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ORIGINAL ARTICLE

Expression of threonine-biosynthetic genes in mammalian cells and transgenic mice

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Abstract Threonine is a nutritionally essential amino acid (EAA) for the growth and development of humans and other nonruminant animals and must be provided in diets to sustain life. The aim of this study was to synthesize threonine in mammalian cells through transgenic techniques. To achieve this goal, we combined the genes involved in bacterial threonine biosynthesis pathways into a single open reading frame separated by self-cleaving peptides (2A) and then linked it into a transposon system (*piggyBac*). The plasmids pEF1a-IRES-GFP-E2F-his and pEF1a-IRES-GFP-M2F-his expressed *Escherichia coli* homoserine kinase and threonine synthase efficiently in mouse cells and enabled cells to synthesize threonine from homoserine. This biosynthetic pathway occurred with a low level of efficiency in transgenic mice. Three transgenic mice were identified by Southern blot from 72 newborn mice, raising the possibility that a high level of expression of these genes in mouse embryos might be lethal. The results indicated that it is feasible to synthesize threonine in animal cells using genetic engineering technology. Further work is required to improve the efficiency of this method for introducing genes into mammals. We propose that the transgenic technology provides a promising means to enhance the synthesis of nutritionally EAAs in farm animals and to eliminate or reduce supplementation of these nutrients in diets for livestock, poultry and fish.

Keywords Threonine biosynthesis · Transgene · Homoserine · 2A peptide

Abbreviations

EAA	Nutritionally essential amino acid
HPLC	High-performance liquid chromatography
KHB	Krebs–Henseleit bicarbonate buffer
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction

Introduction

Metabolic pathways for the synthesis of threonine in animal cells were lost during the evolutionary process (Wu 2009). This amino acid has important nutritional and physiological functions in organisms (Wu 2013a). Thus, it must be present in diets to support the growth, development and survival of humans and other nonruminant animals with limited synthesis of threonine in the gastrointestinal tract (Bach et al. 2005; Fenderson and Bergen 1975). For this reason, threonine has been classified as a nutritionally essential amino acid (EAA). The development of transgenic technology provides a method to repair the nonfunctional biochemical pathways in animals which still exist in bacteria and plants (Ward 2000). Research has provided useful examples of powerful possibilities to transfer functional genes from bacteria to animals,

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including the introduction of a cysteine biosynthetic pathway to improve wool growth (Bawden et al. 1995), the transfer of the glyoxylate cycle genes to mice to convert acetate into glucose (Saini et al. 1996), and the construction of phytase transgenes to increase the bioavailability of dietary phytate (Golovan et al. 2001). Increasing the supply of EAA has become another goal in this area and Rees et al. (1990) predicted the possibility of producing transgenic animals capable of synthesizing lysine and threonine.

Threonine is usually a limiting amino acid in the diets of pigs and broilers (Wu et al. 2014). Except for its utilization for supporting normal growth, threonine also improves the immunity function of the animal by promoting the synthesis of intestinal mucin and \plasma r-globulin (Li et al. 2007). The utilization of dietary threonine by the intestine is up to 60 % in piglets (Stoll et al. 1998). Threonine constitutes approximately 30 % of the total amino acids in the protein of the predominant secretory mucin in the human intestinal tract (Gum 1992). Dietary threonine restriction specifically impaired the synthesis of mucins in all segments of the small intestine (Wang et al. 2007, 2010). Furthermore, dietary supplementation with threonine increases antibody production, serum IgG levels, and jejunal mucosal concentrations of IgG and IgA (Li et al. 2007). Therefore, dietary deficiency of threonine indeed impairs the growth of young animals as well as their immunity (Ciftci and Ceylan 2004; Horn et al. 2009; Remond et al. 2009; Wang et al. 2007). Recent studies showed that threonine is critically required for the pluripotency of mouse embryonic stem cells (Shyh-Chang et al. 2013; Ryu and Han 2011; Wang et al. 2009) and is crucial for the growth of mouse embryos.

Threonine is metabolized in two ways: it can be catabolized by threonine dehydratase to yield 2-ketobutyric acid and then transaminated to 2-aminobutyric acid by alanine aminotransferase or degraded by branched-chain a-keto acid dehydrogenase (BCKDH) or pyruvate dehydrogenase (PDH) to propionyl coenzyme A. It also can be catabolized by threonine dehydrogenase to 2-amino-3-ketobutyric acid, which is mainly cleaved to glycine and acetyl CoA by 2-amino-ketobutyrate CoA ligase (Wu 2013b). Threonine dehydrogenase pathway accounts for ~80 % of total threonine degradation in animals (Bird and Nunn 1983; Dale 1978; Darling et al. 1999).

Threonine cannot be synthesized in the body of humans and animals (Wu et al. 2013b). Studies with the biosynthesis of threonine in bacteria showed that the predominant industrial producer of threonine is *Escherichia coli* followed by *Corynebacterium glutamicum*. The demand for L-threonine has provided a strong impetus to improve the productivity of microbial fermentation through genetic manipulation over the past decades. Belonging to the aspartic family of amino acids, L-threonine is synthesized from L-aspartate. Five enzymes, aspartate kinase, aspartyl semialdehyde dehydrogenase, homoserine dehydrogenase, homoserine kinase and threonine synthase, are involved in the biosynthesis pathway, which share common L-lysine and L-methionine competing branches (Fig. 1) (Wu et al. 2013b). In *E. coli*, there are three aspartate kinase isoenzymes designated aspartate kinases I, II and III; aspartate kinase I is the most abundant and is encoded by *thrA*. Aspartate kinases I (AKI-HSDI) and II (AKII-HSDII) are bifunctional enzymes, with both aspartate kinase activity and homoserine dehydrogenase activity (Chassagnole et al. 2001; Katinka et al. 1980). In *C. glutamicum*, aspartate kinase encoded only by *lysC* and homoserine dehydrogenase encoded by *hom* are monofunctional enzymes (Eikmanns et al. 1993). In both *E. coli* and *C. glutamicum*, aspartyl semialdehyde dehydrogenase is encoded by *asd*, homoserine kinase is encoded by *thrB* and threonine synthase is encoded by *thrC*.

Engineering of mouse cells transfected with threonine-synthetic genes was done in the 1990s by Rees et al. (1992, 1994) and Rees and Hay (1993, 1995). In contrast to the relatively simple one- or two-gene pathways, it is technically more complex and difficult to assemble bacterial genes into a single fragment in preparation for gene transfer. In the studies cited above, *E. coli thrB* (homoserine kinase) and *thrC* (threonine synthase) genes were

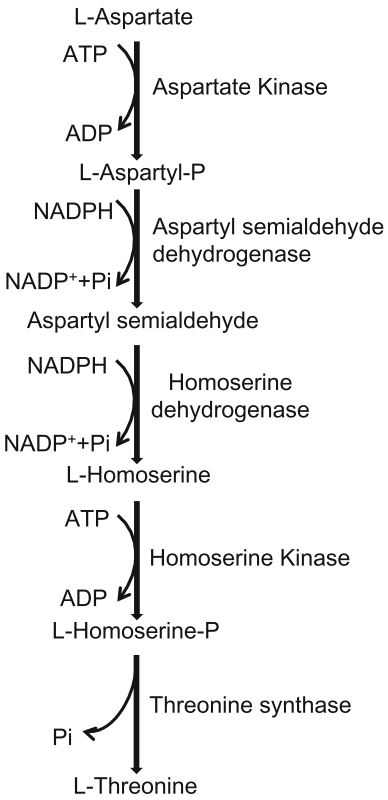

Fig. 1 The pathway of L-threonine biosynthesis in bacteria

cloned into plasmid pSVL to yield pSVthrBC with two SV40 promoters before each coding sequence. Plasmid pSVthrA/asd expressing *E. coli thrA* (aspartokinase I/homoserine dehydrogenase I) and *C. glutamicum asd* (aspartic semialdehyde dehydrogenase) genes was constructed in the same way. These plasmids were transfected into mouse 3T3 cells and those able to synthesize threonine by culturing cells in threonine-free medium were selected. Despite the expression of the enzymes and the survival of transgenic cells in threonine-free medium, there was an imbalance in the expression of each gene because of the separate promoters.

To date, several approaches have been proposed to overcome the need for multiple promoters within a multigene complex, in which the 'self-cleaving' 2A peptide method is the most attractive choice (Szymczak et al. 2004; Trichas et al. 2008). It provides the advantage of reliable and equimolar expression of multiple genes, because these peptides allow multiple proteins to be encoded as polyproteins and dissociate into component proteins on translation. It has been shown to function in a wide variety of cells, including yeast, plants, insects and mammals (de Felipe et al. 2006). In the current work, we linked threonine-biosynthetic genes from *E. coli* and made synthetic sequences after optimizing for mammals by self-cleaving peptides (2A) and then cloned the genes into a transposon plasmid with one EF1a promoter. Because of equimolar expression by the 2A peptides, we labeled the last gene of each plasmid with a his- or a flag-tag for testing protein expression by Western blots. Thus, we attempted to introduce the threonine-biosynthetic pathway into mammalian cells and mice with stoichiometric amounts of protein expression, so as to eliminate the need for supplemental threonine in animal diets.

Materials and methods

Construction of expression vectors

Plasmid pEF1a-PB was a gift from Dr. Sen Wu (China Agricultural University, China). The *Swa*I and *Spe*I IRES-GFP element was amplified by PCR and ligated into pEF1a-PB to yield pEF1a-IRES-GFP, which was the backbone of all the plasmids listed below. The genes coding for aspartokinase I/homoserine dehydrogenase I (*thrA,* NP_414543.1), aspartic semialdehyde dehydrogenase (*asd,* NP_417891.1), homoserine kinase (*thrB,* NP_414544.1) and threonine synthase (*thrC,* NP_414545.1) were amplified by PCR from the *E. coli* genome, then cloned into a T-vector and confirmed by sequencing. Fragments containing parts of the 2A peptide sequence in each gene and his-tag sequence in *thrC* were amplified from T-vectors carrying correct sequences and then constructed into the 2A peptide-linked four- or two-factor cassettes by fusion PCR. These cassettes were combined into pEF1a-IRES-GFP to yield pEF1a-IRES-GFP-E4F-his and pEF1a-IRES-GFP-E2F-his. The sequences of four genes in *E. coli* and three genes in *C. glutamicum* (*lysC*, YP_224551.1; *hom*, YP_225473.1; *asd*, YP_224552.1) were optimized for animals using the software available at website http://www.jcat.de/Start.jsp. These genes were linked with the 2A peptide to yield sequences of *Nhe*I-M4F-his-*Swa*I and *Nhe*I-*C.Glu*-M3F-flag-*Hin*dIII, which were synthesized by BGI, China. The cassettes were combined into pEF1a-IRES-GFP to yield pEF1a-IRES-GFP-M4F-his and pEF1a-IRES-GFP-*C.glu*-M3F-flag. Plasmids pEF1a-IRES-GFP-M2F-his and pEF1a-IRES-GFP-MthrA-Masd-flag were constructed as follows. Cassettes of *Nhe*I-M2F-his-*Swa*I and *Nhe*I-MthrA-Masd-flag-*Swa*I were amplified by PCR from the pEF1a-IRES-GFP-M4F-his plasmid and then combined into pEF1a-IRES-GFP. All primers are given in Table 1.

Cell culture and transfection

The C2C12 cells and the NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % (v/v) fetal bovine serum (FBS, Gibco) and maintained at 37 °C in a humidified environment with a 5 % (v/v) CO_2 atmosphere. *piggyBac* transposon (3 μg) and 1 μg of *piggyBac* transposase expression vectors were co-transfected into 10^6 cells by electroporation (Lonza). At 24 h after transfection cells were cultured for 7 days in medium containing 500 g/ml G418 (Sigma-Aldrich).

Assays for synthesis of threonine in mammalian cells

Krebs–Henseleit bicarbonate (KHB) buffer (pH 7.4 at 37 °C) was prepared with the following final concentrations: 119 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.8 mM KCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$ and 5 mM glucose (Wu et al. 1994). The buffer was equilibrated with 95 % O_2 and 5 % CO_2 before use.

C2C12 cells and NIH-3T3 cells (1×10^6/flask) were incubated for 3 h at 37 °C in 1 ml of oxygenated KHB buffer with or without 2 mM aspartate or homoserine. Then 200 μl of $HClO_4$ was added to the culture medium. The acidified medium was neutralized with 100 μl of K_2CO_3. Whole-cell extracts were collected individually into 1.5-ml tubes and centrifuged at 12,000×*g* for 5 min. The supernatant fluid was collected for analysis of amino acids using our established HPLC method (Dai et al. 2013).

Table 1 PCR primer sequences

Primer name	Primer sequence
Primers for amplification of *E. coli* genes	
EthrA F	ATGCGAGTGTTGAAGTTCGGCG
EthrA R	TCAGACTCCTAACTTCCATGAG
EthrB F	ATGGTTAAAGTTTATGCCCCGG
EthrB R	TTAGTTTTCCAGTACTCGTGCGC
EthrC F	ATGAAACTCTACAATCTGAAAG
EthrC R	TTACTGATGATTCATCATCAAT
Easd F	ATGAAAAATGTTGGTTTTATCGGC
Easd R	TTACGCCAGTTGACGAAGCATCC
Primers for construction of pEF1a-IRES-GFP-E4F-his	
NheI-EthrA F	CTAGCTAGCGCCACCATGGGGATGCGAGTGTTGAAGTTCGGCG
2A-EthrA R	CAACATCGCCAGCGAGTTTCAACAAAGCGTAGTTAGTACATTGCCCACTACCGACTCCTAACTTCCATGAGAGG
2A-Easd F	GCTTTGTTGAAACTCGCTGGCGATGTTGAAAGTAACCCCGGTCCTATGAAAAATGTTGGTTTTATCG
2A-Easd R	GGCCTCGAGCGGGGGCCCTGGGTTGGACTCCACGTCTCCCGCCAACTTGAGAAGGTCAAAATTCAAAGTCTGTTTCACGCCACTTCCCGCCAGTTGACGAAGCATCCGACG
2A-EthrB F	CCCGCGGCCGCAGCCACCATGGGGATGGTTAAAGTTTATGCCCCGG
2A-EthrB R	CTCCACGTCGCCGCAGGTCAGCAGGCTGCCCCTGCCCTCGCCGCTGCCGTTTTCCAGTACTCGTGCGCCC
2A-EthrC F	CAGCCTGCTGACCTGCGGCGACGTGGAGGAGAACCCCGGCCCCATGAAACTCTACAATCTGAAAG
BamHI-EthrC R	CGCGGATCCTTAATGGTGATGGTGATGATGCTGATGATTCATCATCAATTTAC
Primers for construction of pEF1a-IRES-GFP-E2F-his	
NheI-EthrB F	CTAGCTAGCGCCACCATGGGGATGGTTAAAGTTTATGCCCCG
BamHI-EthrC R	CGCGGATCCTTAATGGTGATGGTGATGATGCTGATGATTCATCATCAATTTAC
Primers for construction of pEF1a-IRES-GFP-M2F-his	
Nhe-MthrB F	GTGAAGCTAGCGCCACCATGGGGATGGTGAAGGTGTACGCCCCCGCC
swaI-MthrC R	GATTTAAATTTAATGGTGATGGTGATGATGCTGGTGGTTCATCATCAGCTTCCTC
Primers for construction of pEF1a-IRES-GFP-MthrA-Masd-flag	
NheI-MthrA F	CTAGCTAGCGCCACCATGGGGATGAGGGTGCTGAAGTTCGGCG
swaI-flag-Masd R	CCAGATTTAAATTTACTTATCGTCGTCATCCTTGTAATCGGCCAGCTGCCTCAGCATCCT
Primers for RT-PCR	
RT-MthrB F	AGCGAGCCCAGGGAGAACA
RT-MthrB R	GCTGTAGCAGGCGTGGATGAA
RT-MthrC F	TGCTGAAGCTGGACTTCGTG
Rt-MthrC R	TTGTTGGGCTGGCTCACG
Primers for quantitative real-time PCR	
Fabpi F	GACTGCTGGTCCTCCTACAGGAT
Fabpi R	ATTTGCACCCAACCAATGGA
M2F-QPCR F	GCGTGAAACAGACTTTGA
M2F-QPCR R	GTGGTCCTTCAGGTTGTA

In some experiments, 1×10^6 cells were lysed by freezing in liquid nitrogen and thawing at 37 °C in a water bath three times. The whole-cell lysates were then transferred into 1 ml of oxygenated KHB buffer containing 5 mM NADPH and 5 mM ATP, with or without 2 mM aspartate. The mixture was incubated for 3 h at 37 °C, acidified and neutralized for the analysis of amino acids as described above.

Generation and screening of transgenic mice

The pEF1a-IRES-GFP-M2F and *piggyBac* transposase expression vector were co-microinjected at a 3:1 (w/w) ratio into the pronuclei of fertilized Kunming white mouse eggs. Genomic DNA was isolated from tail tips of 3- to 4-week-old mice. The presence of the transgenes was

identified by both PCR and Southern blot. The primer pairs specific to the optimized sequence of *MthrB* and *MthrC*:

5′-AGCGAGCCCAGGGAGAACA-3′
5′- GCTGTAGCAGGCGTGGATGAA-3′;
5′- TGCTGAAGCTGGACTTCGTG-3′
5′-TTGTTGGGCTGGCTCACG-3′

were used for preliminary screening by PCR, which yielded 477 and 787 bp products, respectively. Genomic DNA (5 μg) was digested with *Nco*I and hybridized with a 477 bp [α-^{32}P]dCTP-labeled probe amplified from the transgene vector:

forward primer 5′-AGCGAGCCCAGGGAGAACA-3′
reverse primer 5′- GCTAGCAGGCGTGGATGAA-3′

The transgenic mouse was mated to wild-type mice for propagation.

Animal care and use

These studies were approved by China Agriculture University (Beijing, China). All the procedures were performed in accordance with the guiding principles for the care and use of laboratory animals.

Western blot analysis

Cells and tissues of transgenic mice were lysed in ice-cold immunoprecipitation (IP) lysis buffer (Beyotime) with 1 mM phenylmethanesulfonyl fluoride (Sino-American Biotec) then centrifuged at 12,000×*g* for 10 min at 4 °C. Total proteins were separated by SDS-PAGE (10 % (w/v) polyacrylamide gel) and then transferred electrophoretically to nitrocellulose membranes (Amersham Pharmacia). After blocking with 5 % (v/v) milk for 1 h the membranes were incubated with primary antibody (anti-his tag, anti-flag tag; Abcam) for 1 h and treated with peroxidase-conjugated secondary antibodies for 1 h. Signals were detected using an enhanced chemiluminescence detection system.

RT-PCR and quantitative real-time PCR

Total RNA was isolated from mouse tissues using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (2 μg) was treated with transcriptase using oligo(dT) primers (Promega). Two pairs of primers were used to detect transgene expression. Quantitative real-time PCR was done with a LightCycler 480II system (Roche) and amplification was done using SYBP Green I in a total volume of 15 μl for 40 cycles of 94 °C for 10 s, 60 °C for 15 s and 72 °C for 10 s. The copy number of the transgene was determined using a standard curve established by a standard set of mixtures representing 1, 2, 4, 8, 16, 32 and 64 copies of plasmid DNA in 10 ng of wild-type mouse genomic DNA. The housekeeping gene encoding fatty acid-binding protein (*Fabpi*) was used as an internal control. All data were collected from three independent experiments. Primers are given in Table 1.

Statistical analysis

Data expressed as mean ± SEM were analyzed using one-way analysis of variance (ANOVA) or the *t* test. When the *F* test was significant in ANOVA, the differences among means were determined by the Student–Newman–Keul multiple comparison. $P < 0.05$ was taken to indicate statistical significance.

Results

Expression and function of the whole threonine-biosynthetic pathway in mammalian cells

The threonine-biosynthetic genes in *E. coli* or synthesized after optimizing for mammals were combined into a single open reading frame separated by self-cleaving peptides (2A) and placed into the pEF1a-IRES-GFP transposon plasmid (designated pEF1a-IRES-GFP-E4F-his, pEF1a-IRES-GFP-M4F-his) (Fig. 2a). C2C12 or NIH-3T3 cells (1×10^6) were electroporated with 3 μg *piggyBac* vectors and 1 μg *piggyBac* transposase expression vector (C2C12-E4F, C2C12-M4F and NIH-3T3-E4F, NIH-3T3-M4F, respectively). Protein expression was analyzed by observing green fluorescent protein (GFP) with a fluorescence microscope and by Western blot. The sequences optimized for mammals were expressed in C2C12 and in NIH-3T3, whereas the genes of *E. coli* were not expressed (Fig. 2b, c). After culturing C2C12, NIH-3T3, C2C12-M4F and NIH-3T3-M4F cells (1×10^6) for 3 h in KHB buffer with or without 2 mM aspartate, the concentration of threonine in cell extracts was measured by HPLC. The results showed the concentration of threonine in transgenic cells provided with 2 mM Asp (C2C12-M4F, 10.60 ± 0.18 μM; NIH-3T3-M4F, 6.54 ± 0.41 μM) was not increased significantly compared to control non-transgenic cells (C2C12, 9.95 ± 0.54 μM; NIH-3T3, 4.26 ± 0.71 μM) (Table 2).

We asked whether ATP and NADPH used in the *E. coli* threonine-synthetic pathway were essential in our systems. We added 5 mM NADPH and 5 mM ATP to cell lysates in KHB buffer. Lysed C2C12, NIH-3T3, C2C12-M4F and NIH-3T3-M4F cells were cultured for 3 h in KHB buffer with or without 2 mM aspartate. The results showed no significant increase in threonine production even in the presence of NADPH and ATP (from 3.33 ± 0.08 to

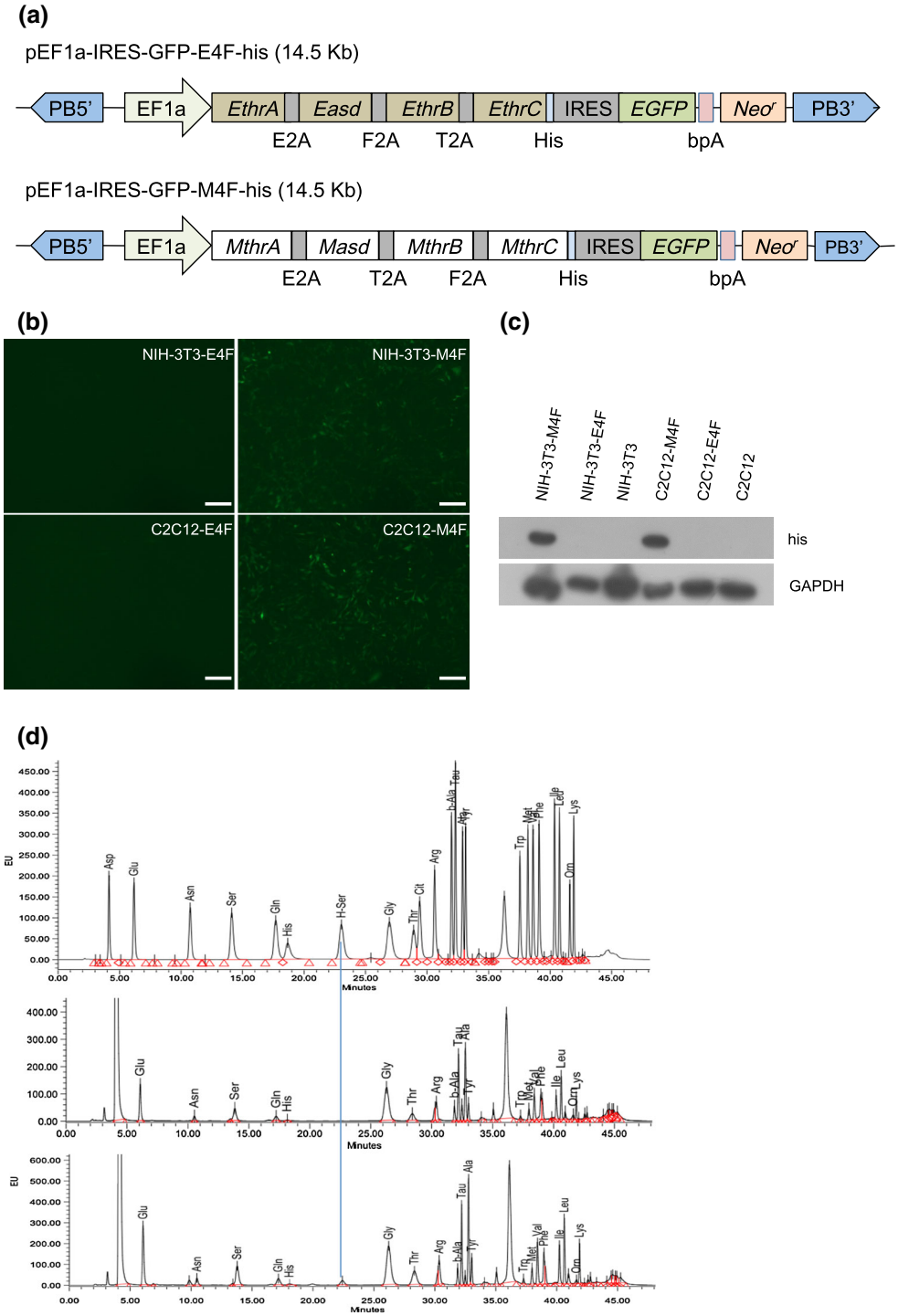

Fig. 2 Introduction and expression of the whole threonine-biosynthetic pathway in mammalian cells. **a** Schematic of pEF1a-IRES-GFP-E4F-his and pEF1a-IRES-GFP-M4F-his vectors used to deliver the genes involved in the whole threonine-biosynthetic pathway. Genes amplified from *E. coli* (*EthrA, Easd, EthrB* and *EthrC*) and optimized for mammals (*MthrA, Masd, MthrB* and *MthrC*) were linked by 2A peptides, and these cassettes of factors and 2A peptides were termed E4F and M4F. Expression of the factors was driven by the constitutively active EF1a promoter. PB5′ and PB3′ were terminal repeats of the *piggyBac* transposon; IRES-GFP was a reporter gene; Neo^r was a neomycin resistance gene; bpA was a bovine growth hormone polyadenylation signal; and His was a his-tag sequence. **b** GFP expression in cells carrying E4F and M4F at day 7. *Scale bar* 250 μm. **c** Western blot analyses of his-tag-labeled *thrC* expression in cells carrying E4F and M4F. **d** Homoserine production by lysates of C2C12-M4F cells provided with NADPH, ATP and aspartate. *Upper* authentic homoserine standard (10 μM). *Middle* extract of control C2C12-M4F cells provided with aspartate but not with NADPH or ATP. *Lower* extract of C2C12-M4F cells provided with NADPH, ATP and aspartate. The *blue line* shows the peak for homoserine (color figure online)

Table 2 Concentration of threonine (μM) in cell extracts plus KHB medium after 3 h incubation of cells transfected with the whole threonine-biosynthetic pathway

Buffer	C2C12	C2C12-M4F	NIH-3T3	NIH-3T3-M4F
KHB	7.19 ± 0.36	7.82 ± 1.27	2.76 ± 0.36	3.98 ± 0.53
KHB + 2 mM Asp	9.95 ± 0.54	10.6 ± 0.18	4.26 ± 0.71	6.54 ± 0.41

The results were from three experiments ($n = 3$) and are expressed as mean ± SD

Table 3 Concentration of threonine (μM) in whole-cell extracts plus KHB medium after lysates of cells were incubated with 5 mM NADPH and 5 mM ATP

Buffer	C2C12	C2C12-M4F	NIH-3T3	NIH-3T3-M4F
KHB + 5 mM NADPH + 5 mM ATP	3.47 ± 0.18	3.96 ± 0.29	3.76 ± 0.50	4.10 ± 0.50
KHB + 5 mMA NDPH + 5mM ATP + 2 mM Asp	3.33 ± 0.08	4.27 ± 0.16	3.93 ± 0.56	4.70 ± 0.25

The results were from three experiments ($n = 3$) and are expressed as mean ± SD

Table 4 Concentration of threonine (μM) synthesized from homoserine in cells transfected with genes for the whole threonine-biosynthetic pathway

Buffer	C2C12	C2C12-M4F	NIH-3T3	NIH-3T3-M4F
KHB	11.0 ± 1.27	11.2 ± 0.83	3.95 ± 0.15	7.08 ± 0.13
KHB + 2 mM homoserine	14.9 ± 0.23	142.3 ± 1.29	5.32 ± 0.76	114.7 ± 0.13

The results were from three experiments ($n = 3$) and are expressed as mean ± SD

4.27 ± 0.16 μM in the C2C12 group; from 3.93 ± 0.56 to 4.70 ± 0.25 μM in the NIH-3T3 group) (Table 3). However, peaks representing homoserine appeared in the HPLC trace of transgenic cells provided with NADPH, ATP and aspartate, but homoserine was not found in the control cell extracts (Fig. 2d). The results described above suggested homoserine could be synthesized from aspartate in an ATP- and NADPH-dependent manner.

Threonine synthesis from homoserine in mammalian cells

To test whether a high concentration of homoserine could stimulate the synthesis of threonine, we cultured 1×10^6 C2C12, NIH-3T3, C2C12-M4F or NIH-3T3-M4F cells for 3 h in KHB buffer with or without 2 mM homoserine. The results indicated the concentration of threonine in extracts of transgenic cells incubated with homoserine was increased from 14.9 ± 0.23 and 5.32 ± 0.76 μM in control non-transgenic C2C12 and NIH-3T3 cells, respectively, to 142.3 ± 1.29 and 114.7 ± 0.13 μM in transgenic C2C12-M4F and NIH-3T3-M4F cells, respectively. There was a 9.6- and 21.6-fold increase in C2C12-M4F and NIH-3T3-M4F cells, respectively (Table 4). Thus, a large amount of threonine was synthesized from homoserine in these mammalian cells.

To compare the efficiency of *E. coli* genes and synthetic genes optimized for mammals, we linked each source of genes encoding homoserine kinase and threonine synthase with the 2A peptide and constructed pEF1a-IRES-GFP-E2F-his and pEF1a-IRES-GFP-M2F-his plasmids (Fig. 3a). We co-electroporated these vectors with *piggyBac* transposase expression vectors into C2C12 and NIH-3T3 cells at a 3:1 (w/w) ratio (C2C12-E2F, C2C12-M2F, NIH-3T3-E2F and NIH-3T3-M2F). Protein expression was determined by observing GFP and Western blots (Fig. 3b, c). When the cells (1×10^6) were cultured for 3 h in KHB buffer with or without 2 mM homoserine, threonine was formed readily from homoserine using *E. coli* genes and synthetic genes optimized for mammals. In the C2C12 group, the rate of threonine synthesis was much greater in response to transfection with pEF1a-IRES-GFP-E2F-his (15.8-fold increase from 12.8 ± 0.82 to 202.5 ± 6.51 μM) compared to pEF1a-IRES-GFP-M2F-his (9.6-fold increase from 12.8 ± 0.82 to 122.8 ± 12.5 μM). The results were diametrically opposed in the NIH-3T3 group; i.e., an 8.3-fold increase from 7.73 ± 0.45 to 64.4 ± 4.45 μM for transfection with pEF1a-IRES-GFP-E2F-his and a 19.8-fold increase from 7.73 ± 0.45 to 152.9 ± 27.3 μM for transfection with pEF1a-IRES-GFP-M2F-his (Table 5).

Threonine synthesis from homoserine in transgenic mice

Considering the contamination of *E. coli* in the environment, it was difficult to identify the transgenic mice

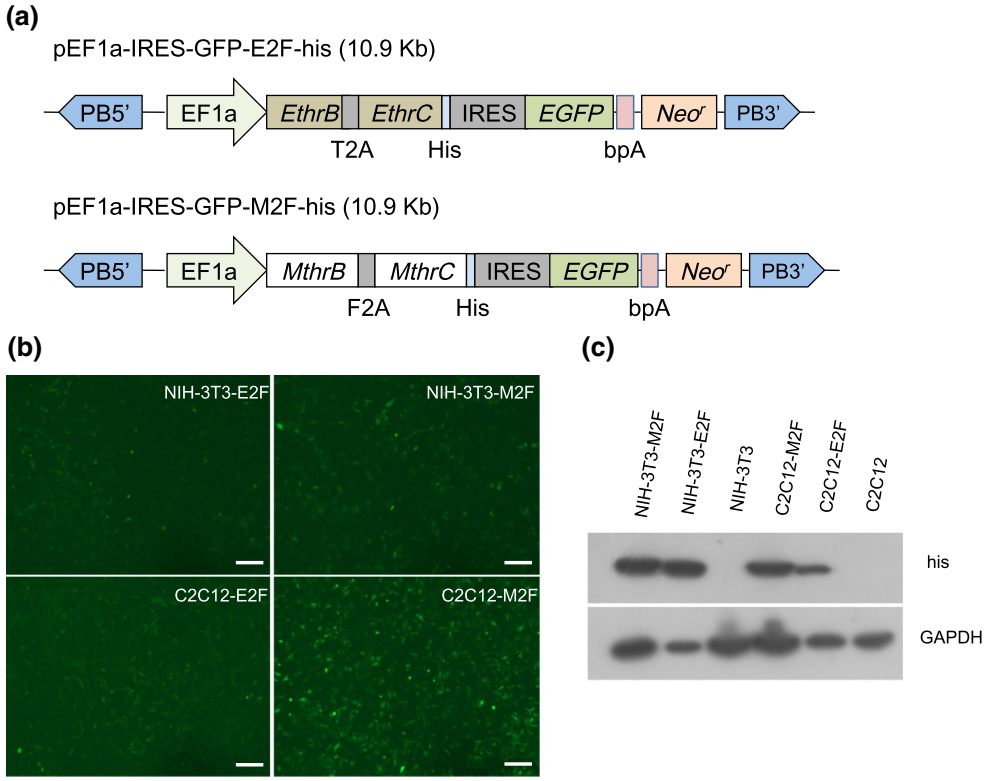

Fig. 3 Threonine synthesis from homoserine in mouse cells. **a** The *piggyBac* transposon vectors used to deliver *E. coli* genes *thrB* and *thrC* (termed E2F), and optimized genes *MthrB* and *MthrC* (termed M2F). The backbone of these vectors was as shown in Fig. 2a. **b** GFP expression in cells transfected with pEF1a-IRES-GFP-E2F-his and pEF1a-IRES-GFP-M2F-his at day 7. *Scale bar* 250 μm. **c** Western blot analyses of his-tag-labeled *thrC* expression in these cells

Table 5 Concentration of threonine (μM) in whole-cell extracts plus KHB medium of cells transfected with pEF1a-IRES-GFP-E2F-his and pEF1a-IRES-GFP-M2F-his

Buffer	C2C12	C2C12-E2F	C2C12-M2F	NIH-3T3	NIH-3T3-E2F	NIH-3T3-M2F
KHB	5.62 ± 0.83	5.91 ± 0.30	2.17 ± 0.17	0.61 ± 0.01	9.85 ± 2.23	7.69 ± 0.95
KHB + 2 mM homoserine	12.8 ± 0.82	202.5 ± 6.51	122.8 ± 12.5	7.7 ± 0.45	64.4 ± 4.45	152.9 ± 27.34

The results were from three experiments ($n = 3$) and are expressed as mean ± SD. The incubation medium contained 2 mM homoserine or none

carrying *E. coli* genes *thrB* and *thrC*. Therefore, we generated transgenic mice carrying synthetic genes that optimized *thrB* and *thrC* genes for mammals. One male (No. 3) and two female (No. 16 and No. 26) transgenic founders were identified by PCR and the Southern blot from 72 newborn mice (Fig. 4a, b). Quantitative real-time PCR was used to determine the copy number of these transgenic mice and the results showed all of the animals carried only one copy of foreign genes. To investigate whether foreign genes were expressed in transgenic mice, the RNA and protein isolated from heart, liver, spleen, lung, kidney, small intestine, large intestine and muscle of F1 adult mice from different lines were analyzed by RT-PCR and Western blots, which indicated the level of expression of the foreign genes was too low to be detected (date not show).

Homoserine synthesis from aspartate in mammalian cells

To verify that aspartokinase I/homoserine dehydrogenase I and aspartic semialdehyde dehydrogenase were present and cleaved by the 2A peptide in animal cells, we placed the fragment of a synthetic *MthrA-Masd* sequence with a flag-tag at the N terminus into the pEF1a-IRES-GFP plasmid (designated pEF1a-IRES-GFP-MthrA-Masd-flag; Fig. 5a) and co-electroporated it into C2C12 and NIH-3T3 cells with *piggyBac* transposase expression vector. The results of GFP expression and Western blot showed the two polypeptides were expressed and cleaved with a high degree of efficiency (Fig. 5b, c). Thus, expression and cleavage of aspartokinase I/homoserine dehydrogenase I

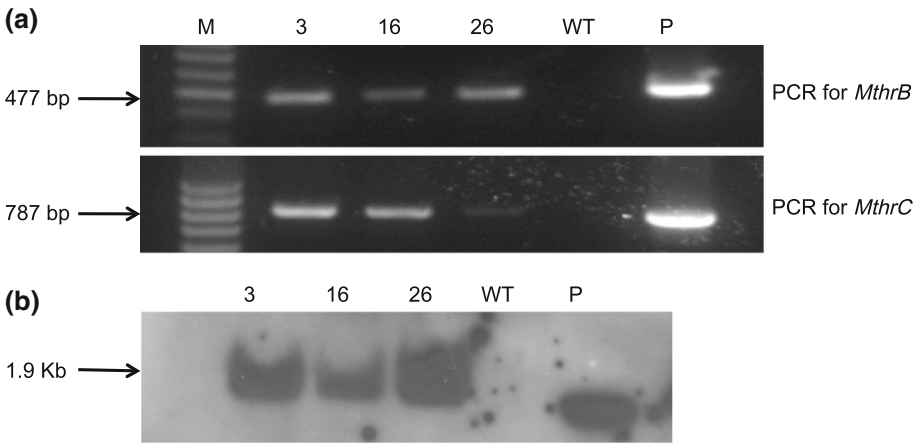

Fig. 4 Generation and molecular characterization of the M2F transgenic mice. **a** Foreign genes identified by PCR in the transgenic mice. The 477 bp product was a fragment of *MthrB* and the 787 bp product was a fragment of *MthrC*. *M* 100 bp DNA ladder, *p* positive plasmid control, and *WT* negative wild-type control. **b** Southern blot analysis of *M2F* integration in the transgenic mice. Mice genome and pEF1a-IRES-GFP-M2F-his plasmid were digested by *Nco*I and hybridized with a 477 bp-labeled probe. An expected 1.9 kb band was detected in mice No. 3, 16 and 26

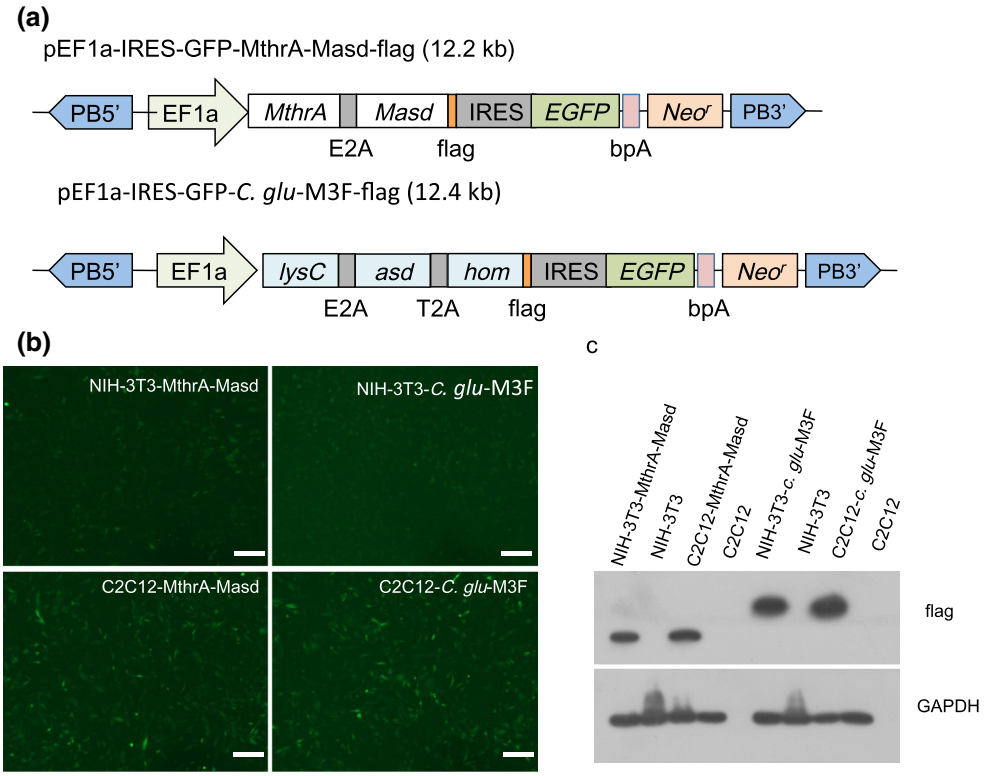

Fig. 5 Homoserine synthesis from aspartate in mouse cells. **a** Schematic of the transposon vectors used to convert L-aspartate to L-homoserine in *E. coli* and *C. glutamicum*. The backbone of these vectors was as shown in Fig. 2a. *C. glutamicum* genes *lysC*, *asd* and *hom* were optimized for mammals and linked with 2A peptide (termed *C. glu*-M3F). **b** Expression of green fluorescent protein (GFP) in cells transfected with pEF1a-IRES-GFP-*C. glu*-M3F-flag and pEF1a-IRES-GFP-MthrA-Masd-flag at day 7. *Scale bar* 250 μm. **c** Western blot analysis of expression of flag-tag-labeled genes in cells

and aspartic semialdehyde dehydrogenase were not the main reason for a low level of homoserine synthesis. Enzyme activity might be another factor to be taken into consideration.

Aspartokinase and homoserine dehydrogenase, which were present as the bifunctional AKI-HSDI with two different catalytic domains, were key enzymes in *E. coli*. This was in contrast to *C. glutamicum*, in which they were two

separate monofunctional polypeptides. It was possible that the pEF1a-IRES-GFP-MthrA-Masd-flag plasmid could not catalyze the two reactions efficiently. Therefore, we linked synthetic sequences of *C. glutamicum* genes *lysC*, *asd* and *hom* optimized for mammals with 2A peptides, which were transferred into the pEF1a-IRES-GFP plasmid (designated pEF1a-IRES-GFP-*C.glu*-M3F-flag; Fig. 5a). Protein expression and homoserine synthesis were detected as described above. The results showed the enzymes could be expressed and cleaved in our systems (Fig. 5b, c) but no synthesis of homoserine from aspartate was detected (date not shown).

Discussion

In the long history of evolution, specific gene combinations are progressively removed or replaced, resulting in the loss of many biochemical pathways that still exist in simple organisms other than the animals. When such animals become the genetic base for selection of specific production, their restricted biosynthetic capabilities will result in specific requirements for certain nutrients. Development of transgenic technology has allowed the transfer of genes across species boundaries, and it provides a method for the repair of non-functional biochemical pathways in animals. In this area, repair for synthesis of so-called nutritionally EAAs has received considerable attention, because animals are able to synthesize most of the compounds of intermediary metabolism (Wu et al. 2013a, b).

Threonine is quantitatively one of the most important EAA for mammals, birds and fish (Wu 2009). In commercial animal production, large amounts of crystalline threonine must be added to the feed to achieve optimal growth performance and health of the animals (Wu 2010), which will increase costs and environmental boundaries for the industry. Here, we tried to introduce all four genes involved in the threonine-biosynthetic pathway into mammalian cell lines and mice. We combined all of the genes into one vector with 2A peptides between each gene and under the control of a single EF1a promoter and we used a his or a flag-tag at the N terminus of the last gene to determine protein expression by Western blot. The 2A sequences are relatively short peptides ($\sim$20 amino acids long, depending on the virus of origin) containing the consensus motif Asp-Val/Ile-Glu-X-Asn-Pro-Gly-Pro. A single open reading frame encodes a large polyprotein, which was co-translationally cleaved between Gly and Pro, leaving the remains of the short 2A peptides fused to the 'upstream' protein and proline added to the 'downstream' protein. The cleavage results in multiple, discrete proteins in essentially equimolar quantities. Therefore, a Western blot specific to the last gene of each plasmid can represent the expression of all the genes. In our systems, the plasmid pEF1a-IRES-GFP-E4F-his containing four genes involved in the whole pathway amplified from the *E. coli* genome was not expressed in animal cells, but the plasmid pEF1a-IRES-GFP-E2F-his linkage of two genes (*thrB* and *thrC*) in one vector could be expressed efficiently. Perhaps translation of the four-gene mRNA stopped at the forward part for unknown reason, which was not consistent with the results reported by Rees and Hay (1995). We used synthetic genes optimized for mammals and they were expressed efficiently. In work by Wang et al. (2009) and in this study mouse embryonic fibroblast cells were not sensitive to threonine deprivation. Thus, survival of cells in a threonine-free medium was not used as an indicator of threonine availability. Rather, we determined threonine synthesis in the cells directly by measuring its concentration in whole-cell extracts plus culture medium.

In our systems, threonine could be synthesized efficiently from homoserine in NIH-3T3 and C2C12 cells transfected with both pEF1a-IRES-GFP-E2F-his and pEF1a-IRES-GFP-M2F-his, but the levels of efficiency of the two plasmids were not similar. In C2C12 cells, more threonine could be synthesized by pEF1a-IRES-GFP-E2F-his, whereas opposite results were obtained for NIH-3T3 cells. However, when transgenic mice carrying synthetic *MthrB* and *MthrC* genes were generated, only three founders were identified and the expression of genes was not detected in any tissue of these animals. The number of transgenic animals produced was very small compared to other genes in our laboratory (Bi et al. 2012; Wei et al. 2011), even with the same plasmid backbone (unpublished data). This result could be explained if high levels of expression of the threonine-biosynthetic genes in mouse embryos were lethal. The only transgenic animals obtained were those in which the genes had been inserted into the region that prevented their expression. There is a need for a promoter that regulates expression of the genes only after the birth of the animal.

To answer the question of whether the growth performance of mice was affected when threonine was absent from the diet and if so whether the impairing of growth could be rescued by transgene and/or providing homoserine, we designed two isonitrogenous and isocaloric diets, which contained no threonine or 100 % requirement of threonine for the growth of mice. Twenty-five-day-old transgenic and non-transgenic mice of the F2 offspring from the same transgenic founder (No. 3 and 26, respectively) were identified by PCR and Southern blot and divided into three groups, respectively ($n = 5$). One group of mice was fed diets containing 100 % of the theoretical threonine requirement and the other two groups were fed the threonine-devoid diets, but one group with 2 mM homoserine in drinking water. We weighed the mice every

2 days and collected blood samples (~20 μl) on day 7. Non-transgenic mice fed with diets containing 100 % requirement of threonine had similar weight gain and similar concentration of threonine in blood, when compared with the wild-type mice fed with normal diets. However, the weights and threonine concentrations of non-transgenic mice fed threonine-devoid diets were reduced gradually and mice receiving homoserine lost less weight, compared with the mice that did not receive homoserine. It is possible that bacteria in the lumen of the small intestine may be able to convert a limited amount of homoserine into threonine. Experiments conducted in the transgenic mice groups produced similar results (date not show). A low level of expression of the genes was an important reason. By measuring the concentrations of amino acids in blood, we found that there was no significant difference in homoserine concentrations between the mice provided with 2 mM homoserine in drinking water and the mice receiving no homoserine in drinking water (date not show). It is possible that homoserine was degraded rapidly in vivo. Further studies are warranted to investigate the metabolism of homoserine in animals.

When introducing the whole metabolic pathway into animal cells, we could not detect significant synthesis of threonine from aspartate and positively identified the formation of homoserine when NADPH and ATP were provided to C2C12 cells. After transferring synthetic genes involved in *E. coli* homoserine-biosynthetic pathways into animal cells we could detect the expression of genes in the cells, indicating gene expression and self-cleavage were not responsible for the low level of homoserine synthesis. It is possible that the bifunctional polypeptide AKI-HSDI cannot catalyze the two reactions efficiently. Therefore, we constructed a plasmid carrying the genes for the optimized *C. glutamicum* homoserine pathway in which aspartate kinase and the homoserine dehydrogenase were monofunctional; however, we could not detect more homoserine synthesis. A possible explanation is that not all the enzymes were activated properly under our experimental conditions. For example, variation of temperature, pH and inhibition factors will affect the reactions, and measurement of the kinetic parameters of these reactions did not yield consistent values. Besides, the structure and activity ratios of these enzymes could be different between animals and bacteria, which might affect the production of homoserine.

In summary, a significant amount of threonine could be synthesized from homoserine in mammalian cells transfected with a plasmid carrying *E. coli* genes or synthetic genes optimized for mammals coding for homoserine kinase and threonine synthase. Expression of this threonine-synthetic pathway in transgenic mice is limited. By introducing the whole metabolic pathway into cells, formation of homoserine from aspartate could be detected when NADPH and ATP were available. Transferring the homoserine-synthetic pathway from *E. coli* or *C. glutamicum* into mammalian cells did not increase the level of homoserine production in these systems. Further studies of transgenic mouse embryos and kinetics of each enzyme reaction are needed.

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Conflict of interest The authors declare that they have no conflict of interest.

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