Molecular phenotyping uncovers differences in basic housekeeping functions among closely related species of hares (*Lepus* spp., Lagomorpha: Leporidae)

**Running title: Molecular-level species differences among hares**

Kateryna Gaertner1‡, Craig Michell2,3‡, Riikka Tapanainen2, Steffi Goffart2, Sina Saari1, Manu Soininmäki2, Eric Dufour1‡ and Jaakko L. O. Pohjoismäki2†‡

†Corresponding author: jaakko.pohjoismaki@uef.fi

‡These authors contributed equally

1Mitochondrial Bioenergetics and Metabolism, Faculty of Medicine and Health Technology, FI-33014 Tampere University, Finland

2Department of Environmental and Biological Sciences, FI-80101 University of Eastern Finland, Finland

3Red Sea Research Center, Division of Biological and Environmental Science and Engineering, King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia

# Abstract

Speciation is a fundamental evolutionary process, which results in genetic differentiation of populations and manifests as discrete morphological, physiological and behavioral differences. Each species has had its own evolutionary trajectory, formed by many types of selection pressures and random drift, making the association of genetic differences between the species with the phenotypic differences extremely difficult. In the present study, we have used an *in vitro* model to analyze in depth the genetic and gene regulation differences between fibroblasts of two closely related mammals, the arctic/subarctic mountain hare (*Lepus timidus* Linnaeus) and the temperate steppe-climate adapted brown hare (*Lepus europaeus* Pallas). We discovered the existence of a species-specific expression pattern of 1,623 genes, manifesting in differences in cell growth, respiration, and metabolism. Interspecific differences in the housekeeping functions of fibroblast cells suggest speciation acts on fundamental processes, even in these two interfertile species. Our results help to understand the molecular constituents of a species difference on cellular level, which could contribute to the maintenance of the species boundary.

**Keywords**

Speciation; transcriptome; genome; metabolism; mammal; evolution.

# Introduction

Species are the central units for taxonomy, evolution and biodiversity research. Species are commonly defined by emphasizing reproductive isolation between them, also known as the biological species concept (Dobzhansky, 1937; Mayr, 1942). Although the concept is not fully comprehensive regarding e.g., asexual organisms and the many examples of gene flow across the species barrier (Abbott et al., 2013; Chan & Levin, 2005; Giska et al., 2019; Hamilton & Miller, 2016; Harrison & Larson, 2014; Hedrick, 2013; Mallet, Besansky, & Hahn, 2016; Soubrier et al., 2016; Todesco et al., 2016; Wolf, Lindell, & Backstrom, 2010), it remains a valid generalization for most animal species. Species are products of evolutionary processes, which result in genetic differentiation of originally similar populations of a common ancestor (Wolf et al., 2010). When given enough time, this differentiation will contribute to the formation of a reproductive barriers maintaining the species separation.

While random genetic drift operates in all populations and contributes to the phenotypic differences between isolated populations, natural selection is a more potent driver of differentiation through local adaptation and specialization (Arnegard et al., 2014; Schluter & Conte, 2009; Wolf et al., 2010). If the two species are genetically incompatible or their hybrids maladapted, natural selection can reinforce the reproductive barrier by favoring individuals that are accurate in avoiding pairing with the wrong species. However, the situation can be more complex in nature. For example, in the Nordic countries an expansive species, the (European) brown hare (*Lepus europaeus* Pallas, Lagomorpha: Leporidae), is gradually establishing itself in regions formerly dominated by the mountain hare (*Lepus timidus* Linnaeus). These two species hybridize and produce fertile offspring, resulting in notable allele sharing (Levanen, Thulin, Spong, & Pohjoismaki, 2018; Pohjoismaki, Michell, Levanen, & Smith, 2021). Similar gene flow is known to occur also between other, more related members of the *Lepus* genus (Ferreira et al., 2020; Ferreira et al., 2021; Giska et al., 2019; Marques, Farelo, et al., 2017; Melo-Ferreira, Farelo, et al., 2014; Melo-Ferreira, Seixas, Cheng, Mills, & Alves, 2014).

Hybridization between the two hare species is interesting, as mountain hare and brown hare are separated by three million years of evolution (Ferreira et al., 2021) and differ remarkably in their biology. The mountain hare is characteristically an arctic/subarctic tundra to taiga forest specialist, highly adapted to snowy environments with its wide snowshoe feet and white winter pelage. It has an impressive, almost continuous distribution across the Palearctic, from Ireland to the Russian Far East, which has persisted through the last ice age (Ferreira et al., 2021; Smith et al., 2017). In contrast, the brown hare originates from temperate, steppe-like climate and was pushed to several different refugia in Europe and Near East during the last glacial maximum (Fickel et al., 2008). The two species have followed radically different evolutionary paths, with multiple selective pressures driving the evolution of the observed morphological, physiological and behavioral differences (Fickel et al., 2008; Reid, 2011; Zimova et al., 2020). While some of these differences are obvious, such as the color of the winter pelage, whose evolution can be understood using modern genomic approaches (Ferreira et al., 2020; Ferreira et al., 2017; Giska et al., 2019; Jones et al., 2018), almost nothing is known about the species differences in the most fundamental cellular housekeeping functions. Housekeeping functions of the cells include the basic gene regulation and expression, maintenance of cellular homeostasis and steady-state metabolism (Eisenberg & Levanon, 2013). For instance, arctic hares and snowshoe hares are typically showing depressed basal metabolic rate during the winter season, which is not present in more temperate species (Sheriff, Kuchel, Boutin, & Humphries, 2009).

It is generally assumed that the housekeeping functions are conserved across closely related taxa due to their fundamental importance for the normal function of the organism. Understanding the cellular level species differences not only helps to understand elusive physiological adaptations, but also sheds light to the maintenance of the species barrier. The incompatibility in the housekeeping functions of two species might be contributing to the hybrid disadvantage (Abbott et al., 2013; Arnegard et al., 2014; Burton & Barreto, 2012; Wolf et al., 2010), eventually reinforcing the species separation. This is especially interesting for hares, as despite the recurrent gene flow between many hare species (Ferreira et al., 2021; Jones et al., 2018; Levanen, Thulin, et al., 2018; Marques, Farelo, et al., 2017; Melo-Ferreira, Boursot, Suchentrunk, Ferrand, & Alves, 2005; Melo-Ferreira, Seixas, et al., 2014; Pohjoismaki et al., 2021), no hybrid species have emerged, unlike e.g., in the case of the European bison (Soubrier et al., 2016).

In the present study, we have analyzed in depth the genetic and gene regulation differences in the fibroblasts of brown hares and mountain hares and correlated these with the phenotypic dissimilarities between the cells. We discovered the existence of a species-specific expression pattern of 1,623 genes, manifesting in differences in cell growth, respiration, and metabolism. Our results demonstrate that some species-specific adaptations can be observed at the level of single cells and confirm plasticity in some fundamental housekeeping functions of cells, such as in the genetic circuits controlling cell proliferation. Although the physiological traits of cultured fibroblasts are limited compared to the whole organism, cell models offer many interesting and ethically sustainable opportunities to conduct research on wild animals.

# Materials and methods

All sample metadata, sequence data as well as final transcriptome data are available through Dryad and SRA depositories (see the section data accessibility and benefit sharing).

## Sampling

Four mountain hares and four brown hares were collected by hunting from seven different locations in central parts of southern Finland (Fig. 1A, Table 1). The specimens were instantly killed using 12/76-gauge shotguns with 40 g charge, lead-free 2.75 mm tungsten (UnA) shells or a .22WMR rifle with 1.92 g Hornady V-Max® ammunition at a maximum distance of 40 m. The sampling had minimal impact on the populations and no impact on their habitats. Because both species are legal game animals in Finland and the hunting followed the regional hunting seasons and legislation (Metsästyslaki [Hunting law] 1993/615/5§), the sampling adheres to the ARRIVE guidelines and no ethical assessment was required. Sampling did not involve International Trade in Endangered Species of Wild Fauna and Flora (CITES) or other export of specimens, as defined by the Convention on Biological Diversity (CBD).

## Generation of immortalized fibroblast cell lines

A small piece of abdominal skin (3 mm × 5 mm) was removed from the freshly killed specimen and placed in a 15 ml vial with high glucose Dulbecco's Modified Eagle Medium (DMEM, Biowest), supplemented with 10 % fetal bovine serum and 100 μg/ml Primocin® antimicrobial agent (InvivoGen, Toulouse France). The sample was kept at ambient temperature until arrival at the laboratory, generally within 48h of the sampling. The skin biopsies were cut by scalpel into smaller pieces, placed on 35 mm cell culture dishes with DMEM containing fetal bovine serum and Primocin and incubated at 37 °C, 100 % humidity and 7,5 % CO2. After 4-5 days, the skin pieces were removed, and the growth medium changed to DMEM containing Penicillin and Streptomycin. Fibroblasts attached to the cell culture plate were cultivated until ca. 70 % confluency and then transfected with a mammalian expression vector containing the large T-antigen gene (Addgene # #21826), using Lipofectamine 3000 according to the manufacturer’s instruction. When reaching confluency, the fibroblasts were reseeded at 5 % confluency. After six such passages the fibroblast cell lines were frozen as liquid nitrogen stocks. Cells used for the experiments were derived from these stocks to minimize genotypic drift under culture conditions.

## RNA isolation and sequencing

Total RNA was extracted from each hare cell line using TRI-Reagent® (Sigma-Aldrich) according to the manufacturer’s instruction and quantified by NanoDrop. Poly(A)+ mRNA was extracted from the total RNA using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (Item Number: E7490S, NEB) following the manufacturer’s protocol. The purified mRNA was sent to the Institute for Molecular Medicine Finland (FIMM) for library preparation and sequencing on the NovaSeq 6000. The sequencing libraries were preparing using the Illumina TruSeq stranded mRNA library preparation kit (Item Number:20020594, Illumina) and then 2x150bp paired end sequenced on the Illumina NovaSeq using a S1 flowcell with v 1.5 chemistry.

## *De novo* transcriptome assembly

Sequencing adapters and low quality base pairs were trimmed and removed using Trimmomatic version 0.39 (Bolger, Lohse, & Usadel, 2014) with the following options enabled: ILLUMINACLIP: TruSeq3-PE.fa:2:30:10:2:keepBothReads; LEADING:5; TRAILING:5; MINLEN:50. The trimming of the reads was confirmed and inspected using FastQC version 0.11.8 (Andrews, 2010) followed by multiQC version 1.10.1 (Ewels, Magnusson, Lundin, & Kaller, 2016) to compile the data.

*De novo* transcriptome assemblies were created for each species using Trinity version 2.13.0 (Haas et al., 2013) on the Puhti server of the Center for Scientific computing Finland (CSC). Prior to assembly the reads from the four biological replicates were combined to increase the diversity of the final transcriptome assembly. Then Trinity was run with the following parameters: --seqType fq; --max\_memory 124G; --left Letim.P1.fastq.gz; --right Letim.P2.fastq.gz; --SS\_lib\_type RF; --CPU 8; --normalize\_max\_read\_cov 100; --full\_cleanup; --output trinity\_run\_out; --grid\_exec sbatch\_commandlist\_trinity. After the transcriptome assembly, the assembly statistics were calculated using TrinityStats.pl.

## Validation and transcriptome filtering

To validate the *de novo* transcriptome assemblies, the trimmed reads used to assemble the transcriptome were mapped back on to their respective assembly using bowtie2 version 2.4.4 (Langmead & Salzberg, 2012). As a large number of transcripts were assembled for each species, we used three different filters to reduce the number of misassembled and poor-quality transcripts in the data. Firstly, we removed lowly expressed genes from the transcriptomes using the align\_and\_estimate\_abundance.pl (Li & Dewey, 2011) and filter\_low\_expr\_transcripts.pl scripts included with the Trinity package. Secondly, we reduced the redundancy of the transcriptomes by clustering similar transcripts at 95% similarity with CD-HIT version 4.8.1 (Fu, Niu, Zhu, Wu, & Li, 2012). Finally, miss-assembled and incomplete transcripts were removed from the transcriptomes using TransRate version 1.0.3 (Smith-Unna, Boursnell, Patro, Hibberd, & Kelly, 2016).

Completeness of the transcriptomes was assess by identifying and comparing single copy orthologs from the transcriptomes against the general (Metazoa\_odb10, Download date: 2021-02-17) and lineage specific (Glires\_odb10, Download Date: 2021-02-19) databases using BUSCO version 5.2.2 (Waterhouse et al., 2018).

## Functional annotation of transcriptomes

The functional annotation of the transcriptomes was performed following the Trinotate pipeline (Bryant et al., 2017). TransDecoder version 5.5.0 was used to identify open reading frames with a minimum length of 200 amino acids (-m 200). The identified transcripts and predicted proteins were compared against the SwissProt protein database using diamond version 2.0.4.142 with the parameters: --max-target-seqs 1; --outfmt 6; --more-sensitive. Protein domains, signal proteins and transmembrane proteins were mined from the protein sequences using hmmer version 3.1 (Finn, Clements, & Eddy, 2011), SignalP version 4.1 (Petersen, Brunak, von Heijne, & Nielsen, 2011) and, tmhmm version 2.0 (Krogh, Larsson, von Heijne, & Sonnhammer, 2001) respectively. RNAmmer version 1.2 (Lagesen et al., 2007) was used to identify rRNA genes in the transcriptome using the wrapper script RnammerTranscriptome.pl provided in the Trinotate pipeline. For each species, the output from each analysis was compiled in the Trinotate SQlite database.

## Transcript genotyping

To infer the genotypes of each sample, we identified genetic variants by mapping the reads onto the pseudo-reference genome (Genbank accession: GCA\_009760805.1) (Marques et al., 2020) using STAR version 2.7.8 (Dobin et al., 2013). Variants were then called following the recommendation of the GATK 4.0 pipeline for RNAseq data (Brouard, Schenkel, Marete, & Bissonnette, 2019). The raw variant file was filtered using VCFtools(Danecek et al., 2011). Following the removal of indels, missing data, and variants with an MAF < 0.05, we obtained 1 biallelic site for each contig. The resulting VCF file was analyzed for basic population genomic parameters using Poppr2(Kamvar, Tabima, & Grunwald, 2014). The ancestry coefficient of each sample was estimated using the sparse non-negative matrix factorization (sNMF) method in LEA(Frichot & Francois, 2015).

## DNA isolation, sexing, mtDNA genotyping and DNA copy number analysis

Total DNA was extracted from the cells using the peqGOLD Blood and tissue DNA mini kit (VWR Life Science). The sex of the cell lines was confirmed using PCR-RFLP for the *ZFX* + *ZFY* loci (Fontanesi et al., 2008; Levänen, Pohjoismäki, & Kunnasranta, 2019) and the species specificity of the mitochondrial DNA (mtDNA) was checked with PCR-RFLP of the mitochondrial *CYTB* gene region (Alves, Ferrand, Suchentrunk, & Harris, 2003; Melo-Ferreira et al., 2005). Mitochondrial DNA copy number was measured with quantitative Real-Time PCR (qPCR) using TaqMan™ chemistry. Primers and TaqMan™ probes (Metabion International AG, Panegg, Germany) were designed to be fully compatible for both species, targeting *16S* gene of the mtDNA as well as *SDHa* for the single-copy nuclear locus:

Lepus-16S-F: 5´-ACC CCG CCT GTT TAC CAA-3´

Lepus-16S-R: 5´-ATG CTA CCT TTG CAC GGT CA-3´

Lepus-16S-probe: 5´-6-Fam-TGC CTG CCC AGT GAC AAA CGT-BHQ-1-3´

Lepus-SDHa-F: 5´-CCT GCC TGG CAT TCC TGA GA-3´

Lepus-SDHa-R: 5´-ATT GGC TCC TTG GTG ACG TC-3´

Lepus-SDHa-probe: 5´-Hex-GCC ATG ATC TTC GCG GGT GTG-BHQ-1-3´

The qPCR program had an initial denaturation step of 3 min 95 °C followed by 40 cycles of 15 s 95 °C, 15 s 54 °C and 15 s 72°C (read).

## Differential gene expression

As most allelic variants from the two species were initially identified as different genes by the annotation algorithms (likely due to the species difference in the sequences), defining a common gene set between the two species was essential. To do this we identified one-to-one reciprocal best BLAST hits. Firstly, the final transcriptome of *L. timidus* was compared against the final transcriptome of the *L. europaeus*. Then the reciprocal BLAST analysis was performed by comparing the transcriptome of L. *europaeus* to the *L. timidus* transcriptome. Transcripts identified as reciprocal best BLAST hits as determined by the bit score (python script available on request) were then extracted from the final transcriptomes into species-specific fasta files. The trimmed reads for all samples were then mapped onto the one-to-one reciprocal best BLAST hits of *L. timidus* and then *L. europaeus* using bowtie2 version 2.4.4 with the following parameters --all --min-score L,-0.1,-0.1. The resulting alignment files were then clustered and quantified using Corset version 1.09 (Davidson & Oshlack, 2014). The raw count table was then analyzed for differentially expressed genes using the Integrative Differential Expression Analysis for Multiple Experiments (IDEAMEX) webtool (Jimenez-Jacinto, Sanchez-Flores, & Vega-Alvarado, 2019). Visualization of the RNAseq was then performed in R using ggplot2 (Ginestet, 2011).

**Cell culture**

Fibroblasts were maintained under standard cell culture conditions at 37°C, 5% CO2 in a humidified incubator. Cells were grown in high-glucose (HG, 4.5 g/l) or low-glucose (LG, 1 g/l) Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, #D6546 and #D5546 respectively). Media were supplemented with 10% heat inactivated fetal bovine serum (Sigma-Aldrich, # F7524), 1% L-glutamine, and 1% penicillin–streptomycin. Unless otherwise stated, culture media were changed every three days. Cells were dissociated with 1x trypsin–EDTA (Gibco, # 15400054) for 5 minutes after rinsing with Dulbecco′s Phosphate Buffered Saline (PBS) (Sigma-Aldrich, #D8537). Trypsin was inhibited by the addition of fresh growth medium before mechanical resuspension and pelleting of the cells at 250 × g for 3 minutes. Cell concentrations were measured using EVETM automatic cell counter.

## Cell growth measurements

**Nuclei counting**

Cells were seeded into 48 well plates at 3×103 cells/well, in quadruplicate. Every 24 hours following plating, fibroblasts from one of the plates were fixed in 4% paraformaldehyde (PFA) (RT, 20 min.), washed with PBS and stained in 1 µg/ml Hoechst solution (Invitrogen, # H1399, RT, 10 min.). Cells were rinsed twice with PBS before imaging with an Olympus IX51 Inverted Phase Contrast Fluorescence Microscope (DAPI filter, EM 420, EX 360/370, 1 sec. exposure). For each well, four images, aligned alongst the longest width, were captured at 10 x magnification with an interval of one visual field between each image. Images were processed in Fiji-ImageJ-64bit software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018). Nuclei were separated from the background by thresholding. Clumped nuclei were split by applying Watershed algorithm. Automatic particle counting was applied to count nuclei excluding partial nuclei at the images’ edges and debris. Population doubling times were derived from the exponential growth phase of the obtained growth curves. Outliers were removed using the ROUT method (FDR set to 0.1%)(Motulsky & Brown, 2006). PDTs were compared using Mann–Whitney U test. Growth curves were compared using a CGGC permutation test (Elso et al., 2004) and a two-way ANOVA (Genotype \* Time point).

**Cell counting**

Cells were seeded as described for nuclei counting. After 24 hours, one plate was placed into the onstage incubator of EVOS® FL cell imaging system adjusted to the standard culture conditions. A time-lapse routine was created with (built-in Time Lapse Wizard tool). For each well, three 10x imaging fields were randomly picked. Phase contrast images were captured at these locations every 8 hours over a period of 96 hours. Images from every third timepoint (24, 48, etc.) were processed and analyzed using Fiji-ImageJ. Brightness and contrast were automatically adjusted. Cells were manually counted in each image using a multi-point tool. Analysis was done as described for nuclei counting.

## Cell cycle measurements

Each fibroblast line was seeded in three 6-well plates and grown until 80% confluency. Cells were then blocked in the G1/S phase by incubation for 18 hours in the presence of aphidicolin (2.5 µg/ml of in growth medium) (Sigma-Aldrich, # A0781) except for the two control wells. To resume cell cycle progression, aphidicolin-containing medium was removed by two washes with PBS and replaced by growth medium. Every three hours starting from T0 (removal of aphidicolin), samples from three wells were collected at a density of 1×106 cells/ml and ethanol fixed (70% ethanol in ice-cold PBS) for 24 hours at +4°C. Samples were then rinsed with ice-cold PBS, treated with RNase A (1 mg/ml in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl; RNASEA-RO, Roche) at 37°C for 30 minutes and stained for 30 minutes at room temperature, in the dark, with 10 µg propidium iodine (PI; 20 µg/ml in PBS) per 1×106 cells (Sigma-Aldrich, #P4864). PI staining was analysed from 2×104 events per sample (EX 561 /EM 585) using Cytoflex S Flow Cytometer. Cell cycle analysis was performed using CytExpert software. Cycling cells were gated using the area and hights of the PI channel to exclude doublets and prevent G2 overestimation. Percentages of cells in G1, S, and G2 phases were calculated from the histogram of cell count vs PI staining area after visually inspection for accuracy. Comparison of cell cycle progression was done in GraphPad Prism 9 using two-way ANOVA.

## Cell migration measurements

The migratory potential of ﬁbroblasts was assessed with wound healing assays (Jonkman et al., 2014). Cells were grown in two 12-well plates to form confluent monolayers. Each cell line was represented in one of the wells of each plate. Manual scratching was performed with a sterile 200 µl micropipette tip to produce a linear cell-free zone in the centre of each well. Cells were washed three times with PBS to remove debris, and fresh growth medium was added. Each plate was then maintained in the microscope stage of either an EVOS® FL (repeat 1) or a Cell-IQ® (repeat 2) automatic cell imaging system adjusted to the standard cell culture conditions. Migration of the fibroblasts into the “wound” area was measured by phase contrast at 4 × magnification. For each well, three image fields were captured at 1 hour time interval for 48 hours. Each field included the “wound” area and both edges of the wound. The images were analysed in Fiji-ImageJ software. Light intensity variations were ﬂat-ﬁeld corrected. Brightness and contrast were automatically adjusted. The length of the wound (*l*) was measured using a straight-line selection tool. Wound areas were quantified at each time point using MRI Wound Healing tool (Volker Baecker, Montpellier RIO Imaging, Montpellier, France). For each series of images, the wound area *versus* time was plotted. Comparative R-Squared analysis was used to define the longest time interval during which cell migration was linear. Slope values were extracted from the linear function and converted into wound closure rate (). The rates were compared between genotypes and repeats using two-way ANOVA. The delay before the start of linear cell phase (T0-linear – T0) was used to estimate the lag-time (i.e. wound closure delay) and compared between genotypes using Mann-Whitney U test.

## Mitochondrial mass measurements

Cells from each cell line were grown until 70-80% confluency. After 1 hour of exposure to fresh media in normal culture conditions, cells were resuspended in PBS at 1×106 cells/ml and split into seven aliquots of 0.5×106 cells (to generate six stained and one unstained sample). Three of the aliquots were stained with NAO under standard cell culture conditions for 20 min (25 nM nonylacridine orange; Invitrogen, #A1372). In the three other aliquots mitochondria were uncoupled with FCCP (50 μM, 3 min) prior to NAO staining. Intensity of NAO staining was measured from 2×104 events per sample (EX 488 /EM 525) in a Cytoflex S Flow Cytometer. Data was processed in FlowJo™ software (Version 10.7.2. Ashland, OR: Becton, Dickinson and Company; 2021) excluding cell-doublets from the analysis. From the resulting NAO signal intensities outliers were removed using the ROUT method (FDR: 1%)(Motulsky & Brown, 2006). Comparisons were done using Mann-Whitney U test.

## Mitochondrial membrane potential measurement

Cells were collected and distributed in Eppendorf tubes as described for the NAO staining procedure. Each sample was divided into two to three technical repeats. Staining was performed in the presence of 50 μM Verapamil (Sigma-Aldrich, # V4629)(Morganti, Bonora, Ito, & Ito, 2019). To estimate membrane potential, a subset of the aliquots was stained with TMRM (tetramethylrhodamine, methyl ester, 20 nM, Invitrogen, # T668) under standard cell culture conditions for 30 min. In a second subset of aliquots, mitochondrial membrane potential was collapsed by FCCP (50 μM, 3 min) before TMRM staining. In the last subset of the aliquots, mitochondrial membrane potential was allowed to reach its maximum level using Oligomycin treatment (5 μg/mL, 3 min) before TMRM staining. Untreated cells were used as negative controls. Cell suspensions were analysed with Cytoflex S Flow Cytometer. The intensity of TMRM staining was measured from 2×104 events per sample (EX 561 /EM 585). Data was analysed in FlowJo software. Cell-doublets were excluded from the analysis. Corrected fluorescence intensity was calculated as (TMRM signal intensity) – (TMRM + FCCP signal intensity). Sample were excluded if (TMRM + Oligomycin signal intensity) < (TMRM signal intensity). Comparison was done using unpaired two-tailed Student’s t-test.

**Mitochondrial morphology**

Cells were seeded in 35mm glass bottom poly-d-lysine coated plates (MatTek, # P35GC-1.5-14-C) at 2×105 cells/plate and grown in normal culture conditions until 70% confluency. The growth medium was refreshed 1 hour before cell staining. Cells were then incubated in MitoTracker Deep Red FM (50 nM, Invitrogen, cat. #M22426) + NucBlue (2 drops/ml, Invitrogen, #R37605) in growth medium at 37°C and 5% CO2 for 10 min. Cells were washed twice with PBS and maintained in PBS during imaging. Imaging was performed using Olympus IX51 Inverted Phase Contrast Fluorescence Microscope at EX 360/70, EM 420, exposure time 1 second to visualise cell nuclei, and EX 620/60, EM 700/75, exposure time 1 second to image mitochondria. Images were analysed in Fiji-ImageJ software. Brightness and contrast were automatically adjusted.

**Mitochondrial respiration measurements**

Cells were grown until 70-80% confluency in HG media. Media was refreshed 1 hour prior to cell collection. For each measure, 5×106 cells were pelleted and resuspended in 50 µL of respiration buffer (225 mM Sucrose, 75 mM mannitol, 10 mM KCl, 10 mM KH2PO4, 5 mM MgCl2, 10 mM TRIS pH 7.4, 1 mg/ml of bovine serum albumin BSA). Cell suspensions were immediately added into the oxygen-calibrated chamber of Hansatech Oxytherm respirometer pre-equilibrated with 500 µl of respiration buffer. Cells were permeabilised with digitonin (55 µM). Mitochondrial respiration was then assessed by additions of respiratory substrates and inhibitors in the following order: pyruvate + glutamate + malate (5 mM each, Sigma-Aldrich respectively #P8574, #G5889, #M7397), ADP (1 mM, Calbiochem #117105), succinate (5 mM, Sigma-Aldrich #W327700), oligomycin (1 µM, Sigma-Aldrich #75351), FCCP (1 µM, Sigma-Aldrich # C2920), rotenone (300 nM, Sigma-Aldrich #R8875), antimycin A (90 ng/ml, Sigma-Aldrich #A8674), ascorbate + N,N,N′,N′-tetramethyl-p-phenylenediamine (700 µM, Sigma-Aldrich #A4034 + 300 µM, Sigma-Aldrich #T7394), and potassium cyanide (200 μM, Sigma-Aldrich #60178). Oxygen consumption (pmol.sec−1.ml−1) is presented per million of cells. Respirometry data were analysed with two-way ANOVA (Genotype \* Compound) with interactions.

## Protein preparation and Western blotting

Cells were grown at 80% confluence in 10 cm2 dishes, resuspended, washed with ice-cold DPBS and lysed in ice-cold RIPA buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate) supplemented with cOmplete EDTA-free Protease Inhibitor Cocktail (Roche, # 04693132001). Samples were maintained on ice for 30 min and debris was eliminated by centrifugation (13 000 × g, 20 min, +4°C). Protein supernatants were stored at -80 °C. Protein concentration was measured with BCA assay (Pierce BCA Protein Assay Kit, #23225) using EnVision® 2105 (Abs. 562 nm) plate reader. 30 µg of protein per sample was loaded in Criterion™ TGX Stain-Free™ precast gels (Biorad, #5678093). Spectra Multicolor Broad Range (Thermo Scientific™, #26634) was used as a molecular size ladder. Gel electrophoresis was performed at 100 V for 80 min. Proteins were transferred onto nitrocellulose membranes (Biorad, #1704158, #1704159) using the Transblot-Turbo Transfer System (Biorad, #1704150). Blots were washed in Tris-buffered saline-Tween buffer (TBS-T) and blocked with TBS-T + 5% milk for 1 hour under agitation at RT. Blots were probed at +4 ºC overnight in blocking buffer with one of the following primary antibodies: beta-Actin IgG rabbit polyclonal (1:1000; NB600-505; Novus Biologicals), COX IV IgG rabbit polyclonal (1:2000; ab16056; Abcam), SDHA IgG1 mouse monoclonal (1:10000; ab14715; Abcam), Tom20 IgG2a λ mouse monoclonal (1:200; sc-17764; Santa Cruz), Vdac1 mouse monoclonal (1:1000; SAB5201374; Sigma-Aldrich), Vinculin mouse monoclonal (1:10000; V9264; Sigma-Aldrich). After incubation, blots were washed with TBS-T and exposed for 2 hours at RT in the dark to the relevant peroxidase-labelled secondary antibody diluted in 2.5% milk TBS-T buffer: goat anti-rabbit IgG (1:10000; PI-1000; Vector Laboratories) or horse anti-mouse IgG (1:10000; PI-2000; Vector Laboratories). Blots were washed with TBS and incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, #34096) for 5 minutes in the dark. Images were obtained with ChemiDoc XRS+ Imaging System (Biorad, #1708265) using auto-exposure. Band intensities were quantified in Image Lab software and normalised to the intensities of reference proteins (beta-Actin and Vinculin). Data were analysed with unpaired two-tailed Student’s t-test.

# Results

## Genotype and ancestry validation of the hare cell lines

All the sampled cell lines (LE for *Lepus europaeus* or brown hare and LT for *Lepus timidus* or mountain hare) originate from sympatric hare populations across southern Finland (Fig. 1A, Table 1). As mountain hares frequently hybridize with brown hares, producing fertile hybrids and therefore forming potential hybrid continuums in the populations of both species, we first wanted to confirm the genotypes and the ancestry of the cell lines. For the nuclear DNA genotyping, we used 9,056 SNPs in 519,066–549,582 transcripts obtained from the RNAseq data (Fig. 1B, S-Tables 1–2), while the mitochondrial DNA (mtDNA) identity and sex of the cell lines was checked using PCR-RFLP.

The cell lines of the two species formed distinct and species-specific clusters in the PCA analysis of the SNP differences; the brown hare samples clustered notably tight compared to the mountain hares (Fig. 1B). This difference can be attributed to the low levels of heterozygosity in brown hares compared to the mountain hare samples (Fig. 1C). The clustering of the samples also did not correlate with the geographic origin of the animals, giving confidence that the cell lines represent independent and unrelated biological replicates. In ancestry coefficient analysis each cell line fit uniformly under the assigned species with no or minimal evidence of ancestral hybridization (Fig. 1D). The only cell lines showing any evidence of mixed ancestry were the mountain hare cell lines LT5 (~3%) and LT6 (~1%) as well as brown hare cell line LE3 (~1%). All cell lines had conspecific mtDNA, and the male to female ratio was 3:1 in mountain hares and 1:1 in brown hares (Table 1).

## Differential gene expression between species

We next looked at the global gene expression differences between the cell lines. When all 400,000+ “operational genes” i.e., including all allelic variants and isoforms that were different enough to be counted as separate genes by the algorithm, were compared (S-Table 2), mountain hare and brown hare cell lines formed species-specific clusters (Fig. 1E–F). Notably, the intraspecific variance in mRNA expression levels was similar in both species (Fig. 1F) and did not correlate with the genotypic variability (Fig. 1B) or with the sex of the cell line. The species-specific expression pattern was considerably weaker when the 16,689 orthologous genes were compared (S-Table 2, Fig. 1E). In this analysis, the gene expression pattern of the brown hare cell line LE1 was closely matching that of the mountain hare cells. Notably, LE1 was also shifted towards the mountain hares in the heatmap clustering of the global transcriptome (Fig. 1E), although the coding sequence genotype of the cell line did not show any ancestral mountain hare influence (Fig. 1D).

As much of the observed total transcriptome differences could be attributed to species-specific isoforms and allelic variants, more detailed differential expression analyses were performed only with the orthologues (S-Table 2). Gene ontology (GO)-term analysis of the gene expression of the orthologues revealed 1,623 differentially expressed genes, belonging to over hundred genetic processes or cellular components that showed significant (p < 0.001) difference between the two species (S-Table 3). The most notable difference between the two species was that the brown hares showed higher expression of genes involved in basic housekeeping functions of cells, such as oxidative energy metabolism, cell migration and cell cycle regulation (Fig. 2A). For example, several genes related to e.g. mitochondrial function (*PDK4*, *FMC1*, *ATPK*, *ACADL* and *PPARG*) had higher expression levels in brown hares (Fig. 2B). In contrast, many genes showing higher expression in mountain hare were orphan or master regulators of cell functions, such as the L-xylulose reductase enzyme (*DCXR*), the transcriptional inactivator *PAGR1* (Zhang, Sun, Cho, Chow, & Simons, 2013), the regulator of cell fate, proliferation and differentiation *NOTC3* (Choy et al., 2017), as well as the high-fat diet associated hormonal regulator *ENHO* (Jasaszwili, Billert, Strowski, Nowak, & Skrzypski, 2020).

## Mountain hare and brown hare fibroblast mitochondria show dramatically different coupling while being otherwise very similar

The enrichment of mitochondria-related gene expression in brown hare cells is interesting, as enhanced oxidative metabolism might reflect a general physiological difference in energy expenditure and utilization between the species. However, we did not observe any significant differences in mitochondrial mass (Fig. 3A), cell respiration (Fig. 3C–D), mtDNA copy number (Fig. 3E) or selected mitochondrial protein levels (Fig. 3E). However, mountain hare fibroblasts showed dramatically higher mitochondrial membrane potential (Fig. 3B), indicating a stronger coupling of the respiratory chain in mountain hares compared to brown hares.

## Cell proliferation and migration is faster in brown hares

The observed differences in gene expression related to cell cycle and cell migration regulation indicate that the fibroblasts of two different species might differ in their proliferative and regenerative capacity. We could confirm this in cell culture, as the brown hare cells showed significantly faster cell doubling time (Fig. 4A) as well as wound closure rate (Fig. 4B) compared to the mountain hares. Maintaining cells in low glucose medium, which generally promotes oxidative metabolism over glycolysis, reduced the variation between the cell lines, but did not influence the overall species difference (Fig. 4A). Curiously, although the proliferation and migration of mountain hare cells during wound closure was slower, they showed a smaller delay in the initiation of the wound closure than the brown hares (Fig. 4B).

## Differences in cell proliferation and migration can be attributed to cell cycle differences

While GO-term analysis can identify differences in gene expression patterns, it seldomly reveals anything about their phenotypic consequences. The fact that brown hare cells proliferate faster (Fig. 4A) also means that they express genes related to cell cycle stronger (Fig. 2A). As immortalized fibroblasts should proliferate at their maximum capacity under cell culture conditions, we wanted to understand the biological reason behind the species difference in more detail. The cell cycle consists of the primary growth phase G1, where cells grow in size after mitosis. Once the cells are large enough, they enter S-phase, where the chromosomal DNA is duplicated, which is followed by the G2- or secondary growth-phase preceding mitosis (M-phase). By synchronizing the cells, we were able to show that while there was no difference in the G1-phase between the two species, while the mountain hare cells entered S-phase faster but took longer in the G2-phase than the brown hare cells (Fig. 5). This difference was not explained by the size of the cytoplasm required to grow before the mitosis, as all cells were relatively variable in size and shape, regardless of the species (Fig. 6, S-Fig. 1).

# Discussion

While species-specific adaptations to ecological niches, manifesting as utilization of food resources and discrete phenotypic features, such as the white winter pelage and the snowshoes of the mountain hare, are often obvious for any observer, very little is known about the species differences in the hardwiring of cells. This is not only important in understanding potential mechanisms of genetic compatibility of species hybrids, but also serves notice to studies generalizing findings from one species (typically human, mouse or other model organism) across a wider spectrum of biodiversity.

## Fibroblasts come with flavors

Our motivation for this study was twofold. Primarily we were interested whether cell lines from two closely related and interbreeding species of hares showed marked differences in key cellular functions, which would help to understand possible constraints of species hybridization, but also to detect signatures of adaptation, such as metabolic specialization. While focusing on cell lines leads to the omissions of many features relevant to the physiology and metabolism of the whole organism, any observed species differences in fibroblasts should be robust and universally applicable. Secondly, we wanted to apply some of the common methods in cell biology for comparative phenotyping of cells from non-model organisms and test the utility of isolated fibroblasts for such a purpose, as they are easy to establish and maintain and widely used.

It should be noted that the comparison of the transcriptomes of two species is not trivial: the genetic differences between the two species cluttered the gene annotation pipeline, as evident from the large number of detected genes in the first pass of the data analysis (S-Table 2). Our approach to compare only the orthologue genes provided a more robust set of common genes to compare, although it likely missed interesting genes that are expressed in a strict species-specific manner. It is also noteworthy that the number of orthologous genes found by using reciprocal BLAST search differed from the polypeptide-encoding gene count based on the predicted ORFs. This is due to the fact that some truncated transcript sequences do not contain ORFs, while others might be present in one species but not in the another.

While many of the features showed variability between the cell lines, including the cell morphology (Fig. 6), the species separation existed also when the samples were clustered based on the levels of orthologous transcripts (Fig. 1G). We believe that the species difference is genuine and not caused by variation in the cell type. While several subtypes of dermal fibroblasts are known from mammals (Jiang & Rinkevich, 2018), any cell line deviating from the others would have been visible as an outlier in the analysis due to the cell type-specific gene expression patterns. If one imagines that the separation of the cell lines along the first component axis would represent different cell types, these form a continuum along this axis (Fig. 1G). This is not compatible with the idea of having discrete fibroblast subtypes with typical expression patterns (Buechler et al., 2021), but would fit well with individual differences between the specimens. Furthermore, it is likely that the used isolation method, sampling sections of the skin as well as the culture conditions favor some fibroblast types over others. We also observed no segregation of the samples based on the sex of the animal (Table 1).

## Genetic diversity *vs.* phenotypic diversity among Finnish hares

Although the sampled animals come from a geographic region where the two hare species live in sympatry (Fig. 1A, Table 1), none of the cell lines showed notable hybrid ancestry based on the global transcript sequence genotypes (Fig. 1B, D). The genotyping also confirmed previous observations finding much lower genetic diversity in the Finnish brown hares than in the mountain hares (Fig. 1B, C) and that most of the genetic variation seen in the brown hares originates from mountain hares (Levanen, Thulin, et al., 2018; Pohjoismaki et al., 2021). The low genetic diversity, especially the lack of private alleles, in brown hare is likely explained by a founder effect due to the species relatively recent northern expansion, further strengthened by genetic drift in the small pioneering populations. (Pohjoismaki et al., 2021). The observed allele sharing between the species might be explained by frequent hybridization with mountain hare at the expansion front, which steadily decreases when the brown hare population gets established (Levanen, Thulin, et al., 2018).

While the sample set of eight cell lines is small for a population genetic analysis, the lack of genetic structure among the specimens is remarkable. For example, the mountain hare sample LT1 from Ilomantsi, representing typical boreal taiga forest location, is very similar to the LT4 specimen from Vesilahti, some 420 km apart and representing mostly agricultural landscapes (Table 1, Fig. 1A, B, D). Interestingly, none of the brown hare cell lines from Eastern Finland showed noticeable ancestral hybridization with mountain hare (Fig. 1D), although hybridization is relatively common in this region due to the strong mountain hare populations (Levanen, Kunnasranta, & Pohjoismaki, 2018). However, we cannot rule out that this might be caused by our analysis being performed with coding sequence variation, therefore breaking the assumption of neutrality underlying such population genetic analyses. In this case, it might be that differential selection pressures are maintaining the species difference, inviting future studies to compare coding vs non-coding variation in sympatric populations of the two hares.

Although the brown hare cells showed little genetic diversity compared to the mountain hares (Fig. 1B), the two species showed similar variability at the level of gene expression (Fig. 1F, G). While it is plausible that the brown hares retain more genetic variation in the control regions of genes, it is unlikely that there would be no correlation between non-coding and coding variation in the hare genomes. The results are likely to reflect the fact that gene expression is influenced by several intrinsic as well as environmental factors, giving it a good degree of plasticity even under controlled cell culture conditions. As the RNA was isolated from non-synchronized cells with similar confluency, fluctuations caused by cell-cell interaction should have averaged out. In contrast, cell-cycle dependent gene expression may underlie some of the species differences, as mountain hare cells spend more time G2 but less in S phase (Fig. 5)

## Adaptations and noise – molecular constituents of a species difference?

One of the differentially regulated cellular components picked up in the transcriptome analysis were mitochondria (Fig. 2). We found this potentially interesting, as energy expenditure and its fine tuning by mitochondrial metabolism might show correspondence with the requirements of environmental adaptation between arctic vs temperate species. However, we found no correlation with mitochondria-related gene expression and mitochondrial mass, cell respiration under standard conditions, mtDNA copy number or with the levels of various mitochondrial proteins (Fig. 3). In fact, the only mitochondrial parameter showing significant species difference was mitochondrial membrane potential (MMP), which was higher in mountain hare cells (Fig. 3B). While elevated mitochondrial biogenesis in brown hares could be related to the faster cell doubling time of the cells (Fig. 4A), also necessitating the need for faster organelle renewal, the membrane potential difference may have more complex roots. Under physiological conditions coupling is mainly sustained by the influx of electrons through the ETC, which generates the H+ gradient across the inner mitochondrial membrane. Uncoupling is the result of ATP production, but also protein and ion transport across the mitochondrial inner membrane, as well as proton leak and illicit electron transfer to non-ETC components. The MMP therefore reflects the balance between coupling and uncoupling mechanisms, and MMP variation may indicate differences in the cellular and mitochondrial metabolism of the cells.

Of note, the proton gradient causing the membrane potential can be vented out as heat using mitochondrial uncoupling protein 1 (UCP1), although this mechanism is almost exclusively restricted to brown adipocytes (Chouchani, Kazak, & Spiegelman, 2019; Cioffi et al., 2009; Nowack, Giroud, Arnold, & Ruf, 2017). One of the aspects of uncoupling, whether through UCP1 or other mechanisms, is that part of the energy of the proton gradient is released as heat. The observation that the mountain hare-specific UCP1 alleles are under positive selection in Finnish brown hares (Pohjoismaki et al., 2021), warrants for further testing of this hypothesis in the future.

It should be noted that phenotypic features such as membrane potential are likely not to be controlled at the level of gene expression, but rather result from functional allelic variants of genes encoding for inner mitochondrial membrane proteins (mitochondrial membrane transport machineries, ion channels, ANT and ATPV as well as RC complexes). This is likely to be the case also with most other phenotypic species differences and is evident from the fact that while there are small overall differences in the expression of orthologous genes (Fig. 1G), the overall allelic difference between the species very distinct (Fig. 1F). The more defined a phenotypic difference is, the more possible it is to dissect it detail. For the membrane potential example, it should be straight-forward to perform a sequence comparison for a subset of proteins mentioned above, using e.g., MitoCarta as reference (Rath et al., 2021). Such a comparison should reveal potentially interesting functional differences between the species, narrowing down the candidate genes and allowing their experimental testing in cell culture.

The fact that the brown hare cells express more genes related to mitotic cell cycle and cell migration (Fig. 2A) shows that these cells are more frequently in mitosis than mountain hare cells but does not explain why the difference exists. By measuring cell doubling and wound healing speed we could confirm the association of gene expression with cell proliferation phenotype (Fig. 4) as well as identify the differences at the specific steps of the cell cycle. Specifically, mountain hare cells enter S phase faster than the brown hares but take longer to complete the G2 phase (Fig. 5). The faster progression from G1 to S phase could explain the shorter lag time for mountain hares in the wound healing assay, although a considerably longer G2 phase makes the overall process slower (Fig. 4B). Generally, slower G2 phase could mean that mountain hare cells need more time than brown hares to build up cytoplasm, cell organelles or storages for the mitosis. From the adaptational perspective, this could either reflect a metabolic difference (in metabolic storage and/or biosynthetic activities) between the species or that the mountain hare cells maintain quantitatively and/or qualitatively better cytosolic components. In both cases, this could be an adaptation to cold climate and poor-quality food during winter months. This topic could be addressed in further detail through a global metabolome analysis.

While the results reported here mainly provide hypotheses for future studies, a key takeaway from our observations is that many essential housekeeping functions of the cells show considerable plasticity. One could assume that skin fibroblasts from closely related species perform their relatively small number of tasks similarly and that individual variation due to age or genotype would be more relevant than the species difference, but this seems not to be the case. The species difference in the fibroblast phenotype is interesting, as it is generated by the same genotype, which produces the species differences in more specialized tissues, such as skeletal muscle, liver, brown fat and neurons. For example, metabolism differences in neurons or skeletal muscle should be recapitulated, albeit probably not at the same scale, also in the superficially simpler fibroblasts.

## Using cultured cells for wild animal research

Cultured cells isolated from animals are often believed to be poorly fit for population studies and to have limited value in terms of physiological representativity, compared to e.g., whole animal or *ex vivo* tissue samples. However, they can offer interesting opportunities to address specific questions in the field of ecology and evolutionary biology. First, it is possible to use ear clips or other minimally invasive skin biopsies to isolate and culture fibroblasts, allowing sampling of rare or endangered animals while minimizing the harm to natural populations. Based on our results, the cell lines originating from three to four individuals would be enough to differentiate between intra- and interspecies variation. However, even smaller sampling might give a biased impression of the species difference, as the comparison of LE1/LT1 with LE1/LT5 shows (Fig. 1G

Secondly, cultured cells can be preserved frozen for almost indefinite time as well as expanded to large scale cultures providing an excellent source of high quality RNA as well as DNA for ‑omics purposes. For example, the RNA-sequencing results provided with this paper represents the most comprehensive transcriptome data provided for mountain hare (Marques, Ferreira, et al., 2017; Marques et al., 2020) and brown hare (Amoutzias et al., 2016) thus far, hopefully being a useful resource for future studies. Furthermore, living cells growing on a monolayer also make library preparations for chromosome-scaffolded reference genomes using Hi-C (Kadota et al., 2020; Yamaguchi et al., 2021) and long read sequencing technologies straightforward.

Thirdly, methods in cell and molecular biology provide exciting opportunities to experimentally test genotype to phenotype association. For example, it should be possible to narrow down candidate genes behind many cell traits and critically test their influence on the trait using CRISPR-Cas9 knockout and/or transgenic expression of alternative variants. It is also possible to program fibroblasts to induced pluripotent stem cells (iPSC) (Malik & Rao, 2013) and differentiate these iPSC to various other cell types with more characteristics than fibroblasts (Liu, David, Trawczynski, & Fessler, 2020). To date, the generation and differentiation of iPSCs can be bought as a commercial service, therefore not requiring specialized in-house skills. Altering cell culture conditions (e.g. changing the incubation temperature, limiting the source of energy in culture media or exposing the cells to specific stresses) allows to test cellular responses to environmental changes under controlled conditions.

In conclusion, the differences between two closely related hare species clearly manifested as phenotypic differences in a cell model, which are likely to reflect biologically meaningful, adaptive features of the animals. Understanding the molecular mechanisms behind these cellular differences will help to shed light on the constituents of species boundaries, genetic basis of adaptation and genotype to phenotype correlations.

# Acknowledgements

We would like to thank Mr Lauri Peippo (Parikkala, Finland), Mr Jukka Pusa (Joensuu, Finland) and Mr Jari Kokkonen (Kontiolahti, Finland) for providing the samples LT6, LE1 and LE3, respectively. This study belongs to the xHARES consortium funded by the R’Life initiative of the Academy of Finland, grant number 329264.

# *This research paper is dedicated to the home country of the first author, Ukraine, and all people who stand to protect world peace, democracy, and freedom.*

# References

Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J., Bierne, N., . . . Zinner, D. (2013). Hybridization and speciation. *J Evol Biol, 26*(2), 229-246. doi:10.1111/j.1420-9101.2012.02599.x

Alves, P. C., Ferrand, N., Suchentrunk, F., & Harris, D. J. (2003). Ancient introgression of Lepus timidus mtDNA into L-granatensis and L-europaeus in the Iberian Peninsula. *Molecular Phylogenetics and Evolution, 27*(1), 70-80. doi:10.1016/S1055-7903(02)00417-7

Amoutzias, G. D., Giannoulis, T., Moutou, K. A., Psarra, A. M., Stamatis, C., Tsipourlianos, A., & Mamuris, Z. (2016). SNP Identification through Transcriptome Analysis of the European Brown Hare (Lepus europaeus): Cellular Energetics and Mother's Curse. *Plos One, 11*(7), e0159939. doi:10.1371/journal.pone.0159939

Andrews, S. (2010). FastQC: A quality control tool for high throughput sequence data [Online].

Arnegard, M. E., McGee, M. D., Matthews, B., Marchinko, K. B., Conte, G. L., Kabir, S., . . . Schluter, D. (2014). Genetics of ecological divergence during speciation. *Nature, 511*(7509), 307-311. doi:10.1038/nature13301

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics, 30*(15), 2114-2120. doi:10.1093/bioinformatics/btu170

Brouard, J. S., Schenkel, F., Marete, A., & Bissonnette, N. (2019). The GATK joint genotyping workflow is appropriate for calling variants in RNA-seq experiments. *J Anim Sci Biotechnol, 10*, 44. doi:10.1186/s40104-019-0359-0

Bryant, D. M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M. B., Payzin-Dogru, D., . . . Whited, J. L. (2017). A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors. *Cell Reports, 18*(3), 762-776. doi:10.1016/j.celrep.2016.12.063

Buechler, M. B., Pradhan, R. N., Krishnamurty, A. T., Cox, C., Calviello, A. K., Wang, A. W., . . . Turley, S. J. (2021). Cross-tissue organization of the fibroblast lineage. *Nature, 593*(7860), 575-579. doi:10.1038/s41586-021-03549-5

Burton, R. S., & Barreto, F. S. (2012). A disproportionate role for mtDNA in Dobzhansky-Muller incompatibilities? *Mol Ecol, 21*(20), 4942-4957. doi:10.1111/mec.12006

Chan, K. M. A., & Levin, S. A. (2005). Leaky prezygotic isolation and porous genomes: Rapid introgression of maternally inherited DNA. *Evolution, 59*(4), 720-729.

Chouchani, E. T., Kazak, L., & Spiegelman, B. M. (2019). New Advances in Adaptive Thermogenesis: UCP1 and Beyond. *Cell Metab, 29*(1), 27-37. doi:10.1016/j.cmet.2018.11.002

Choy, L., Hagenbeek, T. J., Solon, M., French, D., Finkle, D., Shelton, A., . . . Siebel, C. W. (2017). Constitutive NOTCH3 Signaling Promotes the Growth of Basal Breast Cancers. *Cancer Res, 77*(6), 1439-1452. doi:10.1158/0008-5472.CAN-16-1022

Cioffi, F., Senese, R., de Lange, P., Goglia, F., Lanni, A., & Lombardi, A. (2009). Uncoupling proteins: a complex journey to function discovery. *Biofactors, 35*(5), 417-428. doi:10.1002/biof.54

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., . . . Genomes Project Analysis, G. (2011). The variant call format and VCFtools. *Bioinformatics, 27*(15), 2156-2158. doi:10.1093/bioinformatics/btr330

Davidson, N. M., & Oshlack, A. (2014). Corset: enabling differential gene expression analysis for de novo assembled transcriptomes. *Genome Biol, 15*(7), 410. doi:10.1186/s13059-014-0410-6

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., . . . Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics, 29*(1), 15-21. doi:10.1093/bioinformatics/bts635

Dobzhansky, T. (1937). *Genetics and the Origin of Species*. New York: Columbia University Press.

Eisenberg, E., & Levanon, E. Y. (2013). Human housekeeping genes, revisited. *Trends Genet, 29*(10), 569-574. doi:10.1016/j.tig.2013.05.010

Elso, C. M., Roberts, L. J., Smyth, G. K., Thomson, R. J., Baldwin, T. M., Foote, S. J., & Handman, E. (2004). Leishmaniasis host response loci (lmr1-3) modify disease severity through a Th1/Th2-independent pathway. *Genes Immun, 5*(2), 93-100. doi:10.1038/sj.gene.6364042

Ewels, P., Magnusson, M., Lundin, S., & Kaller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics, 32*(19), 3047-3048. doi:10.1093/bioinformatics/btw354

Ferreira, M. S., Alves, P. C., Callahan, C. M., Giska, I., Farelo, L., Jenny, H., . . . Melo-Ferreira, J. (2020). Transcriptomic regulation of seasonal coat color change in hares. *Ecol Evol, 10*(3), 1180-1192. doi:10.1002/ece3.5956

Ferreira, M. S., Alves, P. C., Callahan, C. M., Marques, J. P., Mills, L. S., Good, J. M., & Melo-Ferreira, J. (2017). The transcriptional landscape of seasonal coat colour moult in the snowshoe hare. *Mol Ecol, 26*(16), 4173-4185. doi:10.1111/mec.14177

Ferreira, M. S., Jones, M. R., Callahan, C. M., Farelo, L., Tolesa, Z., Suchentrunk, F., . . . Melo-Ferreira, J. (2021). The Legacy of Recurrent Introgression during the Radiation of Hares. *Syst Biol, 70*(3), 593-607. doi:10.1093/sysbio/syaa088

Fickel, J., Hauffe, H. C., Pecchioli, E., Soriguer, R., Vapa, L., & Pitra, C. (2008). Cladogenesis of the European brown hare (Lepus europaeus Pallas, 1778). *European Journal of Wildlife Research, 54*(3), 495-510. doi:10.1007/s10344-008-0175-x

Finn, R. D., Clements, J., & Eddy, S. R. (2011). HMMER web server: interactive sequence similarity searching. *Nucleic Acids Res, 39*(Web Server issue), W29-37. doi:10.1093/nar/gkr367

Fontanesi, L., Tazzoli, M., Pecchioli, E., Hauffe, H. C., Robinson, T. J., & Russo, V. (2008). Sexing European rabbits (Oryctolagus cuniculus), European brown hares (Lepus europaeus) and mountain hares (Lepus timidus) with ZFX and ZFY loci. *Mol Ecol Resour, 8*(6), 1294-1296. doi:10.1111/j.1755-0998.2008.02167.x

Frichot, E., & Francois, O. (2015). LEA: An R package for landscape and ecological association studies. *Methods in Ecology and Evolution, 6*(8), 925-929. doi:10.1111/2041-210x.12382

Fu, L., Niu, B., Zhu, Z., Wu, S., & Li, W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics, 28*(23), 3150-3152. doi:10.1093/bioinformatics/bts565

Ginestet, C. (2011). ggplot2: Elegant Graphics for Data Analysis. *Journal of the Royal Statistical Society Series a-Statistics in Society, 174*, 245-245. doi:DOI 10.1111/j.1467-985X.2010.00676\_9.x

Giska, I., Farelo, L., Pimenta, J., Seixas, F. A., Ferreira, M. S., Marques, J. P., . . . Melo-Ferreira, J. (2019). Introgression drives repeated evolution of winter coat color polymorphism in hares. *Proc Natl Acad Sci U S A, 116*(48), 24150-24156. doi:10.1073/pnas.1910471116

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., . . . Regev, A. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat Protoc, 8*(8), 1494-1512. doi:10.1038/nprot.2013.084

Hamilton, J. A., & Miller, J. M. (2016). Adaptive introgression as a resource for management and genetic conservation in a changing climate. *Conservation Biology, 30*(1), 33-41. doi:10.1111/cobi.12574

Harrison, R. G., & Larson, E. L. (2014). Hybridization, introgression, and the nature of species boundaries. *J Hered, 105 Suppl 1*, 795-809. doi:10.1093/jhered/esu033

Hedrick, P. W. (2013). Adaptive introgression in animals: examples and comparison to new mutation and standing variation as sources of adaptive variation. *Mol Ecol, 22*(18), 4606-4618. doi:10.1111/mec.12415

Jasaszwili, M., Billert, M., Strowski, M. Z., Nowak, K. W., & Skrzypski, M. (2020). Adropin as A Fat-Burning Hormone with Multiple Functions-Review of a Decade of Research. *Molecules, 25*(3). doi:10.3390/molecules25030549

Jiang, D., & Rinkevich, Y. (2018). Defining Skin Fibroblastic Cell Types Beyond CD90. *Front Cell Dev Biol, 6*, 133. doi:10.3389/fcell.2018.00133

Jimenez-Jacinto, V., Sanchez-Flores, A., & Vega-Alvarado, L. (2019). Integrative Differential Expression Analysis for Multiple EXperiments (IDEAMEX): A Web Server Tool for Integrated RNA-Seq Data Analysis. *Front Genet, 10*, 279. doi:10.3389/fgene.2019.00279

Jones, M. R., Mills, L. S., Alves, P. C., Callahan, C. M., Alves, J. M., Lafferty, D. J. R., . . . Good, J. M. (2018). Adaptive introgression underlies polymorphic seasonal camouflage in snowshoe hares. *Science, 360*(6395), 1355-1358. doi:10.1126/science.aar5273

Jonkman, J. E., Cathcart, J. A., Xu, F., Bartolini, M. E., Amon, J. E., Stevens, K. M., & Colarusso, P. (2014). An introduction to the wound healing assay using live-cell microscopy. *Cell Adh Migr, 8*(5), 440-451. doi:10.4161/cam.36224

Kadota, M., Nishimura, O., Miura, H., Tanaka, K., Hiratani, I., & Kuraku, S. (2020). Multifaceted Hi-C benchmarking: what makes a difference in chromosome-scale genome scaffolding? *Gigascience, 9*(1). doi:10.1093/gigascience/giz158

Kamvar, Z. N., Tabima, J. F., & Grunwald, N. J. (2014). Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ, 2*, e281. doi:10.7717/peerj.281

Krogh, A., Larsson, B., von Heijne, G., & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol, 305*(3), 567-580. doi:10.1006/jmbi.2000.4315

Lagesen, K., Hallin, P., Rodland, E. A., Staerfeldt, H. H., Rognes, T., & Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res, 35*(9), 3100-3108. doi:10.1093/nar/gkm160

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat Methods, 9*(4), 357-359. doi:10.1038/nmeth.1923

Levanen, R., Kunnasranta, M., & Pohjoismaki, J. (2018). Mitochondrial DNA introgression at the northern edge of the brown hare (Lepus europaeus) range. *Annales Zoologici Fennici, 55*(1-3), 15-24.

Levänen, R., Pohjoismäki, J., & Kunnasranta, M. (2019). Home ranges of semi-urban brown hares (Lepus europaeus) and mountain hares (Lepus timidus) at northern latitudes. *Annales Zoologici Fennici, 56*, 107–120.

Levanen, R., Thulin, C. G., Spong, G., & Pohjoismaki, J. L. O. (2018). Widespread introgression of mountain hare genes into Fennoscandian brown hare populations. *Plos One, 13*(1). doi:ARTN e0191790

10.1371/journal.pone.0191790

Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *Bmc Bioinformatics, 12*. doi:Artn 323

10.1186/1471-2105-12-323

Liu, G., David, B. T., Trawczynski, M., & Fessler, R. G. (2020). Advances in Pluripotent Stem Cells: History, Mechanisms, Technologies, and Applications. *Stem Cell Rev Rep, 16*(1), 3-32. doi:10.1007/s12015-019-09935-x

Malik, N., & Rao, M. S. (2013). A review of the methods for human iPSC derivation. *Methods Mol Biol, 997*, 23-33. doi:10.1007/978-1-62703-348-0\_3

Mallet, J., Besansky, N., & Hahn, M. W. (2016). How reticulated are species? *Bioessays, 38*(2), 140-149. doi:10.1002/bies.201500149

Marques, J. P., Farelo, L., Vilela, J., Vanderpool, D., Alves, P. C., Good, J. M., . . . Melo-Ferreira, J. (2017). Range expansion underlies historical introgressive hybridization in the Iberian hare. *Scientific Reports, 7*. doi:Artn 40788

10.1038/Srep40788

Marques, J. P., Ferreira, M. S., Farelo, L., Callahan, C. M., Hacklander, K., Jenny, H., . . . Melo-Ferreira, J. (2017). Mountain hare transcriptome and diagnostic markers as resources to monitor hybridization with European hares. *Sci Data, 4*, 170178. doi:10.1038/sdata.2017.178

Marques, J. P., Seixas, F. A., Farelo, L., Callahan, C. M., Good, J. M., Montgomery, W. I., . . . Melo-Ferreira, J. (2020). An Annotated Draft Genome of the Mountain Hare (Lepus timidus). *Genome Biology and Evolution, 12*(1), 3656-3662. doi:10.1093/gbe/evz273

Mayr, E. (1942). *Systematics and the origin of species from the viewpoint of a zoologist*. New York: Columbia University Press.

Melo-Ferreira, J., Boursot, P., Suchentrunk, F., Ferrand, N., & Alves, P. C. (2005). Invasion from the cold past: extensive introgression of mountain hare (Lepus timidus) mitochondrial DNA into three other hare species in northern Iberia. *Mol Ecol, 14*(8), 2459-2464. doi:10.1111/j.1365-294X.2005.02599.x

Melo-Ferreira, J., Farelo, L., Freitas, H., Suchentrunk, F., Boursot, P., & Alves, P. C. (2014). Home-loving boreal hare mitochondria survived several invasions in Iberia: the relative roles of recurrent hybridisation and allele surfing. *Heredity, 112*(3), 265-273. doi:10.1038/hdy.2013.102

Melo-Ferreira, J., Seixas, F. A., Cheng, E., Mills, L. S., & Alves, P. C. (2014). The hidden history of the snowshoe hare, Lepus americanus: extensive mitochondrial DNA introgression inferred from multilocus genetic variation. *Mol Ecol, 23*(18), 4617-4630. doi:10.1111/mec.12886

Morganti, C., Bonora, M., Ito, K., & Ito, K. (2019). Electron transport chain complex II sustains high mitochondrial membrane potential in hematopoietic stem and progenitor cells. *Stem Cell Res, 40*, 101573. doi:10.1016/j.scr.2019.101573

Motulsky, H. J., & Brown, R. E. (2006). Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. *Bmc Bioinformatics, 7*. doi:Artn 123

10.1186/1471-2105-7-123

Nowack, J., Giroud, S., Arnold, W., & Ruf, T. (2017). Muscle Non-shivering Thermogenesis and Its Role in the Evolution of Endothermy. *Front Physiol, 8*, 889. doi:10.3389/fphys.2017.00889

Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods, 8*(10), 785-786. doi:10.1038/nmeth.1701

Pohjoismaki, J. L. O., Michell, C., Levanen, R., & Smith, S. (2021). Hybridization with mountain hares increases the functional allelic repertoire in brown hares. *Sci Rep, 11*(1), 15771. doi:10.1038/s41598-021-95357-0

Rath, S., Sharma, R., Gupta, R., Ast, T., Chan, C., Durham, T. J., . . . Mootha, V. K. (2021). MitoCarta3.0: an updated mitochondrial proteome now with sub-organelle localization and pathway annotations. *Nucleic Acids Res, 49*(D1), D1541-D1547. doi:10.1093/nar/gkaa1011

Reid, N. (2011). European hare (Lepus europaeus) invasion ecology: implication for the conservation of the endemic Irish hare (Lepus timidus hibernicus). *Biological Invasions, 13*(3), 559-569. doi:10.1007/s10530-010-9849-x

Schluter, D., & Conte, G. L. (2009). Genetics and ecological speciation. *Proc Natl Acad Sci U S A, 106 Suppl 1*, 9955-9962. doi:10.1073/pnas.0901264106

Sheriff, M. J., Kuchel, L., Boutin, S., & Humphries, M. M. (2009). Seasonal Metabolic Acclimatization in a Northern Population of Free-Ranging Snowshoe Hares, Lepus Americanus. *Journal of Mammalogy, 90*(3), 761-767. doi:Doi 10.1644/08-Mamm-a-247r.1

Smith, S., Sandoval-Castellanos, E., Lagerholm, V. K., Napierala, H., Sablin, M., Von Seth, J., . . . Dalen, L. (2017). Nonreceding hare lines: genetic continuity since the Late Pleistocene in European mountain hares (Lepus timidus). *Biological Journal of the Linnean Society, 120*(4), 891-908.

Smith-Unna, R., Boursnell, C., Patro, R., Hibberd, J. M., & Kelly, S. (2016). TransRate: reference-free quality assessment of de novo transcriptome assemblies. *Genome Res, 26*(8), 1134-1144. doi:10.1101/gr.196469.115

Soubrier, J., Gower, G., Chen, K., Richards, S. M., Llamas, B., Mitchell, K. J., . . . Cooper, A. (2016). Early cave art and ancient DNA record the origin of European bison. *Nat Commun, 7*, 13158. doi:10.1038/ncomms13158

Todesco, M., Pascual, M. A., Owens, G. L., Ostevik, K. L., Moyers, B. T., Hubner, S., . . . Rieseberg, L. H. (2016). Hybridization and extinction. *Evolutionary Applications, 9*(7), 892-908. doi:10.1111/eva.12367

Waterhouse, R. M., Seppey, M., Simao, F. A., Manni, M., Ioannidis, P., Klioutchnikov, G., . . . Zdobnov, E. M. (2018). BUSCO Applications from Quality Assessments to Gene Prediction and Phylogenomics. *Mol Biol Evol, 35*(3), 543-548. doi:10.1093/molbev/msx319

Wolf, J. B., Lindell, J., & Backstrom, N. (2010). Speciation genetics: current status and evolving approaches. *Philos Trans R Soc Lond B Biol Sci, 365*(1547), 1717-1733. doi:10.1098/rstb.2010.0023

Yamaguchi, K., Kadota, M., Nishimura, O., Ohishi, Y., Naito, Y., & Kuraku, S. (2021). Technical considerations in Hi-C scaffolding and evaluation of chromosome-scale genome assemblies. *Mol Ecol, 30*(23), 5923-5934. doi:10.1111/mec.16146

Zhang, Z., Sun, Y., Cho, Y. W., Chow, C. C., & Simons, S. S., Jr. (2013). PA1 protein, a new competitive decelerator acting at more than one step to impede glucocorticoid receptor-mediated transactivation. *J Biol Chem, 288*(1), 42-58. doi:10.1074/jbc.M112.427740

Zimova, M., Giery, S. T., Newey, S., Nowak, J. J., Spencer, M., & Mills, L. S. (2020). Lack of phenological shift leads to increased camouflage mismatch in mountain hares. *Proc Biol Sci, 287*(1941), 20201786. doi:10.1098/rspb.2020.1786

# Data Accessibility and Benefit-Sharing Statement

**Genetic data:**

All data are available through Dryad depository under Gaertner, Kateryna et al. (2022), Molecular phenotyping uncovers differences in basic housekeeping functions among closely related species of hares (Lepus spp., Lagomorpha: Leporidae), Dryad, Dataset, <https://doi.org/10.5061/dryad.p8cz8w9sm>

Reviewer link to the dataset is:

<https://datadryad.org/stash/share/odi_wPGLHCvOqn9cLRxVN6KIRrY0kTi38wTl-i9f7o0>

Raw sequence reads are deposited in the SRA under the access numbers given in S-Table 1. Reviewer link to the dataset in SRA depository is:

<https://dataview.ncbi.nlm.nih.gov/object/PRJNA826339?reviewer=e3h1ov8q50i74mn799farm602v>

**Sample metadata:**

Metadata is presented in Table 1 as well as stored in the SRA (see S-Table 1).

**Research material availability:**

The authors are willing to share the cell lines, reagents, laboratory notes and advice upon reasonable request for non-commercial purposes.

**Benefits Generated:**

All collaborators are included as co-authors, the results of research have been shared with the provider communities and the broader scientific community. More broadly, our group is committed to international scientific partnerships, as well as institutional capacity building. The contributions of all individuals to the research, including hunters, are described in the METHODS and ACKNOWLEDGEMENTS. All data have been shared with the broader public via appropriate biological databases.

# Author contributions

Designed research: JLOP, ED, KG, CM; Performed research: KG, CM, ED, RT, SG, SES, JP;

Contributed new reagents or analytical tools: CM, SG, MS; Analyzed data: KG, CM, ED, RT, JP; Wrote the paper: JP, ED, KG, CM, SG.

# Tables

Table 1. Species, locality data, sex and mtDNA haplotype of the cell lines used in the study.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Species** | **Cell line ID** | **Collection locality** | **Longitude;Latitude** | **Sex** | **mtDNA** |
| *Lepus timidus* | LT1 | Ilomantsi | 63.0609;30.6248 | Male | *timidus* |
| LT4 | Vesilahti | 61.2671;23.4845 | Female | *timidus* |
| LT5 | Outokumpu | 62.7595;28.9765 | Male | *timidus* |
| LT6 | Ruokolahti | 61.3390;28.8804 | Male | *timidus* |
| *Lepus europaeus* | LE1 | Liperi | 62.6207;29.4478 | Male | *europaeus* |
| LE2 | Outokumpu | 62.6212;29.0733 | Female | *europaeus* |
| LE3 | Kontiolahti | 62.6508;29.7507 | Female | *europaeus* |
| LE4 | Vesilahti | 61.2937;23.4590 | Male | *europaeus* |

# Figures

**Fig. 1.** Overview of the sample distribution, genotypes and transcriptomes of the hare cell lines. (A) Collection loci of the hare specimens. (B) PCA plot of the sample genotypes based on 9,056 SNPs in 519,066–549,582 transcripts. Insert Venn-diagram illustrates private and shared alleles between the two species. (C) Distribution plot of heterozygosity levels among the two species, four cell lines each. Horizontal line denotes the mean heterozygosity. (D) Ancestry coefficient plot of the eight cell lines. Mountain hare contribution to the ancestry in blue and brown hare in yellow. (E) Heatmap clustering of the eight cell lines based on the relative expression levels of the 16,689 orthologous genes. (F) PCA plot based on the relative expression levels of all transcripts. (G) PCA plot based on the relative expression levels of orthologous transcripts. LE = *Lepus europaeus*, LT = *Lepus timidus*.

**Fig. 2.** Species specific gene expression in hare fibroblasts. Left: Examples of differentially expressed GO-terms between the two species. Right: A volcano plot showing differentially expressed genes. Vertical blue line indicates 2-fold difference and horizontal the *p*-value of 0.001. Some example genes are indicated.

**Fig. 3.** Comparison of the main mitochondrial parameters between mountain hare and brown hare cell lines. (A) Mitochondrial mass. FCCP is uncoupling chemical used as a control to show that the mitochondrial staining is working. (B) Mitochondrial membrane potential. (C) Comparison of cell respiration rates. (D) Explanation of the experimental conditions for (C). (E) Mitochondrial DNA (mtDNA) copy number and levels of example mitochondrial proteins as quantified from Western blots. Data are presented as mean ± SD, n = 27; two-tailed Student's unpaired t-test, except for (C), where n = 16 and two-way ANOVA was used; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. LE = *Lepus europaeus*, LT = *Lepus timidus*.

**Fig. 4.** Cell proliferation and migration. (A) Cell doubling times in high and low glucose media. (B) Delay time before the wound closure starts (left) and final wound closure rate (right). Wound closure rate measured using two different approaches (see methods) that are indicated as their own scatter diagrams. (C) Example image of a wound closure assay showing notable cell proliferation and migration after 21 h. Data are presented as mean ± SD, for delay analysis n = 24, Mann Whitney U test; and for closure rate n = 12, two-way ANOVA; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

**Fig. 5.** Cell cycle comparison between mountain hare and brown hare fibroblasts. (A) Cell cycle phase distribution in the two species before and after synchronization. (B) Comparison of G1, S and G2 phase duration. Data are presented as mean ± SD, n = 24, Mixed-effects model (REML); \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.

**Fig. 6.** Examples of the size and morphology of the cell lines. Scale bar 50 μm. See S-Fig. 1 for more details.