The role of Vitamin C in fibrosis as illustrated for collagen synthesis and epigenetics

D. A. Seijkens, R.A. Bank
University of Groningen, Groningen, June 2015

Abstract

In this review, the role of vitamin C in fibrosis is reviewed, illustrated for collagen synthesis and epigenetics. Vitamin C is essential for the human diet to prevent scurvy. This due to impairment of several collagen-associated enzymes in absence of vitamin C. Fibrosis is a disease known for excessive collagen accumulation, but in order to effectively synthesize collagen, vitamin C is needed. Recent studies have also elucidated an important new role for vitamin C. It enhances specific epigenetics modifying enzymes and has also been reported to play a role in the induction and enhancement of pluripotent stem cells. Because of all these roles vitamin C has, it should be considered to be added to culture media when studying fibrosis. Researchers should consider the concentration of vitamin C in the investigated tissue in vivo. Vitamin C could also have several possible therapeutic purposes in the future, these should however be further investigated, especially for fibrosis.

INDEX

Introduction

Because of its antioxidant properties, vitamin C is frequently added as a supplement in food and beverages. Because of this, it seems surprising that it's rarely included in cell culture media. (Table 1) Only a few research groups do culture their cells with vitamin C, also known as L-ascorbic acid, mostly because of the vital role it plays in collagen synthesis and therefore in fibrosis. (Bastiaansen-Jenniskens et al., 2008; Hosper et al., 2013; Zuurmond et al., 2005) Most eukaryotic organisms synthesize vitamin C themselves. Because of the vital importance of vitamin C in collagen synthesis, as well as its antioxidant capacities, it seems odd that primates and humans have gotten rid of this treat over the ages. (Wheeler, 2015) The most important reason for researchers to add vitamin C when researching fibrosis *in vitro* is that without it, fibroblasts are unable to produce proper collagen structures.

Reference	Medium/supplement	L-ascorbic acid
Sigma Aldrich	DMEM/Ham's F12	×
	M2	×
	M16	×
	MEM-α	50 mg/l
	CMRL-1066	50 mg/l
	Medium 199	0.0566 mg/l
	RPMI 1640	×
Life Technologies	CMRL	50 mg/l
	Advanced DMEM	2.5 mg/l
	RPMI 1640	×
	GMEM	×
	Ham's F12	×
	Neurobasal	×
	Leibovitz's L-15	×
	DMEM	×
(Monfort, 2013)	N2 supplement	×

Table 1 - Some examples of frequently used culture media and their vitamin C concentrations

Vitamin C is mostly known as an antioxidant, but research has shown its role as a cofactor for several enzymes, related and unrelated to collagen synthesis. These enzymes are called alpha-ketoglutarate-dependent dioxygenases. The function of vitamin C in these enzymes, usually involves the chemical reduction of copper or iron, thereby increasing the pro-oxidant capacities of these metals. Vitamin C is a chiral molecule. The L-oriented molecule is what we call vitamin C, while its D-oriented enantiomer is known as D-ascorbic acid. While both molecules possess the same antioxidant properties, they do not share the same biological functions. (Figure 1A) (Buettner, 1997) The biological function of vitamin C rests on its ability to donate 2 electrons in order to reduce for the enzymes used in this review iron. (Figure 1B) Vitamin C is vital for returning iron into its reduced state when no substrate is present for the enzyme. If iron cannot be reduced by either vitamin C or a substrate, the enzyme will become and remain inactive. (Figure 1C) D-Ascorbic acid cannot fulfill this co-factor function.

In vivo the lack of vitamin C causes a disease known as scurvy, which was frequently found on the ships of many travelers and explorers, dating back to the early thirteenth century. Its symptoms were even described by Hippocrates in the second book of his 'Prorrheticorum'. (Lind, 1772) Research has pointed to the importance of lysyl hydroxylases and prolyl hydroxylases in proper collagen synthesis and the importance of vitamin C in the activity of these enzymes. Both enzymes are involved in the post-translational modifications of procollagen, essential for proper collagen structuring. (Myllyharju, 2004)

Because vitamin C influences collagen synthesis, it plays an important role in fibrosis. In fibrosis, excessive collagen is produced when trying to restore organs and this leads to tissue remodeling and scarring. It is believed that the excessive collagen cannot be degraded

properly and therefore the amount of collagen degraded is lower than the amount produced, which results in excessive collagen accumulation. Studies have shown that without vitamin C, collagen cannot be synthesized properly and because of that restricting vitamin C intake after a heart attack per instance, might cause the fibroblasts that cause fibrosis in the heart to produce less collagen and therefore reduce excessive synthesis of collagen associated with fibrosis.

Over the last decade the field of epigenetics has been greatly improved. It has been reported that several alpha-ketoglutarate-dependent dioxygenases can influence DNA methylation, Histone modifications and can even influence DNA and RNA repair.

Because of this vitamin C might have great impact on transcription of several fibrosis related and unrelated genes and therefore might play a role in fibrosis other than influencing collagen synthesis.

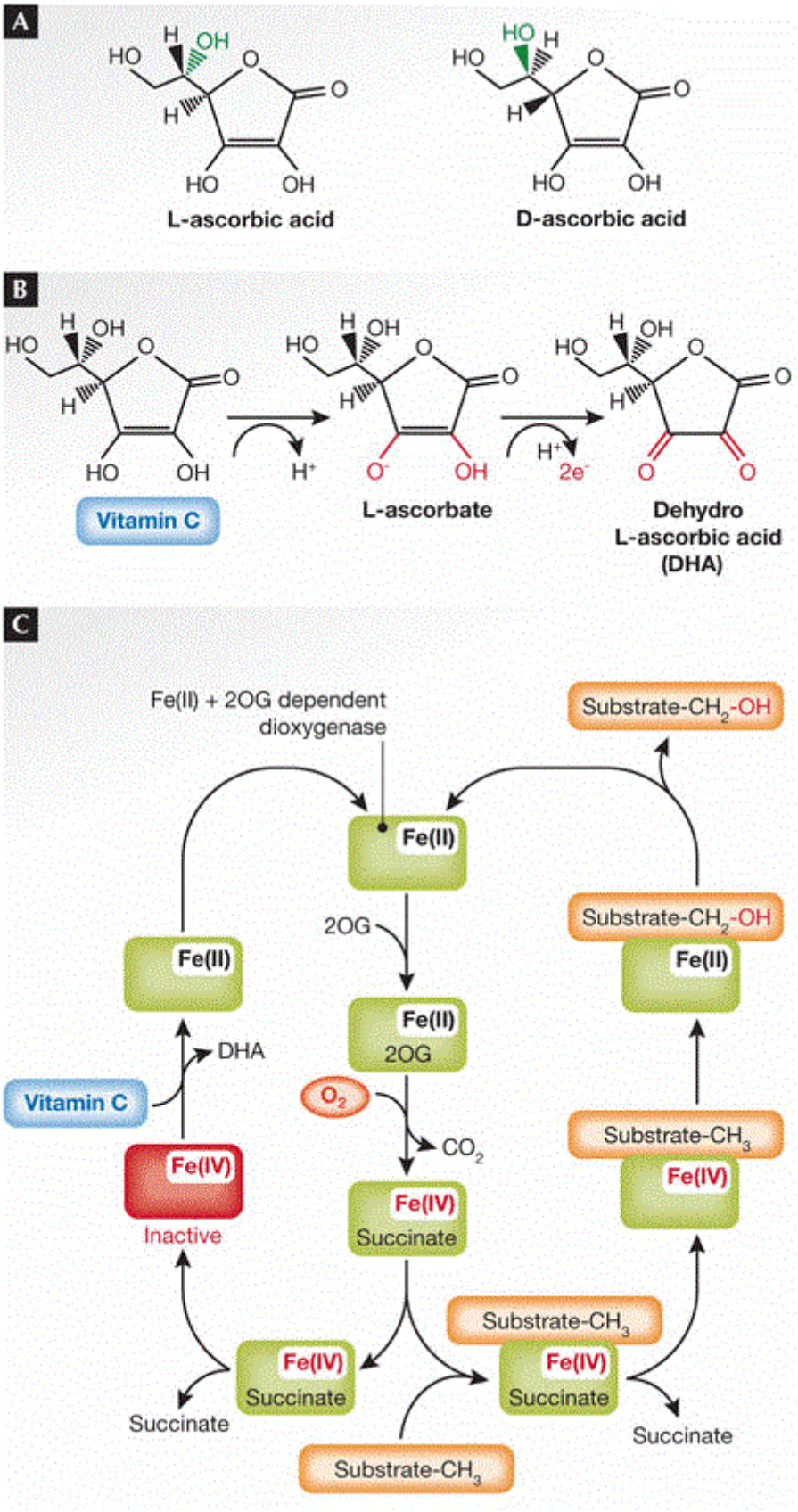

Figure 1 - (A) Vitamin C (L-ascorbic acid) and its enantiomer D-ascorbic acid. (B) Oxidation of vitamin C into Dehydro-L-ascorbic acid allows vitamin C to donate 2 electrons. (C) A schematic overview of alpha-ketoglutarate dependent dioxygenase catalysis and enzyme reactivation by vitamin C. (Monfort, 2013)

Because of the great variety of effects vitamin C can have on fibroblasts, its functions in fibroblasts and fibrosis should be reviewed. The aim of this paper is therefore to review the role of vitamin C in fibrosis, illustrated for collagen synthesis and epigenetics, to predict whether or not it should be incorporated in cell culture medium and could play a role in inhibiting or even preventing fibrosis.

The influence of vitamin C in scurvy

To illustrate the role of vitamin C in fibrosis for collagen synthesis, the best example to look at is when not enough vitamin C or even no vitamin C at all is present in the human body. An intake of less than 10 mg is considered to be low enough to cause scurvy. This would result in a plasma concentration of less than 10mM. The recommended daily amount (RDA) of vitamin C intake however is 75 mg/day for women and 90 mg/day for men. Exceeding the RDA does not seem to affect healthy individuals. This might be because vitamin C uptake reduces when intake is increased, from about 90% when intake is around daily recommended amount, while it is about 50% when intake is 1g or higher. The effects on individuals with certain diseases or conditions have not been defined. Scurvy is defined as having pathologically low plasma levels of vitamin C which cause connective tissue damage, blood vessel fragility, fatigue and eventually death. (Grosso et al.)

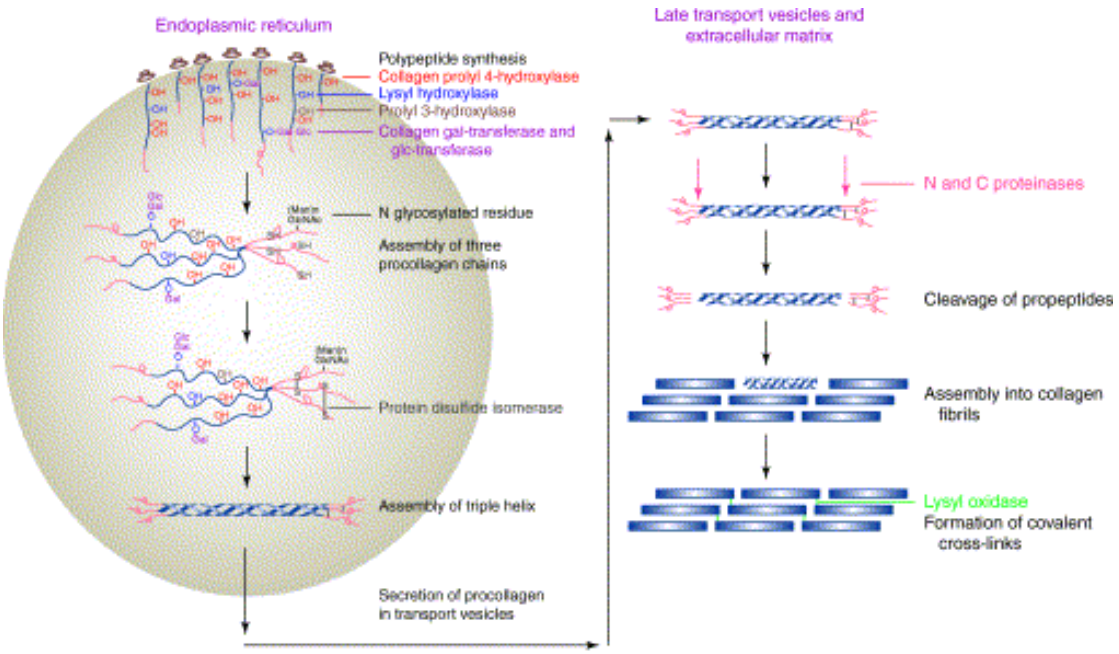

Figure 2- A schematic view of the role of collagen modifying enzymes. (Myllyharju et al., 2004)

Vitamin C is known to be an essential cofactor for alpha-ketoglutarate-dependent dioxygenases such as prolyl and lysyl hydroxylases. These enzymes are important for the hydroxylation of lysine and proline in all types of collagen by donating required electrons. (Grosso et al.) With scurvy in mind it is important to note that without this hydroxylation collagen cannot obtain a triple helical structure, which causes formed collagen to be unstable and unable to self-assemble any further. (Figure 2) This does not only cause connective tissue damage, as collagen is the most important protein in connective tissue,

but it is also the reason blood vessels become more fragile. (Hirschmann and Raugi) It influences the three main layers blood vessels are made up of: The tunica adventitia, tunica media and tunica intima. The tunica adventitia is made up of mostly fibroblasts and connective tissue, which is mostly collagen. The lack of collagen structures also causes the endothelial cells of the tunica intima to protrude into the lumen and erythrocytes to migrate into the tunica intima and media. The lack of collagen could also explain the fatigue found in scurvy patients as most skeletal muscles contain a lot of connective tissue and this connective tissue plays a role in the connection of skeletal muscles to bones and therefore the energy transmission from the skeletal muscles to the skeleton. Due to damage in the connective tissue the overall power and energy generated might be reduced, as well as the effectiveness of the transmission of the energy from the skeletal muscles. (Pearson)

Vitamin C synthesis in humans

Two different pathways in the synthesis of vitamin C in eukaryotes have been reported. One pathway uses the terminal enzyme L-gulonolactone oxidase (GULO) and is present in most animal lineages. The alternative pathway uses L-galactonolactone dehydrogenase (GLDH) as its terminal enzyme and is present in most land plants and *Euglena*, a genus of single-celled flagellate protists. Both pathways use different routes to synthesize an aldonolactone precursor, L-gulonolactone (L-GulL) or L-galactonolactone (L-GalL). (Figure 3) Although GULO is able to oxidize both L-GulL and L-GalL, GLDH can only oxidize L-GalL. Both enzymes also reside in different cell compartments. GULO localizes in the lumen of the ER, while GLDH is a part of three mitochondrial electron transport complex I subcomplexes.

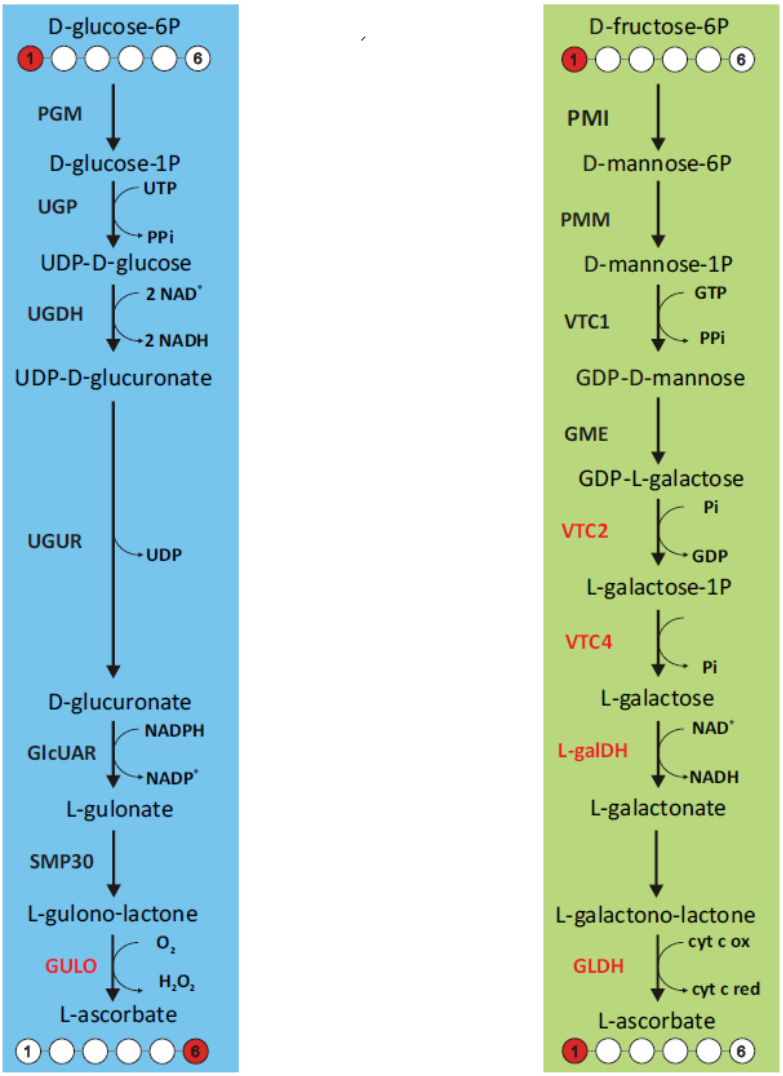

Figure 3 - A schematic view of vitamin C synthesis through the GULO and GLDH pathways. (Wheeler, 2015)

The most important function of vitamin C from an evolutionary point of view seems to be its antioxidant capacity. GULO uses O2 as an electron acceptor to synthesize vitamin C. By doing so, hydrogen peroxide (H2O2) is produced, which is one of many reactive oxygen species (ROS). This would mean in order to reduce ROS through vitamin C synthesis, ROS would also be produced. This seems rather ineffective. Therefore it is not at all surprising to see that the GULO enzyme seemed to disappear when another, more effective antioxidant pathway was introduced. The superoxide dismutase (SOD) pathway has been found to be inversely related to GULO activity in many organisms today. Along with the more effective antioxidant synthesis in humans themselves, their intake of vitamin C now originates from the GLDH pathway, which uses cytochrome C instead of O2 and produces no ROS. This might also contribute in keeping ROS low in humans, while sufficient vitamin C plasma levels can be maintained. (Wheeler, 2015; Nandia, 1997) Although the studies discussed above point to possible explanations, the reason for primates to lose the ability to synthesize vitamin C remains unknown.

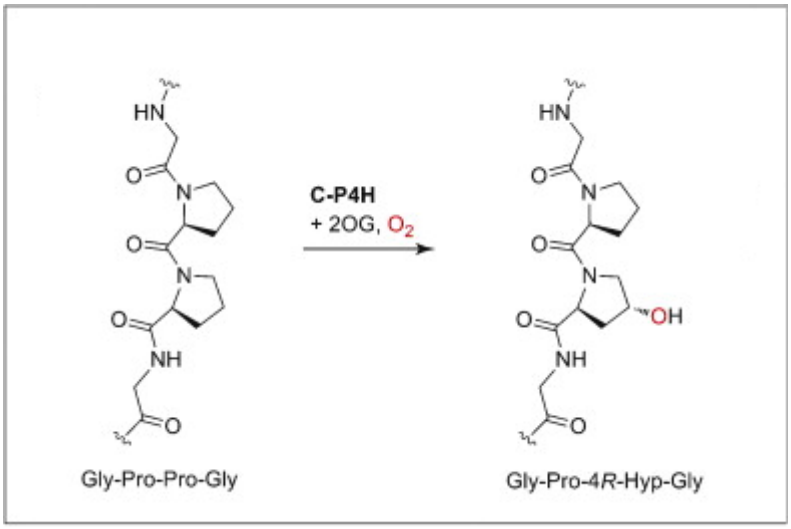

Figure 5 - A schematic view of the chemical reaction performed by prolyl 4-hydroxylases. (Myllyharju et al., 2005)

Collagen prolyl hydroxylases and their associated diseases

An important group of enzymes in the synthesis of collagen are the prolyl hydroxylases. They are present in the lumen of the endoplasmatic reticulum (ER). All of these enzymes are alpha-ketoglutarate-dependent dioxygenases, these use 2-oxoglutarate (2OG), as well as both vitamin C and ferrous iron as a cofactor. The vitamin C helps reversing oxidation of iron and by doing so, prevents auto-inactivation of the enzyme. When vitamin C is not present, reduction of the Fe (III) to Fe (II) will not take place and the enzymes become inactive. There are two different types of prolyl hydroxylases involved in collagen synthesis. Prolyl 4-Hydroxylases modify almost all proline to 4-hydroxyproline (4-Hyp) in the Y position of G-X-Y repeat in vertebrates. (Figure 4) Prolyl 3-hydroxylases hydroxylate proline to 3-hydroxyproline in the X position of a G-X-(4-

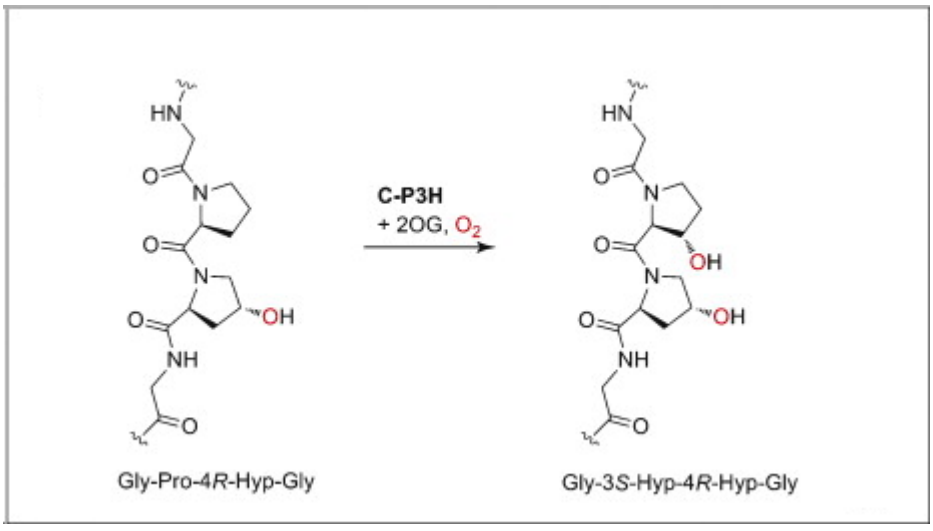

Figure 4 - A schematic view of the reaction performed by prolyl 3-hydroxylase, with the 4-hydroxylated proline already present. (Myllyharju et al., 2005)

Hyp) sequence, which means 3-hydroxylation occurs after 4-hydroxylation. (Figure 5) 4-hydroxylation is important for the folding of the newly synthesized collagen polypeptides and provides thermal stability to the triple helical structure of collagen. (Ishikawa, 2013)

Collagen prolyl 4-hydroxylases are a tetramer of two α and two β subunits. The β-subunit is identical to protein-disulfide isomerase (PDI), while there are three different known isoforms of the α-subunit (α (I), α (II), α (III)) (Kukkola, 2003). The β-subunit (PDI) is encoded by the P4HB gene, which can be found on chromosome 17, base-pairs (bp) 81,843,159-81,860,694 reverse strand, which makes it about 17 kbp long. It contains 11 exons, which encode the 508 amino acid enzyme PDI. (EC: 5.3.4.1) (Ensembl; Uniprot) Even though there are three α-subunits known for the prolyl 4-hydroxylases, these subunits only combine with another of the same subunit to create a prolyl 4-hydroxylase (Annunen 1997). Because of this, the eventual proteins are usually referred to as P4H1-3, with the 1-3 being the isoform of the α-subunit. What's rather interesting about the tetramer structure of the Prolyl 4-hydroxylases is that it has been reported that not only it is essential for proper collagen synthesis, expression of collagen polypeptide chains is essential for production of a stable prolyl 4-hydroxylase tetramer. (Vuorela, 1997)

The gene coding for α-subunit 1 is known as P4HA1. This gene can be found on chromosome 10, bp 73,007,217-73,096,974 reverse strand, which makes it about 89 kbp long. It contains 16 exons which encode the 534 amino acid Prolyl 4-hydroxylase subunit alpha-1. (P4HA1; EC: 1.14.11.2) The gene coding for α-subunit 2 is known as P4HA2. This gene can be found on chromosome 5, bp 132,191,838-132,295,315 reverse strand, which makes it about 103 kbp long. It contains 24 exons which encode the 535 amino acid Prolyl 4-hydroxylase subunit alpha-2. (P4HA2; EC: 1.14.11.2) The gene coding for α-subunit 3 is known as P4HA3. This gene can be found on chromosome 11, bp 74,235,801-74,311,657 reverse strand, which makes it about 75 kbp long. It contains 18 exons which encode the 544 amino acid Prolyl 4-hydroxylase subunit alpha-3. (P4HA3; EC: 1.14.11.2) (Ensembl; Uniprot)

The α(I)2β2 type I tetramer is the main form and is found in most cells and tissues. It represents 90% of enzyme activity in most cell types (liver, heart and kidney p. e.). The α(II)2β2 type II tetramer has been reported to be essential in capillary endothelium and differentiated bone cells. (Annunen, 1998) The α(III)2β2 type III tetramer has been found to be most highly expressed in placenta, adult liver, and fetal skin, although much lower than the α(I) and α (II) type P4H's. (Kukkola, 2003)

The gene coding for prolyl 3-hydroxylase 1 is known as P3H1. This gene can be found on chromosome 1, bp 42,746,335-42,767,084 reverse strand, which makes it about 20 kbp long. It contains 15 exons which encode the 736 amino acid prolyl 3-hydroxylase 1. (P3H1; EC: 1.14.11.7) A defect in the P3H1 gene is known to be the cause of Osteogenesis Imperfecta (OI) type VIII. This is a genetic bone disease that causes severe growth deficiency, extreme skeletal undermineralization, white sclerae and bulbous metaphyses (Large end portions of the bones). (Cabral, 2007)

The gene coding for prolyl 3-hydroxylase 2 is known as P3H2. This gene can be found on chromosome 3, bp 189,956,728-190,122,437 reverse strand, which makes it about 165 kbp long. It contains 18 exons which encode the 708 amino acid enzyme prolyl 3-hydroxylase 2 (P3H2; EC: 1.14.11.7) A mutation in the P3H2 gene has been reported to cause autosomal-recessive high-grade axial myopia. Myopia causes visual impairment and blindness, while

also causing macular degeneration, retinal detachment, premature cataract and even glaucoma. (Mordechai, 2011)

The gene coding for prolyl 3-hydroxylase 3 is known as P3H3. This gene can be found on chromosome 12, bp 6,828,410-6,839,851 forward strand, which makes it about 11 kbp long. It contains 14 exons which encode the 736 amino acid enzyme prolyl 3-hydroxylase 3. (P3H3; EC: 1.14.11.7) (Ensembl; Uniprot)

P3H1 mainly modifies type I collagen. Genetic mutations in P3H1 are known to cause severe OI in both mice and humans. (Pokidysheva, 2013) P3H2 has mostly been associated with the fibrillar collagen types (I, II, and V/XI) (Fernandes, 2011) But it has also been reported that type VI collagen is the main substrate for this enzyme. The interaction of this type of collagen with platelet-specific glycoprotein VI (GPVI) plays a role in some cancers. This, combined with the findings that P3H2 is downregulated in breast cancer cells by epigenetic inactivation, leads to believe that P3H2 plays an important role in the interaction between type VI collagen and platelets in the vascular system. (Pokidysheva, 2013) Not much is known about specific functions for P3H3. It has been reported it is expressed weakly in heart, lung, ovary and skeletal muscle cells. (Ansari-Lari, 2015)

Lysyl hydroxylases and their associated diseases

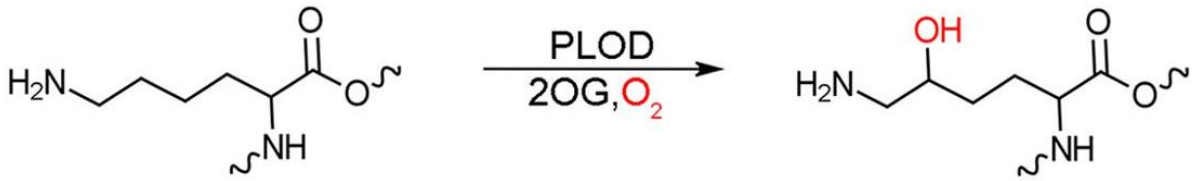

Figure 6 - A schematic view of the reaction performed by lysyl hydroxylases.

Lysyl hydroxylases (LH) play an important role in collagen synthesis. Like the prolyl 4-hydroxylases, all of these enzymes are alpha-ketoglutarate-dependent dioxygenases. The genes coding for these enzyms are known as procollagen-lysine, 2-oxoglutarate 5-dioxygenases (PLODs). The effect of vitamin C is the same in these enzymes. So far, three different LH isoforms have been identified, LH1, LH2 and LH3, encoded by PLOD1, PLOD2 and PLOD3 respectively, which all contribute to collagen synthesis through hydroxylation of different lysyl residues in different procollagens. They hydroxylate lysyl residues in the Y positions of repeating -X-Y-Gly- collagen sequences which serve as sites of attachment for carbohydrate units (either galactose or glucosyl-galactose) and play an essential role in the formation of intra- and intermolecular collagen cross-links. (Figure 6) (Risteli, 2004)

The PLOD1 gene can be found on chromosome 1, bp 11,934,205-11,975,538 forward strand, which makes it about 41 kbp long. It contains 19 exons which encode the 727 amino acid enzyme lysyl hydroxylase 1 (LH1, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1; EC 1.14.11.4) (Giunta, 2005; Ensembl; Uniprot). PLOD1 is expressed in almost every human tissue. PLOD1 has been known to modify lysine in the triple helical region. (Ishikawa, 2013) PLOD1 deficiency is believed to be the main cause for kyphoscoliotic Ehlers-Danlos Syndrome (EDS, formerly known as EDS VI) (Ha, 1994). Symptoms of this disease include hyperextenisble skin, easy bruising and thin scarring; joint slackness; extreme muscular hypothonia at birth; progressive early life onset scoliosis. There is no known cure for this disease. Treatment consists of managing symptoms through surgery, if needed, physical

therapy and bracing to support unstable joints. Most genetic causes for this disease involve single base substitutions or deletions. (Ensembl; Uniprot)

The PLOD2 gene can be found on chromosome 3, bp 146,069,440-146,163,653 reverse strand, which makes it about 94 kbp long. This LH isoform has isoforms of its own, produced by alternative splicing. The first isoform of PLOD2 is also known as PLOD2a and it contains 24 exons which encode the 737 amino acid enzyme PLOD 2a (LH2a, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2a, EC: 1.14.11.4). The second isoform is also known as PLOD2b and it contains an extra 21 amino acid exon between the 13th and 14th exon of PLOD 2a. Because of this, the enzyme PLOD 2b (LH2b, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2b, EC: 1.14.11.4) is made up of 758 amino acids. (Yeowell, 1999; Ensembl; Uniprot) These isoforms seem to be tissue specific in humans. Almost all tissues express both PLOD2a and PLOD2b, with the exception of skin, lung, dura and aorta, which seem to express PLOD2b exclusively. (Yeowell, 1999) PLOD2 has been known to modify lysine in the telopeptide region and is the only LH known to be able to do so. (Ishikawa, 2013) A disease associated with defective PLOD2 genes is Bruck syndrome. This disease is known to cause osteoporosis, fragile bones, short stature and joint contractures. (Van der Slot, 2003) Overexpression of LH2b has also been reported to cause defective collagen fibrillogenesis and matrix mineralization. (Pornprasertsuk, 2004)

The PLOD3 gene can be found on chromosome 7, bp 101,205,977-101,218,420 reverse strand, which makes it about 12 kbp long. (Ensembl) It contains 17 exons, which encode the 738 amino acid enzyme PLOD 3(LH3, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3, EC: 1.14.11.4; Uniprot). PLOD3 is mostly associated with biosynthesis of type IV and VI collagen. (Salo, 2008) PLOD3 can also act as both a galactosyltransferase and a glucosyltransferase, but the galactosyltransferase activity of PLOD3 is marginal. These studies implicate biological importance of O-linked sugars for collagen synthesis, but further functions remain unknown. (Ishikawa 2013)

The alpha-ketoglutarate-dependent dioxygenases involved in epigenetic modifications

So far, three major families that are alpha-ketoglutarate-dependent dioxygenases have been found to influence the epigenome. The Jumonji C (JmjC)-domain-containing enzymes (JHDM) family, the ten-eleven translocation (TET) family and the ABH (alkylation repair homologue) family. The JCDM family is a group of histone demethylases, the TET family is a group of DNA demethylases and the ABH family can actually do both, as they can serve as a demethylase for methylated H2A histones as well as both single and double stranded DNA.

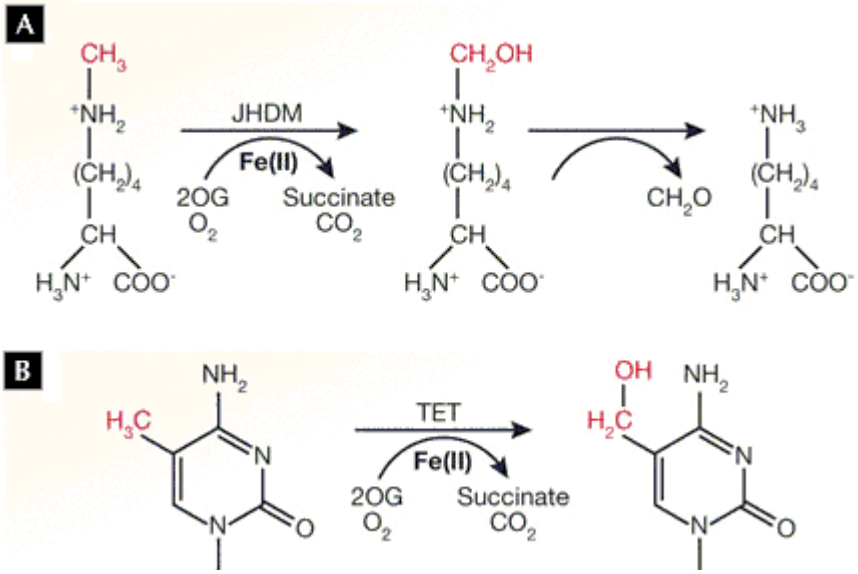

Figure 7- (A) Demethylation by JmjC-domain-containing enzymes. (B) Demethylation by TET enzymes

The JHDM family functions in the demethylation of lysine groups in histone proteins in a comparable way as lysyl hydroxylase and prolyl hydroxylase do, requiring Fe (II), vitamin C and 2OG. Although most histone demethylases which remove methyl-lysine modifications only remove mono- and dimethyl groups, some of these JHDMs can also demethylate trimethylated lysines. The reaction catalyzed by JHDM is performed in two steps. First, hydroxylation converts the methyl group into an unstable intermediate, which contains a hydroxyl group. Afterwards, formaldehyde is spontaneously eliminated and so the demethylation is complete. (Figure 7A) The JMCD family can be divided into several subfamilies which all contain the JmjC-domain as their active site, binding Fe (II), vitamin C and 2OG. The different members of the subfamilies perform the same demethylation reactions, but all on different lysines on their target histone and methylation state. The size of the methyl-binding pocket is decisive for its specificity for mono-, di- or trimethyl-modified substrates. Defects in these enzymes have reported to be the cause of many different developmental phenotypes.

The TET family of DNA demethylases has three different isoforms, TET 1-3. They convert 5-methyl-cytosine (5mC) into 5-hydroxymethyl-cytosine (5hmC). (Figure 7B) All enzymes contain a double-stranded β-helix (DSBH) fold, which consist of eight β-strands as well as one carboxylate (glutamate or aspartate) and two histidines that can bind Fe (II), that bind Fe (II) and 2OG in the catalytic site. TET 1 and 3 also have a zinc-finger domain, which might help binding to other proteins or DNA. A mutation in the gene for TET 2, which is also a supposed tumor surpressor gene, has been reported to contribute to myeloproliferative neoplasm (MPN). MPN families have shown distinct clinical entities, the most dominant being essential primary myelofibrosis (Fibrosis of the bone marrow; PMF), Thrombocythemia (Overproduction of thrombocytes; ET) and Polycythemia Vera (Overproduction of erythrocytes; PV). (Saint-Martin, 2009) The finding that a mutation in TET2 is associated with fibrosis in the bone marrow, indicates that TET2 plays a role in fibrosis. TET 3 has been reported to play an important role in the activation of Hepatic stellate cells (HSC). HSCs are known to express α-SMA, a known marker for myofibroblasts, as well as producing extra-cellular matrix (ECM) in the liver. While downregulation of TET3 has been reported to increase the fibrosis associated HSCs, TET3 has also been reported to be important in preventing kidney fibrosis. Both in vitro and in mouse models, it was shown that BMP7 causes normalization of RASAL1 (which encodes for rasGAP-activating–like protein 1, a suppressor of Ras-GTP function) methylation and that this process is dependent on TET3-mediated hydroxymethylation. RASAL1 hypermethylation is thought to play an important role in kidney fibrosis. (Zhang, 2014; Tampe, 2015)

The ABH family contains 9 different members in mammals. ABH1-8 and FTO (fat mass and obesity associated) all have very varying functions. Although ABH proteins have been reported to have an important role in DNA repair, they appear to also have a role in repairing RNA after damage by alkylating agents. The mechanisms used by the ABH family are similar to those of JHDMs and they use metal catalysis to oxidize stable methyl adducts attached to nitrogen of heterocyclic bases. ABHs however have nucleic acid binding domains which makes them more accessible to chemical reactions. ABH2 and ABH3 are known to help DNA repair through oxidation of 1-methyladenine and 3-methylcytosine. In vitro, ABH2 has a strong preference for dsDNA, while ABH3 prefers to demethylate RNA. FTO is believed

to mediate repair of 3-methyl-thymidine. Some ABH proteins have no catalytic activity. ABH1 per example, lacks significant DNA/RNA repair activity, but is very important in early development in mice. Mutations in ABH1 interfere with differentiation of trophoblasts in mice and increases the number of trophoblast stem cells. ABH1 is known to deacetylate histones by binding to Mrj, an essential factor for trophoblasts, and demethylate histone H2A. (Monfort et al., 2013)

Vitamin C enhances induced pluripotent stem cell generation and quality

As mentioned above, vitamin C plays a role in several epigenetic modifications. Because of this, it is an extremely interesting substance for induced pluripotent stem (iPS) cell generation from fibroblasts. The generation of iPS cells usually increases the level of ROS, which suggests vitamin C's antioxidant properties contribute to its effect. When other antioxidants are used however, no enhanced reprogramming can be observed. It can therefore be assumed that the activity of the alpha-ketoglutarate dependent dioxygenases is more important. Vitamin C also prevents activation of the INK4/ARF locus, which is not only a barrier for iPS cell reprogramming, but also associated with increased expression in senescence. In essence, the INK4/ARF locus is expressed in somatic cells and not in iPS and embryonic stem cells. (Monfort, 2013; Esteban, 2010; Li, 2009) Specifically, vitamin C enhances Jhdm1b, a histone demethylase and key regulator of Lys 36 demethylation in reprogramming. It also cooperates with Oct4 to activate miR-302/367, a micro RNA cluster which regulates pluripotency by inhibiting genes essential for embryonic differentiation and development. Its expression is lost early in differentiation. (Figure 8) (Wang, 2011)

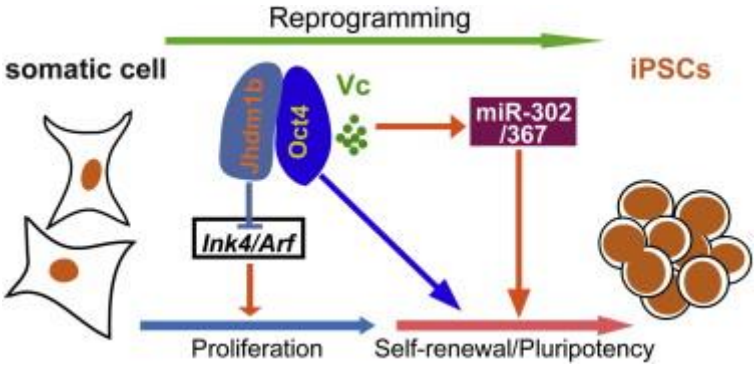

Figure 8- Schematic view of iPSC induction enhancement by vitamin C (Wang 2011)

Vitamin C has also been shown to convert pre-iPS cells, which are trapped in an intermediate stage of reprogramming, to functioning iPS cells. By examining the epigenetic factors that impede pluripotency, repressive H3 Lys 9 methylation on transcription factor genes that are needed for iPS reprogramming have been found. Presence of vitamin C however, decreases H3 Lys 9 dimethylation, causing expression upregulation. In this system, inhibition of a spectrum Lys-9 specific methyltransferases synergizes with vitamin C, resulting in improved efficiency of reprogramming and kinetics. (Chen, 2013) Considering all of this, alpha-ketoglutarate-dependent dioxygenases play an important role in reprogramming. Culturing without vitamin C therefore limits the activity of these enzymes and because of that, it interferes with epigenetic adjustment patterns.

Usage of vitamin C in cell-biological fibrosis research

Vitamin C is not commonly present in cell culture media right now (Table 1), even though its well documented importance in collagen synthesis. The biggest reason for this is probably that only a small sample of the researchers using culture media investigate collagen synthesis and that this fraction could just add it themselves. However, due to the widespread enzymes involved in epigenetics it activates, one might now consider it to be incorporated in standard media. But to really compare the effects of vitamin C in vivo and in vitro, the concentration of vitamin C should be adapted to the cells that are used accordingly, as not all tissues contain identical amounts of vitamin C in vivo. The highest concentrations of vitamin C are found in corneal epithelial tissue and neuronal tissue, while the lowest concentrations are found in bronchoalveolar lavage and red blood cells. (Table 2) These varying concentrations should be considered when investigating different types of fibrosis, liver and kidney fibrosis per example, or even other diseases.

Also, for the sake of fibrosis research, it is very important to note that vitamin C directly influences transcript stability of collagen types I and III. This treat might very well also contribute to the enhancing effects of vitamin C on collagen synthesis. It also directly influences the ECM by not only stabilizing collagen transcripts, but also decreasing the stability of elastin transcripts. Increases of elastin have long been associated with several types of fibrosis. (Hoff et al., 1999; Arrigoni et al., 2001; Pellicoro et al. 2012) Unpublished results also show that vitamin C also significantly enhances expression of α-smooth muscle actin, an important marker for myofibroblast differentiation and ACTA2 transcription in human dermal fibroblasts. This also indicates an important role for vitamin C in fibroblast differentiation. (Unpublished results by the matrix medical biology research group of the UMCG Groningen, the Netherlands. Bank, 2015)

Tissue	Ascorbate (mmol/kg wet tissue)
Adrenal glands	1.7–2.3
Pituitary gland	2.3–2.8
Liver	0.8–1
Spleen	0.8–1
Pancreas	0.8–1
Kidneys	0.28–0.85
Skeletal muscle	0.17–0.23
Brain	0.74–0.85
Placenta	0.23–0.72

Tissue	Ascorbate (mM)
Plasma (healthy)	0.04–0.08
Red blood cell	0.04–0.06
Neutrophil	1.2
Lymphocyte	4.0
Monocyte	3.2
Platelet	3.7
Cerebral spinal fluid	0.15–0.25
Neuron	10
Glial cells	1
Lens	2.5–3.4
Corneal epithelium	12.5
Aqueous humor	0.4–1.1
Alveolar macrophage	0.32
Bronchoalveolar lavage	0.04–0.06
Saliva	0.04–0.05

Table 2- The concentration of vitamin C in several tissues and cell types. (Du, 2012)

There are actually different ways to add vitamin C to cells in culture. The most obvious and easiest one is to just add vitamin C to the culture media. However, addition of ascorbate-2-phosphate, as well as dehydroascorbic acid (DHA) will also cause increased vitamin C concentrations in the cell. Ascorbate-2-phosphate is believed to be hydrolyzed by

esterases on the membrane resulting in vitamin C being transported into cells, while DHA can rapidly be transported into cells through glucose transporters in a matter of minutes. It is then reduced into vitamin C by several biological reductants as well as enzyme systems. When studying biological effects of vitamin C in cultures, it should be considered that addition of vitamin C causes H2O2 production, while the addition of ascorbate-2-phosphate does not have this effect, resulting in more oxidative stress in the cell culture. The downside of this process is that the uptake of vitamin C through ascorbate-2-phosphate takes some more time compared to culturing with regular vitamin C.
(Du, 2012)

Discussion and perspectives

This review gave insight in the role of vitamin C in fibrosis, illustrated by collagen synthesis and epigenetics. Vitamin C is an essential molecule in humans to prevent scurvy, a disease that was commonly found on ships of explorers dating back to the early 13th century. The lack of vitamin C causes impairment of many collagen-associated enzymes which together cause scurvy. But defects in just one of these genes can cause significant problems, which vary from embryonic lethality to Ehlers-Danlos Syndrome or Osteogenesis Imperfecta. The vitamin C associated enzymes directly involved in collagen synthesis are the prolyl 3- and 4-hydroxylases and the lysyl hydroxylases. All of these enzymes rely on vitamin C to reducing iron back into its reduced state and hereby reactivating the enzymes.

The variety of epigenetic effects that are mediated by vitamin C implicate a much larger role for vitamin C than its role as an antioxidant and important cofactor for collagen modifying enzymes. The findings that vitamin C enhances iPS cells derived from fibroblasts, might have interesting implications for wound healing. It is well known that fetal fibroblasts are able to induce scarless healing, with almost no functional losses. It could very well be that vitamin C will play an important role in inducing scarless healing in adult fibroblasts and therefore helping reducing or curing fibrosis. But to further evaluate the role vitamin C has on fibrosis related genes and their transcription and expression, further research in several types of fibroblasts should be done. There might even be a role for vitamin C in enzymes that do not require it directly for enzyme function, as it might influence transcription or expression of these enzymes.

Even though the variety of influences vitamin C has on cells should be considered in cell-biological research, incorporating it in standard media should not be recommended. Firstly, because of the fact that the concentration of vitamin C differs per tissue, it would be ineffective to add a standard amount to all culture media. This because the concentration of vitamin C which is to be used to culture with should be the amount which is found in vivo. Also, there are several methods to get vitamin C into the cell, all with their own advantages and disadvantages. Researchers should consider which method should work best for their research.

Although vitamin C plays such a wide variety of roles, using it for therapeutic reasons comes with several difficulties. Because the uptake of vitamin C reduces with increasing intake, it might be hard to administer amounts high enough to have significant effect. This is supported by the fact that increasing intake above RDA does not affect healthy individuals. However, in patients with nonalcoholic steatohepatitis a treatment of vitamin C and vitamin E has been reported to improve fibrosis. This not only points to effective therapeutic possibilities for vitamin C, but also specifically towards therapeutic possibilities for fibrosis. (Harrison et al., 2003)

References

All Ansari-Lari M., Muzny D. M., Lu J., Lu F., Lilley C. E., Spanos S., Malley T., Gibbs R. A., *A Gene-rich Cluster between the CD4 and Triosephosphate isomerase Genes at Human Chromosome 12p13 Genome Res. 1996 Apr;6(4):314-26.*

Annunen P., Autio-Harmainen H., Kivirikko K. I., *The novel type II prolyl 4-hydroxylase is the main enzyme form in chondrocytes and capillary endothelial cells, whereas the type I enzyme predominates in most cells J. Biol. Chem., 273 (1998), pp. 5989–5992*

Annunen P., Helaakoski T., Myllyharju J., Veijola J., Pihlajaniemi T., Kivirikko K. I., *Cloning of the Human Prolyl 4-Hydroxylase α Subunit Isoform α(II) and Characterization of the Type II Enzyme Tetramer THE α(I) AND α(II) SUBUNITS DO NOT FORM A MIXED α(I)α(II)β2 TETRAMER J Biol Chem. 1997 Jul 11;272(28):17342-8.*

Arrigoni O., De Tullio M. C., *Ascorbic acid: much more than just an antioxidant. doi:10.1016/S0304-4165(01)00235-5 (2001)*

Buettner G. R., Jurkiewicz B. A., *Catalytic Metals, Ascorbate and Free Radicals: Combinations to Avoid. Radiat. Res. 145, 532-541 (1996).*

Cabral W. A., Chang W., Barnes A. M., Weis M., Scott M. A., Leikin S., Makareeva E., Kuznetsova N. V., Rosenbaum K. N., Tifft C. J., Bulas D. I., Kozma C., Smith P. A., Eyre D. R., Marini J. C. *Prolyl 3-hydroxylase 1 deficiency causes a recessive metabolic bone disorder resembling lethal/severe osteogenesis imperfecta. Nature Genet. 39: 359-365 (2007)*

Chen J., Liu H., Liu J., Qi J., Wei B., Yang J., Liang H., Chen Y., Chen J., Wu Y., Guo L., Zhu J., Zhao X., Peng T., Zhang Y., Chen S., Li X., Li D., Wang T., Pei D., *H3K9 methylation is a barrier during somatic cell reprogramming into iPSCs Nature Genetics 45, 34–42 doi:10.1038/ng.2491 (2013)*

Du J., Cullen J. J., Buettnera G. R., *Ascorbic acid: Chemistry, biology and the treatment of cancer doi:10.1016/j.bbcan.2012.06.003 (2012)*

Ensembl database, consulted May and June 2015

Esteban M. A., Wang T., Qin B., Yang J., Qin D., Cai J., Li W., Weng Z., Chen J., Ni S., Chen K., Li Y., Liu X., Xu J., Zhang S., Li F., He W., Labuda K., Song Y., Peterbauer A., Wolbank S., Redl H., Zhong M., Cai D., Zeng L., Pei D., *Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. Cell Stem Cell. 2010 Jan 8;6(1):71-9. doi: 10.1016/j.stem.2009.12.001. Epub (2009)*

Fernandes R. J., Farnand A. W., Traeger G. R., Weis M. A., Eyre D. R., *A role for prolyl 3-hydroxylase 2 in post-translational modification of fibril-forming collagens. J Biol Chem. 2011 Sep 2;286(35):30662-9. doi: 10.1074/jbc.M111.267906. Epub (2011)*

Giunta C., Randolph A., Steinmann B., *Mutation analysis of the PLOD1 gene: An efficient multistep approach to the molecular diagnosis of the kyphoscoliotic type of Ehlers-Danlos syndrome (EDS VIA) (2005).*

Grosso G., Bei R., Mistretta A., Marventano S., Calabrese G., Masuelli L., Giganti M.G., Modesti A., Galvano F., Gazzolo D., *Effects of vitamin C on health: a review of evidence. Front Biosci (Landmark Ed) 18:1017-29. (2013)*

Harrison S. A., Torgerson S., Hayashi P., Ward J., Schenker S., *Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. The American Journal of Gastroenterology 98, 2485–2490; doi:10.1111/j.1572-0241.2003.08699.x (2003)*

Ha V. T., Marshall M. K., Elsas L. J., Pinnell S. R., Yeowell H. N., *A patient with Ehlers-Danlos syndrome type VI is a compound heterozygote for mutations in the lysyl hydroxylase gene. (1994)*

Hirschmann J. V. and Raugi G. J., *Adult scurvy J Am Acad Dermatol. Dec;41(6):895-906; quiz 907-10. (1999)*

Hoff C. R., Perkins D. R., Davidson J. M., *Elastin gene expression is upregulated during pulmonary fibrosis. Connect Tissue Res. ;40(2):145-53. (1999)*

Ishikawa Y., Bächinger H. P*., A molecular ensemble in the rER for procollagen maturation. Biochim Biophys Acta. 2013 Nov;1833(11):2479-91. doi: 10.1016/j.bbamcr.2013.04.008. Epub (2013)*

Jackson C. M., *Vitamin C : Nutrition, Side Effects, and Supplements (2011)*

Jacob R. A., *Vitamin C Function and Status in Chronic Disease Nutr Clin Care. 2002;5:66–74 (2002)*

Kukkola L., Hieta R., Kivirikko K. I., Myllyharju J., *Identification and Characterization of a Third Human, Rat, and Mouse Collagen Prolyl 4-Hydroxylase Isoenzyme The Journal of Biological Chemistry, 278, 47685-47693. (2003)*

Li H., Collado M., Villasante A., Strati K., Ortega S., Cañamero M., Blasco M. A., Serrano M., *The Ink4/Arf locus is a barrier for iPS cell reprogramming (2009)*

Lind J., *a Treatise on the Scurvy (1753)*

Mordechai S., Gradstein S., Pasanen A., Ofir R., El Amour K., Levy J., Belfair N., Lifshitz T., Joshua S., Narkis G., Elbedour K., Myllyharju J. and Birk O. S., *High Myopia Caused by a Mutation in LEPREL1, Encoding Prolyl 3-Hydroxylase 2 Am J Hum Genet. 2011 Sep 9;89(3):438-45. doi: 10.1016/j.ajhg.2011.08.003. Epub (2011)*

Myllyharju J., *Intracellular post-translational modifications of collagens. Topics in Current Chemistry, 247, 115–147. doi:10.1007/b103821 (2005).*

Myllyharju J., Kivirikko K. I., *Collagens, modifying enzymes and their mutations in humans, flies and worms Trends Genet. 2004 Jan;20(1):33-43.*

Nandia A., Mukhopadhyaya C. K., Ghosha M. K., Chattopadhyaya D. J., Chatterjeea I. B., *Evolutionary Significance of Vitamin C Biosynthesis in Terrestrial Vertebrates Free Radic Biol Med. 1997;22(6):1047-54.*

Pellicoro A., Aucott R. L., Ramachandran P., Robson A. J., Fallowfield J. A., Snowdon V. K., Hartland S. N., Vernon M., Duffield J. S., Benyon R. C., Forbes S. J., Iredale J. P., *Elastin accumulation is regulated at the level of degradation by macrophage metalloelastase (MMP-12) during experimental liver fibrosis. Hepatology. 2012 Jun;55(6):1965-75. doi: 10.1002/hep.25567. Epub (2012)*

Pokidysheva E., Boudko S., Vranka J., Zientek K., Maddox K., Moser M., Fässler R., Ware J., Bächinger H. P., *Biological role of prolyl 3-hydroxylation in type IV collagen. Proc Natl Acad Sci U S A. 2014 Jan 7;111(1):161-6. doi: 10.1073/pnas.1307597111. Epub (2013)*

Ponticos M., Silverthorn, *Human Physiology. (2010)*

Pornprasertsuk S., Duarte W. R., Mochida Y., Yamauchi M., *Overexpression of Lysyl Hydroxylase-2b Leads to Defective Collagen Fibrillogenesis and Matrix Mineralization DOI: 10.1359/JBMR.04102 (2004)*

Risteli M., Niemitalo, O., Lankinen, H., Juffer, A.H., Myllylä, R. *Characterization of collagenous peptides bound to lysyl hydroxylase isoforms. J. Biol. Chem. (2004)*

Saint-Martin C., Leroy G., Delhommeau F., Panelatti G., Dupont S., James C., Plo I., Bordessoule D., Chomienne C., Delannoy A., Devidas A., Gardembas-Pain M., Isnard F., Plumelle Y., Bernard O., Vainchenker W., Najman A., Bellanné-Chantelot C., and the French Group of Familial Myeloproliferative Disorders, *Analysis of the Ten-Eleven Translocation 2 (TET2) gene in familial myeloproliferative neoplasms Blood. 2009 Aug 20;114(8):1628-32. doi: 10.1182/blood-2009-01-197525. Epub (2009)*

Uniprot database, consulted May and June 2015

Tampe B., Tampe D., Zeisberg E. M., Müller G. A., Bechtel-Walz W., Koziolek M., Kalluri R., Zeisberga M., *Induction of Tet3-dependent Epigenetic Remodeling by Low-dose Hydralazine Attenuates Progression of Chronic Kidney Disease EBioMedicine. 2015 Jan;2(1):19-36.*

Van der Slot A. J., Zuurmond A. M., Bardoel A. F., Wijmenga C., Pruijs H. E., Sillence D. O., Brinckmann J., Abraham D. J., Black C. M., Verzijl N., De Groot J., Hanemaaijer R., Te Koppele J. M., Huizinga T. W., Bank R. A., *Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. J Biol Chem. 2003 Oct 17;278(42):40967-72. Epub (2003)*

Vuorela A., Myllyharju J., Nissi R., Pihlajaniemi T., Kivirikko K. I., *Assembly of human prolyl 4-hydroxylase and type III collagen in the yeast Pichia pastoris: formation of a stable enzyme tetramer requires coexpression with collagen and assembly of a stable collagen requires coexpression with prolyl 4-hydroxylase EMBO J. 16(22):6702-12. (1997)*

Wang T., Chen K., Zeng X., Yang J., Wu Y., Shi X., Qin B., Zeng L., Esteban M. A., Pan G., Pei D., *The Histone Demethylases Jhdm1a/1b Enhance Somatic Cell Reprogramming in a Vitamin-C-Dependent Manner Cell Stem Cell. 2011 Dec 2;9(6):575-87. doi: 10.1016/j.stem.2011.10.005. Epub (2011)*

Wheeler G., Ishikawa T., Pornsaksit V., Smirnoff N., *Evolution of alternative biosynthetic pathways for vitamin C following plastid acquisition in photosynthetic eukaryotes Elife. Mar 13;4. doi: 10.7554/eLife.06369. (2015)*

Yeowell H. N., Walker L. C., *Tissue specificity of a new splice form of the human lysyl hydroxylase 2 gene Matrix Biol ;18(2):179-87. (1999)*

Zhang Q., Xu M., Qu Y., Hu J., Li Z., Zhang Q., Lu L., *TET3 mediates the activation of human hepatic stellate cells via modulating the expression of long non-coding RNA HIF1A-AS1 Int J Clin Exp Pathol. 2014 Oct 15;7(11):7744-51. eCollection (2014)*