementation of cell growth medium with extracellular Tβ4 increased

$[T\beta4]_i$ concentration slightly under normal conditions (Figure 2J) and significantly under starvation conditions (Figure 3J).

Free $[Ca^{II}]_i$ ions were not present in cells growing under normal (Figure 2A) or starvation conditions (Figure 3A), as measured by Fura-2 staining, with the exception of cells growing both under starvation conditions and supplemented with Ca^{II}/Tβ4 complexes (Figure 3M). The cytoplasmic localization of calcium ions (the nucleus localization marked in blue with DAPI) was superimposable with the cytoplasmic localization of Tβ4 (Figure 3P) suggesting internalization of Ca^{II}/Tβ4 complexes, rather than free calcium ions and free Tβ4 separately. The positive Fura-2 staining upon complex formation with calcium ions, suggest low stability of Ca^{II}/Tβ4 complexes.

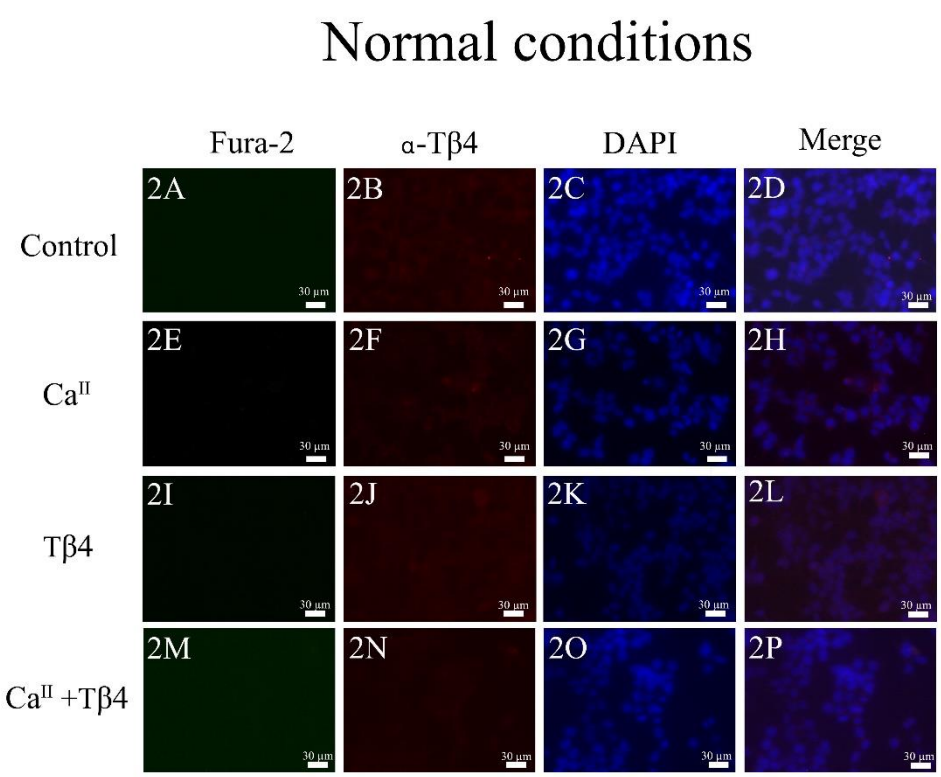

Figure 2. Qualitative extracellular Tβ4 and calcium levels under normal conditions. Tβ4 was labelled with fluorescent immunostaining (red), while free calcium was measured with Fura-2 (green). DAPI (blue) fluorescent marker was used for the nucleus staining.

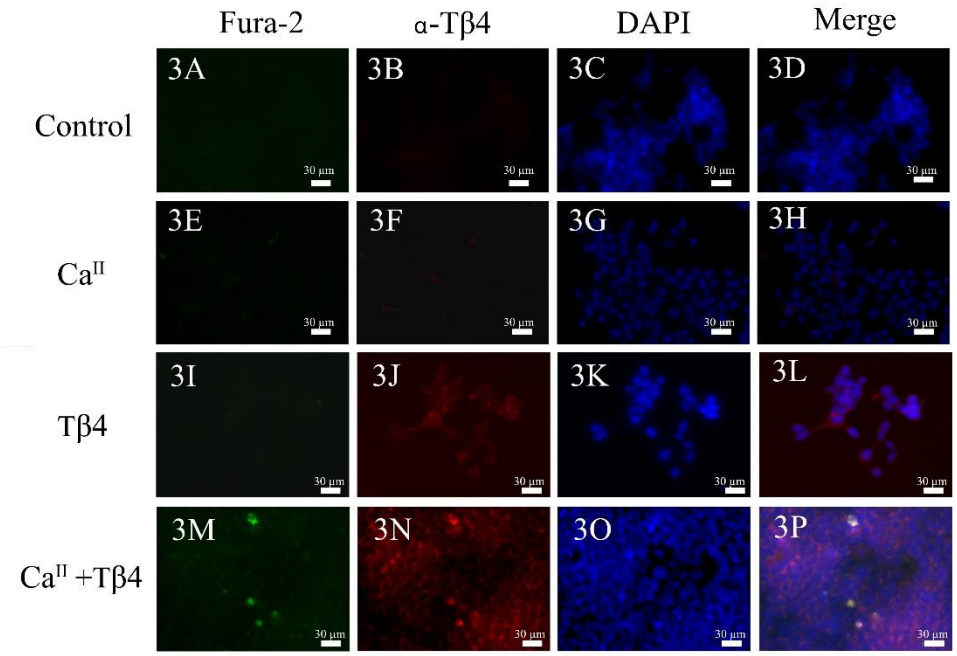

Figure 3. Qualitative extracellular Tβ4 and calcium levels under starvation conditions. Tβ4 was labelled with fluorescent immunostaining (red), while free calcium was measured with Fura-2 (green). DAPI (blue) fluorescent marker was used for the nucleus staining.

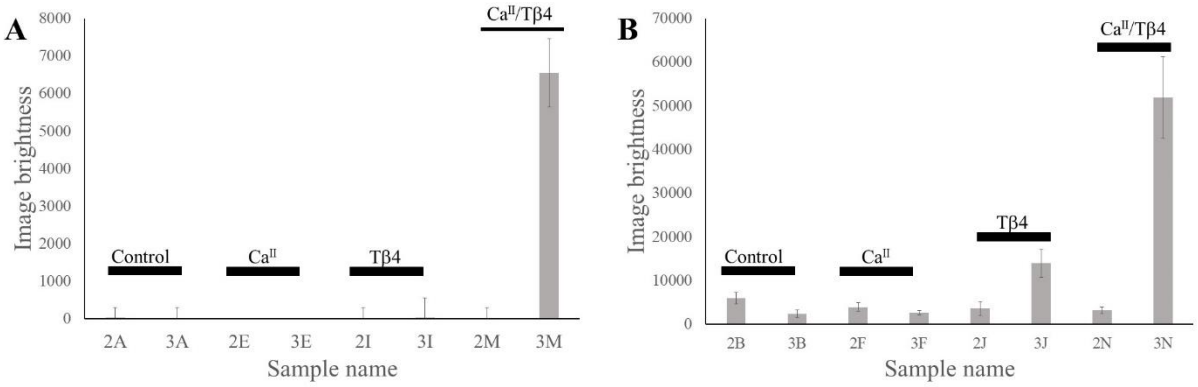

Figure 4. Quantitative analysis of Tβ4 and calcium labelling. The image brightness (Figures 2 and 3) was measured using ImageJ[28] (according to the procedure described in reference[29])and is plotted for free calcium ions (3A) and Tβ4 (3B). Statistical comparison of the results in the different conditions was performed, with 5.0 GraphPad Prism software, by the unpaired t-test. The data were analysed also with one-way ANOVA as well as the multicomparison Tukey's test. P values < 0.05 was considered significant

Discussion

New molecular clinical test are now available[30, 31] and new biological approach are able to expand the number of diagnostic strategies[32, 33].

Intracellular calcium ($[Ca^{II}]_i$) is a key cell migration regulator, which activates transcription of proliferation and motility genes. Altered calcium signaling, cell proliferation and migration, and decreased apoptosis contributes to metastasis[16] and drug-resistance[34]. Thymosin β4 actively contributes to all these processes[35], but the biochemical pathways are still not defined.

At the subcellular level, Tβ4 is localized in the cytoplasm and nucleus, and can translocate between compartments in some conditions[13]. For instance, starvation and other stress conditions alter the expression of Tβ4 in HepG2 cells (2-fold increase of mRNA expression respect to the normal conditions) and lead to peptide translocation from the cytoplasm into the nucleus[36-38].

Extracellular Tβ4 is released by cells and found in large amounts in extracellular fluids. *In vitro* cellular experiments (under normal conditions) showed that extracellular Tβ4 is internalized rapidly, even in the range of minutes, and promotes cell migration, proliferation, apoptosis and inflammation[26]. The recent studies by Yoon et al.[39] showed that not only wildtype Tβ4, but also its derived fragments (1-15; 12-26; 23-43 peptides) including those without an actin binding motif, stimulate migration and invasion of SKOV ovarian cancer cells in the serum deprived medium (as a migration and invasion stimuli).

In pregnant rats, Tβ4 treatment during gestational time increases the fetal growth, and accelerates the development of newborn[40].

Under the normal cell-growth conditions, the wildtype Tβ4, as well as mutants deprived of the N-terminal acetylated tetrapeptide AcSDKP or the acting-binding region KLKKTET, enhance endothelial cell migration without increasing ($[Ca^{II}]_i$) concentration[26]. However, the contradictory data, which show Tβ4- and AcSDKP-stimulation of $[Ca^{II}]_i$ increase from intracellular Ca^{II} pool, can also be found[41].

The data presented here show the first time that cell starvation conditions decrease intracellular Tβ4 concentration, which can be restored by extracellular Tβ4 supplementation. The concentration of free intracellular calcium ions is too low to be detected by Fura-2 staining in cells growing under normal and starvation conditions. The supplementation of cell growing medium with free calcium ions or Tβ4 (both under normal and starvation conditions) do not increase the free $[Ca^{II}]_i$. Unexpectedly, cell starvation significantly increases internalization of extracellular Ca^{II}/Tβ4 complexes.

The low stability of Ca^{II}/Tβ4 complexes supports the hypothesis that Tβ4 could be calcium transporter, moving Ca^{II} inside the cell under starvation conditions, rather than sequestering metal ions.

The increase of intracellular calcium and Tβ4 activates a number of signaling processes, which lead to metastasis[16] and resistance to ferroptotic oxidative stress[14]. Further studies on the calcium-trafficking activity of Tβ4 could help to decipher the biochemical pathways of cell proliferation and migration under starvation conditions.

Conclusions

New biological approach are necessary in order to expand the number of clinical diagnostic tests. Understanding invasiveness signals and why some cells are endowed with a greater capacity to initiate metastases than others is of significant clinical relevance. Calcium ions and Thymosin β4 peptide increase cell migration, proliferation, and resistance to stress conditions. Increased uptake of Tβ4 and its calcium complexes under starvation conditions could be an initial signal for cell invasion. Further understanding the Ca^{II}/Tβ4 pathway will likely be an important step towards controlling metastases and switching off their pathological functions.

Methods

Reagents

Thymosin β4 was obtained from Regene RX, Rockville, MA, USA. anti-Thymosin β4 antibody was from Peninsula Laboratories, LLC and goat anti-rabbit IgG conjugated with Alexa Fluor 594 was from Invitrogen. DAPI dye was obtained from Sigma Aldrich.

Cell culture

Commercial 293T human kidney embryonic cells (catalog code HTL03003) were obtained from the Istituto Nazionale per la Ricerca sul Cancro c/o CBA (ICLC, Genova). The cell cultures were prepared following previously described procedures [42]. After 24 h of growth with the complete medium, the cells were grown under different experimental conditions with and without bovine serum: $CaCl_2$, Thymosin β4 (final concentration 10 μM), Thymosin β4 with calcium chloride (10 μM; 1:1 molar ratio). All solutions were fresh and daily prepared.

The final 10 μM Tβ4 concentration was chosen on the base of cell viability and morphology data in relation to different peptide concentrations [43].

Fluorescence microscopy

A ZOE Live Imaging System was used for cell imaging in variable fluorescence wavelengths. The Objective 20x plan achromatic (Numerical aperture 0.40) and Camera: Monochrome camera, 12 bit CMOS, 5 megapixels were used for image data acquisition. Image acquisition software ZOE Fluorescent Cell Imager Galileo Stand-alone Android operating system was used. Each data image was 2592x1944 px resolution. The adherence cell culture was fixed with methanol before the fluorescence staining. ImageJ[28] software was used to quantify the fluorescence intensity according to the procedure described in reference[29]. Each experiment was repeated 3 times for the statistical analysis. For each experiment, 3 images were taken, and fluorescence was quantified.

Immunostaining

For immunocytochemistry cells on coverslips were pre-treated with normal goat serum for 30 min. Cells were then incubated for 60 min at room temperature with the commercial validated rabbit anti-Thymosin β4 antibody, diluted 1:200 (Peninsula Laboratories, LLC; https://www.bma.ch/antibodies?q=thymosin). Then the cells were washed three times with PBS and subsequently incubated for 1 h with a goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594; Invitrogen; ref. A11012; https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11012) diluted 1:500. Nuclei were counterstained with

DAPI Dye (Sigma Aldrich). Negative controls were routinely performed for each experiment, incubating the samples with non-immune serum and then with an appropriate secondary antibody.

Fura-2 calcium staining

Intracellular Ca^{II} was measured with the Fura-2 AM Calcium Assay kit (Sigma Aldrich) according to the manufacturer's protocols. Cells were plated in different experimental conditions and after 24 h the medium was removed, and the cells were gently washed two times with PBS and loaded with Flura-2 AM (1 μg/μL) in 1 mL cell culture medium. Incubation continued for 30 min at 37 °C/5% CO_2. After incubation the dye was removed, and the cells were washed twice with PBS.

References

[1] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100(1) (2000) 57-70.

[2] R.L. Siegel, K.D. Miller, H.E. Fuchs, A. Jemal, Cancer statistics, 2021, CA: a cancer journal for clinicians 71(1) (2021) 7-33.

[3] C.A. Aktipis, A.M. Boddy, R.A. Gatenby, J.S. Brown, C.C. Maley, Life history trade-offs in cancer evolution, Nature Reviews Cancer 13(12) (2013) 883-892.

[4] C. García-Jiménez, C.R. Goding, Starvation and pseudo-starvation as drivers of cancer metastasis through translation reprogramming, Cell metabolism 29(2) (2019) 254-267.

[5] Y.S. Chang, E. di Tomaso, D.M. McDonald, R. Jones, R.K. Jain, L.L. Munn, Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood, Proceedings of the National Academy of Sciences 97(26) (2000) 14608-14613.

[6] Y. Kang, K. Pantel, Tumor cell dissemination: emerging biological insights from animal models and cancer patients, Cancer cell 23(5) (2013) 573-581.

[7] I.S. Harris, A.E. Treloar, S. Inoue, M. Sasaki, C. Gorrini, K.C. Lee, K.Y. Yung, D. Brenner, C.B. Knobbe-Thomsen, M.A. Cox, Glutathione and thioredoxin antioxidant pathways synergize to drive cancer initiation and progression, Cancer cell 27(2) (2015) 211-222.

[8] M.C. Sanders, A.L. Goldstein, Y.-L. Wang, Thymosin beta 4 (Fx peptide) is a potent regulator of actin polymerization in living cells, Proceedings of the National Academy of Sciences 89(10) (1992) 4678-4682.

[9] K.M. Malinda, H.K. Kleinman, G.S. Sidhu, H. Mani, K. Banaudha, R.K. Maheshwari, A.L. Goldstein, Thymosin β4 accelerates wound healing, Journal of Investigative Dermatology 113(3) (1999) 364-368.

[10] D.S. Grant, W. Rose, C. Yaen, A. Goldstein, J. Martinez, H. Kleinman, Thymosin β4 enhances endothelial cell differentiation and angiogenesis, Angiogenesis 3(2) (1999) 125-135.
[11] M.-C. Tang, L.-C. Chan, Y.-C. Yeh, C.-Y. Chen, T.-Y. Chou, W.-S. Wang, Y. Su, Thymosin beta 4 induces colon cancer cell migration and clinical metastasis via enhancing ILK/IQGAP1/Rac1 signal transduction pathway, Cancer letters 308(2) (2011) 162-171.
[12] J.-W. Lee, Y.-K. Ryu, Y.-H. Ji, J.H. Kang, E.-Y. Moon, Hypoxia/reoxygenation-experienced cancer cell migration and metastasis are regulated by Rap1-and Rac1-GTPase activation via the expression of thymosin beta-4, Oncotarget 6(12) (2015) 9820.
[13] J.I. Lachowicz, M. Jaremko, L. Jaremko, G. Pichiri, P. Coni, M. Piludu, Metal coordination of thymosin β4: Chemistry and possible implications, Coordination Chemistry Reviews 396 (2019) 117-123.
[14] J.I. Lachowicz, G. Pichiri, M. Piludu, S. Fais, G. Orrù, T. Congiu, M. Piras, G. Faa, D. Fanni, G. Dalla Torre, Thymosin β4 Is an Endogenous Iron Chelator and Molecular Switcher of Ferroptosis, International Journal of Molecular Sciences 23(1) (2022) 551.
[15] G. Sosne, P. Qiu, A.L. Goldstein, M. Wheater, Biological activities of thymosin ß4 defined by active sites in short peptide sequences, The FASEB Journal 24(7) (2010) 2144-2151.
[16] N. Prevarskaya, R. Skryma, Y. Shuba, Calcium in tumour metastasis: new roles for known actors, Nature Reviews Cancer 11(8) (2011) 609-618.
[17] F.M. Tonelli, A.K. Santos, D.A. Gomes, S.L.d. Silva, K.N. Gomes, L.O. Ladeira, R.R. Resende, Stem cells and calcium signaling, Calcium signaling (2012) 891-916.
[18] A. Reali, M. Puddu, M.C. Pintus, M.A. Marcialis, G. Pichiri, P. Coni, D. Manus, A. Dessì, G. Faa, V. Fanos, Multipotent stem cells of mother's milk, Journal of Pediatric and Neonatal Individualized Medicine (JPNIM) 5(1) (2016) e050103-e050103.
[19] S.E. Webb, A.L. Miller, Calcium signalling during embryonic development, Nature Reviews Molecular Cell Biology 4(7) (2003) 539-551.
[20] A.B. Lansdown, Calcium: a potential central regulator in wound healing in the skin, Wound repair and regeneration 10(5) (2002) 271-285.
[21] T. Subramaniam, M.B. Fauzi, Y. Lokanathan, J.X. Law, The role of calcium in wound healing, International Journal of Molecular Sciences 22(12) (2021) 6486.
[22] E. Hannappel, G.-J. Xu, J. Morgan, J. Hempstead, B. Horecker, Thymosin beta 4: a ubiquitous peptide in rat and mouse tissues, Proceedings of the National Academy of Sciences 79(7) (1982) 2172-2175.
[23] S. Farsinejad, Z. Gheisary, S. Ebrahimi Samani, A.M. Alizadeh, Mitochondrial targeted peptides for cancer therapy, Tumor Biology 36(8) (2015) 5715-5725.

[24] W.J. Lee, G.R. Monteith, S.J. Roberts-Thomson, Calcium transport and signaling in the mammary gland: targets for breast cancer, Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 1765(2) (2006) 235-255.

[25] E.Z. White, N.M. Pennant, J.R. Carter, O. Hawsawi, V. Odero-Marah, C.V. Hinton, Serum deprivation initiates adaptation and survival to oxidative stress in prostate cancer cells, Scientific reports 10(1) (2020) 1-18.

[26] A. Selmi, M. Malinowski, W. Brutkowski, R. Bednarek, C.S. Cierniewski, Thymosin β4 promotes the migration of endothelial cells without intracellular Ca2+ elevation, Experimental cell research 318(14) (2012) 1659-1666.

[27] D. Groden, Z. Guan, B. Stokes, Determination of Fura-2 dissociation constants following adjustment of the apparent Ca-EGTA association constant for temperature and ionic strength, Cell calcium 12(4) (1991) 279-287.

[28] (https://imagej.nih.gov/ij/).

[29] (https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html).

[30] P. Coni, A. Mateddu, L. Kuqi, G. Pichiri, A. Occhinegro, D. Ratto, G. Orrù, LncRNA colon cancer-associated transcript 1 (CCAT1) in ovarian cancer, (2018).

[31] F. Medas, P. Coni, F. Podda, C. Salaris, F. Cappellacci, G. Faa, P.G. Calò, Evaluation of accuracy of one-step nucleic acid amplification (OSNA) in diagnosis of lymph node metastases of papillary thyroid carcinoma. Diagnostic study, Annals of Medicine and Surgery 46 (2019) 17-22.

[32] A.H. Emwas, M. Alghrably, M. Dhahri, A. Sharfalddin, R. Alsiary, M. Jaremko, G. Faa, M. Campagna, T. Congiu, M. Piras, M. Piludu, G. Pichiri, P. Coni, J.I. Lachowicz, Living with the enemy: from protein-misfolding pathologies we know, to those we want to know, Ageing Res Rev 70 (2021) 101391.

[33] C. Gerosa, G. Faa, D. Fanni, M. Manchia, J. Suri, A. Ravarino, D. Barcellona, G. Pichiri, P. Coni, T. Congiu, Fetal pro-gramming of COVID-19: May the barker hypothesis explain the susceptibility of a subset of young adults to develop severe disease, Eur. Rev. Med. Pharmacol. Sci 25 (2021) 5876-5884.

[34] D. Büsselberg, A.-M. Florea, Targeting intracellular calcium signaling ([Ca2+] i) to overcome acquired multidrug resistance of cancer cells: a mini-overview, Cancers 9(5) (2017) 48.

[35] A.L. Goldstein, Thymosin β4: a new molecular target for antitumor strategies, Oxford University Press, 2003, pp. 1646-1647.

[36] M. Piludu, M. Piras, G. Pichiri, P. Coni, G. Orrù, T. Cabras, I. Messana, G. Faa, M. Castagnola, Thymosin beta 4 may translocate from the cytoplasm in to the nucleus in HepG2 cells following serum starvation. An ultrastructural study, PloS one 10(4) (2015) e0119642.
[37] G. Pichiri, P. Coni, S. Nemolato, T. Cabras, M.U. Fanari, A. Sanna, E. Di Felice, I. Messana, M. Castagnola, G. Faa, Cellular trafficking of thymosin beta-4 in HEPG2 cells following serum starvation, PloS one 8(8) (2013) e67999.
[38] P. Coni, M. Piras, A. Mateddu, M. Piludu, G. Orru, A. Scano, T. Cabras, V. Piras, J.I. Lachowicz, M. Jaremko, Thymosin β4 cytoplasmic/nuclear translocation as a new marker of cellular stress. A Caco2 case study, RSC advances 10(21) (2020) 12680-12688.
[39] H.J. Yoon, Y.L. Oh, E.-J. Ko, A. Kang, W.K. Eo, K.H. Kim, J.Y. Lee, A. Kim, S. Chun, H. Kim, Effects of thymosin β4-derived peptides on migration and invasion of ovarian cancer cells, Genes & Genomics 43(8) (2021) 987-993.
[40] G. Faa, M. Piras, L. Mancuso, P. Coni, G. Pichiri, G. Orrù, D. Fanni, C. Gerosa, G. Cao, R. Taibi, Thymosin beta-4 prenatal administration improves fetal development and halts side effects due to preterm delivery, (2021).
[41] W.Q. Huang, B.H. Wang, Q.R. Wang, Thymosin β4 and AcSDKP inhibit the proliferation of HL-60 cells and induce their differentiation and apoptosis, Cell biology international 30(6) (2006) 514-520.
[42] J.I. Lachowicz, V.M. Nurchi, G. Crisponi, I. Cappai, R. Cappai, M. Busato, A. Melchior, M. Tolazzi, M. Peana, E. Garribba, para-Aminosalicylic acid in the treatment of manganese toxicity. Complexation of Mn 2+ with 4-amino-2-hydroxybenzoic acid and its N-acetylated metabolite, New Journal of Chemistry 42(10) (2018) 8035-8049.
[43] J.I. Lachowicz, G. Pichiri, M. Piludu, S. Fais, G. Orru, T. Congiu, M. Piras, G. Faa, D. Fanni, G. Dalla Torre, X. Lopez, K. Chandra, K. Szczepski, L. Jaremko, M. Ghosh, A.H. Emwas, M. Castagnola, M. Jaremko, E. Hannappel, P. Coni, Thymosin beta4 Is an Endogenous Iron Chelator and Molecular Switcher of Ferroptosis, Int J Mol Sci 23(1) (2022).