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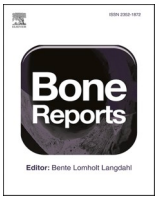

Amino acid metabolism in skeletal cells

Claire-Sophie Devignes, Geert Carmeliet, Steve Stegen *

Laboratory of Clinical and Experimental Endocrinology, Department of Chronic Diseases and Metabolism, KU Leuven, O&N1bis, Herestraat 49 box 902, 3000 Leuven, Belgium

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ABSTRACT

Amino acid metabolism regulates essential cellular functions, not only by fueling protein synthesis, but also by supporting the biogenesis of nucleotides, redox factors and lipids. Amino acids are also involved in tricarboxylic acid cycle anaplerosis, epigenetic modifications, next to synthesis of neurotransmitters and hormones. As such, amino acids contribute to a broad range of cellular processes such as proliferation, matrix synthesis and intercellular communication, which are all critical for skeletal cell functioning. Here we summarize recent work elucidating how amino acid metabolism supports and regulates skeletal cell function during bone growth and homeostasis, as well as during skeletal disease. The most extensively studied amino acid is glutamine, and osteoblasts and chondrocytes rely heavily on this non-essential amino acid during for their functioning and differentiation. Regulated by lineage-specific transcription factors such as SOX9 and osteoanabolic agents such as parathyroid hormone or WNT, glutamine metabolism has a wide range of metabolic roles, as it fuels anabolic processes by producing nucleotides and non-essential amino acids, maintains redox balance by generating the antioxidant glutathione and regulates cell-specific gene expression *via* epigenetic mechanisms. We also describe how other amino acids affect skeletal cell functions, although further work is needed to fully understand their effect. The increasing number of studies using stable isotope labelling in several skeletal cell types at various stages of differentiation, together with conditional inactivation of amino acid transporters or enzymes in mouse models, will allow us to obtain a more complete picture of amino acid metabolism in skeletal cells.

1. Introduction

Bone development, remodeling and repair are metabolically highly demanding processes. The regulatory role of cytokines, hormones and related signaling pathways on skeletal cell function has been well established, but recent evidence also shows the importance of cell metabolism as a control mechanism. The maintenance of cellular properties like proliferation and extracellular matrix synthesis is closely intertwined with energy metabolism, which comprises a series of metabolic pathways that generate energy in the form of adenosine triphosphate (ATP) from nutrients such as carbohydrates and lipids (Karner and Long, 2018; Kushwaha et al., 2018). Amino acid metabolism complements glucose and fatty acid-driven bioenergetic pathways during bone anabolism, as one of its major roles is to provide the molecular building blocks for protein synthesis, which is especially important for extracellular matrix-synthesizing cells like osteoblasts and chondrocytes. Not surprisingly, there is a significant requirement for amino acids within the skeleton, although some regional differences may exist that are linked to specific bone cell behavior.

There are nine essential amino acids, defined as those whose carbon skeleton cannot be synthetized by the cell and have to be obtained *via* the diet and the intestinal microbiota. On the other hand, non-essential amino acids can also be synthetized from exogenous sources, thereby providing more flexibility for cells to ensure an adequate supply. Some non-essential amino acids are categorized as conditionally essential or semi-essential, because their synthesis is limited in certain (pathological) conditions, for example during prematurity, infection or injury (Reeds, 2000) (Table 1).

In addition to protein synthesis, amino acids can also be used for other cellular processes (Fig. 1), either *via* direct incorporation in macromolecules such as nucleotides or after enzymatic breakdown and fueling other metabolic pathways (*i.e.* anaplerosis). First, they provide metabolic intermediates for the biosynthesis of lipids, nucleotides and the nucleotide coenzymes NAD^+ and $NADP^+$. Indeed, in addition to the ribose-5-phosphate generated by the pentose phosphate pathway, nucleotide biosynthesis depends on feeder pathways that provide

* Corresponding author at: Clinical and Experimental Endocrinology, KU Leuven, O&N1bis, Herestraat 49 box 902, 3000 Leuven, Belgium.
E-mail address: steve.stegen@kuleuven.be (S. Stegen).

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Table 1
List of amino acids (AA) categorized according to essentiality. Three- and one-letter codes are included. Non-proteinogenic (NP) amino acids that have been studied in bone are also listed.

Essential AA	Non-essential AA	Conditionally essential AA
Leucine – Leu - L	Alanine – Ala - A	Glycine – Gly - G
Isoleucine – Ile - I	Asparagine – Asn - N	Arginine – Arg - R
Valine – Val - V	Aspartate – Asp - D	Glutamine – Gln - Q
Phenylalanine – Phe - F	Glutamate – Glu - E	Tyrosine – Tyr - Y
Threonine – Thr - T	Serine – Ser - S	Cysteine – Cys - C
Lysine – Lys - K	Homocysteine (NP)	Proline – Pro - P
Tryptophan – Trp - W		Taurine (NP)
Histidine – His - H		
Methionine – Met - M		

carbon or nitrogen precursors, including the amino acids glutamine, aspartate, serine and glycine (Lane and Fan, 2015). Furthermore, tryptophan is used to generate quinolinate, the *de novo* precursor for the pyridine ring of NAD^+ and its phosphate derivative $NADP^+$.

Second, glutamate, glycine and cysteine are crucial for maintaining cellular redox balance by synthesizing glutathione (GSH), the most potent non-enzymatic cellular antioxidant. Moreover, serine-driven one-carbon metabolism *via* the folate cycle contributes, amongst others, to the generation of NADPH, which converts glutathione back to its reduced form and is critical for redox regulation under hypoxic conditions (Lieu et al., 2020; Ye et al., 2014). Third, in certain conditions, amino acids can serve as anaplerotic substrates that drive tricarboxylic acid (TCA) cycle activity to sustain mitochondrial ATP production or lipogenesis. Most studied is the anaplerotic metabolism of glutamine that generates α-ketoglutarate (αKG) and subsequently fuels the TCA cycle. The oxidation of (branched-chain) amino acids in the TCA cycle is also important for generating acetyl-CoA to support lipid biosynthesis (Lieu et al., 2020). Fourth, in addition to their direct integration into biosynthetic reactions, amino acids and their derivates are also fundamental in mediating epigenetic modifications and thus gene transcription. For example, DNA and histone methylation are regulated by metabolites from the methionine cycle/one-carbon metabolism, including methionine, serine and glycine (Mentch and Locasale, 2016). Similarly, histone acetylation events that promote gene transcription require acetyl-CoA, which can be derived from (iso)leucine, valine or glutamine. Fifth, catabolism of specific amino acids, such as glutamate and arginine, generates metabolic precursors that support collagen synthesis (proline) and cell proliferation (polyamines). Finally, amino acids are also precursors of bioactive amines such as neurotransmitters or hormones, and are thus involved in cell-to-cell communication. For example, the non-essential amino acid glutamate is a major excitatory neurotransmitter and also functions as a precursor of gamma-aminobutyric acid (GABA), its inhibitory counterpart. Thus, amino acid metabolism is complex and highly interconnected with other pathways, and may therefore regulate cell function at different levels.

In this review, we will summarize the current knowledge of amino acid metabolism in skeletal cells, its regulation by lineage-specific (transcription) factors and signaling pathways, and how it is altered in skeletal disease.

2. Amino acid metabolism in bone cells

Bone development, maintenance and repair are achieved by the concerted action of several types of skeletal cells that differ in function and location. Osteoblasts are bone-forming cells and derive from mesenchymal precursor cells referred to as skeletal stem and progenitor cells (SSPCs) (Kurenkova et al., 2020). Under normal physiological conditions, early SSPCs remain relatively quiescent, but they are able to rapidly proliferate and differentiate upon exogenous stimuli or injury. Phenotypic markers are often used to target SSPC populations in different anatomical niches such as the bone marrow, the periosteum and the epiphysis and these markers include, but are not limited to, Leptin receptor (LepR), Nestin (Nes), Paired-related homeobox 1 (Prrx1) or parathyroid hormone-related protein (PTHrP) (Kurenkova et al., 2020). Early committed osteoprogenitors, generally defined by Osterix expression, are still able to proliferate but they also deposit a typical type 1 collagen-rich extracellular matrix. More mature, osteocalcin-expressing osteoblasts do not proliferate but one of their major functions is matrix deposition and its subsequent mineralization. At the final stages of differentiation, some osteoblasts become embedded in the mineralized bone matrix and are referred to as osteocytes. These long-living cells acquire other functions, including the regulation of phosphate/calcium homeostasis and bone remodeling, by secreting factors such as fibroblast growth factor 23 and receptor activator of nuclear factor κ-B ligand (RANKL), respectively (Robling and Bonewald, 2020). The second major skeletal cell type are bone-resorbing osteoclasts. These cells degrade the mineralized bone matrix by a dual mechanism: the local acidification dissolves the bone mineral and the produced catalytic

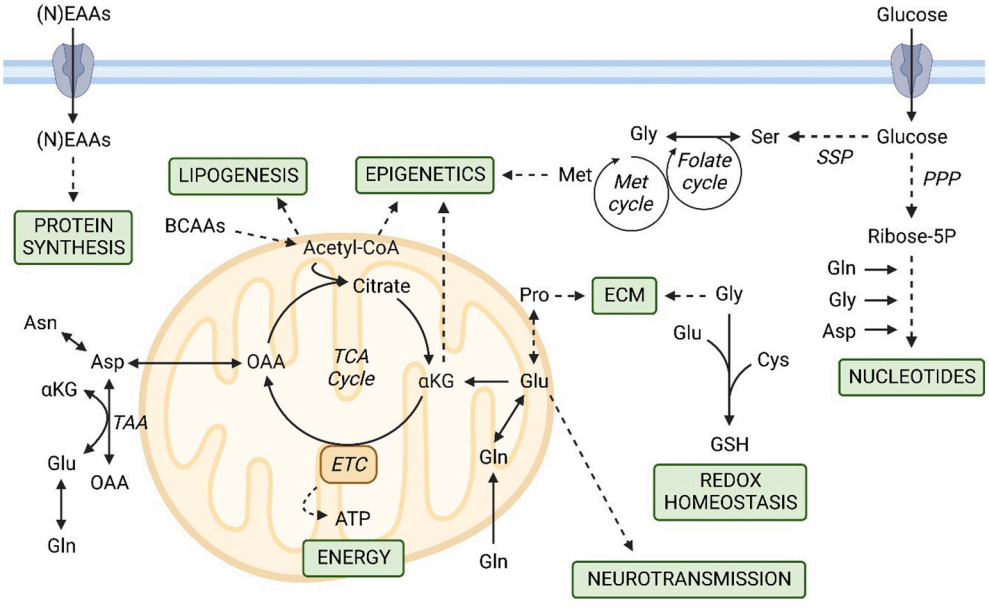

Fig. 1. General overview of amino acids metabolism. Scheme showing the main amino acid-fueled and amino acid-generating metabolic pathways (in italic) and cellular processes (in green boxes). Dotted lines indicate a pathway consisting of several reactions. Abbreviations: αKG: α-ketoglutarate, BCAAs: branched chain amino acids, (N)EAAs: (non-)essential amino acids, ECM: extracellular matrix, ETC: electron transport chain, GSH: glutathione, OAA: oxaloacetic acid, PPP: pentose phosphate pathway, SSP: serine synthesis pathway, TAA: transaminase, TCA cycle: tricarboxylic acid cycle, THF: tetrahydrofolate. Three-letter abbreviations are defined in Table 1.

enzymes degrade the extracellular matrix (Rucci and Teti, 2016). Osteoclasts are derived from proliferative monocyte/macrophage precursor cells, which upon activation with osteoblast/osteocyte-derived factors like RANKL form multinucleated cells that acquire bone-resorbing capacity.

Metabolic programs, including those fueled by amino acids, are intimately linked to specific cell functions, and recent studies indicate that these pathways may differ between proliferating progenitors *versus* differentiating cells. Moreover, the supply of amino acids is related to the type and density of blood vessels in the microenvironment, which is especially relevant for osteoblasts and osteoclasts that reside close to the vasculature (Stegen and Carmeliet, 2018). Interestingly, when comparing amino acid levels in bone marrow plasma to peripheral blood, significant differences were observed. For example, glutamate and aspartate are amongst the lowest amino acids in the circulation, but their respective levels were 20- to 70-fold higher in bone marrow plasma. On the other hand, glutamine and tryptophan were relatively less abundant in bone marrow (van Gastel et al., 2020a), suggesting that these amino acids may be actively consumed in the bone microenvironment. Together, these observations indicate that bone cells may display specific amino acid requirements depending on their functional properties, lineage allocation and differentiation status, which may be, at least partially, controlled by the local microenvironment. Below, we will summarize recent findings how amino acid metabolism regulates osteoblast and osteoclast function.

2.1. Amino acid metabolism in osteolineage cells

2.1.1. The pleiotropic metabolic roles of glutamine

In addition to the importance of carbohydrates and lipids in regulating skeletal cell properties, recent studies reveal a multifaceted role for amino acid metabolism in bone cells. General amino acid-sensitive pathways such as mammalian target of rapamycin (mTOR) or the integrated stress response (ISR) are known to regulate osteolineage cell function, and the reader is referred to recent literature for more information (Chen and Long, 2018; Elefteriou et al., 2006; Hu et al., 2020; Rached et al., 2010). However, less is known about the direct contribution of individual amino acids and amino acid-metabolizing pathways. Most studies focus on glutamine, which is the most abundant amino acid in the circulation, and is further discussed below.

Most osteolineage cells, including skeletal progenitors in the bone marrow and the periosteum, as well as osteoblasts, take up exogenous glutamine in normal conditions predominantly by SLC1A5, a Na^+-dependent neutral amino acid exchanger that can also transport asparagine, alanine, serine and threonine (Chen et al., 2019; Huang et al., 2017; Sharma et al., 2021; Shen et al., 2021; Stegen et al., 2021; Stegen et al., 2020; Stegen et al., 2016; Yu et al., 2019). Other transporters, including SLC7A7 and SLC38A2, may facilitate glutamine uptake upon anabolic stimulation or during bone malignancy (Chiu et al., 2020; Shen et al., 2021). As expected, depletion of extracellular glutamine strongly impairs osteogenic cell function *in vitro*, largely because of a combination of impaired TCA cycle anaplerosis, protein synthesis or glutathione generation. In proliferating osteoblast progenitors, glutamine uptake is regulated by a cellular sensing mechanism controlled by general control nonderepressible 2 (GCN2). In conditions of amino acid stress or increased demand, such as the high protein synthesis required for osteoblast proliferation and matrix production, GCN2 is activated by the presence of uncharged tRNA and phosphorylates the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α). In turn, this leads to an increase in the translation of the transcription factor ATF4, which stimulates the expression of amino acid transporters such as SLC1A5, likely to provide sufficient glutamine to support SSPC proliferation in a feed-forward mechanism (Hu et al., 2020). During osteoblast differentiation, SLC1A5 levels further increase and its expression is stimulated by osteoanabolic signals such as WNT and PTH (Sharma et al., 2021; Shen et al., 2021; Stegen et al., 2021). *In vivo*, ablation of *Slc1a5* in Osterix-expressing osteolineage cells impairs intramembranous bone formation, while endochondral ossification is only transiently affected. Mechanistically, SLC1A5 facilitates glutamine and, to a lesser extent, asparagine import that is necessary to support amino acid biosynthesis required for osteoblast proliferation and matrix production (Sharma et al., 2021) (Fig. 2). Intriguingly, while glutamine is a critical nutrient during all stages of osteogenesis, asparagine appears more important for terminal osteogenic differentiation. Further studies are therefore required to elucidate the role of asparagine metabolism in osteogenic cells.

Once taken up by the cell, glutamine metabolism is generally initiated by the enzyme glutaminase (GLS), which converts glutamine into glutamate that is further deaminated to form αKG. Other enzymes, mostly involved in nucleotide synthesis, can also generate glutamate from glutamine, but their functional role in osteoblast lineage cells is currently unknown. Conditional ablation of GLS in the early skeletal lineage using *Lepr*-Cre or *Prrx1*-Cre transgenic mice results in a decrease in SSPC proliferation and bone formation, and is associated with increased marrow adiposity (Yu et al., 2019). At the cellular level, differentiation of bone marrow stromal cells is shifted towards adipocytes at the expense of osteogenic differentiation, indicating that GLS is required for osteoblast lineage commitment. The decrease in proliferation is linked to a deficit in transaminase-dependent αKG synthesis, although the mechanism by which αKG regulates SSPC proliferation requires further investigation. Similarly, whether and how glutamine-derived αKG regulates SSPC lineage specification remains unknown, but might involve epigenetic regulation of gene expression as observed in growth plate chondrocytes (Stegen et al., 2020). Finally, we could demonstrate that in skeletal progenitors from the periosteum, the hypoxia-inducible transcription factor HIF-1α induces GLS-driven glutamine metabolism to support a glutathione-dependent antioxidant mechanism that safeguards cell survival during bone regeneration (Stegen et al., 2016) (Fig. 2).

GLS-mediated catabolism is also important in more lineage-committed osteogenic progenitors, as genetic inactivation (*Osterix*-Cre mice) results in decreased bone mass, which is caused by impaired biosynthesis but also cell survival of osteogenic cells (Stegen et al., 2021). Although not analyzed in detail, the decrease in bone mass is also in this model associated with increased bone marrow adiposity. In osteoprogenitors, GLS-mediated glutamine catabolism supports nucleotide and amino acid synthesis that is necessary for proliferation and matrix synthesis, whereas glutamine-derived glutathione prevents the accumulation of harmful reactive oxygen species (ROS) (Fig. 2). Supplementation of αKG fully prevents the detrimental effects of GLS deletion on osteoblast anabolism by restoring NEAA and nucleotide synthesis, whereas GSH supplementation rescues the defect in cell survival. Intriguingly, while GLS-mediated glutamine catabolism is important in early osteolineage cells, deletion of GLS in mature osteocalcin-expressing osteoblasts does not affect bone properties (Yu et al., 2019). These observations suggest that mature osteoblasts metabolize glutamine *via* other pathways, or that other nutrients such as glucose or fatty acids can compensate for the loss of GLS, but these hypotheses require further testing.

In addition to its role as a proteogenic amino acid and metabolic precursor, exogenous or glutamine-derived glutamate can also function as an excitatory neurotransmitter through binding on ionotropic (*i.e.* ligand-gated ion channel) or metabotropic receptors (*i.e.* inducing G-protein signaling cascade). Several osteogenic cell types, including bone marrow SSPCs, osteoblasts and osteocytes express SLC1A3, a glutamate-aspartate transporter (also known as GLAST) involved in glutamate recapture from the extracellular space (Mason et al., 1997; van Gastel et al., 2020a), and osteoblasts and osteoclasts also express the ionotropic *N*-methyl-D-aspartate glutamate receptor (NMDAR) (Patton et al., 1998). *In vitro*, glutamate can induce osteoblast differentiation through NMDAR or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor signaling. In rats, local injections of an AMPA agonist

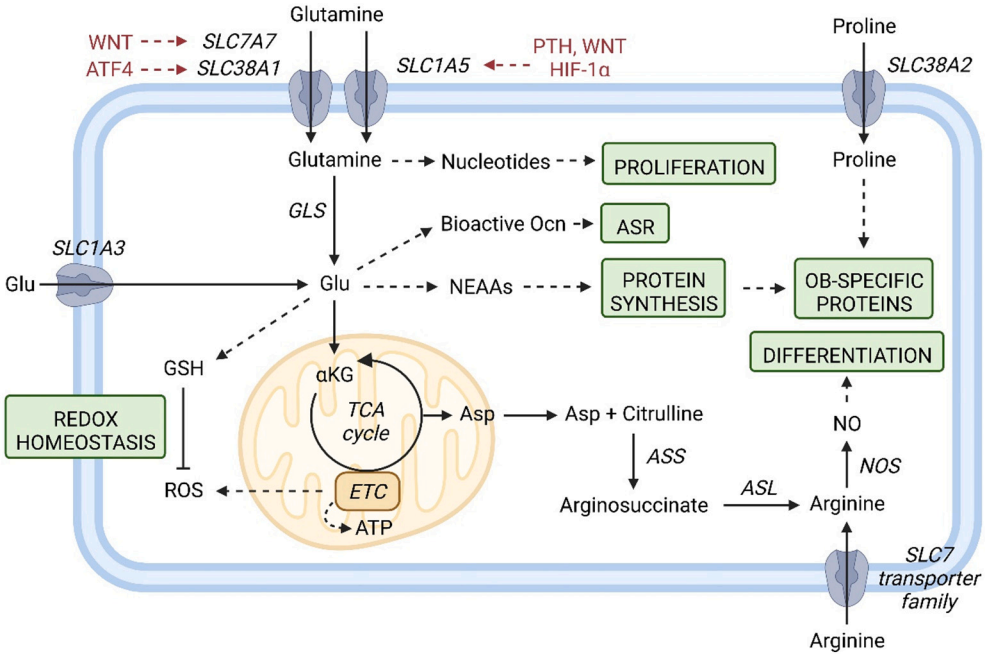

Fig. 2. Metabolism and function of amino acids in osteoblast lineage cells. Scheme showing the main cellular processes (in green boxes) supported by amino acids in osteoblasts. Dotted lines indicate a pathway consisting of several reactions. Transporters or key enzymes are in italic. Transcription factors and signaling pathways that promote the expression of amino acid transporters are depicted in red. Abbreviations: αKG: α-ketoglutarate, ASL: argininosuccinate lyase, ASR: acute stress response, ASS: argininosuccinate synthase, ATF4: cyclic AMP-dependent transcription factor ATF-4, (N)EAAs: (non-)essential amino acids, ETC: electron transport chain, GLS: glutaminase, GSH: glutathione, HIF: hypoxia-inducible factor, NOS: nitric oxide synthase, OB: osteoblast, Ocn: osteocalcin, PTH: parathyroid hormone, ROS: reactive oxygen species, TCA: tri-carboxylic acid cycle. Three-letter abbreviations are defined in Table 1.

in the subcutaneous tissue surrounding the tibia results in increased bone mass (Lin et al., 2008), although the underlying mechanism was not fully investigated. Interestingly, systemic *Slc1a3* knockout mice do not display a bone phenotype (Gray et al., 2001), whereas deletion of *Nmdar1* in osteocalcin-expressing cells results in severe skeletal defects (Skerry, 2008). A more recent study revealed an additional role for SLC1A3-mediated glutamate uptake in regulating the acute stress response in rodents and human. Mechanistically, stress exposure favors glutamate uptake by osteoblasts, resulting in the secretion of bioactive osteocalcin as glutamate competitively inhibits gamma-carboxylase activity. In turn, uncarboxylated osteocalcin blocks the activity of parasympathetic neurons, thereby leaving the sympathetic tone unhampered and allowing for a fight-or-flight response (Berger et al., 2019) (Fig. 2). Together, these observations indicate that glutamate-mediated signaling is an important regulator of bone properties and even whole-body physiology, but several questions remain: which cells generate the required glutamate, how it is recycled in the local microenvironment and what are the molecular mechanisms that mediate the effects of glutamate on bone cells.

2.1.2. *Proline, necessary for synthesis of osteoblast-specific proteins*

In addition to glutamine, other amino acids are also involved in sustaining the high anabolic needs of matrix-synthesizing osteoblasts. A recent study by the Karner lab revealed an important role for proline in osteoblasts (Shen et al., 2022). This non-essential amino acid has different metabolic functions ranging from extracellular matrix synthesis and modification, to bioenergetics and redox homeostasis (Elia et al., 2018; Krane, 2008; Vettore et al., 2021). Although proline can be generated in osteoblasts from glutamine (Stegen et al., 2021), the majority is taken up *via* SLC38A2 and its consumption is increased during osteogenic differentiation (Shen et al., 2022). In contrast to glutamine, which is likely used as a general amino acid precursor (Sharma et al., 2021; Stegen et al., 2021), most of proline is directly incorporated in osteoblast-associated proteins such as runt-related transcription factor 2 (RUNX2), Osterix and type 1 collagen, with little metabolism in other metabolic pathways such as the TCA cycle. Accordingly, limiting proline availability mainly affects the production of proline-rich osteoblastic proteins, whereas glutamine deprivation causes activation of the ISR with a decrease in global protein synthesis (Sharma et al., 2021; Shen et al., 2022) (Fig. 2). Whether chronic proline starvation has cellular effects is unknown. Still, this relatively modest reduction in osteoblast-related proteins is physiologically relevant, as deletion of *Slc38a2* in Osterix-expressing cells negatively impacts osteoblast differentiation and bone development (Shen et al., 2022). Interestingly, type X collagen remodeling is delayed in mutant mice. As *Osterix*-Cre also targets hypertrophic chondrocytes (Chen et al., 2014), these data suggest that loss of SLC38A2 impairs chondrocyte function during endochondral ossification. Further studies are therefore necessary to delineate the importance of proline uptake *versus* glutamine-mediated *de novo* synthesis during chondrogenesis (Stegen et al., 2020).

2.1.3. *Amino acid metabolism: beyond glutamine and proline*

A number of studies have suggested a regulatory role for other amino acids in osteogenic cells, although the molecular mechanism is often not fully understood or validated *in vivo*. Methionine is an essential amino acid that is critical for protein synthesis, as it is encoded by the start codon during protein synthesis, but also functions as a metabolic precursor of S-adenosylmethionine (SAM), which is used as a cofactor by DNA and histone methyltransferases and thereby regulates gene expression. Dietary methionine restriction is gaining interest over the last decade, as it may extend life-span and reduce malignancy by decreasing oxidative stress (Ables et al., 2016). However, methionine restriction negatively affects bone properties, which is, at least partially, caused by impaired osteoblast differentiation (Ouattara et al., 2016; Plummer et al., 2017). Whether these defects result from epigenetic changes or from detrimental metabolic alterations remains to be studied.

The tryptophan degradation pathway produces kynurenine metabolites with important effects on the brain, gut and skeletal muscle, but also on the skeleton (Cervenka et al., 2017). In patients, kynurenine serum levels increase with age, which are correlated with osteoporotic bone loss (Forrest et al., 2006). In accordance, kynurenine administration decreases bone mass in adult mice (Pierce et al., 2020). However, also deletion of indoleamine dioxygenase, the rate-limiting enzyme in the pathway, prevents bone loss (Vidal et al., 2015), arguing that regulation of skeletal cell function by kynurenine-derived metabolism is complex and requires further study.

Another amino acid that might be involved in osteoblast anabolism is taurine, a non-proteogenic amino acid that is predominantly synthesized by the liver (Hansen and Grunnet, 2013). Administration of taurine

stimulates osteoblast differentiation *in vitro*, and feeding rats a high-taurine diet protects against ovariectomy-induced bone loss (Choi and DiMarco, 2009). In a model of vitamin B12 deficiency that is associated with skeletal defects, a taurine-supplemented diet increases IGF1 synthesis in the liver that results in increased osteoblast proliferation and rescues the bone phenotype (Roman-Garcia et al., 2014). A recent study shows that osteocytes synthetize taurine, which may be involved in regulating osteocyte viability and sclerostin expression, although *in vivo* evidence is lacking (Prideaux et al., 2020).

Arginine is a conditionally essential amino acid that is also implicated in bone biology (van't Hof and Ralston, 2001). It can be taken up primarily *via* the SLC7 transporter family or generated *via* a step-wise process that involves the formation of arginosuccinate from citrulline and aspartate by arginosuccinate synthase, which is subsequently cleaved by arginosuccinate lyase (ASL) that generates arginine and fumarate. The importance of arginine metabolism for skeletal cell function is primarily attributed to its role in nitric oxide (NO) synthesis, as loss of ASL in osteoblasts (*Osteocalcin*-Cre) results in decreased bone mass caused by reduced osteoblast differentiation (Jin et al., 2021) (Fig. 2). This defect in osteoblast function is associated with metabolic changes, as decreased NO production downregulates glycolysis, although the contribution of NO-induced metabolic alterations *versus* direct effects of NO on osteoblasts and surrounding cells remains to be investigated.

Together, these studies indicate that osteolineage cells can both consume and synthesize the requisite amino acids to support their anabolic functions during bone formation.

2.2. Metabolic interaction between bone marrow stromal cells and hematopoietic or leukemic cells

In adult life, the bone marrow is the main site of hematopoiesis. Hematopoietic homeostasis, including self-renewal, proliferation, differentiation and migration of hematopoietic stem and progenitor cells, is primarily ensured by bone marrow stromal cells through cell-bound or secreted molecules (Calvi and Link, 2015). Recent studies also suggest a regulatory role for cell metabolism, although insight is still limited, especially concerning amino acids. The essential amino acid valine can support the proliferation and maintenance of hematopoietic stem cells (HSCs), as shown by *in vivo* and *in vitro* models of valine deprivation (Taya et al., 2016). Interestingly, bone marrow-derived endothelial cells, osteoblasts and stromal cells secrete valine *in vitro*, suggesting that cells from the niche maintain HSCs through the secretion of specific amino acids but this hypothesis requires further testing *in vivo*.

In hematological malignancies such as leukemia, which is characterized by rapid expansion of abnormal white blood cells, the metabolic crosstalk between bone marrow stromal cells and leukemia cells is disturbed and likely contributes to disease progression. Acute leukemia occurs when less mature, fast-developing become dysfunctional cells called blasts as they leave the bone marrow. By contrast, chronic leukemia occurs when leukocytes develop more slowly, potentially taking years to cause symptoms. In a model of chronic lymphocytic leukemia (CLL), Zhang et al. have shown that bone marrow stromal cells modulate the redox status of CLL cells, thereby promoting their survival and drug resistance. Mechanistically, stromal cells take up cystine and convert it into cysteine, which is then used by CLL cells to generate GSH. Blocking cystine uptake *in vivo* depletes intracellular GSH levels in CLL cells specifically, resulting in increased drug-induced cytotoxicity and decreased leukemia burden (Zhang et al., 2012). In a model of acute myeloid leukemia (AML), van Gastel and colleagues showed that, in addition to cell-intrinsic changes in glutamine metabolism, AML cells also depend on aspartate derived from bone marrow stromal cells to persist after chemotherapy treatment. Specifically, glutamine-derived aspartate is predominantly provided by bone marrow stromal cells and fuels nucleotide synthesis in chemotherapy-resistant AML cells. In accordance, blunting glutamine metabolism or pyrimidine synthesis selects against residual leukemia-initiating cells and thereby impairs leukemia progression in mice (van Gastel et al., 2020a). Together, these studies suggest that alterations in stromal cell amino acid metabolism are an important driver of leukemia progression, and that targeting these metabolic pathways, albeit in combination with known cytotoxic drugs, may be an appealing strategy to treat this disease.

2.3. Amino acid metabolism in osteoclasts

In contrast to osteogenic cells, fewer studies have investigated amino acid metabolism in bone-resorbing osteoclasts. Similar to osteoblasts, amino acid-sensing factors like mTOR are important for osteoclast formation and survival (Dai et al., 2017; Glantschnig et al., 2003; Hu et al., 2016; Sugatani and Hruska, 2005), especially in the setting of osteolytic bone metastasis (Abdelaziz et al., 2014; Hussein et al., 2012; Mercatali et al., 2016), although conflicting data exist (Huynh and Wan, 2018; Y. Zhang et al., 2017). However, how specific amino acids and amino acid-metabolizing pathways regulate osteoclast differentiation and function is even less well understood, especially *in vivo*, despite long-standing knowledge that these cells express many amino acid transporters.

During *in vitro* differentiation, osteoclasts take up a considerable amount of amino acids, including glutamine, arginine, serine, and branched-chain amino acids (BCAAs) such as (iso)leucine and valine, and their withdrawal from the culture medium manifestly affects osteoclast formation and/or function (Brunner et al., 2020; Go et al., 2022; Indo et al., 2013; Pollari et al., 2011). Glutamine metabolism appears to be primarily sustained *via* its uptake through SLC1A5, as genetic or pharmacological blockade of this transporter reduces intracellular glutamine levels and impairs osteoclastogenesis *in vitro* (Indo et al., 2013; Tsumura et al., 2021). Supplementation of cell-permeable αKG fully rescues the phenotypic defects caused by SLC1A5 inhibition (Indo et al., 2013), although it remains unknown how glutamine is metabolized in osteoclasts, which metabolic role(s) it fulfills and how it functions *in vivo*.

In a recent study, Brunner and colleagues have shown that the extracellular availability of the conditionally essential amino acid arginine is also critical for RANKL-induced osteoclastogenesis (Brunner et al., 2020). Although its uptake is not altered upon RANKL treatment, extracellular arginine supports TCA cycle activity and oxidative phosphorylation, which are both critical metabolic regulators of osteoclast formation (Arnett and Orriss, 2018; Da et al., 2021). Interestingly, supplementation of the TCA cycle intermediate αKG does not rescue the defect in osteoclastogenesis caused by arginine depletion (Brunner et al., 2020), suggesting that other arginine-derived metabolites, including NO, polyamines or proline (Brandi et al., 1995; Bronte and Zanovello, 2005; Klein-Nulend et al., 2014; Yamamoto et al., 2012), may be involved. Another amino acid that regulates osteoclastogenesis is methionine. During osteoclast differentiation, RANKL induces a shift towards oxidative metabolism with increased ATP production, which is accompanied by enhanced synthesis of SAM from methionine. In turn, SAM supports the activity of DNA methyltransferases that regulate osteoclast differentiation *via* epigenetic repression of anti-osteoclastogenic genes (Nishikawa et al., 2015). However, impaired osteoclast formation caused by inhibition of oxidative phosphorylation is not rescued by exogenous SAM, suggesting that the changes in DNA methyltransferase activity are not directly linked to altered ATP production *via* oxidative phosphorylation. On the other hand, a high methionine diet leads to increased circulating homocysteine levels, which cause oxidative stress and osteoclast-mediated bone loss by disrupting OPG and RANKL production in osteoblasts (Vijayan et al., 2013). Finally, two recent studies have shown an important role for branched-chain aminotransferase 1 (BCAT1) in regulating osteoclastogenesis. BCAT1 is the cytoplasmic BCAT isoform that converts BCAAs into branched-chain ketoacids, thereby generating glutamate from αKG. Systemic deletion of BCAT1 in mice results in increased bone mass, which is caused by impaired osteoclastogenesis with no change in

osteoblast-related parameters. Pharmacological inhibition of BCAT1 prevents bone resorption in a mouse model of lipopolysaccharide-induced osteoporosis (Go et al., 2022; Pereira et al., 2020). At the molecular level, inhibition of BCAT1 reduces the expression of the pro-osteoclastogenic transcription factor NFATc1 (Go et al., 2022), although the metabolic link between BCAAs and osteoclast differentiation is not known at present. BCAA metabolism is not only regulated by BCAT activity but also by the principal BCAA transporter SLC7A5 (also known as L-type amino acid transporter 1 or LAT1). Deletion of *Slc7a5* in osteoclasts (*Lysozyme M*-Cre or *Tnfrs11a*-Cre) impairs mTOR signaling, resulting in enhanced osteoclast-mediated bone resorption, although it remains puzzling how impaired mTOR signaling leads to increased cell activity (Ozaki et al., 2019). The apparent phenotypic discrepancy upon inactivation of BCAT1 or SLC7A5 suggests that blocking BCAA uptake *versus* BCAA metabolism causes differential (metabolic) rewiring that in turn results in altered osteoclast behavior although this hypothesis requires further testing.

Taken together, recent studies are only starting to improve our understanding on osteoclast amino acid metabolism. More research is thus needed to demonstrate the clear metabolic link between amino acids and osteoclast function, especially using osteoclast-specific transgenic mouse models that target amino acid transporters and/or amino acid-metabolizing enzymes.

3. Amino acid metabolism in chondrocytes

Another metabolically active skeletal cell type is the chondrocyte, whose anabolic actions are especially evident during bone development and repair. The developing growth plate, through a tightly regulated sequence of chondrocyte proliferation, hypertrophic differentiation and replacement of the cartilaginous extracellular matrix (ECM) by an osseous one, is responsible for the longitudinal growth of the skeleton. These aspects are recapitulated during fracture repair, whereby a cartilage callus is initially formed that is later replaced by bone through the concerted action of bone-resorbing osteoclasts and bone-forming osteoblasts (Hallett et al., 2019). Intriguingly, despite the highly anabolic nature of chondrocytes, cartilage is one of the few avascular tissues in the human body, suggesting that chondrocytes rely on specific modes of nutrient acquisition and removal. For example, while small metabolites like glucose or amino acids reach the centrally localized chondrocytes through passive diffusion, diffusion of free fatty acids and lipids is limited (Torzilli et al., 1998; van Gastel et al., 2020b). Moreover, amino acid recycling *via* autophagy is critical to safeguard chondrocyte homeostasis during nutrient shortage, but also in baseline conditions (Luo et al., 2019). Finally, the absence of blood vessels renders the growth plate hypoxic, especially in the central region, which in turn results in specific metabolic adaptations that facilitate cell survival and function in oxygen-scarce conditions (Stegen and Carmeliet, 2019).

While glucose has been the main nutrient of interest in metabolic studies also in chondrocytes (Hollander and Zeng, 2019; Mobasheri et al., 2017), recent studies started to uncover an important regulatory role for amino acid metabolism. Indeed, key amino acid-sensing signaling pathways and transcription factors such as mTOR and activating transcription factor 4 (ATF4) are critical for chondrocyte function during bone development, likely by integrating nutritional cues to sustain anabolic processes such as proliferation and matrix synthesis (Chen and Long, 2018; Wang et al., 2009). Moreover, specific amino acids and amino acid-metabolizing pathways have been shown to regulate chondrocyte behavior during endochondral ossification and altered amino acid metabolism may be linked to cartilage dysfunction during osteoarthritis, which will be further discussed below.

3.1. Amino acid metabolism regulates chondrocyte function

In line with their pleiotropic metabolic roles, changes in the availability of amino acids such as glutamine, glycine or leucine significantly impact chondrocyte behavior, including survival, proliferation and matrix synthesis (de Paz-Lugo et al., 2018; Handley et al., 1980; Kim et al., 2009; Stegen et al., 2021). We could recently show that in mice, glutamine metabolism regulates multiple cellular properties in growth plate chondrocytes *via* a feedforward process (Stegen et al., 2020). In parallel with the induction of chondrogenesis, the master chondrogenic transcription factor SOX9 also promotes glutamine metabolism by increasing glutamine uptake together with GLS1 mRNA and protein expression. In turn, enhanced glutamine metabolism controls the typical chondrogenic gene expression epigenetically through glutamate dehydrogenase–dependent acetyl-CoA synthesis, which is necessary for histone acetylation of active, chondrocyte-specific gene promoters such as *Type 2 collagen* and *Aggrecan*. Moreover, glutamine-derived aspartate serves as a nucleotide and protein precursor for chondrocyte proliferation and matrix synthesis, whereas glutamine-derived glutathione synthesis avoids the accumulation of harmful ROS and subsequent cell death (Fig. 3). Thus, glutamine metabolism is important for chondrocyte function during bone development and likely also during bone repair, as pharmacological inhibition of GLS1 also impairs the formation of a cartilaginous callus (Stegen et al., 2020).

Glutamine metabolism is thus important for chondrocyte function during endochondral ossification. Importantly, the flux of glutamine into anabolic pathways has to be tightly regulated, as increased glutamine-mediated production of αKG, induced by constitutively active hypoxia-inducible factor 1α (HIF-1α) signaling, stimulates collagen hydroxylation and results in heavily cross-linked collagen. This collagen over-modification impairs osteoclast-mediated degradation of the cartilage matrix at the chondro-osseus junction and ultimately causes skeletal dysplasia that is associated with cartilage remnants and increased trabecular bone mass (Stegen et al., 2019). In the context of cartilage tumors, Zhang and colleagues recently showed that glutamine metabolism differentially regulates tumor development in benign enchondroma and cancer cell survival in malignant chondrosarcoma. While blocking GLS in enchondroma increases the number of tumor-like lesions, genetic ablation of *Gls* in IDH mutant chondrosarcomas results in reduced tumor volume (Zhang et al., 2022). Taken together, these studies highlight glutamine as a central metabolic regulator of chondrocyte function during endochondral bone development, and suggest that alterations in glutamine metabolism are linked to chondrocyte dysfunction and skeletal disease.

In addition to enhanced glutamine metabolism, another important characteristic of many anabolic cell types is *de novo* glucose-dependent synthesis of the non-essential amino acid serine, even in conditions where sufficient extracellular serine is present. We recently uncovered an important role for the serine synthesis pathway (SSP) in regulating chondrocyte proliferation, whereas exogenous serine and its downstream metabolite glycine are less important. Deletion of the rate-limiting enzyme phosphoglycerate dehydrogenase (PHGDH) hinders chondrocyte proliferation through impaired nucleotide synthesis, resulting in decreased long bone growth (Fig. 3). Moreover, pharmacological inhibition of PHGDH impairs cartilaginous callus formation during fracture repair. On the other hand, in conditions of serine starvation, chondrocytes maintain their anabolic functions through ATF4-mediated transactivation of SSP-related enzymes (Stegen et al., 2022). Thus, apart from its physiological role during bone development, *de novo* serine synthesis could also be required to maintain intracellular serine levels necessary to sustain proliferation during fracture repair, when serine supply may be acutely limited.

Taken together, recent evidence shows that glutamine metabolism and *de novo* serine synthesis are essential to endow chondrocyte anabolism during endochondral ossification. A potential role of other amino acids awaits further investigation.

3.2. Altered amino acid metabolism during osteoarthritis

Osteoarthritis (OA) is a chronic degenerative joint disease

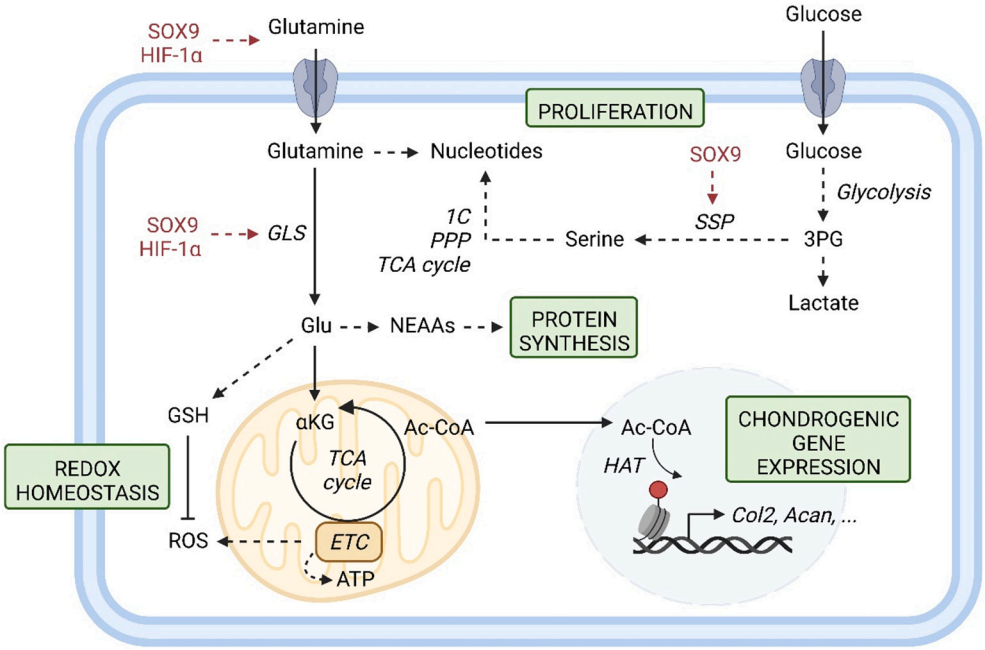

Fig. 3. Metabolism and function of amino acids in chondrocytes. Scheme showing the main cellular processes (in green boxes) supported by amino acids in chondrocytes. Dotted lines indicate a pathway consisting of several reactions. Key enzymes are in italic. Transcription factors that promote the expression of amino acid transporters or metabolic enzymes are depicted in red. Abbreviations: 1C: one-carbon metabolism, 3PG: 3-phosphoglycerate, Acan: aggrecan, Ac-CoA: acetyl-CoA, αKG: α-ketoglutarate, Col2: type 2 collagen, (N)EAA: (non-)essential amino acid, ETC: electron transport chain, GLS: glutaminase, GSH: glutathione, HAT: histone acetyl transferase, PPP: pentose phosphate pathway, ROS: reactive oxygen species, TCA: tri-carboxylic acid cycle. Three-letter abbreviations are defined in Table 1.

characterized by gradual loss of articular cartilage, synovial inflammation, and subchondral bone remodeling (Martel-Pelletier et al., 2016). While the etiology of this disease is multifactorial, recent studies have shown that metabolic alterations in articular chondrocytes, including amino acid metabolism, may be involved (Zheng et al., 2021). Accordingly, mTOR signaling, and more specifically the amino acid-sensitive mTOR complex 1, is upregulated in OA cartilage (H. Zhang et al., 2017; Zhang et al., 2015). The underlying mechanism is not fully understood, but might involve a response to changes in amino acid levels in the microenvironment. Indeed, metabolic analysis using liquid/gas chromatography-based mass spectrometry or NMR reveals differences in amino acid concentrations in OA cartilage, synovial fluid, plasma and urine (Li et al., 2016). Efforts are now ongoing to validate and functionally link these changes to the disease phenotype, in order to use specific amino acid alterations as OA biomarkers (Johnson et al., 2016; Zhai et al., 2010).

In addition to the changes in amino acid levels, amino acid-metabolizing pathways may also be involved in OA pathogenesis. Arginine is the physiological nitrogenous substrate of the inducible NO synthase (iNOS), and iNOS-mediated production of NO from arginine in chondrocytes is associated with OA pathogenesis (Abramson, 2008). In addition, the expression of arginase-2, which converts L-arginine into L-ornithine and urea, is upregulated in OA chondrocytes and is associated with the expression of catabolic enzymes such as matrix metalloproteinase 3 (MMP3) and MMP13 (Choi et al., 2019). Whether and how the effects on MMP expression are mediated by ornithine or its downstream metabolites is still unknown. Disturbed glutamine metabolism may also be involved in altering articular cartilage characteristics. In conditions of impaired glucose uptake, caused by inactivation of the glucose transporter GLUT1, enhanced glutamine-dependent TCA cycle anaplerosis is associated with collagen over-modification, similar as to what is observed in HIF-1α overexpressing growth plate chondrocytes (Stegen et al., 2019; Wang et al., 2021). However, whether glutamine metabolism is altered in OA cartilage and how it contributes to disease progression is still unknown.

Taken together, alterations in amino acid uptake and catabolism are associated with cartilage loss during osteoarthritis. Further research is however warranted to investigate potential causal links, with the ultimate goal of targeting amino acid metabolism as OA treatment.

4. Conclusions and future perspectives

Altogether, several studies have started to investigate the role of amino acid in bone homeostasis and diseases. For some of them, like glutamine, we now have a clearer view of its regulatory effects on bone cell function, while for other amino acids their exact role in different skeletal cell types remains to be elucidated. Glutamine uptake and catabolism are induced by anabolic stimuli such as WNT, ATF4 or HIF, but also by lineage-specific transcription factors such as SOX9. In turn, glutamine-derived metabolites sustain biosynthetic reactions and redox balance, and allow for epigenetic modifications. Proline complements glutamine metabolism by generating osteoblast-specific proteins. Cited studies also provide interesting clues on the role of asparagine, glutamate, methionine, tryptophan, arginine and BCAAs in skeletal cells, but their functional importance, especially *in vivo*, remains to be determined further. The increasing access to metabolomics techniques, together with novel methodologies that interrogate cell metabolism *in vivo* such as *in vivo* isotopic labelling and mass spectrometry imaging, will facilitate new research that will improve our understanding of amino acid metabolism in skeletal cells. In parallel, the use of transgenic mice with cell-specific modulation of metabolic enzymes or transporters is crucial to determine how amino acid cellular metabolism affects bone development and homeostasis. Finally, from a translational point of view, interfering with amino acid metabolism may be an appealing strategy to treat skeletal diseases. A high dietary amino acid intake might be beneficial for bone health, as it was reported to increase bone mineral density (Munger et al., 1999) and reduce fracture risk (Darling et al., 2009). On the other hand, amino acid metabolism may be dysregulated in skeletal diseases such as osteoporosis and osteoarthritis, although further work is needed to link specific metabolic reprogramming to skeletal cell dysfunction and to use these as a therapeutic target.

Declaration of competing interest

None.

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