An arginase-based system for selection of transfected CHO cells without the use of toxic chemicals

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Polyamines have essential roles in cell proliferation, DNA replication, transcription, and translation processes, with intracellular depletion of putrescine, spermidine, and spermine resulting in cellular growth arrest and eventual death. Serum-free media for CHO-K1 cells require putrescine supplementation, because these cells lack the first enzyme of the polyamine production pathway, arginase. On the basis of this phenotype, we developed an arginase-based selection system. We transfected CHO-K1 cells with a bicistronic vector co-expressing GFP and arginase and selected cells in media devoid of l-ornithine and putrescine, resulting in mixed populations stably expressing GFP. Moreover, single clones in these selective media stably expressed GFP for a total of 42 generations. Using this polyamine starvation method, we next generated recombinant CHO-K1 cells co-expressing arginase and human erythropoietin (hEPO), which also displayed stable expression and healthy growth. The hEPO-expressing clones grew in commercial media, such as BalanCD and CHO-S serum-free media (SFM)–II, as well as in a defined serum-free, putrescine-containing medium for at least 9 passages (27 generations), with a minimal decrease in hEPO titer by the end of the culture. We observed a lack of arginase activity also in several CHO cell strains (CHO-DP12, CHO-S, and DUXB11) and other mammalian cell lines, including BHK21, suggesting broader utility of this selection system. In conclusion, we have established an easy-to-apply alternative selection system that effectively generates mammalian cell clones expressing biopharmaceutically relevant or other recombinant proteins without the need for any toxic selective agents. We propose that this system is applicable to mammalian cell lines that lack arginase activity.

The Chinese hamster ovary (CHO) cell line is the workhorse for the production of therapeutic proteins in the biopharmaceutical industry because of their safety record, ability to grow in large-scale suspension cultures and extensive knowledge about their genome, thus facilitating genetic manipulation. Moreover, introduction of essential post-translational human-type modifications (especially glycosylations) are also achievable with this platform. Media formulations, feed strategies, bioprocess development, and gene expression modifications have driven significant increases in final yields over the past years, reaching titer levels of 5–10 g/liter (1). However, stable cell line development and clonal selection still remain as a costly, labor- and time-consuming step (3).

Development of CHO producer cell lines is mainly based on two selection markers: dihydrofolate reductase (DHFR), an enzyme required for nucleotide metabolism, and glutamine synthetase (GS), essential for intracellular glutamine production (4). In both systems, selection occurs in the absence of a vital supplement (thymidine and hypoxanthine for DHFR-deficient cells and glutamine for the GS system), preventing the growth of nontransfected populations. Compared with DHFR selection, the GS system can be equally used to derive parental cells lacking GS activity (such as NS0 myeloma or SP2 hybridoma cell lines) or expressing glutamine synthetase (such as CHO cells), in which case methionine sulfoximine (MSX) selection pressure is applied. However, more stringent selection achieved with GS knockout CHO cells have been reported to double mAb bulk culture productivity compared with parental CHO-K1 (5). Similarly, the −/− DHFR DG44 and DUXB11 cell lines are commonly used for DHFR-selection system. In both cases, highly producing populations are generated by increasing the stringency of the selection process with either methotrexate (MTX), a DHFR inhibitor, or MSX, a GS inhibitor, thus inducing transgene co-amplification. However, several rounds of incrementing MTX concentrations are usually required, resulting in a 5–6-month selecting process (6). To note, increased mutation rates have been reported from the long amplification process involving mutagenic chemicals, resulting in occasions in variations in the amino acid sequence of the desired product (7). Hence, cell line development and pharmaceutical manufacturing in drug-free systems is desirable to sat-
Polyamine starvation as a selection system for CHO cells

Results

CHO-K1 cells require supplementation of putrescine or L-ornithine for healthy growth

During the development of a serum-free medium for CHO cells (SFM-F12) (25), reduction of putrescine supplementation to less than 200 µg/liter was observed to have a dramatic negative effect on cell growth, resulting in a consistent drop on viability to less than 55% at concentrations lower than 100 µg/liter. In SFM-F12 with increased putrescine supplementation up to 200 µg/liter, a substantial negative effect in viable cell density (VCD) and viabilities was still observed (Fig. S1).

Intracellular putrescine production requires the conversion of arginine to L-ornithine, which is then decarboxylated to generate putrescine. To assess which of the two reactions was causing the putrescine-dependent phenotype observed, CHO-K1 cells were cultured for fourteen passages in three conditions: (i) SFM-F12 medium lacking putrescine, (ii) SFM-F12 medium supplemented with L-ornithine (10 µM), and (iii) SFM-F12 control medium (containing 1.08 mg/liter putrescine). Because of the presence of traces of putrescine (81 µg/liter) in the DMEM-F10 formulation (basal media for SFM-F12 medium), a SFM-F10 medium (based in DMEM-F10 medium (1:1 v/v) with 0 µg/liter putrescine) was developed and tested under the same conditions as SFM-F12 to compare effects of medium fully depleted of putrescine. In SFM-F12 (Fig. 1, a, c, e, and f), CHO-K1 cells were observed to display a similar growth profile in both L-ornithine and putrescine supplemented media with consistent viabilities (87–95%) maintained among the four passages. In contrast, cultures in nonsupplemented SFM-F12 (Fig. 1e) displayed negligible growth. Similar to the SFM-F12 results, SFM-F10 L-ornithine and putrescine supplemented cultures displayed similar profiles, with growth observed at each passage and viabilities increased from P2 to P4 (Fig. 1, b and d). Zero growth was observed in CHO-K1 cells in SFM-F10 nonsupplemented medium, with viability decreasing to 15% by P2 (Fig. 1f). It is noteworthy that CHO-K1 cells were grown in SFM-F12 medium supplemented with putrescine prior to the start of the SFM-F10 test, with no previous adaptation to SFM-F10 medium. This may explain the similar growth profiles observed in all three SFM-F10 testing media at P1 and P2 (Fig. 1, b, d, and f).

Replenishment of arginase activity results in healthy growth and can be used to select CHO-K1 cells

In 1982, Hölttä et al. (19) reported a lack of arginase activity in a CHO-K1 cell line in serum-free conditions, resulting in a polyamine-dependent phenotype. In accordance with their findings, it was hypothesized that a lack of arginase expression could be also occurring in our parental cell line. Based on that and, to exploit this phenotype, we designed a selection system for CHO-K1 producer cell lines. As a proof of concept, a commercial arginase-expressing vector, pcDNA3.1-mArg (Addgene), was first transfected and selected in media lacking putrescine (NoP) compared with media with putrescine (P). A negative control, consisting of WT untransfected cells, was also included.

CHO-K1 cells transfected and selected in SFM-F12 medium lacking putrescine resumed cellular growth by passage 6 (day 20), finally reaching VCD and viability profiles similar to the parental control cells in putrescine-containing media by passage 8 (day 26) (Fig. 2, a and b). Correspondingly, an increase in arginase activity was also detected in the transfected and selected cultures (NoP-Arg) from P5 (Fig. 2c). In contrast, expected minimal arginase activity levels were detected in parental cells, with a cessation of growth in media without putrescine (NoP-Neg) from passage 2 (day 9) and viabilities of 60 – 69% observed until the end of the culture. Phenotypic characterization of the selected mixed populations (NoP-Arg) over a 7-day culture period revealed almost identical VCD and viability profiles as parental cells in SFM-F12 medium with putrescine (Fig. S2).

GFP-expressing clones successfully selected in low putrescine containing media

After having demonstrated the ability to select CHO-K1 cells transfected with an arginase expressing vector in media lacking putrescine, the next step was to determine whether the system could be applied to the selection of cells expressing a gene of...
interest. To achieve this aim, GFP was selected for initial tests because of the ease traceability during the transfection process. A bicistronic vector expressing GFP and arginase linked by an IRES and under the control of a CMV promoter was designed for the purposes of this experiment (Fig. S3).

In SFM-F12 medium, healthy mixed populations were successfully obtained by passage 8, recovering VCD and viability profiles similar to the parental control cells (Fig. 3, a and b). GFP expression was first detected at passage 7, with 14.5% GFP-expressing cells observed in mixed population 1, 2.9% in mixed population 2, and 8.1% in mixed population 3. Interestingly, the GFP-expressing population was observed to increase over each passage, reaching an average of 27.2% cells by the end of the experiment (P12) (Fig. 3 c). In contrast and as expected from previous results, the VCD and viability of the untransfected cultures in SFM-F12 medium dropped by passage 2, displaying an average VCD of 0.3–0.6 × 10^6 cells/ml and viability between 57 and 64% until the end of the culture (NoP-Neg). Variability in the viability of the three replicates for the negative control was observed to increase over each passage, reaching an average of 27.2% cells by the end of the experiment (P12) (Fig. 3c). It is noteworthy that mixed population 2 was observed to unstably express GFP from P6 to P10, displaying a dramatic drop in the percentage of GFP-expressing cells by P11, finally resulting in cellular death (P12). Hence, the large error bars displayed at P10 and P11.

Although SFM-F10 medium was observed to outperform SFM-F12 selective medium in terms of percentage of GFP-expressing populations obtained, differences in the composition of both media resulted in maximal viabilities of 80–85% in control parental cells cultured in SFM-F10 medium (P-T, P-Neg) (Fig. S4b), whereas viabilities over 90% were consistently observed in SFM-F12 medium. For this reason, further testing was performed only on SFM-F12 medium.

**Long-term stably expressing clones can be generated by polyamine and L-ornithine starvation**

To assess the stability of the GFP-expressing populations generated, seven clones were isolated and placed in SFM-F12 medium lacking putrescine to monitor their growth (VCD), viability, and GFP expression in terms of mean fluorescence.
intensity (MFI) and at a mRNA level (relative quantification, RQ) for a total of 42 generations (14 passages).

Consistent growth and healthy viabilities were displayed over the 42 generations (Fig. 4, a and b). Clones 7 and 16 were observed as low producers, whereas clone 12 displayed remarkably increased MFI levels. Nevertheless, GFP expression was detected throughout the 42 generations, with an increase in MFI levels relative to passage 1 observed in all clones except clone 11 (Fig. 4c). Stable expression was also confirmed at a transcriptional level (Fig. 5). Because of the design of the expression vector, almost identical GFP and arginase RQ profiles were observed. Interestingly, from passage 4 (12 generations), an increase in the RQ of both GFP and arginase were observed with all clones except clone 11. This effect might be related to the transference of clones from putrescine-containing medium (during single-cell cloning) to SFM-F12 medium depleted of putrescine, i.e. increased stringency of selection.

**Single clones stably expressing a therapeutically relevant recombinant protein can be generated in polyamine and L-ornithine-free media**

To confirm whether the selection system designed would support the generation and preferential survival of clones expressing pharmaceutically relevant therapeutics, a bicistronic vector expressing human erythropoietin (hEPO) was designed and transfected into CHO-K1 cells; cultures were then plated in selective medium (putrescine-free). Successful mixed populations were selected by passage 9, as observed for the detection of hEPO (0.26 mg/liter) and the recovery of VCD and healthy viabilities (Fig. 6). Clones were isolated and seven of them were randomly selected to assess stability in selective medium for a total of 42 generations (14 passages). Stable titer expression was confirmed over the 42 generations (Fig. 7c). Assessment of hEPO and arginase mRNA expression relative to passage 2 was performed on three phenotypically divergent clones: clone 4 (low producer but fast growing), clone 10 (high producer but moderate growth), and clone 18 (medium producer with moderate growth). Both clones 10 and 18 were found to be stable, displaying levels of expression similar to those in passage 2 for at least 36 generations (P12) (Fig. 8). In contrast, the low producer clone 4 displayed a 20% decrease on hEPO mRNA expression at 18 generations (P6), dropping to less than 50% relative to the expression at passage 2 by passage 8. Nonetheless, both hEPO and arginase expression were detected over the 42 generations (P14) (Fig. 7).
To determine whether the application of this selection system may be limited to CHO-K1 cells, several mammalian cell lines (HEK293, SP2, BHK21, Vero, BxPC-3, Capan-2, MiaPaca-2, and PANC-1), including three CHO cell lines, CHO-DP12, CHO-S, and DUXB11, were assessed for arginase activity (Fig. 9). Neither BHK21 nor any of the CHO cell lines tested displayed arginase activity, indicating that the system may be applicable to several parental CHO cell lines. Conversely, the HEK293, Vero lines and the pancreatic BxPC-3 and MiaPaca-2 cell lines displayed arginase activity levels similar to the pcDNA3.1-Arg transfected CHO-K1 cells (Fig. 2c), making these parental cell lines unsuitable for the system. Finally, low levels of activity were observed in SP2, Capan-2, and PANC-1. Whether this activity is low enough to allow application of the arginase-based selection system requires further investigation.

hEPO clones display stable expression in putrescine-containing media

Because of the vital role played by polyamines in the culture of CHO cell lines in serum-free media (19), commercial and chemically defined formulations contain putrescine levels sufficient to supply the metabolic requirements of CHO cells. To address whether clones generated following our polyamine-starvation method would maintain expression in nonselective conditions, clone 4, 10, and 18 were adapted to SFM-F12 medium containing putrescine as well as two commercially available formulations of undisclosed composition: a serum-free medium, CHO-S SFM-II (Gibco), and a chemically defined medium, BalanCD Growth A (Irvine). Clones cultured in SFM-F12 and BalanCD media displayed constant VCD and viability profiles (Fig. 10). In contrast, SFM-II medium was less supportive of healthy cultures, resulting in a decrease in growth from passage 2 (six generations) in two of the clones, reaching consistent VCDs by passage 6 (2.3 × 10⁶ cells/ml, clone 4) and passage 5 (1.5 × 10⁶ cells/ml clone 10). Moreover, in this medium, the viability of clone 10 dropped to 70% by passage 6 and to 60% in clone 18 by passage 7. Stable hEPO expression was observed in BalanCD and SFM-F12 media for clone 10 (up to P9, 27 generations), whereas decreased (23–30% lower) hEPO titers were displayed by passage 8 in clone 18 and clone 4 (40–30% lower) by passage 9 (Fig. 10). In SFM-II medium, both clone 10 and 18 performed similarly to the other two media. However, clone 4 was less stable, with hEPO titer dropping by 60% at passage 6 but maintaining this level until the end of the culture.

To further characterize the capabilities of the system, all three clones were each cultured in both commercial media and SFM-F12 with putrescine for a total of 7 days to allow for high hEPO and, consequently, arginase expression. Healthy growth and hEPO expression was supported at different levels in all media tested, indicating that an increase in the arginase expression had no detrimental effect on CHO cell development (Fig. S5).

**Figure 3.** GFP-expressing populations can be isolated in polyamine-free medium. a–c, VCD (a), viability (b), and percentage of GFP-expressing (c) CHO-K1 cells transfected with 500 ng of GFP-IRES-Arg vector (P-GFP, NoP-GFP). A negative control of cells with no DNA transfected (P-Neg, NoP-Neg) was included. Transfected and nontransfected cells were placed in either SFM-F12 medium supplemented with putrescine (P) or selective SFM-F12 medium (without putrescine, NoP). Triplicate wells were transfected per each condition.
Discussion

With the results reported here, we have demonstrated the efficacy of a newly developed auxotrophic selection system for CHO cells that exploits an arginase-deficiency phenotype observed in several mammalian cells. The method was shown to sustain the survival and growth of arginase-expressing transfected cell pools in a polyamine and L-ornithine–free environment, supporting the isolation of GFP and recombinant therapeutic protein (erythropoietin) expressing single cell–derived populations. Moreover, initial evidence of clonal stability was observed, with the maintenance of VCD, viability, and production profiles for at least 42 generations in selective medium (14 passages). The clones were also observed to express and grow in nonselective commercial media for at least 27 generations.

The routinely used DHFR and GS expression systems, although being described as auxotrophic selection methods, require MTX and MSX drug-selection pressure for the generation and isolation of high producer cell lines (4), resulting in time-consuming amplification processes (reaching up to 5–6 months) mainly for the DHFR system (6), because several rounds are required. Moreover, the DHFR-expressing system is predominately used with CHO cells lacking DHFR expression, thus enabling selection in nucleoside-depleted media (26). On the other hand, despite endogenous expression of glutamine synthetase in CHO cells, selection of parental producer colonies have been isolated in glutamine-free media by MSX pressure. However, the endogenous GS gene has been suggested to display activity at a sufficient levels to allow nonproducer CHO cell survival, reducing the stringency of the GS system (27). Consequently, use of GS-KO CHO-K1 has been found to be optimal (5). In contrast, the arginase-expression system proposed here is a drug-free method applicable, but not limited to, CHO-K1 parental cells because of their inherent lack of arginase activity, which has been further observed as an intrinsic phenotype of several CHO cell lines and some mammalian cells. Interestingly, arginase activity was reported by Hölttä et al. (19) in several sources of serum. Because FBS is still a common supplement used in mammalian cultures, arginase deficiency maybe masked in some cell lines, which indicates the possibility of a broader applicability of the system described here.

Development of cell lines with stable production phenotypes is an essential attribute in the pharmaceutical industry. Hence, several studies have been focused on the characterization of the two predominant selection systems. In 2006, Jun et al. (28) reported decreases of 33–62% in specific productivity of GS-derived high producer clones over the first batch, reaching further lower expression up to passage 6. Similarly, mAb was observed to decrease during the 30 passages assessment of CHO-GS clones in selective and nonselective media (29), whereas lower titer levels were observed after 20 days in a DHFR-derived cell line (10). Further investigation studies have
revealed loss of gene copies, epigenetic modifications, and inefficient or decreased mRNA transcription (8, 9, 30–32) as the main causes of clonal instability, being observed as a widespread issue. Compared with GS and DHFR studies, clones isolated with the arginase system proposed here have displayed stable GFP and hEPO expression at protein and mRNA levels for over 40–53 generations (depending on the clone, 14 passages) in drug-free selection media. Other alternative drug-free selection systems such as OSCAR\(^\text{TM}\) (based on knockout cell lines for hypoxanthine phosphoribosyl transferase), have reported lower stability with a rapid mAb expression decay displayed after 3 weeks in culture (33). Although clonal stability

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**Figure 5. Long-term stable GFP and arginase mRNA expression of isolated GFP-expressing clones in selective medium.** Relative quantification (RQ) of GFP and arginase mRNA levels in seven GFP-expressing clones growing in selective medium for 14 passages (42 generations) was normalized to the mRNA levels in passage 2. The Gapdh endogenous gene was used to standardize the results.
monitoring in industry is performed for periods over 60 generations, initial evidence for stable production has been described for the arginase-expressing method, with further characterization to be performed.

Because of the essential roles of polyamines in the maintenance of cellular wellbeing, it is not surprising to observe regulatory pathways to compensate imbalanced intracellular polyamine content. In response to critical levels of spermidine, spermine, and putrescine, active transport mechanisms participate in the maintenance of polyamine homeostasis. In mammals, polyamine transport systems are still poorly understood, but three models have been suggested: (i) polyamine uptake by...
unidentified membrane permeases, (ii) interaction with heparin sulfate and glypican 1, and (iii) putrescine uptake by a caveolin-1–dependent endocytosis mechanism, which has been associated to SLC3A2 (17). Other solute carrier transporters are also under investigation (reviewed in Ref. 41) and a diamine exporter has been identified to have the ability to export putrescine in CHO (42). In plants and microorganisms, putrescine can be alternatively produced by arginine decarboxylase and agmatinase. In this pathway, arginine is first decarboxylated to agmatine, which is then used as a substrate to generate putrescine (11). Recent evidence of agmatinase activity has been reported in rat liver (16) and kidney (34), as well as brain and other tissues of several mammalian species (35), where agmatine has been described as a potent biological active substance (e.g. acting as a neurotransmitter (15) or a modulator of nitric oxide synthesis or interacting with several receptors, ion channels, or membrane transporters (35)). Consequently, agmatine degradation to putrescine has been suggested as a regulatory system rather than an "alternative" pathway for the production of polyamine (36). In 2014, ornithine decarboxylase knockout ovarian conceptuses were generated and observed to compensate polyamine-deficiency conditions by agmatine production. However, this phenotype was only observed in half of the ornithine decarboxylase knockout ovarian conceptuses, whereas the other half displayed a lack of agmatine production resulting in cessation of cellular development (14). Similarly, we have here observed that removal of putrescine and L-ornithine from media results in a drop of viability and VCD of CHO-K1 parental cells, and this altered phenotype is maintained for at least 40–53 generations (depending on the clone, 14 passages). As a result, investigation of agmatine expression was not performed. Further research is necessary to determine whether the agmatine-derived pathway is active and contributes to polyamine production in CHO cells.

Similar to the findings reported here, a lack of arginase activity and subsequent decreases in cellular growth has been previously reported with CHO cells cultured in absence of putrescine, with cellular death observed after 8–14 days (18, 19) because of the intracellular depletion of the secondary polyamines spermine and spermidine (3). As expected, more stringent conditions obtained in putrescine-depleted media were here observed to outperform the selection efficiency of the low putrescine-containing medium. However, traces of this polyamine in media did not impede isolation of arginase-expressing clones, suggesting potentially easy applicability of this system when paired with common basal media, such as DMEM-F12. It is important to note that the use of this medium would require clonal isolation to remove background populations not expressing the gene of interest (such as cells only expressing arginase or nontransfected populations); in any case, this is a step that is commonly performed to achieve stable high producer clones.

In conclusion, we have presented here evidence for an alternative method for the generation of stable producer CHO-K1 cell lines using an arginase-expressing system in polyamine and ornithine-free media. We have conclusively demonstrated that arginase-expressing selection is efficient, offering a drug-free, cost-effective, and easy-to-apply method for a range of parental mammalian cells displaying lack of arginase activity.
The system may be also used in conjunction with GS or DHFR methods, offering an alternative to antibiotic-based selection for the generation of double transfectants avoiding possible secondary effects from drug selection. To note, the aim of the investigation presented here was to prove the feasibility of the system. Further analysis focused on the isolation of high producing clones and improvement of the system with modifications such as using an attenuated arginase gene, the use of a weak promoter, or knockout of polyamine transporters may lead to a more efficient system and will be further investigated.

**Experimental procedures**

**Media development**

Two SFMs were developed: a low putrescine (SFM-F12 medium) and a putrescine-free formulation (SFM-F10 medium). The commercial DMEM-F12 medium (Sigma–Aldrich, D8437) was used as a basal media for SFM-F12 medium. Because of the presence of putrescine in DMEM-F12 medium formulation (81 μg/liter), a mixture of DMEM high glucose (D5671) and nutrient mixture Ham's F-10 (Sigma–Aldrich, N2147) (1:1) was used for SFM-F10 medium. The latter was further supplemented with HEPES, linoleic acid (L1376), and glucose to mimic DMEM-F12 formulation. Both basal media were further supplemented with sodium selenite (S5261), recombinant insulin (I9279), ethanolic acid (E0135), ammonium iron (III) citrate (F5879), polyvinyl alcohol, L-glutamine (Gibco, 25030024), nonessential amino acids (Gibco, 11140035), and putrescine dihydrochloride (P7505) (25). When indicated, 100 μM l-ornithine or 1 mg/liter putrescine was added to each medium. All media and additives were purchased from Sigma–Aldrich unless otherwise stated.

**Cell culture**

The parental CHO-K1 cell line (ATTC CCL-61) was cultured in SFM-F12 medium supplemented with putrescine. The cells were maintained in suspension culture in an ISF1-X (Climo Shaker) Kuhner incubator at 37 °C, 170 rpm, 5% CO₂, and 80% humidity. The cells were routinely split every 3–4 days and reseeded at 0.2 × 10⁶ cells/ml in 50-ml spin tubes (Sartorius, DF-050MB-SSH) in a 5-ml working volume. Biological triplicates were analyzed for VCD and viability using the ViaCount on a Guava easyCyte HT benchtop cytometer (Merck Millipore). The measurements were performed in technical duplicates (unless stated).

For arginase activity tests, DUXB11 (kindly donated by L. Chasin, Columbia University) were cultured in MEM (Thermo Fisher, 12561056) supplemented with 0.5–2% FBS in T-75 flasks; CHO-DP12 (ATCC, clone #1934) and CHO-S cells were cultured in BalanCD Growth A medium (Irvine Scientific) supplemented with 8 mM L-glutamine (Thermo Fisher, 2503008) in 50-ml spin tubes (Sartorius, DF-050MB-SSH) in a 5-ml working volume; HEK293 were cultured in MEM (Sigma–Aldrich, M5650) supplemented with 2 mM L-glutamine (Thermo Fisher, 2503008), 0.1 mM MEM nonessential amino acid (Biosciences, 11140050), 1 mM sodium pyruvate (Thermo Fisher, 11360070), and 10% heat-inactivated horse serum in T-75 flasks; SP2 (Immune Systems) were cultured in DMEM GlutaMAX (Gibco, 10566-016) supplemented with 10% heat-inactivated FBS in T-25 flasks; BHK-21 (Flow Laboratories Irvine) were cultured in MEM (Sigma–Aldrich, M5650) supplemented with 2 mM L-glutamine (Thermo Fisher, 2503008),
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1% MEM nonessential amino acid (Thermo Fisher, 11140050), 1% sodium pyruvate (Thermo Fisher, 11360070), and 5% fetal calf serum in T-25 flasks; the pancreatic cell lines BxPC-3 (ATCC, CRL-1687), MiaPaca-2 (ATCC, CRL-1420), PANC-1 (ATCC, CRL-1469), and Capan-2 (DSMZ, ACC 244) were cultured in DMEM high glucose (Sigma–Aldrich, D5671) supplemented with 5% fetal calf serum and 2% L-glutamine (Thermo Fisher, 2503008) in T-75 flasks.

Isolation of clonal cells was performed by dilution. A volume of 100 μl/well was added in a 96-well plate at 5 cells/ml. The plates were then incubated uninterrupted at 37 °C and 5% CO2 up to day 7–10, when plates were inspected to identify and mark wells presenting single colonies. The plates were then reincubated at 37 °C. At day 14, 60–70% confluence was observed, and colonies were picked and expanded to larger volumes. First, 1-ml cultures were performed in nonadherent suspension 24-well plates (Greiner Bio-one, 662102). After 2–3 days, successful clones were then placed in 50-ml spin tubes (Sartorius, DF-050MB-SSH) in a 5-ml working volume for stability tests.

For stability tests, the doubling time and generation number of each clone was calculated as per Ref. 37. The clones were assessed for a total of 41–53 generations (for GFP-expressing clones) and 40–51 generations (hEPO-expressing clones), depending on the clone. For simplicity, a doubling time of 24 h was assumed for data discussion, resulting in a total of 42 generations in the experiment.

Vectors

Phusion high-fidelity PCR master mix (Thermo Scientific) was used to obtain the mouse arginase coding sequence (CDS) from pcDNA3.1-mArg1, an internal ribosome entry site (IRES) sequence from pINDUCER10 vector (kindly gifted by Dr. Stephen Elledge, Harvard Medical School Centre of Genetics and Genomics, Boston, MA) and the hEPO CDS from a plenti6.36.3hEPO. The protocol was followed as per the manufacturer’s recommendations, with 10 ng of vector used in a total of 50 μl of reaction volume. Both arginase and IRES fragments were first cloned downstream of a GFP from a modified GFP-expressing vector N44-CSanDI-Hyg (derived from pcDNA5 CMV-d2eGFP (Addgene, 26164), resulting in a GFP-IRES-Arg bicistronic vector controlled by a CMV promoter. For the hEPO-expressing vector, the hEPO CDS replaced the GFP in the GFP-IRES-Arg vector, resulting in a hEPO-IRES-Arg bicistronic vector.

The pcDNA3.1-mArg1 was a gift from Peter Murray (Addgene, plasmid no. 34573), plinducer10-mir–RUP-PHeS was a gift from Stephen Elledge (Addgene plasmid no. 44011) (38), and plenti6.3-hEPO was a gift from Juan Melero-Martin (Addgene plasmid no. 50436) (39). Proof of concept tests were performed using the pcDNA3.1-mArg1 vector.

Transfection and selection

Vector transfections were performed in 1-ml suspension cultures in 24-well tissue-untreated plates (Greiner Bio-one, 662102) SFM-F12 medium with putrescine. The TransIT PRO transfection agent (Mirus Bio, Mir 5740) was used as per the manufacturer’s recommendations with minimal changes. Briefly, the cells were counted and reseeded at 0.5–1 × 10^6 cells/ml in fresh media 24 h prior to transfection. A total of 500 ng of vector were mixed with 1 μl of TransIT PRO and 100 μl of media and incubated for 10 min at room temperature. The cells were resuspended in fresh media at 2 × 10^6 cells/ml, and 900 μl were seeded per well. A total of 100 μl of vector-TransIT PRO complex suspension was then added to each well. The plates were paraffilmed and incubated at 37 °C at 170 rpm with 5% CO2 and 80% humidity in an ISF1-X (Climo Shaker) Kuhner incubator. A negative control for transfection was also included (no vector was added during transfection). Biological triplicate were performed per each transfections. Selection was performed in putrescine-free SFM-F12 medium. The cells were also placed in putrescine-containing SFM-F12 medium as negative controls.

For the GFP-expressing vector, the efficiency of the process was assessed 24 h post-transfection by GFP expression using the Express Plus software of GUAVA easyCyte HT benchtop cytometer (Merck Millipore). To determine the amount of fluorescent cells, negative control cells (zero fluorescence) were gated, and these settings were then used to identify the GFP-positive populations. Fluorescence of dead cells and debris were excluded to avoid false-positive results. Viable cell density and viability were also assessed as previously described.

Arginase activity

One million cells were collected per sample and centrifuged at 1000 rpm. Pellets were then washed with PBS and finally stored at −80 °C until assayed. The arginase activity kit (Sigma–Aldrich, MAK112) was used. Pellets were lysed for 10 min in 100 μl of lysis buffer as per manufacturer’s recommendations, with pepstatin A (Sigma–Aldrich, P5318) and leupeptin (Sigma–Aldrich, L9783). To determine arginase activity, manufacturer’s protocol was followed. Arginase activity (units/L) was determined with the following equation,

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\text{Activity} = \frac{\text{Abs Sple} - \text{Abs B}}{\text{Abs Std} - \text{Abs W}} \times \frac{1 \text{ mm} \times 50 \times 1000}{\text{Sple vol} \times \text{react time}} \tag{Eq. 1}
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where Abs Sple is the absorbance sample at 430 nm; Abs B is the absorbance blank at 430 nm; Abs Std is the absorbance standard at 430 nm; Abs W is the absorbance water at 430 nm; Sple vol is the sample volume (μl); and React time is the reaction time (min).

One unit of arginase corresponds to the amount of enzyme that will convert 1 μmol of l-arginine to ornithine and urea per min at pH 9.5 and 37 °C. Biological triplicates were analyzed per each condition and cell line except for pancreatic cell lines, which were assessed in biological duplicates.

RNA isolation and RT-qPCR performance

RNA was collected by centrifuging 1–5 × 10^6 cells at 1000 rpm at 4 °C for 5 min. Pellets were resuspended in 1-ml TRIzol reagent (Thermo Fisher, 15596018) and stored at −80 °C until assayed. Total RNA isolation was performed as per the manufacturer’s recommendations (Thermo Fisher). RNA quantification and quality were evaluated by NanoDrop
(Thermo Scientific). To remove contaminating DNA, RNA samples were treated with DNase I (Sigma–Aldrich) as per manufacturer’s protocol and stored at −80 °C. cDNA was obtained using the high-capacity cDNA reverse transcription kits (Applied Biosciences), which was followed as per manufacturer’s recommendations. A total of 200 ng of cDNA per reaction well was used for quantitative RT-PCR using Fast SYBR Green Master Mix (Applied Biosystems) and run in a 7500 (Applied Biosystems). The 2× SYBR master mix was prepared with 400 nM primers and nuclease-free water made up to 16 μl/reaction. Relative quantification was measured by the ΔΔCt method with Gapdh as an endogenous control. Technical triplicate wells were run for each clone sample. The sequences of the primers used are as follows (5′ → 3′): Gapdh forward, TG-GCTACGCAACAGAGTGG; Gapdh reverse, GTAGAGGA-GATGATCGGTGT; Arg forward, ACAAGACAGGGCTCCT-TTCA; Arg reverse, TGCCGTGTTCACTAGTCTC; hEPO forward, GCAATGGATGATAAGCCGTCA; hEPO reverse, GCAGTGATTGTTCGGAGTGG; Gapdh forward, GACGA-CGGCACTACAAGAC; and dGFP reverse, TCTTGAAGTGCATGCCCCT.

**ELISA**

For hEPO detection, the protocol previously described by Costello et al. (40) was followed with overnight incubation for capturing antibody and samples (1.5 h).

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