Investigating FKBP65 and Its Role in Type-I Collagen Synthesis

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Abstract

FKBP65 is a 65-kDa protein with four peptidyl-prolyl *cis-trans* isomerization domains followed by two Ca^{2+}-binding domains and ending in a C-terminal HEEL sequence, a putative ER-retention sequence. It is encoded by the gene *Fkbp10*. Our lab first became interested in FKBP65 when it was found to be associated with tropoelastin in the secretory pathway of fetal bovine chondrocytes. Further investigation showed that *Fkbp10* is expressed in a large number of developing tissues but did not correlate with tropoelastin gene (*Eln*) expression, suggesting additional ligands. Further experiments determined that FKBP65 associates with type-I collagen in the cell and that it is very highly expressed in developing bone. Recently, human mutations in *FKBP10* have been linked to Osteogenesis Imperfecta (OI). This finding further implicates FKBP65 in the synthesis and/or assembly of collagen, especially in bone. To investigate the localization of FKBP65 in developing bone, immunohistochemistry was conducted. FKBP65 was found inside the osteoblasts, but also in the osteoid outside the cell. Western blotting of MC3T3 cells, an osteoblast cell line, showed that FKBP65 was not only present in the lysate, but also in the media, indicating that the protein was indeed being secreted. Using α,α'-dipyridyl to disrupt collagen processing and folding, the presence of FKBP65 in the media increased, suggesting that FKBP65 distribution in the lysate, media and matrix was dependent on the folding state of collagen. To determine if FKBP65 could be localized to post-ER compartments, MC3T3 cells were stained by immunofluorescence for FKBP65, HSP47 and type-I collagen in the absence or presence of secretion disrupting agents. α, α-

dipyridyl was also used to cause mis-folding of the collagen. Results showed that FKBP65 did not take on the same pattern as the secreted type-I collagen. Finally, the type-I collagen matrix of primary cells from both OI patients and an FKBP65 knockout mouse were visualized to determine the effect of the absence of FKBP65 on type-I collagen assembly. The extracellular collagen matrix was not largely disrupted by the absence of FKBP65. Overall, these results demonstrate that FKBP65 is a secreted chaperone in type-I collagen synthesis.

Résumé

FKBP65 est une protéine de 65kDa formée de quatre domaines peptidyl prolyl *cis-trans* isomérases, de deux domaines interagissant avec du calcium et d'une séquence présumée de rétention du réticulum endoplasmique HEEL dans le C-terminus. Cette protéine est encodée par le gène *Fkbp10*. Notre laboratoire a précédemment observé l'association de FKBP65 avec tropoélastine dans la voie de sécrétion des chondrocytes bovins fœtaux. D'autres études ont démontré l'expression de *FKBP10* dans de nombreux tissus en développement. Puisque l'expression tissulaire de *FKBP10* et de tropoélastine ne correspondent pas, d'autres ligands doivent interagir avec FKBP65. Comme FKBP65 interagit intracellulairement avec collagène de type I, qu'il est fortement exprimé dans les os en formation et que des mutations du gène *FKPB10* ont été récemment associées avec la maladie ostéogenèse imparfaite, nous émettons l'hypothèse que FKBP65 joue un rôle dans la synthèse et/ou l'assemblée du collagène, en particulier dans le tissus osseux. Pour confirmer notre hypothèse, nous examinons par immunohistochimie la localisation de FKBP65 dans le tissue osseux. FKBP65 est présent dans les ostéoblastes, mais aussi à l'extérieure des cellules dans l'ostéoide. L'immuno-buvardage de type Western des cellules de lignée ostéoblastique MC3T3 a démontré la présence de FKBP65 non seulement dans le lysat mais aussi dans le milieu, indiquant que la protéine est sécrétée. La perturbation du traitement et du pliage de collagène I par α, α'-dipyridyl cause une augmentation du niveau de FKBP65 dans le milieu suggérant que la distribution de FKBP65 dans le lysat, le milieu et la matrice dépend de l'état du

pliage de collagène I. Pour déterminer si FKBP65 est localisé dans les compartiments post-réticulum-endoplasmiques, une immunofluorescence en présence ou absence d'agents perturbant le pliage de collagène I (dont α, α'-dipyridyl) a été réalisée pour les protéines FKBP65, HSP47 et collagène I. Nos résultats démontrent que FKBP65 a un comportement diffèrent de collagène I. Finalement, des fibroblastes primaires provenant de patients souffrant d'ostéogenèse imparfaite et de souris knock-out pour FKBP65 ont été testés par immunofluorescence pour déterminer quels effets à l'absence de FKBP65 sur la matrice extracellulaire collagénique. La formation de la matrice n'est pas affectée par l'absence de FKBP65. En conclusion, ces résultats démontrent que FKBP65 est un chaperon sécrété lors de la synthèse du collagène de type I.

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List of Abbreviations

AA	Ascorbic Acid
BCA	Bicinchoninic acid
BiP	luminal binding protein
BMP	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
CRTAP	Cartilage-associated protein
CyPB	Cyclophilin B
DAPI	4', 6-diamino-2-phenylindole, dihydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DOC	Deoxycholate
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FKBP65	FK506-binding protein 65
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HSP47	Heat-shock protein 47
IF	Immunofluorescence
IgG	Immunoglobulin G
IP	Immunoprecipitation

LEPRE1	leucine- and proline-enriched proteoglycan 1
NEM	N-Ethylmaleimide
OI	Osteogenesis Imperfecta
Osx	Osterix
P/S/G	Penicillin/streptomycin/L-glutamine
P3H1	Prolyl 3-hydroxylase 1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethanesulfonylfluoride
PPIase	Peptidyl-prolyl *cis-trans* isomerase
PPIB	Peptidyl-Prolyl Isomerase B
SDS	Sodium Dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris Buffered Saline
α-MEM	Alpha Minimum Essential Medium
β-GP	β-glycerophosphate
Σ–ACA	Σ–Aminocaproic Acid

Chapter 1: Literature Review

Bone

Bone has many functions in the human body. It anchors, supports and protects the tissues and acts as a mineral reservoir. It is crucial for a normal functioning life. Bone is produced by differentiated cells called osteoblasts that come from different cell lineages depending on bone function and location (Arthur, 1927). Bone is primarily made up of two components: a mineralized component and a non-mineralized component. The mineralized part of bone is made up of hydroxyapatite crystals, whereas the non-mineral component is predominantly type-I collagen, a fibrillar collagen. Primary bone is produced as woven bone that has no overall organization (Dudley & Spiro, 1961). Primary bone can be formed in two ways: *De novo* bone formation in non-mineralized tissues as intramembranous bone formation (Altman & Coe, 2002), or through the deposition of calcified bone onto a cartilage scaffold as endochondral bone formation (Bernard, 1969). Once primary bone is formed, it is subject to normal bone turnover, or secondary bone growth. Woven bone is broken down and an organized system of bone Haversian canals is formed in its place (Crawford, 1940).

Introduction to Collagen

Collagens are one of the most abundant proteins in the human body (Di Lullo et al., 2002). These proteins are characterized by their triple helical quaternary structure and also by their repeating Gly-X-Y triple amino acid sequence (where X is usually proline and Y is usually 4-hydroxyproline) (Altman

& Coe, 2002). This allows the proteins to associate with one another to form rod-like fibers that make up several main structures in the body. Type-I collagen is the most abundant collagen in the human body. It is found in tendons, dermis, arterial walls and scar tissue, and is the main component in the organic portions of bone and teeth (Di Lullo et al., 2002). Bone is made up of mineralized type-I collagen. Type-I collagen is a heterotrimer with two α1(I) chains and one α2(I) chain arranged into a triple helix (Altman & Coe, 2002; Prockop & Kivirikko, 1995). Type-I collagen is assembled as procollagen inside the cell. The three collagen chains associate in the endoplasmic reticulum (ER) and then are secreted into the extracellular matrix (ECM), where the globular domains at each end of the protein are cleaved off. The resulting mature collagen then associates together to form fibers (as reviewed in Mecham, 2001). Several proteins are involved in ensuring that this assembly process proceeds correctly. These proteins are known as collagen chaperones.

In bone, the non-mineralized, newly assembled collagen matrix is called the osteoid (Fell & Robison, 1934). Once the osteoid is formed, the osteoblast releases vesicles containing enzymes which catalyze the mineralization process (Altman & Coe, 2002).

Type-I Collagen in Osteoblasts

Osteoblasts come from multiple, different cell lineages depending on the position and purpose of the bone. There are multiple signal pathways that both inhibit (epidermal growth factor receptor and its ligands (Zhu et al., 2011)) and stimulate (Wnt/β-catenin pathway, see review Westendorf et al,. 2004) osteoblast

differentiation. Intracellularly, Bone Morphogenetic Proteins (BMPs), which are part of the TGF-β superfamily, have strong osteogeneic effects. They are a group of conserved signaling proteins that have the capacity to induce endochondral bone formation. They play a role in both osteoblast differentiation and osteoblast functioning. The different types of BMPs vary in effect and location (as reviewed in Canalis et al., 2003).

All of these different pathways of inducing osteoblast differentiation converge on roughly one path. A key stage of osteoblast and chondrocyte (cells that produce cartilage) formation is the upregulation of the transcription factor Runx2 (Maruyama et al., 2007). Once Runx2 (coded by the gene *RUNX2)* gets upregulated, the transcription factor goes on to upregulate several bone-specific genes including the gene *SP7,* which encodes the osteoblast specific transcription factor Osterix (Osx) (Nakashima et al., 2002). Osx is also crucial for bone development can be upregulated through an entirely separate BMP pathway (Lee et al,. 2003).

RUNX2 and Osx in Osteoblast Differentiation

Runx2 is one of the initial osteogenic markers that get expressed in osteoblast differentiating cells, and upregulates several genes, including *SP7*, *BGLAP* (which encodes Osteocalcin), *COL1A1* and *COL1A2* (Maruyama et al., 2007). Interestingly, over-expression of *RUNX2* functions to downregulate cell proliferation and the expression of *COL1A1* in progenitor cells, but not in mature osteoblasts. This suggests that Runx2 is required in the production of osteoblasts

and immature bone, but in the production of mature bone by fully differentiated osteoblasts (Maruyama et al., 2007). If *Runx2* is disrupted in mice, the resulting animal has no bone or cartilage, highlighting the significance of Runx2 in both endochondral and intramembranous bone formation, in addition to chondroblast differentiation (Komori et al., 2009).

Osx is a transcription factor that can be thought of as downstream of Runx2 and is active in the later stages of osteogenic differentiation. This transcription factor upregulates *BGLAP* and *COL1A1* in differentiating osteoblasts (Nakashima et al., 2002). When *SP7* is knocked out in mice, the animals die at the embryonic stage with no endochondral or intramembranous bone formation. Interestingly, *RUNX2*-expressing preosteoblasts can be found in the animal, as well as fully formed cartilage (Nakashima et al., 2002).

The process of osteoblast differentiation is summarized in figure 1.

Type-I Collagen Post-Translational Processing

The type-I collagen genes, *COL1A1* and *COL1A2*, are upregulated by most osteogenic differentiating transcription factors. Once upregulated, the genes are transcribed and then translated into their respective proteins, COL1A1 or proα1(I) and COL1A2 or proα2(I), and are collectively called procollagen. These newly formed protein chains are produced in the rough ER and have very similar structures to one another. Both the chains have long triple helix-forming domains and similar C-propeptides, but α1(I) has a N-terminal cysteine rich domain,

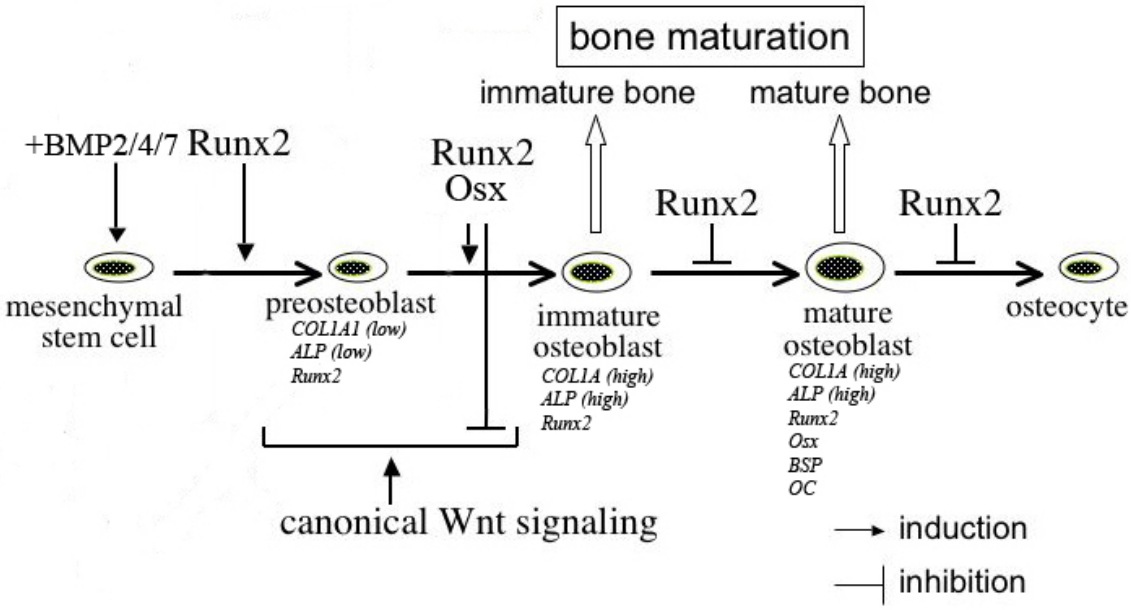


Figure 1 - Summary of osteoblast differentiation. Figure adapted from Zhang 2008 and 2010, Abzhanov et al. 2007 and Komori 2008. The italicized genes names show what genes are being expressing at that particular stage in the differentiating osteoblast. Note that Osx is upregulated by Runx2 and that only Osx inhibits the canonical Wnt signaling pathway to reduce cell proliferation leading to cell differentiation (Zhang, 2008).

whereas the α2(I) chain does not. The triple helix-forming domain has the characteristic collagen Gly-X-Y repeat. The glycine is very important and crucial in the overall structure of collagen because a larger amino acid would not sterically allow the formation of final the rod-like triple helix. Individually, these domains form left-handed helical secondary structures, and then together they form a right-handed triple helical quaternary structure when the three collagen polypeptide chains associate with one another (reviewed in Mecham, 2001). The chains associate with one another through their C-propeptide domains. It is a discontinuous 15 residue variable sequence in the C-propeptide of the collagen polypeptide chain that is responsible for determining chain selection and maintaining the proper chain stoichiometry (Lees et al, 1997).

Once the protein chains are produced, they interact with a variety of protein chaperones and enzymes that perform several post-translational modifications on the nascent protein chains. This process is summarized in figure 2. Briefly, the signal peptide on the polypeptide chains, which directs the production of the protein to the rough ER, is cleaved off. Protein chaperone complexes (prolyl 4-hydroxylase, lysyl hydroxylase and the prolyl 3-hydroxylation complex) hydroxylate certain proline and lysine residues into 4-hydroxyproline, 3-hydroxyproline and hydroxylysine. Some hydroxylysine residues get glycosylated into galactosylhydroxylysine, which predominates in bone, and certain asparagines are glycosylated in the C- and N-propeptide regions through ubiquitous enzymatic machinery that is present in the ER (as reviewed in Myllyharju & Kivirikko, 2004). Once three C-propeptides associate with one another through their recognition sequence, and the quaternary structure of procollagen begins to form, intramolecular and intermolecular cysteine disulfide bonds form. While inside the cell, procollagen is bound to different protein chaperones that prevent early association before all the post-translational modifications occur. These chaperones are HSP47 (Nakai et al, 1992) and possibly, FKBP65 (Ishikawa et al, 2008).

The triple helical quaternary structure of procollagen forms once the three C-propeptides have associated and about 100 proline residues have been hydroxylated into 4-hydroxyproline in each of the chains. The triple helix rod-like structure then forms like a zipper moving towards the N-terminal end of the protein (Engel & Prockop, 1991).

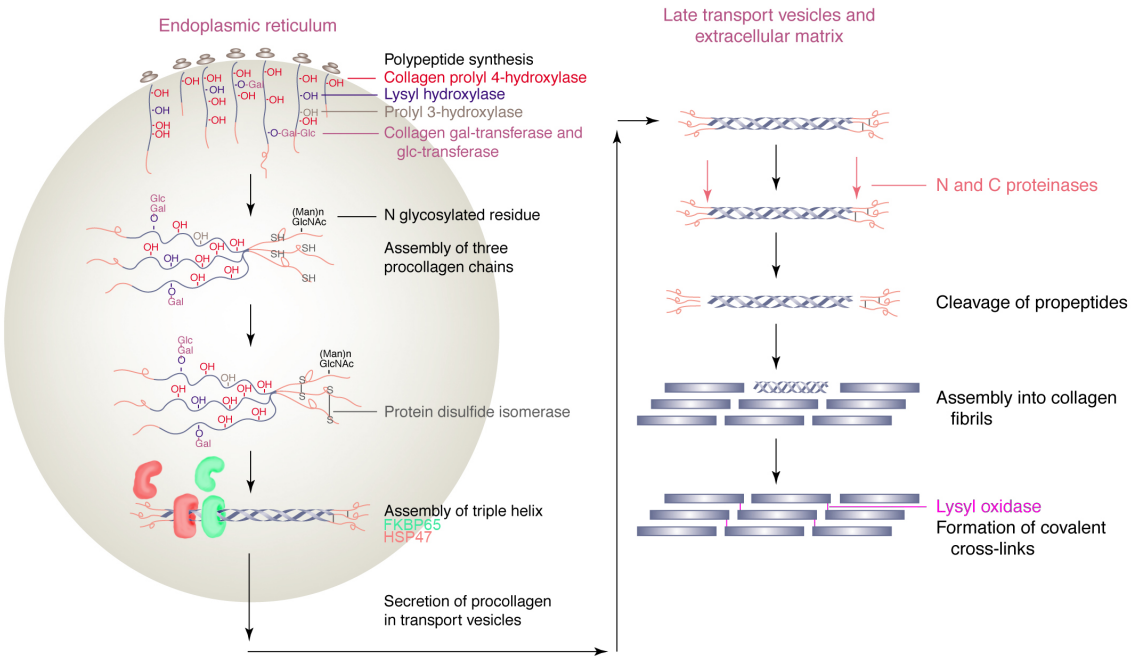


Figure 2 - Summary of type-I collagen synthesis, assembly and secretion. Several associated collagen chaperones and collagen associated proteins are shown. Figure adapted from Myllyharju et al. 2004. The hypothesized function of FKBP65 (green) and HSP47 (red) is shown.

Procollagen moves through the secretory pathway of the osteoblast as it is being processed and it is then secreted through exocytosis into the extracellular environment. Once in the extracellular environment, the procollagen in cleaved twice, once to remove the trimeric globular C-propeptide and then again to remove the trimeric N-propeptide domain (Miyahara et al, 1982). This leaves the rod-like triple helix of collagen flanked on both sides by N- and C-terminal telopeptides, which do not have the Gly-X-Y repeat that is characteristic of the triple helix forming domain of the collagen chains (Kadler et al, 1996). After cleavage, the ~300-nm long triple helical domain begins to self-assemble in a quarter-staggered manner into collagen fibrils. Once self-assembled into fibrils, the individual collagen chains are cross-linked through lysines and hydroxylysines in their telopeptides to lysines and hydroxylysines in the helical

region of neighbouring collagen fibers. This crosslinking is catalyzed by the enzyme lysyl oxidase (Kagan & Trackman, 1991).

Characteristics of Bone Type-I Collagen

In bone, the type-I collagen matrix that makes up the scaffold on which mineralization will occur is slightly different from the type-I collagen that inhabits soft tissues. Even though the genes for the α1(I) and α2(I) chains are the same in both, the post-translational modifications of bone procollagen are different. Type-I collagen in bone has a lower number of hydroxylysines, which affects the crosslinks that can be formed in the maturing matrix (Knott & Bailey, 1998). This is caused by a difference in the proportion of the different lysyl hydroxylase proteins; the family of enzymes that hydroxylate lysine residues in the procollagen chains (Uzawa et al., 1999). This disproportion causes only the lysines in the telopeptides region of the procollagen chains to be hydroxylated. Therefore, the telopeptides of bone type-I collagen have a higher number of hydroxylysine residues, whereas the helical region has a lower number of hydroxylysine residues when compared to soft tissue type-I collagen (Knott & Bailey, 1998). Consequently, once multiple collagen proteins have associated together into fibrils, lysyl oxidase creates lysyl-pyridinoline connections between the collagen proteins in bone, rather than hydroxylysyl-pryridinoline connections which are found in soft tissue collagen (Knott & Bailey, 1998). This happens because type-I collagen in bone is overhydroxylated in the telopeptide region and underhydroxylated in the helical domain. Also interesting is the overall lower

number of pryridinoline connections in bone collagen as compared to soft tissue. To compensate for the lowered amount of crosslinking connections, bone collagen has a high number of pyrrole-type cross-links (Knott & Bailey, 1998). These differences allowed the establishment of clinical assays which determine the rate of degradation of bone collagen as opposed to soft tissue collagen (as summarized in Altman & Coe, 2002).

Mineralization of the Type-I Collagen Matrix

The collagen matrix must be mineralized in order to become bone. Osteoblasts release an enzyme called alkaline phosphatase into the immediate surrounding area which cleaves phosphate groups to increase the local concentration of inorganic phosphate. It also decreases inhibitors of crystal growth such as pyrophosphate. This causes hydroxyapatite, the mineral constituent of bone, to begin to precipitate out of solution and crystalize using the pores found in the collagen fibers between parallel and sequential collagen molecules as nucleation sites (as reviewed in Murshed & McKee, 2010).

Type-I Collagen Chaperones

Prolyl 3-hydroxylation complex

One of the many post-translational modifications that procollagen undertakes is modification of proline into hydroxyproline, occurring at the Y amino acid position of the Gly-X-Y triple repeat. This is a crucial modification as it allows the three polypeptide chains to form a stable triple helix structure (Krane, 2008). Prolyl 4-hydroxylase and lysyl hydroxylase are the (ER) enzymes responsible for the

hydroxylation of most of the prolines and lysines residues in the triple helical domain of procollagen. There is an entire protein complex, though, that is dedicated to modifying the α1(I)Pro986 residue only. It is called the collagen prolyl 3-hydroxylation complex which consists of three proteins: prolyl 3-hydroxylase 1 (P3H1, or leucine- and proline-enriched proteoglycan 1 [LEPRE1]), cartilage-associated protein (CRTAP) and cyclophilin B (CyPB) (Krane, 2008). The function of this one residue modification is still not known.

HSP47, a Different Kind of Type-I Collagen Chaperone

Heat-shock protein 47 (HSP47) is another collagen chaperone. It is an ER-resident protein with a RDEL ER-retention sequence, which functions as a simple binding chaperone for procollagen (Satoh et al., 1996). HSP47 binds to procollagen in the ER and travels with the molecule until the *cis*-Golgi, where HSP47 disassociates from procollagen and returns to the ER (Nakai et al., 1992). The determining factor in the HSP47-procollagen association seems to be the pH of the environment, since HSP47 undergoes major conformational changes at a slightly lower pH. This conformational change causes it to disassociate from procollagen (Thomson & Ananthanarayanan, 2000). HSP47 is thought to keep procollagen from aggregating too early in the secretory pathway (Satoh et al., 1996).

FKBP65, A New Type-I Collagen Chaperone

FKBP65 stands for FK506-binding protein that is 65 kDa in molecular weight, and belongs to the family of immunophilins. The protein, coded for by *FKBP10*, contains a hydrophobic signal peptide at the N-terminus, four peptidyl-prolyl *cis-trans* isomerization (PPIase) domains, two predicted Ca^{2+}-binding motifs, and a C-terminal HEEL sequence (Figure 1). The HEEL sequence is thought to be a modified KDEL, an ER-retention sequence that would retain FKBP65 in the ER (Coss et al., 1995).

Originally, FKBP65 was discovered in the attempt to identify proteins that interacted with tropoelastin inside chondrocytes (Davis et al., 1998). To further investigate this interaction, the expression patterns of *Fkbp10* and the *Eln* were compared in multiple tissues, in both adult and young mice (Patterson et al., 2005). The results showed a mismatch between the expression patterns of the two genes, suggesting that additional ligands for FKBP65 may exist. Histological analysis of developing blood vessels and lung airways showed that FKBP65 colocalized with elastin staining in the surrounding ECM and that FKBP65 was strongly expressed in the smooth muscle cells that produce the elastin (Patterson et al., 2005). In adult tissues, *Eln* is not expressed, and interestingly, *Fkbp10* was also absent except for one tissue, the ovary (Davis, unpublished data). This suggests a potential role of FKBP65 in tissue remodeling, since the ovary is one of the few tissues in the adult that continuously remodeling. Consistent with this notion is that *Fkbp10* expression correlates with ovulation and post-ovulation remodeling (Davis, unpublished data). Similar results were seen in the uterus during gestation and the mammary gland during gestation and involution, when

Fkbp10 expression correlated with that of *Col1a1* during these periods of growth and/or remodeling.

At this point, *Fkbp10* expression had been investigated in normal adult tissues undergoing natural remodeling, therefore, a lung fibrosis model was used to test *Fkbp10* expression during the repair of injured tissue. When mice lungs are exposed to bleomycin, they develop lung fibrosis; an uncontrolled growth of connective tissue. Thus, *Fkbp10* expression was determined in normal mouse lungs and compared to bleomycin-treated lungs. The bleomycin-treated mice developed fibrosis in their lungs and FKBP65 was expressed in the resulting lesion, along with *Eln* and *Col1a1* (Patterson et al., 2005). Interestingly, the pattern of *Fkbp10* upregulation resembled that of *Col1a1* rather than *Eln*.

In the initial, multi-tissue Northern analysis conducted to determine the expression of *Fkbp10* in relation to *Eln*, bone was not included among the tissues examined. *In situ* hybridization for *Fkbp10* were therefore performed on 14.5-day embryonic mice. Surprisingly, there was extremely strong expression for *Fkbp10* in the developing bones (Davis, unpublished data). *Fkbp10* expression was seen in osteoblasts with weaker expression in the proliferating chondrocytes (which express type-II collagen). There was no *Fkbp10* expression observed in the hypertrophic chondrocytes. These *in situ* hybridization results indicated that *Fkbp10* expression was found in areas with both intramembranous and endochondral bone formation, suggesting that FKBP65 could play a major role in bone formation.

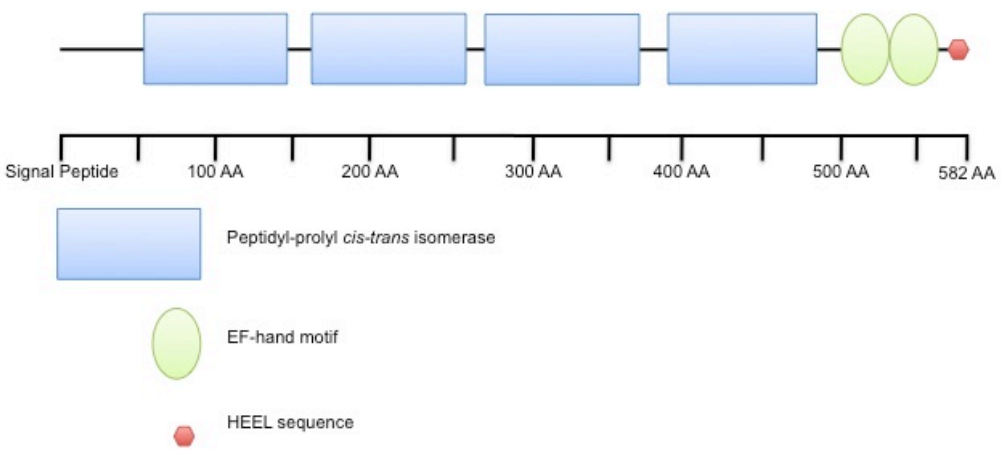


Figure 3 – Schematic of FKBP65 protein structure. Blue rectangles represent peptidyl-prolyl *cis-trans* isomerase domains. The EF-hand motif's that bind calcium are represented by the green circles. The red hexagon indicates the C-terminal HEEL sequence, a putative ER-limiting sequence.

In order to study FKBP65 in bone, sections of adult and developing bone were immunostained for FKBP65. There was no FKBP65 seen in the bone of adult mice, but there was strong staining seen in the developing bone. To mirror the lung-bleomycin experiment, adult bone was broken and allowed to heal. Examination of the presence and distribution of FKBP65 using immunohistochemistry showed that FKBP65 was present in the new bone that was forming on both sides of the break. This showed that FKBP65 was present in developing bone, but not during the normal turnover of bone (Davis, unpublished data)

Osteogenesis Imperfecta

Osteogenesis Imperfecta (OI) is a heritable disorder where the connective tissue, usually associated with type-I collagen, is malformed. The primary symptom of OI

is bone fragility. There are several different kinds of OI and they are currently divided into eleven types, with three unclassified OI-like or collagen-related diseases (Sillence et al., 1979). These types can be grouped into two categories: autosomal dominant and autosomal recessive. Autosomal dominant OI includes Type I through Type V, and Caffey's disease. Common among these types (with the exception of Type V OI) is that they are caused by mutations in the type-I collagen genes *COL1A1* and/or *COL1A2* (as reviewed in Forlino & Marini, 2011). There are varying phenotypes and varying degrees of severity of the phenotype in the patients.

Types VI through XI are autosomal recessive, as are Bruck Syndrome type 2 and disorders caused by osteoblast maturation defects. All of these types are caused by mutations in the proteins associated with collagen production or osteoblast function. Among these types of OI there are three overall categories: Type VI is a mineralization defect caused by an unknown gene mutation; Types VII through IX are 3-hydroxylation defects and are caused by mutations in *CRTAP* (Morello et al., 2006), *LEPRE1* (Cabral et al., 2007), and *PPIB* (van Dijk et al., 2009)*;* Type X and XI are chaperone defects caused by mutations in *SERPINH1* (Christiansen et al., 2010) and *FKBP10* (Alanay et al., 2010). The unclassified OI-like disorders or collagen-related disorders (Bruck Syndrome type 2, Caffey disease, and an unnamed disorder which is caused by osteoblast maturations defects) are caused by mutations in *PLOD2* (lysyl hydroxylase), *COL1A1,* and *SP7* (reviewed in Forlino et al., 2011.)

OI and the Prolyl 3-hydroxylation complex

Mutations in any one of the genes encoding the three proteins (*LEPRE1, CRTAP,* and peptidyl-prolyl isomerase B gene [*PPIB])* of the prolyl 3-hydroxylation complex cause the autosomal recessive form of OI (Cabral et al., 2007; Morello et al., 2006; van Dijk et al., 2009). These particular types of OI (type VII through IX) are characterized by over-processed type-I collagen, which is slow to secrete. Also type-I collagen producing cells have a swollen ER from the back-up of type-I collagen. Knocking out any of these genes in mouse models results in viable mice that mirror the OI phenotype of humans (Choi et al., 2009; Morello et al., 2006; Vranka et al., 2010).

Mutations in SERPINH1 lead to OI

Mutations in the gene encoding HSP47, *SERPINH1,* were first found in a canine form of OI (Drögemüller et al., 2009). In humans, only one patient suffering from OI has been found with mutations in *SERPINH1* (Christiansen et al., 2010). This OI is different from the others and has been designated as Type X. In Type X OI, the collagen is rapidly trafficked from ER to Golgi, suggesting that HSP47 is involved in the regulation of procollagen through the secretory system; however, the actual secretion rate of procollagen by the cells is slower. As expected, the prolyl residue that the prolyl 3-hydroxylation complex hydroxylates, proα1(I)Pro986, is hydroxylated properly in this patient. This shows that the function of HSP47 in procollagen processing is either “downstream” or independent of the prolyl 3-hydroxylation complex. The collagen molecules were

also not overprocessed as in prolyl 3-hydroxylation complex mutants, showing that HSP47 does not function in these post-translational modifications (Christiansen et al., 2010). The stability of the collagen in the *SERPINH1* mutant cells, however, is lower and the collagen shows an increased rate of denaturation (Christiansen et al., 2010). This phenotype suggests that HSP47 is important for the quality control of procollagen.

Interestingly, if *SERPINH1* is disrupted in mice, the resulting knockout is embryonic lethal. These mice have additional defects in basement membranes due to disrupted type IV collagen production (Ishida et al., 2006; Marutani et al., 2004; Matsuoka et al., 2004; Nagai et al., 2000). Since humans with *SERPINH1* mutations don't have this additional phenotype, HSP47 must have an additional role in type IV collagen synthesis in mice.

Mutations in FKBP10 lead to OI and Bruck Syndrome Type 1

The next logical step in investigating the role of FKBP65 in type-I collagen production would be to see the effects of the absence of FKBP65 on collagen synthesis. Development of a FKBP65 knockout mouse was underway when human patients with OI due to mutations in the *FKBP10* gene were discovered (Alanay et al., 2010). The *FKBP10* mutations leading to OI are summarized in figure 4. Preliminary results show that the *Fkbp10* knockout mouse is perinatally lethal (Davis, unpublished data). The knockout mice pups have a physical phenotype of downturned front limbs, which is similar to the Osx knockout mouse (Nakashima et al., 2002).

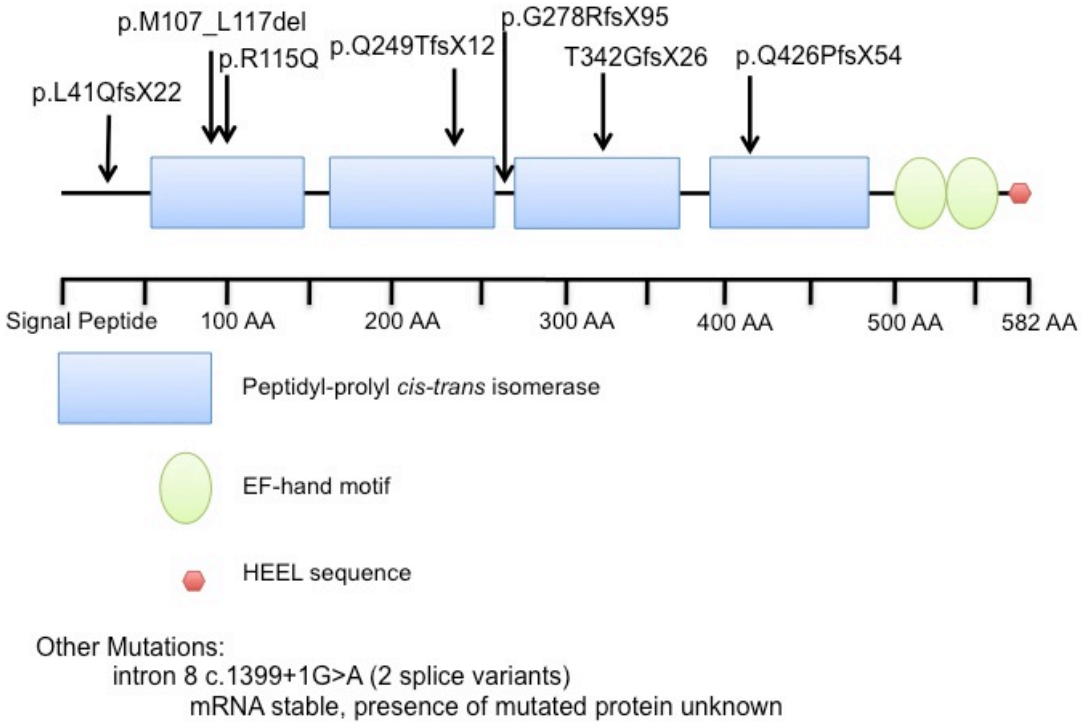


Figure 4 - Summary of FKBP65 mutations. The location of FKBP65 mutations is indicated via their protein mutation code and an arrow to the location of the mutation. The mutations p.M107_L117del (which leads to no FKBP65 protein being translated and autosomal recessive OI) and p.G278RfsX95 (which leads to no FKBP65 protein being translated and autosomal recessive OI) are discussed in Alanay et al., 2010. The mutation p.T342GfsX26 (which leads to a truncated protein being expressed and Bruck syndrome) is discussed in Shaheen et al., 2010. The mutations p.L41QfsX22, p.Q426PfsX54, and p.R115Q (all leading to Bruck syndrome) are discussed in Kelley et al., 2011. The mutation p.Q249TfsX12 (which leads to both OI and Bruck syndrome depending on the patient) is discussed in Shaheen et al., 2011. The splice mutation in intron 8 c.1339+1G>A (which leads to OI and two splice varients) is discussed in Venturi et al., 2011.

Humans with mutations in *FKBP10* have several common OI characteristics. These patients have reoccurring long bone fractures that start in infancy. The breaks lead to severe deformities in the limbs and result in wheelchair use from a young age. Their spines developed kyphoscoliosis, flattening and wedging at a young age. This leads to a tortuous spinal column. Contrary to other types of OI, these patients do not have dentinogenesis imperfecta (brittle teeth), their sclera are normal coloured (as opposed to blue in other OI types) and their hearing is normal (as opposed to impaired in other OI

types). Their fingers and toes are long and they have lax ligaments (as summarized in (Alanay et al., 2010; Kelley et al., 2011; Shaheen et al., 2011)).

Several mutations in *FKBP10* have also been found in patients suffering from Bruck Syndrome type 1 (Kelley et al., 2011; Shaheen et al., 2011; Shaheen et al., 2010a; 2010b). As mentioned above, Bruck Syndrome type 2 is caused by mutations in a lysyl hydroxylase, specifically PLOD2, which functions in the telopeptide region of procollagen and leads to differences in type-I collagen of bone versus soft tissue. Bruck Syndrome is similar to OI and is characterized by osteopenia (thinning of bone mass), congenital joint contractures (permanently stiffened joints that cannot fully extend) with webbing, scoliosis (warping of the spine) and osteoporosis (loss of bone mass due to demineralization) (as summarized in (Forlino et al., 2011)). Bruck Syndrome type 1 has now been attributed to mutations in *FKBP10*. Therefore, depending on the mutation, abnormal FKBP65 can lead to either Type XI OI or Bruck Syndrome type 1. The involvement of *FKBP10* mutations in Bruck Syndrome type 1 also suggests that FKBP65 has a function in tendons or joints since Bruck Syndrome is characterized and differentiated from OI by stiff and inflexible joints (Shaheen et al., 2011).

It is interesting to note that mutations can occur at almost any point in osteoblast differentiation or collagen synthesis that cause disorders which all fall under the umbrella term of OI with largely similar phenotypes.

Clinical Treatments of OI

Clinical treatments of OI are currently mostly palliative. Orthopedic surgery and physical therapy are options used to prevent and repair bone breaks. In those cases of OI with a dental phenotype or hearing loss, steps are taken to minimize pain and discomfort of the patients.

As summarized in the review by Forlino *et al*. 2011, the only current pharmacological therapy is antiresorptive compounds that are given to children to increase the amount of bone matrix. Even if defective, this would lead to a lower amount of bone fractures. The antiresorptive therapy prevents the breakdown and resorption of bone during normal metabolic turnover. There are several issues with this treatment, key among them is the decision regarding the length of the treatment. Some wish to treat children with the antiresorptives until the epiphyseal plate closes to avoid a potential weak spots for future fractures. Also, the antiresorptive consists of bisphosphonates that have a very long half-life in bones, so the absolute minimal cumulative dose is needed. This is difficult to prescribe, since the dose cannot be standardized across all patients and must be calculated and determined for each individual patient. Also, the dosage needs to be monitored and constantly changed as the patient grows older. These antiresorptives may be more effective in OI patients with mutations in *SERPINH1* and *FKBP10* since the type-I collagen in these patients is relatively normal compared to OI patients with mutations in the collagen genes themselves. The exact function of these proteins in relation to the bone production needs to be determined in order to maximize the quality of life of those OI patients with mutations in *FKBP10* and *SERPINH1*. Perhaps, if their function in type-I collagen

was more well-defined, new clinical treatments could be identified which would allow children suffering from OI or Bruck Syndrome type 1 to lead a more comfortable life.

Chapter 2: Experimental Rationale

With the results of the past research done in the lab and the new findings that FKBP65 is linked to OI, it is clear that there is some relationship between FKBP65 and type-I collagen. The nature of this relationship is not yet known. Previous work in our laboratory has shown that *FKBP10* is very highly expressed in developing bone, but the actual distribution of the FKBP65 protein is not yet known. We therefore wanted to investigate the distribution of FKBP65 in developing mice limbs using immunohistochemistry.

To further elucidate the interaction between FKBP65 and type-I collagen in bone, a good *in vitro* model of bone development was needed. For this, we used the MC3T3-E1 subclone 14 immortalized cell line derived from mouse calvaria. These cells are pre-osteoblasts that differentiate into osteoblasts and secrete, assemble and mineralize a type-I collagen matrix in the presence of ascorbic acid (AA), a type-I collagen synthesis co-factor, and β-glycerophosphate (β-GP), a source of inorganic phosphate. Since the MC3T3 cell line fully matures and develops a mineralized matrix over 12 days, we determined the production and distribution of FKBP65 over this time period to find out when FKBP65 may be particularly active. These cells also allowed us to observe the effects of manipulating type-I collagen synthesis and assembly on the amount and distribution of FKBP65 in the cell by using the drug α,α-dipyridyl, which prevents the proper quaternary structure of type-I collagen.

To determine where FKBP65 disassociates from procollagen inside the cell, an experimental protocol used by HSP47 researchers (Satoh et al., 1996)

and previously in our lab on tropoelastin (Davis & Mecham, 1998) was used. Using a variety of secretion disrupting drugs and immunofluorescence microscopy, we were able to determine in which compartments of the cell that FKBP65 and type-I collagen are associated.

Finally, fibroblasts harvested from both FKBP65 OI patients and from a FKBP65 knockout mouse model were stained to observe the effect of the absence of FKBP65 on the production of procollagen and the assembly of the type-I collagen ECM.

Together, these observations will aid in the understanding of a possible functional relationship between FKBP65 and type-I collagen and in the long-term, could potentially lead to treatment targets for individuals suffering from type XI OI.

Chapter 3: Materials and Methods

Cell Culture

Cell Lines

MC3T3-E1 Subclone 14 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured at 37° C and 5% CO_2 in Alpha Minimum Essential Medium (α-MEM) with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate but without ascorbic acid (Invitrogen, Montreal, QC) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin/L-glutamine (P/S/G) (Invitrogen). Media was renewed on cultured cells every 2 to 3 days. MC3T3 cells were used between passages 17 and 21.

Mouse primary fibroblasts (both wildtype and *Fkbp10* knockout) were obtained from the skin of embryonic mice pups. A pregnant mouse dissected and the individual pups removed from within the uterus. The pups were rinsed in phosphate buffered saline (PBS) (Invitrogen) and the skin from their backs was removed and placed into sterile PBS briefly. Afterwards, the skin was placed into 6-well plates and covered with Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% FBS and 1% P/S/G. Media was changed after a week of seeding and cells were passed and expanded three times before being frozen down for storage and use in experiments. *Fkbp10* knockout was confirmed through both Real Time Quantitative Polymerase Chain Reaction (PCR) and Western blotting to assure that no FKBP65 protein was present. Mice primary fibroblasts were used between passages 4 and 9.

FKBP10 mutated human fibroblasts were generously provided by Dr. Deborah Krakow (UCLA, LA, USA). These cells were obtained through skin punch biopsies of the patients. All patient cells were cultured in DMEM (Invitrogen) with 10% FBS and 1% P/S/G at 37° C and 5% CO_2. Patient 381 cells are fibroblasts with base pair insertion (831-832insC) frameshift mutation in the *FKBP10* gene that results in a stop codon downstream from the 3rd peptidyl-prolyl *cis-trans* isomerase domain of FKBP65. PCR confirmed that the resulting mRNA is degraded and no mutated protein is produced. Patient 014 cells are fibroblasts with a 33 base pair in-frame deletion in the *FKBP10* gene resulting in 11 amino acids missing from the full-length protein. PCR and Western blots have confirmed that the mutation is severe enough to cause the mRNA to be degraded and no mutant FKBP65 protein is produced. These cells were only used until passage 7 and then discarded.

Thawing/Passing/Freezing Cells

Cells were thawed in 37° C water bath and then resuspended in 10 ml of full media. In an Eppendorf centrifuge, the cells were spun down for 5 min at 1.1 rcf and the supernatant was removed. The cell pellet was again resuspended in full media and aliquoted into culture dishes. The media was replaced with new full media after 24 hours.

Once cells were confluent and ready for passage, media is removed and the cells were washed with warm, sterile PBS (Invitrogen) 3 times. Trypsin/EDTA (Multicell, 0.25% trypsin and 0.1% EDTA) was added to culture dishes and the cells were incubated at 37° C until the cells detached from the dish surface. The

cells were collected into 15 ml conical flasks (Sarstedt) and diluted with full media (1:5). Cells were spun down in Eppendorf centrifuge for 5 min at 1.1 rcf. The supernatant was removed and the total cell number was calculated with a hematocytometer (Fisher Scientific). The cells were then resuspended in full media and re-plated into new culture dishes (Sarstedt).

Once cells were ready to be frozen down, they were washed with sterile PBS and trypsinized as described above. Once the cells had been spun down and the supernatant removed, the cell pellet was resuspended in freezing media: 70% DMEM for fibroblasts/α-MEM for osteoblasts, 20% FCS, 1% dimethyl sulphoxide (DMSO) (Sigma). The suspended cells were aliquoted into cryogenic vials (Corning) and placed into a Cryo 1°C Freezing Container (Nalgene) at -80° overnight. After 24 hours, the frozen tubes were transferred into liquid nitrogen storage.

Western Blot

Sample preparation

Once samples from individual experiments were resuspended in Laemmli sample buffer, they were boiled for 5 minutes with 0.1 M DTT (Sigma). Samples were stored at -20°C until ready for running.

Samples were loaded and run on 6.25%-10% acrylamide gels via SDS-PAGE. After separation, proteins were transferred onto nitrocellulose membranes (BioRad, Mississauga, Ontario) in 20 mM Tris, 150 mM glycine and 20% methanol for 1 hr at 100V. Membranes were stained with Ponceau (Sigma) stain

to verify proper transfer. The stain was removed from the membranes with multiple washes with distilled water. The membranes were then blocked overnight in 5% dry non-fat milk in TBS with 0.2% Tween-20 at 4° C to prevent non-specific binding.

The next day, membranes were probed with primary antibodies (See table 1 for dilutions) for 1 hour at RT with gentle agitation. Membranes were then washed 3 times (15 minutes, 5 minutes, 5 minutes) in TBS with 0.2% Tween-20. After the washes, the membranes were incubated with secondary antibodies (See table 1 for dilutions) for 1 hour at RT with gentle agitation. Afterwards, the membranes were extensively washed (4 times 30 minutes) in TBS with 0.2% Tween-20 to reduce background.

Once the membranes were sufficiently washed, they were developed with Supersignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) for 5 minutes. Afterwards, the membranes were then ready for exposure to film and development of the Western blot.

Table 1 - Antibodies used in Western blotting.

Antibody	Source	Isotype	Dilution and Diluent
Primary			
FKBP65 (p74)	Davis Lab generated	Rabbit IgG	1:2000 in TBS + 0.2% Tween-20 + 5% milk
FKBP65	BD Transduction, *Cat. No. 610648*	Mouse IgG	1:5000 in TBS + 0.2% Tween-20
FKBP65 (FKBP10)	ProteinTech, Chicago, IL, USA *Cat. No. 12172-1-AP*	Rabbit IgG	1:3000 in TBS + 0.2% Tween-20 + 5% milk
Type-I collagen	CalBiochemistry EMD Biosciences San Diego, CA, USA *Cat No. 234167*	Rabbit IgG	1:10,000 in TBS + 0.2% Tween-20 + 5% milk
HSP57 (SerpinH1, Colligin)	ENZO, Plymouth Meeting, PA, USA *Cat No. ADI-SPA-470-D/F/J*	Mouse IgG	1:10,000 in TBS + 0.2% Tween-20
GAPDH	Cell Signalling, Pickering, ON *Cat. No. 2118*	Rabbit IgG	1:10,000 in TBS + 5% BSA
Secondary			
Peroxidase-conjugated anti-rabbit	Jackson Immune Research, West Grove, PA, USA	Goat IgG	1:5000 in TBS + 0.2% Tween-20
Peroxidase-conjugated anti-mouse	Jackson Immune Research, West Grove, PA, USA	Goat IgG	1:5000 in TBS + 0.2% Tween-20

Immunohistochemistry

Slides of one-day-old wildtype mice limbs, paraffin embedded, and cut longitudinally were provided by Kinsey Lam. The UltraVision LP Value Detection System AP Polymer + fast Red Chromatogen kit (Thermo Scientific) was used to probe the tissue. Slides were rehydrated and deparaffinized with 5 minute sequential baths in CitriSolve (FisherBrand) (3x), 1:1 CitriSolve:Ethanol, 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, and distilled water. Slides were submerged in a heated sodium citrate buffer solution (100 M Sodium Citrate, 0.05% Tween-20, pH 6) for 20 minutes in a 90°-100°C oven and the 20 minutes at room temperature for antigen retrieval. A buffer of TBS + 0.1% Triton X-100 was placed on the slides for 15 minutes and then rinsed in TBS for 2 minutes. The slides were then washed in TBS + 0.1% BSA 3 times for 5 minutes. The samples were blocked using the blocking buffer from an UltraVision LP Value Detection System AP Polymer + fast Red Chromatogen kit (Thermo Scientific) for 5 minutes. Afterwards, the slides were rinsed in TBS + 0.1% BSA 3 times for 5 minutes. The primary antibody, Davis lab generated FKBP65 polyclonal antibody, was placed carefully on one slide at a dilution of 1:200 in TBS + 0.1% BSA. The other slide was incubated without primary antibody as a control. Antibody and control were placed into a humidity chamber overnight at 4° C.

The next day, the excess antibody/buffer was removed by tapping against a KimWipe and the slides were rinsed in TBS + 0.1% BSA 3 times for 5 minutes. The samples were then incubated with the Value Primary Antibody Enhancer (from the kit) for 20 minutes and then rinsed again as before. Anti-Rabbit IgG

Value AP polymer was placed on the samples for 30 minutes and then washed in TBS +0.1% BSA as above. The antibodies were visualized by incubating the slides with Fast Red Tablet solution that contains napthol phosphate substrate for the secondary antibody for 75 seconds each. After the colour reaction had reached an acceptable level, the slides were plunged into distilled water to stop the reaction.

The tissue was counterstained by incubating the samples with hematoxylin for ~30 seconds and then rinsing off the hematoxylin with tap water. The slides are then dipped 10 times in an acid solution (2% glacial acetic acid), 20 times in tap water, placed into bluing solution (2.22% NH_4OH in ethanol) for 1 minute, and finished with a brief rinse in tap water. Slides were mounted using GelTol before being viewed using a Zeiss Axioskop 2 microscope with AxioVision Rel. 4.8 software.

Testing the effects of TGF-β and ascorbic acid on FKBP65 production

MC3T3-E1 Subclone 14 cells were seeded into 4 wells of a 6-well plate at a density of 50,000 cells/cm^2. For 4 days, the cells were either left untreated, or treated with 50 μg of ascorbic acid, or 5 ng/ml of TGF-β, or both ascorbic acid and TGF-β in full media. At the end of day 4, media was removed and the cells were gently washed three times with sterile, warm PBS (GIBCO). One ml of serum-free media was added to each well and the treatment was continued for the duration of day 5.

At the end of day 5, the media was collected and protease inhibitors (4 mM PMSF, 5 mM Σ–ACA, and 5 mM NEM) were added to the samples. The media was then spun at 13,000 rpm for 10 minutes at 4° C to remove cell debris and frozen overnight at -80° C.

The cells were washed gently 3 times with cold PBS and 1 ml of lysis buffer (25 mM Tris-Cl pH 7.5, 250 mM NaCl, 5 mM EDTA pH 7.5, 1% Triton-X-100, and protease inhibitors) was added directly to the cells. The cells were then scraped up into the lysis buffer with a rubber policeman and frozen overnight at -80° C.

Both sets of samples, total lysate and media, were thawed at 4° C turning end on end. Once the total lysate was thawed, it was allowed to spin for another 30 minutes to ensure that all the cells were lysed. The lysate samples were then spun at 13,000 rpm for 10 minutes at 4° C to remove any remaining cell debris. Once finished, the supernatant was removed to a new eppendorf tube and the pellet was discarded. The amount of total protein in the lysate samples was determined via BCA Protein Assay kit (Thermo Scientific). Using the concentrations of the different samples, the amount of protein in all the samples was equalized.

To prepare the media and lysate samples for Western blotting, the samples were acetone precipitated at a ratio of 1:1 cold acetone to sample for 1 hour at -20° C. After 1 hour, the precipitated protein was pelleted by spinning the samples at 10,000 rpm for 10 minutes for 4° C. The supernatant was discarded and the pellets were allowed to briefly air-dry to remove any excess acetone.

Once the pellets were somewhat dry, they were resuspended in 1X Laemmli buffer without DTT. The lysate samples were resuspended in 200 μl of buffer, while the media samples were resuspended in 90 μl. Once evenly resuspended, DTT was added to the samples and they were boiled for 5 minutes. The samples were then briefly vortexed and spun down after which they were stored at -20° C until ready to be run on an SDS-PAGE gel.

Collecting Lysate and Media from Longterm Cultures of MC3T3-E1 Subclone 14 Cells

MC3T3-E1 Subclone 14 cells were seeded in a 6-well plate at a density of 50,000 cells/cm^2. The cells were given one day to attach, this was designated Day 0. Afterwards, the cells were treated with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate in full media continually for 12 days. The media and treatment was refreshed every second day (Days 1, 3, 5, 7, 9 and 11). For days 2, 4, 6, 8, 10 and 12, the treatment was switched to 1 ml of serum-free media for 24 hours in one well only. At the end of those 24 hours, the serum-free well was collected. The serum-free media was collected and protease inhibitors (4 mM PMSF, 5 mM Σ–ACA, and 5 mM NEM) were added to it. It was then promptly spun at 13,000 rpm for 10 minutes at 4° C to remove any cellular contamination. The pellet was discarded and the media was frozen at -80° C until all samples were collected.

The lysate was washed gently 3 times with PBS and then lysis buffer (25 mM Tris-Cl pH 7.5, 250 mM NaCl, 5 mM EDTA pH 7.5, 1% Triton-X-100, and

protease inhibitors) was added and the cells were scrapped up into it with. This was promptly frozen at -80° C until all samples were collected.

Once all days have been collected, both sets of samples, total lysate and media, were thawed at 4° C turning end on end. Once the total lysate was thawed, it was allowed to spin for another 30 minutes to ensure that all the cells were lysed. The lysate samples were then spun at 13,000 rpm for 10 minutes at 4° C to remove any remaining cell debris and the pellet was discarded. The total protein concentration was equalized across all days after the protein concentration was measured via BCA assay.

To prepare the media and lysate samples for Western blotting, the samples were acetone precipitated at a ratio of 1:1 cold acetone to sample for 1 hour at -20° C. After 1 hour, the precipitated protein was pelleted by spinning the samples at 10,000 rpm for 10 minutes for 4° C. The supernatant was discarded and the pellets were allowed to briefly air-dry to remove any excess acetone.

Once the pellets were somewhat dry, they were resuspended in 1X Laemmli buffer without DTT. The lysate samples were resuspended in 200 μl of buffer, while the media samples were resuspended in 90 μl. Once evenly resuspended, DTT was added to the samples and they were boiled for 5 minutes. The samples were then briefly vortexed and spun down after which they were stored at -20° C until ready to be run on an SDS-PAGE gel.

Collecting lysate, media and matrix after α, α-dipyridyl exposure

MC3T3-E1 Subclone 14 cells were seeded in a 6-well plate at the density of 50,000 cells/cm^2 for 6 days in full media. Two wells were left untreated and 4 wells were treated with 5 ng/ml TGF-β, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate up until the 5^{th} day. At the start of the 6^{th} day, media was removed and the cells were gently washed three times with sterile, warm PBS (GIBCO). One ml of serum-free media was added to each well and the treatment was continued for the next 18 hours. Six hours before the end of day 6, 500 μM of α, α-dipyridyl was added to two treated wells.

At the end of day 6, the serum-free media was collected and protease inhibitors (4 mM PMSF, 5 mM Σ–ACA, and 5 mM NEM) were added. Cell debris was spun out of the media at 13,000 rpm for 10 minutes at 4° C and then the media was frozen at -80° C overnight. The cells were gently washed with cold PBS three times and 1 ml of lysis buffer (25 mM Tris-Cl pH 7.5, 250 mM NaCl, 5 mM EDTA pH 7.5, 1% Triton-X-100, and protease inhibitors) was added directly to the wells. The plate was then gently agitated for 30 minutes on ice to allow the cells to lyse. After 30 minutes, the lysate was carefully collected and frozen overnight at -80° C.

The leftover matrix was gently washed three times with cold PBS to remove any lysate contamination. Boiling hot 1X Laemmli buffer (without DTT) added directly to the well, and the matrix was scrapped up with a rubber policeman. DTT is then added, or not, and the matrix sample was boiled for 5 minutes and then stored at -20° C.

To prepare the lysate and media samples for Western blot, the samples were thawed at 4° C turning end over end. Upon thawing, the protein concentration of the lysate was measured via BCA assay and the amount of total protein among the lysate samples was equalized. Then both lysate and media samples were acetone precipitated with a 1:1 ratio of -20° C acetone to amount of sample. After 1 hour, the samples were spun down at 10,000 rpm for 10 minutes at 4°C to pellet the precipitated protein. Once completed, the supernatant was discarded and the pellets were allowed to briefly air-dry to remove any leftover acetone. Once dry, the pellets were resuspended in 1X Laemmli buffer without DTT added. Lysate samples were resuspended in 200 μl of buffer, while the media was resuspended in 90 μl of buffer. DTT is then added and the samples were boiled for 5 minutes and then stored at -20° C overnight. If samples were to be run as non-reduced and reduced, lysate and media samples were evenly separated into ¼ and ¾ aliquots before acetone precipitation. Acetone precipitation proceeded as normal (1:1 ratio was preserved) and the non-reduced samples were resuspended in proportional amounts of Laemmli buffer without a reducing agent, while the reduced samples were.

Verification of the effects of α, α-dipyridyl on FKBP65 secretion

In order to confirm that the effects seen from the above experiment were due to the addition of α, α-dipyridyl, the experiment was repeated as above. The protocol deviates when the α, α-dipyridyl is added to the cell culture. Instead of

adding the α, α-dipyridyl directly to the existing media and collecting it 6 hours later, the media is collected from all wells at the 18 hour mark and new treated serum-free media is placed back onto the cells, this time with α, α-dipyridyl added to 2 of the wells. The two separate sets of media samples are acetone precipitated and prepared for Western blot as described above.

Immunofluorescence

Antibodies and Reagents

Primary antibodies used for solely immunofluorescence are mouse α-GM130 (BD Transduction Laboratories) and mouse α-Golgin-84 (BD Transduction Laboratories). These were used as Golgi markers for mouse and human cells respectively. For extracellular staining, the whole rabbit serum LF-67 (provided by Dr. Larry W. Fisher at the National Institute of Health in Maryland, USA) was used to stain for the collagen fibers.

The secondary antibodies used were goat α-mouse Alexa Fluor 488 (Invitrogen), goat α-rabbit Alexa Fluor 488 (Invitrogen), and Cy3-conjugated goat α-mouse (Jackson ImmunoResearch). 4', 6 –diamino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen) was used for nuclear staining.

Drug Treatments

For the drug-treated experiments, the MC3T3 cells were treated with 50 μg/ml ascorbic acid for 24 hours. For the last 3 hours, the cells were treated with either 10 mM monensin in ethanol or 1 mM bafilomycin in DMSO before being fixed for

immunofluorescence. Otherwise, the MC3T3 cells were treated for 6 hours with 500 mM α, α-dipyridyl in DMSO before being fixed for immunofluorescence.

Protocol

Cells are seeded into 4-well Lab-Tek Chamber slides (Thermo Scientific) and grown until desired confluency. For extra-cellular staining, the cells are plated at high numbers to achieve instant confluency and then are incubated for 6 days to allow for extra-cellular matrix (ECM) growth. For intra-cellular staining, most cell types were grown to 70% confluency except MC3T3 Subclone 14 cells. These cells were grown to <90% confluency to facilitate osteoblast differentiation.

Once cells are at desired confluency, media is removed from the cells and they are rinsed once in PBS and then washed 3 times in more PBS for 5 minutes. All intra-cellular immunofluorescence (IF) experiments were washed and incubated with gentle agitation, while ECM immunofluorescence experiments were washed and incubated on the bench-top to limit the warping of the ECM.

Once PBS washing is finished the cells were fixed in fresh PBS + 2% paraformaldehyde for 30 minutes. The cells are then rinsed twice with PBS + 1% bovine serum albumin – Fraction V (BSA) (Fisher) + 0.1% saponin (Sigma) to remove all traces of PBS + paraformaldehyde. The cells were then permeabilized by two washes of PBS + BSA + saponin of 15 minutes each. ECM immunofluorescence experiments skipped this step, and were only washed 3 times in PBS + 1% BSA for 5 minutes to remove the fix and prevent permeabilization of the cell membranes.

The primary antibody was diluted in the PBS + BSA + saponin solution (or the PBS + BSA solution for extracellular immunofluorescence experiments) and left at room temperature (See table 2 for dilutions). For secondary controls, the cells were incubated without any primary antibody. After 1 hour, the primary antibody was removed and the cells were washed 3 times in PBS + BSA + saponin for 5 minutes each (again, the extracellular IF experiments were washed in PBS + BSA only). Secondary antibody was added to the cells (See table 2 for dilutions) and left for 1 hour at room temperature in a light-proof environment to prevent bleaching of the fluorophores.

The cells were washed again twice in the same wash buffer for 5 minutes in a light-proof environment and then DAPI stained for three minutes. The cells were given a final wash in PBS and mounted with VectaShield mounting media for Fluorescence (Vector Laboratories). Coverslips (Fisher) were sealed with clear nail polish (Revlon) and slides were stored at 4° C in darkness.

Slides were viewed on a Zeiss Axioskop 2 microscope with AxioVision Rel. 4.8 software and FILTERS

Table 2 - Antibody dilutions used for immunofluorescence.

Antibodies used for intracellular staining were diluted in PBS + BSA + Saponin. Antibodies used for extracellular staining were diluted in PBS + BSA only. Company names can be found in *Antibodies and Reagents* section above.

Antibody	Dilution
Primary - *Intracellular*	
Polyclonal α-FKBP65 (Davis' lab generated)	1:200
α-HSP47	1:200
α-Type-I collagen	1:500
α-GM130 (Golgi marker – Mouse)	1:600
α-Golgin (Golgi marker – Human)	1:200
Primary - *Extracellular*	
LF-67	1:500
Secondary	
AlexaFluor 488 goat α-mouse	1:200
AlexaFluor 488 goat α-rabbit	1:200 (1:400 for extracellular staining)
Cy3-conjugated AffiniPure F(ab')$_2$ frag goat α-mouse	1:200
DAPI (nuclear staining)	1:5000

Chapter 4: Results

FKBP65 antibody specificity

To determine which of the three available FKBP65 antibodies would be most suited for experimentation, all three antibodies were tested via Western blotting. As described above, six cell lines (MC3T3-E1 subclone 14 cells, wildtype mouse primary fibroblasts, FKBP65 knockout mouse primary fibroblasts, wildtype human primary fibroblasts, FKBP65-null Patient 381 human primary fibroblasts, and FKBP65-null Patient 014 human primary fibroblasts) were plated and treated with 50 μg/ml ascorbic acid for 24 hours in full media. After collecting the samples, they were Western blotted with the various FKBP65 antibodies and GAPDH as a loading control. A summary of the FKBP65 antibodies and the antigens used to generated them can be found in Figure 2.

The ProteinTech polyclonal α-FKBP65 used human FKBP65 as the antigen, therefore it is far more sensitive for human FKBP65 (Figure 3A, lane 5) than mouse FKBP65 (Figure 3A, lane 1 and 2) with only faint non-specific bands. In fact, the FKBP65 from the MC3T3-E1 subclone 14 cells (Figure 3A, lane 1) is only very faintly detected. This makes this particular antibody less than ideal for most *in vivo* experiments involving MC3T3 murine osteoblast cells.

The BD Transduction monoclonal α-FKBP65 antibody was developed using mouse FKBP65 as an antigen. As a monoclonal antibody, this antibody should be more specific than polyclonal antibodies as it would detect only one epitope. The Western blot of FKBP65 with the monoclonal α-FKBP65 shows otherwise. Though the monoclonal does seem to be more sensitive towards

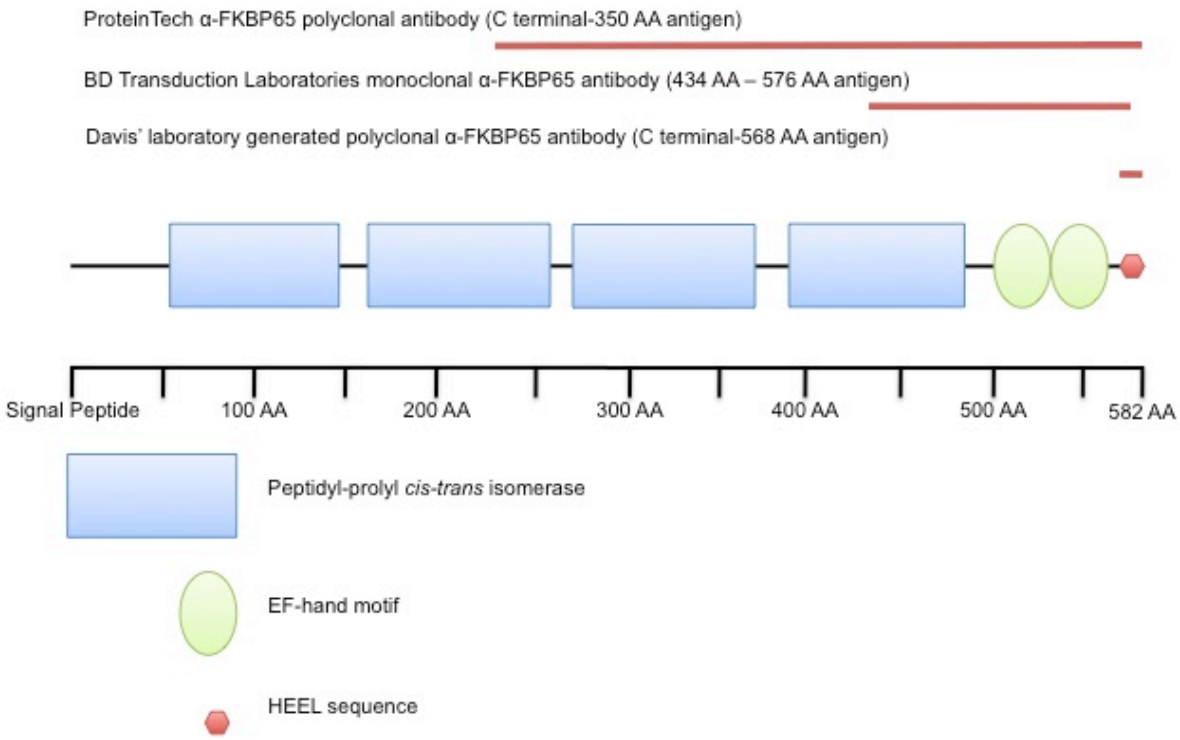


Figure 5 - Schematic of FKBP65 protein structure and Antibody Epitopes. Blue rectangles represent peptidyl-prolyl *cis-trans* isomerase domains. The EF-hand motif's that bind calcium are represented by the green circles. The red hexagon indicates the C-terminal HEEL sequence, a putative ER-limiting sequence. The red bars above the schematic represent the epitopes of the three available FKBP65 antibodies. The ProteinTech FKBP65 polyclonal antibody was generated using the last 350 amino acids of human FKBP65 as an antigen. The BD Transduction Laboratories monoclonal anti-FKBP65 antibody was generated using the 434th amino acid to the 576th amino acid of mouse FKBP65. The lab-generated polyclonal FKBP65 antibody was created using the synthetic polypeptide C-LKSDEDQERVHEEL (Davis 1998), which are the last 14 amino acids of the mouse protein.

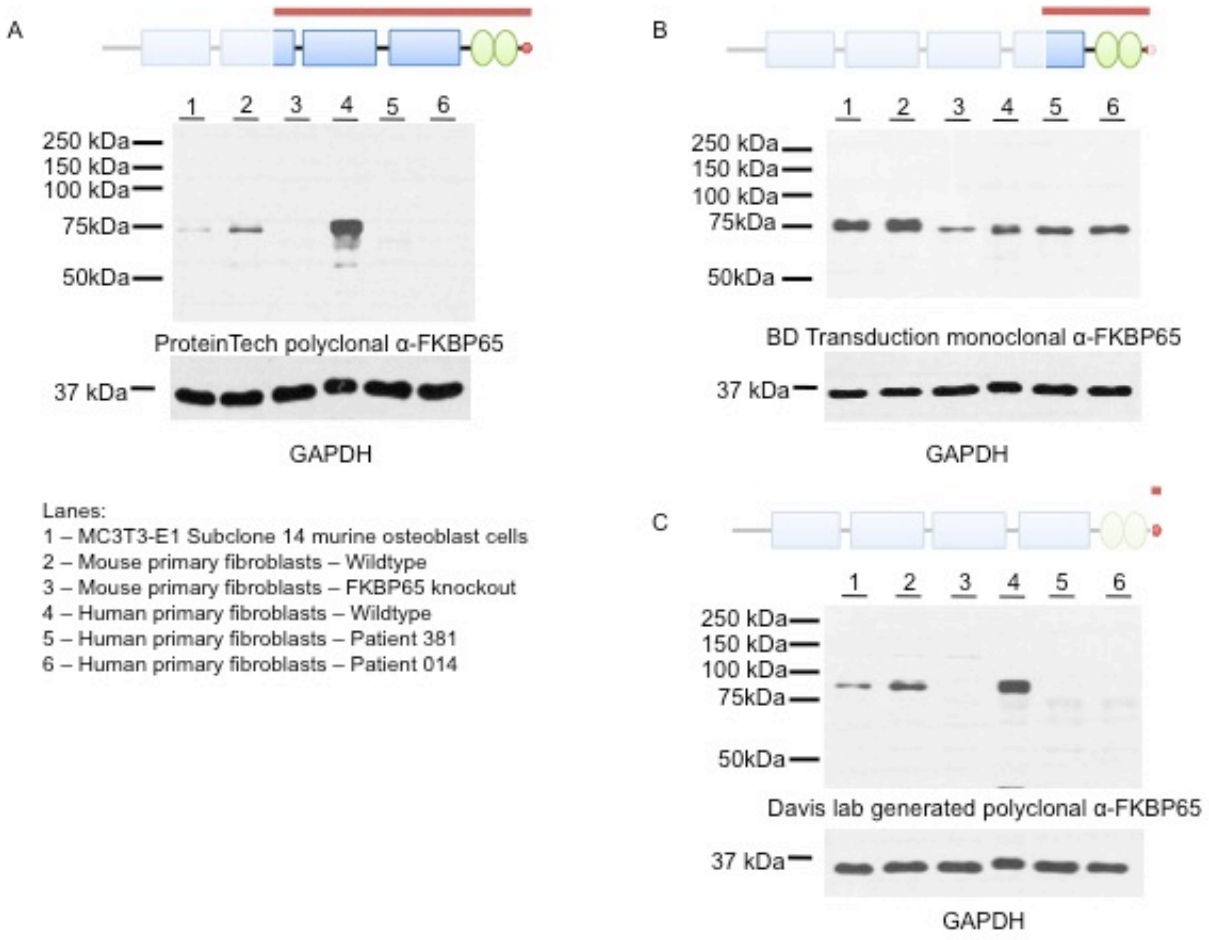


Figure 6 - Western Blot testing of FKBP65 antibodies for specificity. FKBP65 antibodies were tested via Western blot to determine the most specific antibody to be used in all subsequent experiments. All antibodies were used to probe 6 samples of lysate. Lane 1 is the lysate from MC3T3-E1 Subclone 14 mice osteoblast cells. Lane 2 is the lysate from wildtype mice primary fibroblasts, while Lane 3 mice primary fibroblasts from the FKBP65 knockout mouse. Lane 4 is the lysate from Human primary skin fibroblasts. Lane 5 and 6 contain the lysates from human primary skin fibroblasts of OI patients with mutations in the *FKBP10* gene that results in the mRNA being degraded and no protein being produced. All cell types were treated with 50 μg/ml ascorbic acid for 24 hours before collection. Equal amounts of total protein were loaded in each lane with GAPDH acting as a loading control. (A) The ProteinTech polyclonal anti-FKBP65 antibody Western blot. (B) The BD Transduction monoclonal anti-FKBP65 antibody Western blot. (C) The Davis lab generated polyclonal anti-FKBP65 antibody Western blot.

mouse FKBP65 than human (Figure 3B, lanes 1 and 2 vs. lane 4), the monoclonal has a strong non-specific band at the same molecular weight as FKBP65. This can be seen in the FKBP65-null cell lines (Figure 3B, lanes 3, 5 and 6). The monoclonal antibody's non-specificity towards FKBP65 renders it unusable in all future experiments.

The Western blot using the Davis lab generated polyclonal α-FKBP65 showed the required specificity; there are no bands in the lanes containing lysate from FKBP65-null cells (Figure 3C, lanes 3, 5 and 6). But also, the antibody is sensitive enough for both mouse FKBP65 and human FKBP65. Interestingly, this antibody seems to be more sensitive for human FKBP65 even though the antigen used to generate it was from mice. It could be that that even though equal amounts of total protein was loaded into the wells, that the human fibroblasts had a higher proportion of FKBP65 protein in the lysate.

Looking at all three Western blots, it was determined that the Davis lab generated polyclonal α-FKBP65 was the most ideal antibody to use in all future experiments. This antibody was more specific to FKBP65 than the monoclonal BD Transduction antibody and it was more cross-reactive across species than the ProteinTech polyclonal antibody.

Immunohistochemical Staining of FKBP65 in Growing Bone

Previous research conducted in our lab had showed the expression of *Fkbp10* in growing bone, but the actual distribution of FKBP65 had not been visualized in this tissue. Slides of one-day-old wildtype mouse limbs that had been cut

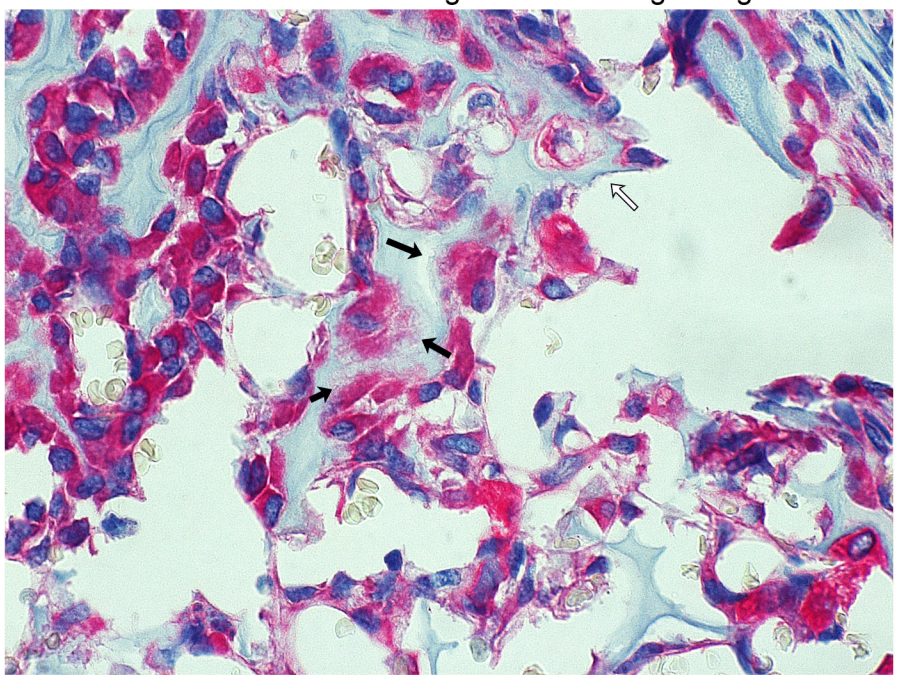
obliquely were stained for FKBP65 using the UltraVision LP Value Detection System AP Polymer plus fast Red Chromatogen kit from Thermo Scientific. Each slide had two sections, therefore one was probed with Davis lab generated α-FKBP65 antibody and the other was left unprobed without primary antibody as a secondary control.

The red staining (Figure 4A) shows the presence of FKBP65 protein in the tissue. The osteoblasts in the growing bone have their cytoplasms heavily stained, showing the presence of FKBP65 inside the cell. Also interesting is the presence of FKBP65 in the newly formed osteoid (Figure 4A, black arrows). This suggests that FKBP65 is secreted and incorporated into the forming bone. The lack of staining deep in the bone suggests that FKBP65 is only present in assembling bone and not in mature bone. The lack of staining on bone not near osteoblasts (Figure 4A, white arrow) shows that the staining seen is not an artifact.

Also, the secondary control tissue, which was exposed to the Fast Red substrate for the same period of time as Figure 4A, shows almost no red staining in a similar tissue. There is some faint red staining in the lumen of blood vessels and in-between surface osteoblasts and the bone surface, but that is probably due to secondary antibody and substrate being caught up in the tissue. The lack of staining in Figure 4B shows that the secondary antibody and substrate has no cross-reactivity with the tissue itself and the staining seen in Figure 4A is specific for FKBP65.

Immunohistochemical staining of FKBP65 in growing bone

A



Secondary staining control

B

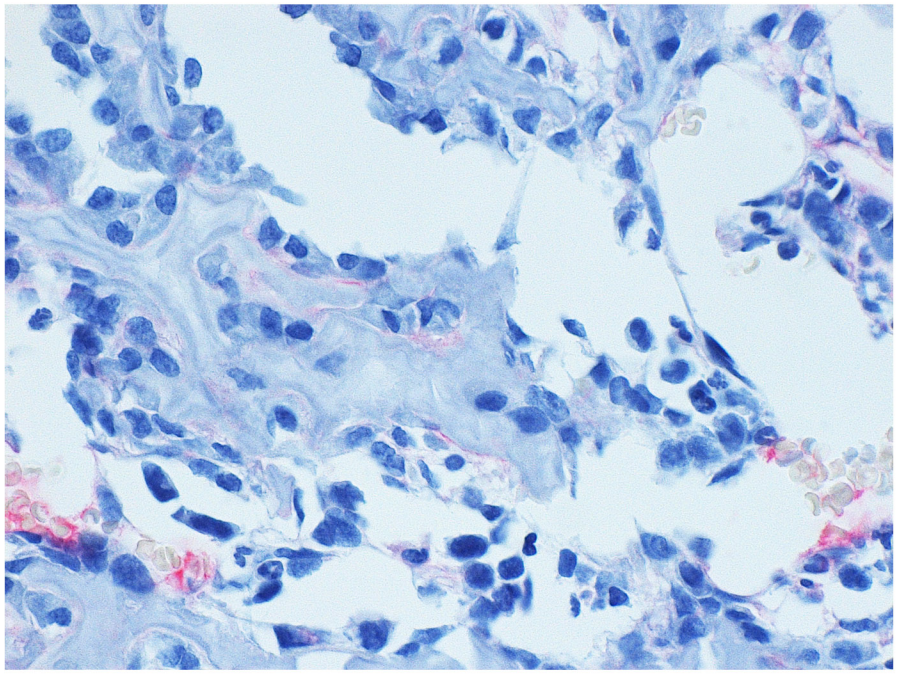


Figure 7 - Immunohistochemistry on growing bone. 1 day old mice sections of growing limb bone spicules were probed with (A) Davis-lab generated α-FKBP65 polyclonal antibody or (B) left un-probed as a control and then visualized. In (A), the presence of FKBP65 outside the osteoblasts in the newly formed bone osteoid is indicated with black arrows. The presence of FKBP65 in the bone is only in near vicinity to an osteoblast cell, as bone surfaces without osteoblasts do not have FKBP65 staining (white arrow). (B) A secondary control to confirm the specificity of the FKBP65 staining in (A).

These results confirm the presence of FKBP65 in bone and suggest a role for FKBP65 in type-I collagen synthesis and bone formation. These results also suggest that the HEEL sequence at the end of the C-terminus is not an ER-retention signal and the FKBP65 is secreted into the extracellular space and incorporated into the ECM.

Testing the Presence of FKBP65 in the Lysate and Media of MC3T3-E1 Subclone 14 cells

To study FKBP65 in osteoblasts, MC3T3-E1 subclone 14 cells were used. They are an immortalized pre-osteoblast cell line that differentiates into mature osteoblasts that secrete, assemble, and mineralize a type-I collagen matrix in 12 days when given ascorbic acid (a type-I collagen synthesis co-factor) and β-glycerophosphate (a source of inorganic phosphate). For four days, these cells were either left untreated, or treated with TGF-β or ascorbic acid, or TGF-β and ascorbic acid in full media. At the end of four days, the lysate and media were collected and equal amounts of total protein loaded and Western blotted for FKBP65 (Figure 5).

When the MC3T3 cells were left untreated, there was no FKBP65 in the media and there was a lower amount in the lysate. When given TGF-β for four days, the amount of FKBP65 in the lysate increased and a faint band appeared in

the media. TGF-β is a growth factor that directly affects and upregulates type-I collagen production, so FKBP65 production being upregulated by TGF-β as well gives yet another link between FKBP65 and type-I collagen production.

When the cells were given only ascorbic acid for four days, the amount of FKBP65 in the lysate is even greater than TGF-β. The amount of FKBP65 being secreted is greatly increased as well. This result shows and even greater connection between type-I collagen production and FKBP65. Ascorbic acid is a co-factor in type-I collagen synthesis and is also involved in upregulating several genes involved in type-I collagen production. If there is no ascorbic acid, lysines and prolines in procollagen do not get hydroxylated properly. Ascorbic acid would have had no effect on FKBP65 if it were not involved with type-I collagen somehow.

When the cells were given both ascorbic acid and TGF-β for the four days, the amount of in FKBP65 in the lysate is still quite substantial. However, this treatment results in the most amount of FKBP65 being secreted out into the media.

It was previously thought that the C-terminal HEEL sequence was sufficiently similar to the ER-retention sequence KDEL to keep FKBP65 inside the ER, but these results show otherwise. These results definitively show that FKBP65 does get secreted into the media by osteoblast cells. Though this is just one time point in the 12 day differentiating process of the cells. The next step would be to monitor FKBP65 inside and outside the cell for the entire 12 days.

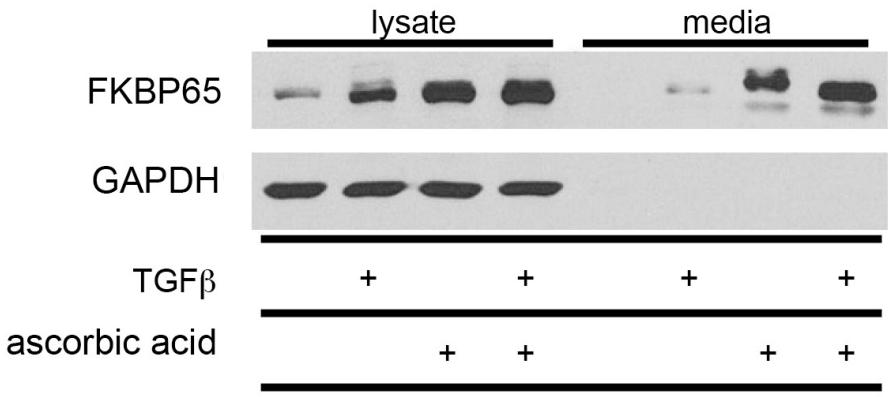


Figure 8 - Testing for FKBP65 secretion via Western blot. A Western blot showing the effects of ascorbic acid and TGF-β, individually and cumulatively, on the amounts of FKBP65 protein in the lysate and media of MC3T3 cells treated for six days. GAPDH is being used as both a loading control and a secretory control.

As the lysate was scrapped up from the tissue culture dish, the matrix that had formed was also scrapped up and collected. It is hard to tell what proportion of matrix proteins remain in the lysate portion as, depending on the buffer, different components would become soluble or insoluble and be spun out with the cell debris. But the matrix component should be taken into consideration when investigating the distribution of FKBP65 in different conditions.

Observing FKBP65 Distribution among Lysate and Media during Differentiation and Mineralization

As MC3T3-E1 Subclone 14 cells proliferate, differentiate and secrete, assemble and mineralize a type-I collagen matrix in 12 days, the amount of FKBP65 during all 12 days in the lysate and media could change. To investigate this, MC3T3-E1

Subclone 14 were cultured with ascorbic acid and β-glycerophosphate for 12 days. The lysate and media was collected every second day and Western blots for type-I collagen and FKBP65 were conducted.

FKBP65 in the lysate seem to stay constant over the entire 12 days, but the FKBP65 that had been secreted into the media is different (Figure 6). FKBP65 starts being secreted from Day 2 and continues to get secreted in greater amounts until it peaks on Day 6. There is less FKBP65 in the media on Day 8 and then absolutely none on Days 10 and 12. MC3T3 cells first go through a proliferative stage and then a collagen matrix forming stage and finally a mineralizing stage. With the absence of FKBP65 in the media for the last four days, it is possible that FKBP65 doesn't have a role in the mineralizing stage.

Type-I collagen seems to stay constant in the media for all 12 days. There are three major bands for type-I collagen in the media. From higher molecular weight to lower molecular weight, the bands are: procollagen, partially processed collagen with one of the propeptide domains cleaved off, and mature collagen which has both of the propeptide domains removed. The presence of type-I collagen in the media of Day 10 and 12 show that collagen can be secreted without FKBP65 as well. The presence of mature collagen in the lysate samples is likely matrix contaminants. Both the lysate and matrix was scrapped up and some soluble components of the matrix dissolved into the buffer and the insoluble matrix was spun out with the cell debris.

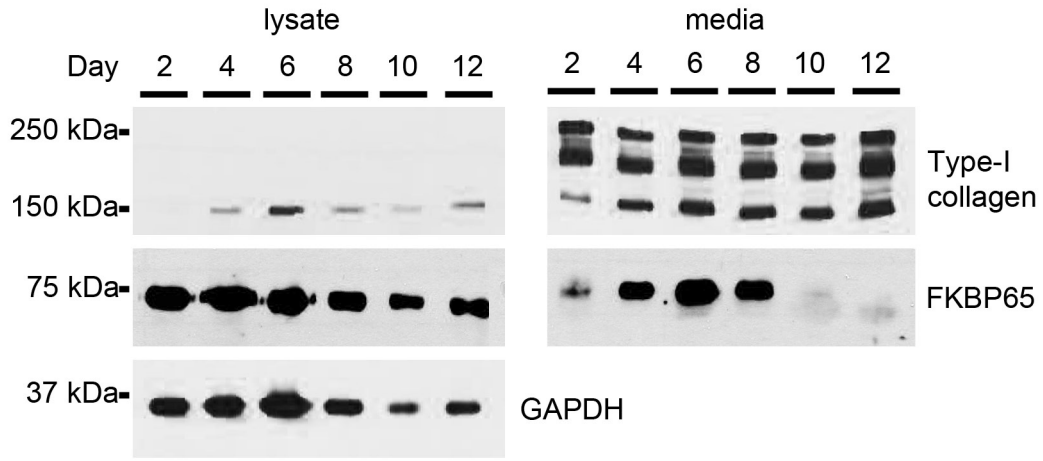


Figure 9 - Observing FKBP65 distribution among lysate and media during MC3T3 proliferation and mineralization. MC3T3-E1 Subclone 14 cells were allowed to grow, differentiate and mature over 12 days in total media plus ascorbic acid and β-glycerophosphate. Total lysate and media was collected every second day and acetone precipitated so Western blots of FKBP65 (lysate and media), type-I collagen (lysate and media) and GAPDH (lysate as a loading control) could be conducted. For type-I collagen, the upper band is procollagen, the middle bands are the intermediate, partially-cleaved type-I collagen, and the lower band is mature collagen.

Investigating the Effects of Collagen Quality on the Distribution of FKBP65

If type-I collagen and FKBP65 are interacting, it would be interesting to see what the effect of misfolded collagen would be on FKBP65. To interfere with the folding of type-I collagen, MC3T3 cells were treated with α,α-dipyridyl. α,α-dipyridyl prevents the hydroxylation of proline residues specifically on collagen. This prevents collagen from forming the signature triple helix quaternary structure. MC3T3 cells were either left untreated or treated with TGF-β, ascorbic acid and β-glycerophosphate for 6 days. For the last 6 hours of Day 6, α,α-dipyridyl was added directly to the media. In this particular experiment, the lysate, media and

matrix were collected individually and both reducing and non-reducing gels were run.

Figure 7A shows that when the cells are left untreated, there is a very small amount of FKBP65 in the lysate and matrix and a rather large amount in the media. Type-I collagen doesn't seem to be produced at all as ascorbic acid is missing and it is a crucial co-factor in its synthesis. With the addition of TGF-β, ascorbic acid and β-glycerophosphate, type-I collagen synthesis occurs normally. There are no procollagen bands in the lysate, most likely due to the rapid secretion of procollagen into the media. There is a mature collagen contamination from the matrix fraction in the lysate. In the media, the procollagen is rapidly processed into its partially processed and mature forms, and a thick band appears in the matrix fraction. FKBP65 appears in a greater amount in the lysate and it almost disappears from the media. However a thick band appears on in the matrix fraction.

When the cells are treated with α, α-dipyridyl for 6 hours, the effects of the drug can be seen in the Western blot. A procollagen band appears in the lysate in addition to several lower molecular weight bands that could possibly be degradation products. In the media, the procollagen band is present as well. The intermediary processed collagen bands appear to be lighter in weight as the lack of hydroxylation has reduced the overall molecular weight of the protein. There is a less intense mature collagen band that is most likely collagen that was produced by the cells in the 18 hours before the addition of α, α-dipyridyl. FKBP65 is affected by the α, α-dipyridyl as well. The amount of FKBP65 remains

the same in the lysate, but a very dark band appears in the media. There is no decrease in the matrix bands of both type-I collagen and FKBP65 due to the presence of 6 days growth.

These results show that the distribution of FKBP65 among the lysate, media and matrix is directly linked to the folding state of type-I collagen. It seems that when type-I collagen is being properly synthesized, FKBP65 is secreted along side of procollagen and incorporated into the matrix. Without ascorbic acid, type-I collagen synthesis cannot occur, and FKBP65 is secreted into the media and it remains there. When the cells producing collagen are treated with α, α-dipyridyl, FKBP65 reappears in the media. This suggests that the FKBP65 being secreted with the misfolded procollagen cannot assemble in the matrix and therefore collects in the media.

As a proof of concept, the protocol was adjusted slightly and media was collected both before and after the addition of α, α-dipyridyl and blotted for FKBP65 (Figure 7B). These results show that the increase of FKBP65 in the media seen only when α, α-dipyridyl is added.

Interestingly, HSP47 which is another type-I collagen chaperone (Figure 7A), doesn't seem to affected by of the different treatments and it only appears in the lysate and not the media or matrix.

This experiment was also run out on a non-reducing gel (Figure 7A) and the FKBP65 non-reducing Western blot shows all matrix bands have disappeared, as well as most of the media bands, except for the increase seen in the media with the addition of α, α-dipyridyl. In the lysate, FKBP65 is decreased

in the α, α-dipyridyl treated band. These results show that FKBP65 is disulfide bonded to something that is affected by α, α-dipyridyl treatment.

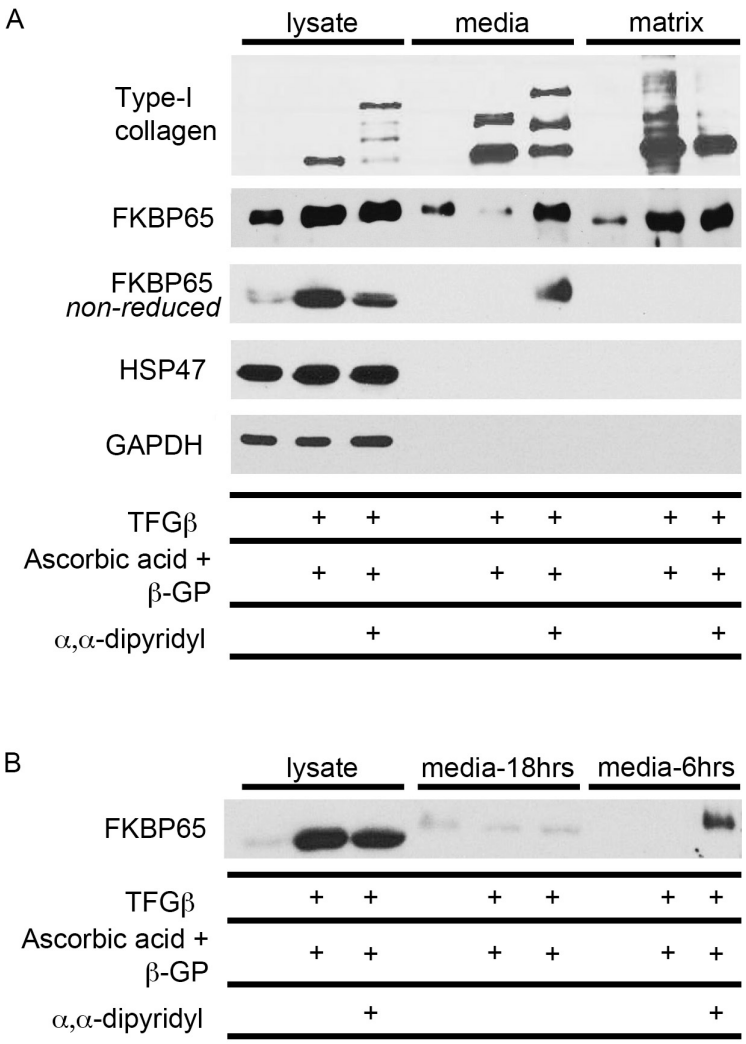


Figure 10 - Investigating the effect of collagen assembly quality on FKBP65 distribution.

(A) These Western blots show the amounts of FKBP65, HSP47, type-I collagen and GAPDH protein in the three cellular components: lysate, media and ECM. MC3T3 cells were given three different conditions in order to manipulate collagen production. Cells were maintained in normal media (without ascorbic acid) for six days as a control; or they were treated with 50 μg/ml ascorbic acid, 10mM β-glycerophosphate, and 5 ng/ml TGF-β for the entirety of the six days; or they were treated with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 5 ng/ml TGF-β for six days and then with 500 μM α, α-dipyridyl for the last eight hours of day six before collection of fractions. Samples were then prepared either via acetone precipitation (media and lysate) or through direct collection (matrix, see Materials and Methods) and run as reducing gels (FKBP65, GAPDH, HSP47, type-I collagen) or as a non-reducing gel (FKBP65). (B) Here, the media was collected before the addition of α, α-dipyridyl and 6 hours after α, α-dipyridyl was added. The two sets of media samples and lysate samples were Western blotted to show the distribution of FKBP65 in the lysate and the two media time points.

Visualizing FKBP65 and Type-I Collagen in the Secretory Pathway

MC3T3 cells that have been treated for 24 hours with ascorbic acid

As previous experiments have shown, FKBP65 seems to be secreted from osteoblast cells. It would be, therefore, possible to visualize FKBP65 in the later parts of the secretory pathway via immunofluorescence. MC3T3 cells were treated with ascorbic acid for 24 hours before being fixed for immunofluorescence.

Under these conditions, when looking at FKBP65 and HSP47 (Figure 8A), HSP47 (which is an ER-resident protein) seems to completely co-localize with FKBP65, but there are some areas of the cell where FKBP65 does not co-localize with HSP47. These areas are not the Golgi because when looking at FKBP65 and GM130 (a Golgi marker) (Figure 8B), GM130 and FKBP65 do no co-localize. These results show that FKBP65 does not seem to be in the Golgi, as delineated by GM130. The partial co-localizing with HSP47 does suggest though, that FKBP65 could be not just in the ER.

When looking at type-I collagen in this condition (Figure 8 C and D), it has the characteristic Golgi staining. GM130 has a very similar pattern and the two overlap (Figure 8D). When comparing HSP47 and type-I collagen (Figure 8C), the two have no areas of overlapping even though HSP47 is a well-known type-I collagen chaperone. This is because type-I collagen is very rapidly moved from the ER to the Golgi. These results are typical of type-I collagen inside the cell.

Control MC3T3 cells

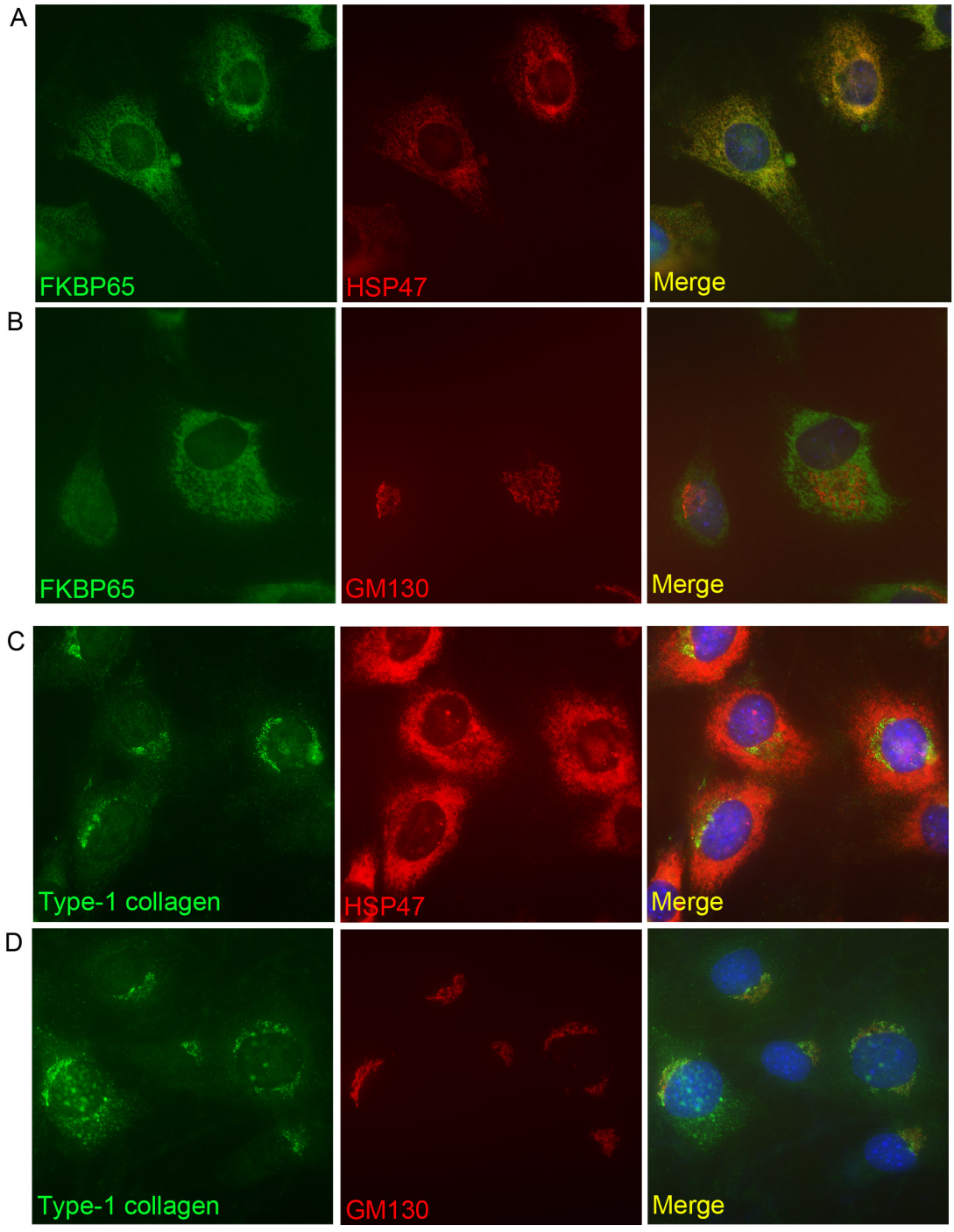


Figure 11(A-D) - Visualizing FKBP65 in the secretory pathway via immunofluorescence. The position of various proteins in relation to one another have been visualized via immunofluorescence in MC3T3 cells treated with 50 μg/ml ascorbic acid for 24 hrs. To determine the position and function of FKBP65 in the secretory pathway, cells were also treated with two secretory disrupting drugs, monensin and bafilomycin. Nuclei were visualized with DAPI staining. Panels (A) through (D) are MC3T3 cells that haven't been treated with secretion disrupting drugs as a control. (A) FKBP65 protein has been labeled with green fluorescence (left) and HSP47, an ER-resident protein involved in type-I collagen production, is labeled in red (centre). The merge of the two (right) show any colocalizing of signals in yellow with blue DAPI nuclear staining. (B) FKBP65 is labeled in green again (left) with the Golgi marker GM130 labeled in red (centre) and merge (right). (C) Type-I collagen protein is labeled with green fluorescence (left) with HSP47 labeled in red (centre) with the merge of the two with DAPI staining on the right. (D) Type-I collagen (green) immunofluorescence staining with GM130 Golgi marker in red (centre) and merge (right).

Visualizing FKBP65 in the monensin-treated secretory pathway via immunofluorescence

Under normal conditions, FKBP65 did not appear in the Golgi of MC3T3 cells, even though we know that these cells will secrete FKBP65. If these cells were treated with monensin for three hours before fixation, it would disrupt the secretory pathway at the *cis*-Golgi and this would cause all secreted proteins to build up in the early part of the secretory pathway. This could then allow us to see FKBP65 and type-I collagen in the stopped up Golgi. Monensin is a monovalent ionophore that disrupts the pH gradient of the secretory pathway at the *cis*-Golgi.

MC3T3 cells were treated with ascorbic acid for 24 hours and with monensin for three hours before being fixed for immunofluorescence. FKBP65 and HSP47 (Figure 8E) seem to have similar co-localization pattern as the control cells. And, exactly as control, FKBP65 and the Golgi marker do not have any areas of overlap. But, type-I collagen in the treated cells (Figure 8G and 8H) does have a different pattern when compared to control. The nice perinuclear cap form has dissipated into punctate structures. The type-I collagen still doesn't

Monensin-treated MC3T3 cells

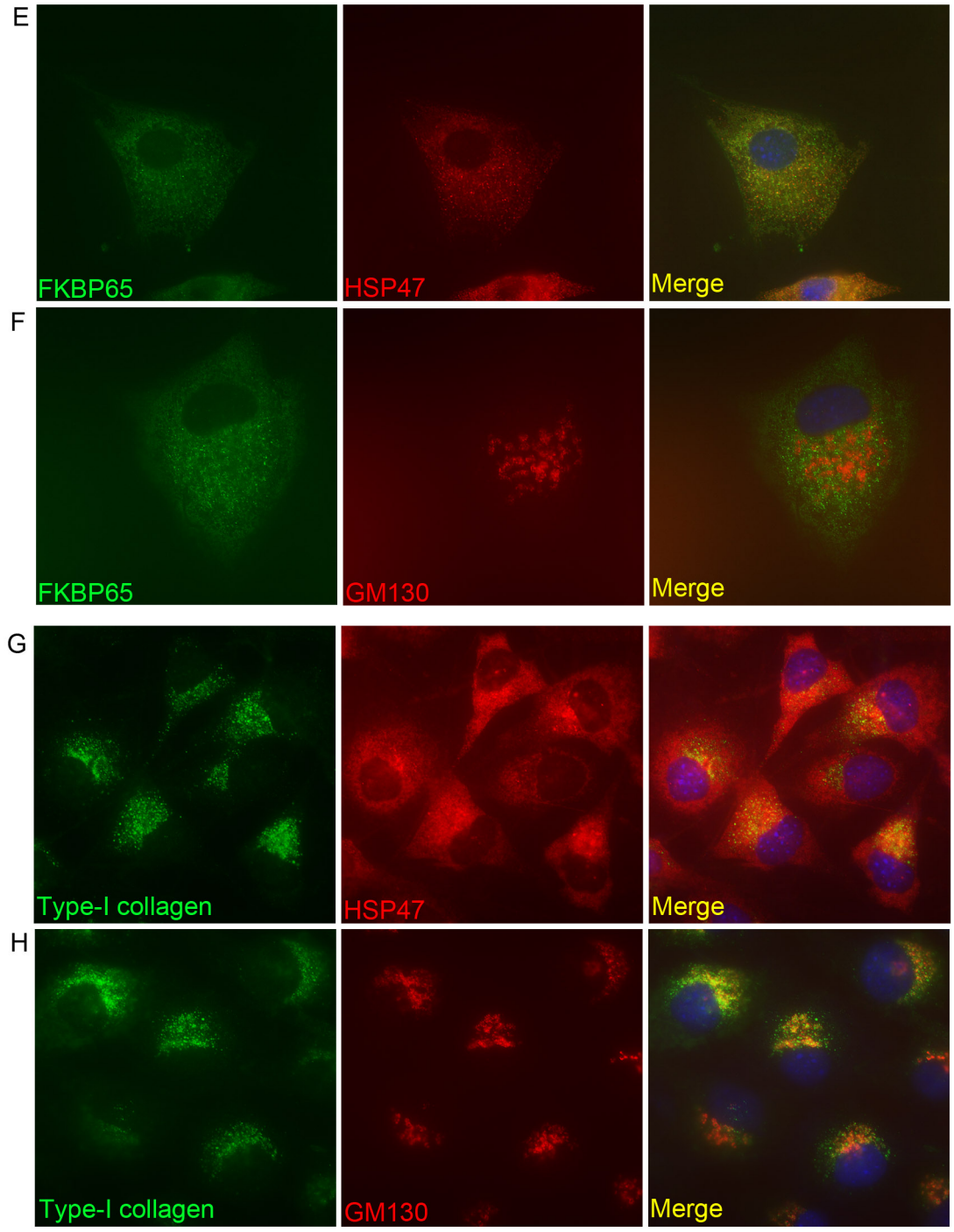


Figure 11(E-H) - Visualizing FKBP65 in the monensin-treated secretory pathway via immunofluorescence. Panels (E) through (H) are MC3T3 cells that were treated for 3 hours with 10 μM monensin in addition to the 24-hour incubation with 50 μg/mL ascorbic acid. Monensin disrupts the secretory pathway after the *cis*-Golgi causing proteins to back up in the secretory pathway. (E) FKBP65 protein visualized with green fluorescence (left) and HSP47 with red fluorescence (centre) shows their respective positions inside the drug treated cells (right, merge). (F) FKBP65 is in green again (left) with the Golgi marker GM130 in red (centre). Rows (G) and (H) show type-I collagen in green (left) with (G) HSP47 labeled with red fluorescence (centre) and (H) the Golgi marker GM130 in red (centre) as well. The merge shows the colocalization of signals between green-labeled proteins and red. The resulting yellow shows any convergence of signal.

colocalize with HSP47 (Figure 8G) and now the GM130 (Figure 8H) only partially colocalizes with type-I collagen, with the rest of type-I collagen in small punctate vesicles.

These results show that the desired effect of sequestering secreted proteins in the early part of the secretory pathway did not happen as the pattern of type-I collagen and FKBP65 are not similar and FKBP65 did not colocalize with GM130 while type-I collagen did.

Visualizing FKBP65 in the Bafilomycin-Treated Secretory Pathway Via Immunofluorescence

Bafilomycin disrupts H+/ATPase pumps which in turn disrupts the pH gradient of the secretory pathway. Bafilomycin disrupts the secretory pathway enough to stop secretion at the *trans*-Golgi and at endosomal vesicles. This should cause secreted proteins to stop and build up in the Golgi. As FKBP65 and type-I collagen are secreted proteins, this would cause them both to build up in the later parts of the secretory pathway and have similar patterns to one another.

Bafilomycin-treated MC3T3 cells

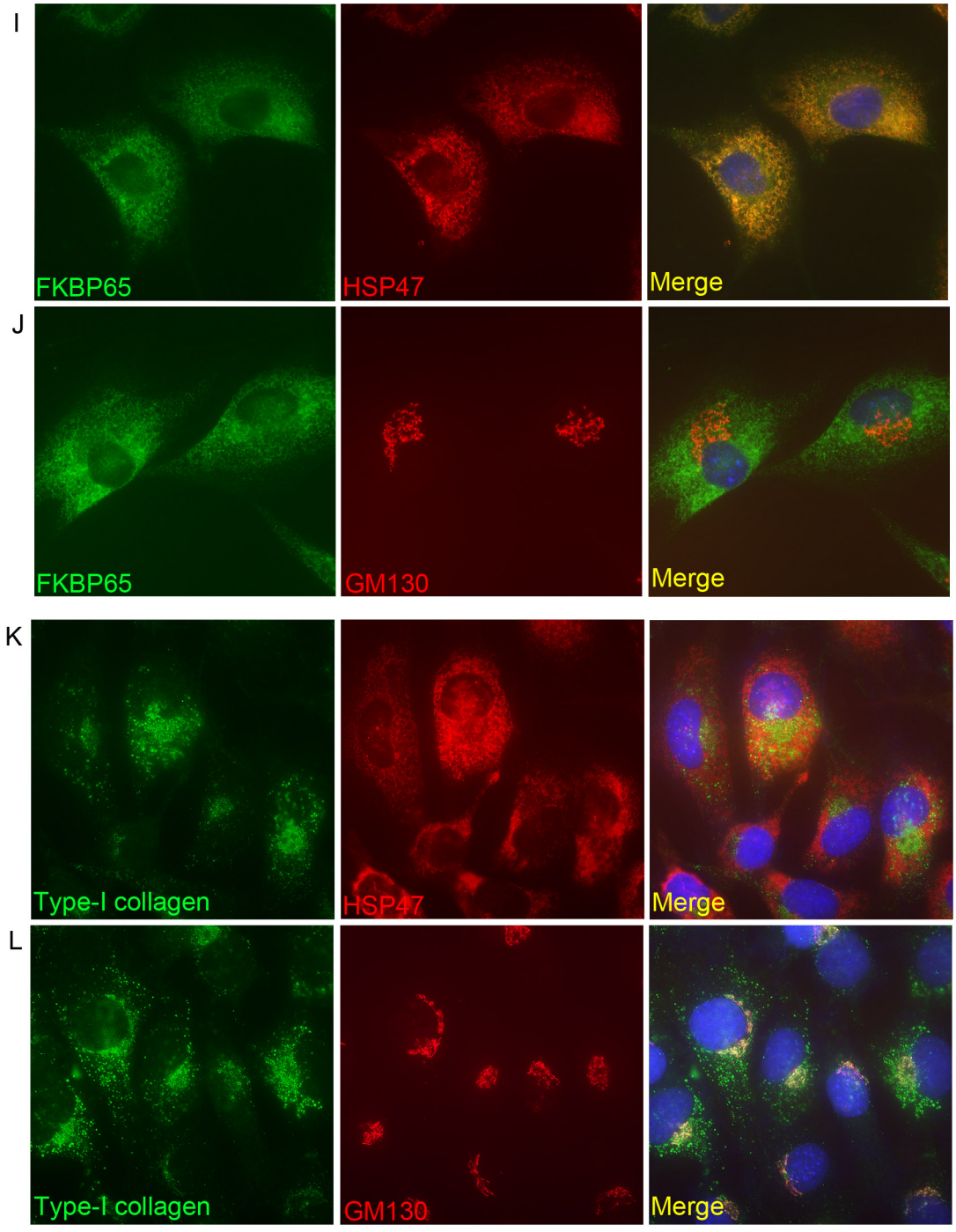


Figure 11(I-L) - Visualizing FKBP65 in the bafilomycin-treated secretory pathway via immunofluorescence. Rows (I) through (L) are MC3T3 cells that were treated for 24 hrs with 50 μg/ml ascorbic acid and were given 1 μM bafilomycin for the last three hours and then fixed for immunofluorescence. Bafilomycin causes the secretory system to be disrupted at the *trans*-Golgi. Rows (I) through (J) show the distribution of FKBP65 (green) with respect to (I) HSP47 and (J) GM130 (both red). Rows (K) and (L) show the distribution of type-I collagen (green) with (K) HSP47 and (J) GM130 (both red) in the treated cells. The nuclei are visualized via DAPI staining in the merged image (right).

MC3T3 cells were treated with ascorbic acid for 24 hours and bafilomycin for 3 hours, after which the cells were fixed for immunofluorescence. FKBP65 and HSP46 (Figure 8I) have similar merged pattern as the control cells and monensin-treated cells. Though the Golgi shadow is more pronounced in the HSP47 staining pattern alone. There are more distinct areas of FKBP65 without HSP47 as compared to control cells. Yet still, FKBP65 and GM130 do not have any areas of colocalization (Figure 8J).

Type-I collagen is clearly being affected by the bafilomycin drug treatment, as the perinuclear staining in control cells is almost gone in the treated cells. Similar to control cells, there still is no colocalization between type-I collagen and HSP47 (Figure 8K) and the Golgi marker GM130 (Figure 8L) now is only colocalizing with a very small proportion of the type-I collagen fluorescence. The rest of the type-I collagen seems to be in punctate vesicles around the cell.

These results showed that the desired effect of the bafilomycin treatment did not happen. FKBP65 is still not colocalizing with GM130 even when treated with bafilomycin, while the pattern of type-I collagen is quite different from control, it still colocalizes with GM130.

α,α-dipyridyl treated MC3T3 cells

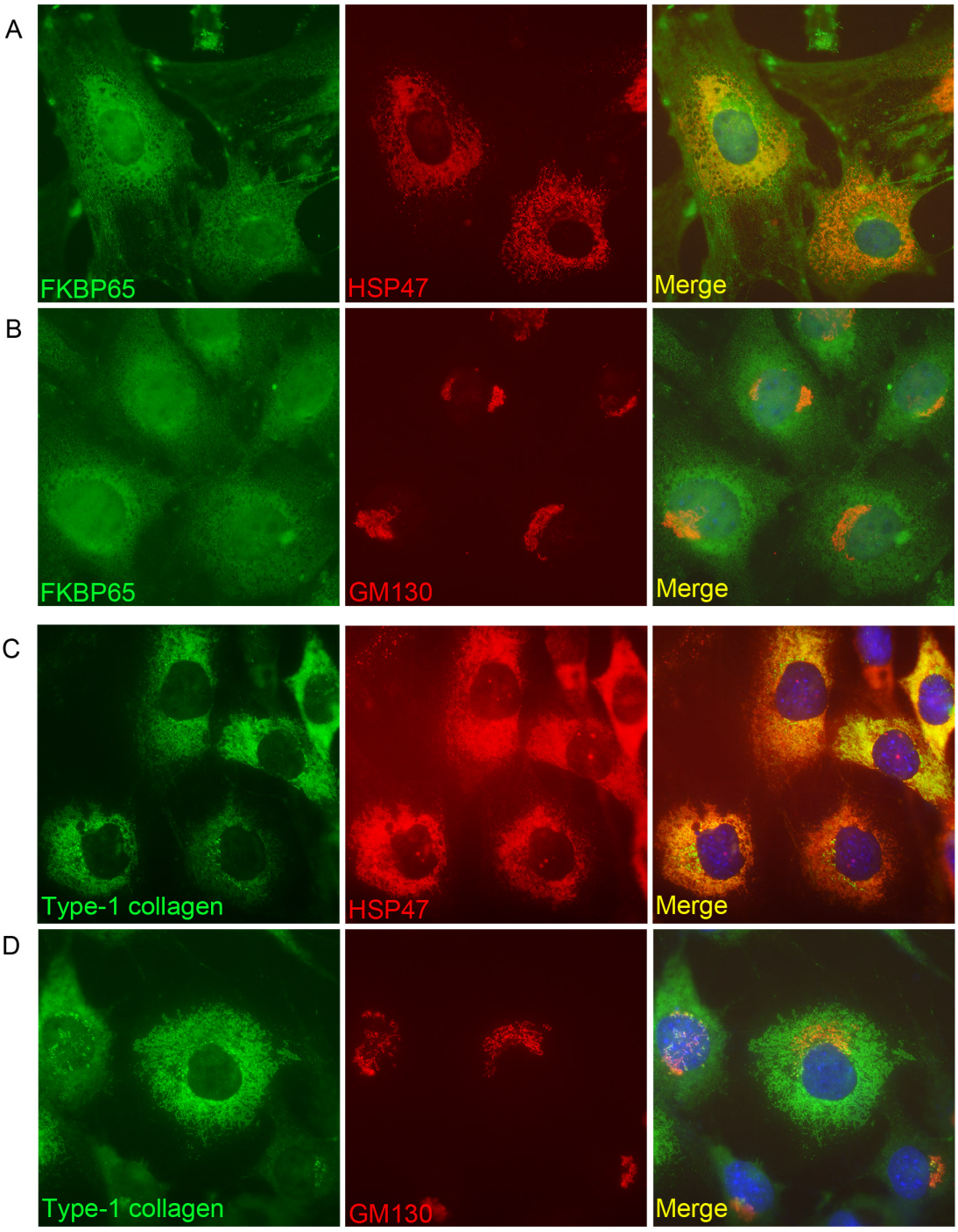


Figure 12 - Visualizing the effects of α, α-dipyridyl on the movement of FKBP65 through the secretory pathway using immunofluorescence. MC3T3-E1 Subclone 14 cells were treated for 24hrs with 50 μg/ml ascorbic acid. 500 μM α, α-dipyridyl was added to the cells in the last 6 hours. Afterwards the cells were fixed for immunofluorescence. α, α-dipyridyl prevents the proper hydroxylation of type-I collagen from occurring. This causes the collagen to be backed up in the secretory pathway and to be folded incorrectly. Rows (A) and (B) show the distribution of FKBP65 (green) inside these cells in relation to (A) HSP57 and (B) GM130, a Golgi marker (both red). Rows (C) and (D) show type-I collagen inside the cells with (C) HSP47 and (D) GM140 (both red). The merged images (right column), show any colocalization between the red and green signals in yellow. The nuclei are visualized via DAPI staining.

Visualizing the Effects of α, α-dipyridyl on the Movement of FKBP65 Through the Secretory Pathway using Immunofluorescence

In order to see what is happening to FKBP65 inside MC3T3 cells when they have been treated with the collagen-disrupting drug α, α-dipyridyl, these cells were treated with ascorbic acid for 24 hours and α, α-dipyridyl for the last 6 hours.

FKBP65 is affected by the treatment of α, α-dipyridyl (Figure 9A and 9B), as the staining pattern looks quite different from the control (Figure 8A and 8B). When FKBP65 and HSP47 are merged (Figure 9A), HSP47 only colocalizes with some of the FKBP65. When looking at FKBP65 and GM130, the signals seem to overlap but not colocalize (Figure 9B). These results suggest that FKBP65 is really affected by the treatment of α, α-dipyridyl, but the actual distribution of FKBP65 inside the treated cells is unknown as it doesn't colocalize completely with either the Golgi marker GM130 or the ER-resident protein HSP47.

As α, α-dipyridyl prevents the hydroxylation of type-I collagen, the triple helical quaternary structure cannot form. This results in procollagen remaining inside the ER as it cannot fold properly. HSP47 now colocalizes with the type-I collagen that is backed up inside the ER (Figure 9C) and GM130 also colocalizes with the type-I collagen that is in the Golgi (Figure 9D). These results are

consistent with similar experiments done by other groups (see (Satoh et al., 1996)).

Immunofluorescence of Human Wildtype Skin Fibroblasts and Human *FKBP10*-mutant OI Patient Skin Fibroblasts

In order to test the specificity of the α-FKBP65 antibody used for the previous immunofluorescence experiments, human wildtype and *FKBP10*-mutated skin fibroblasts were treated with ascorbic acid for 24 hours before being fixed for immunofluorescence (Figure 10). FKBP65 can be seen in the wildtype skin fibroblasts, but no green fluorescence can bee seen at all in the FKBP65-null patient fibroblasts. FKBP65 green fluorescence is combined with the red Golgi marker Golgin, which does appear in the patient cells as well. The nuclei have been stained with DAPI.

The cells were also double stained for type-I collagen (green) and HSP47 (red). The wildtype cells show the characteristic pattern where the type-I collagen only appears in the Golgi and doesn't colocalize with HSP47. Patient 014 fibroblasts are the same as the control wildtype fibroblasts. However, though most of Patient 381 had similar merged patterns as wildtype and Patient 014, there were a few cells were the collagen was colocalizing with HSP47 as well as appearing in the Golgi. This showed that in these cells the processing of type-I collagen is delayed due to the lack of FKBP65.

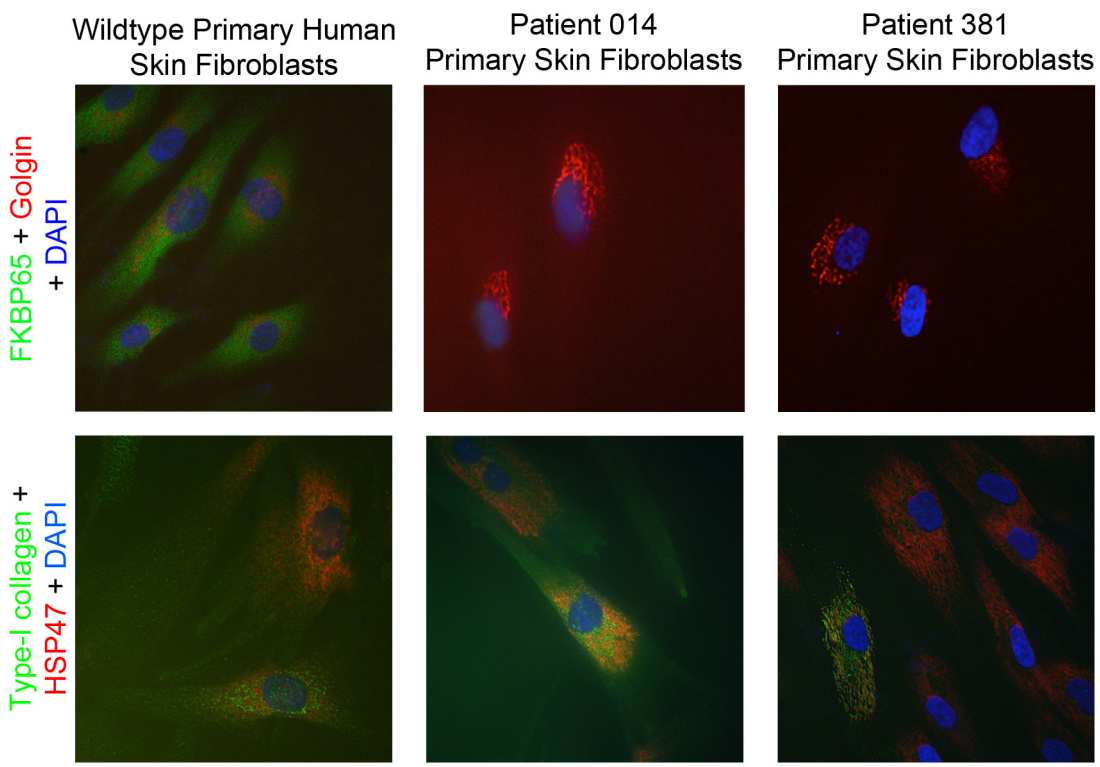


Figure 13 - Immunofluorescence of human wildtype skin fibroblasts and human FKBP65-mutant OI patient skin fibroblasts. Wildtype, Patient 014, and Patient 381 primary skin fibroblasts were treated with 50 μg/ml ascorbic acid for 24 hours and then fixed for immunofluorescence. The top row are the merged images of the three cell types labeled for FKBP65 (green), the Golgi marker golgin (red), and the nuclei (via DAPI staining, blue). The bottom row are merged pictures of the three cell types fluorescently labeled for type-I collagen (green), HSP47 (red), and the nuclei (via DAPI staining, blue). Patient 014 has an in frame eleven amino acid deletion in the *FKBP10* gene. Patient 381 has an insertion mutation in *FKBP10*. The resulting frameshift leads to a premature stop codon. In both cases, the mutations lead to mRNA degradation and no protein being translated.

Observing the Effect of FKBP65 Disruption on Type-I Collagen

Embryonic skin fibroblasts were cultured from the skin of embryonic mice, both wildtype and FKBP65 Knockout mice pups. Once cultured, a basic Western blot was performed. The Western blot (Figure 11A) showed that there was no FKBP65 protein being picked up by the α-FKBP65 antibody at all, and that HSP47 doesn't seem to be effected by the knockout of FKBP65. Interestingly, there seems to be more type-I collagen present in the media of the knockout skin

fibroblasts when compared to the wildtype. In humans, the lack of FKBP65 has actually caused a delay in type-I collagen secretion (Alanay et al., 2010), so it is possible that in the mouse type-I collagen secretion is delayed as well, but then type-I collagen assembly into the matrix is delayed too.

To observe the type-I collagen matrix that is formed by these two cell types (wildtype and FKBP65 knockout), the fibroblast cells were plated at equal cell density and were cultured for 6 days with ascorbic acid. At the end of 6 days, the cells were fixed and the type-I collagen ECM was visualized via immunofluorescence (Figure 11B and 11C). The FKBP65 knockout fibroblasts do produce a noticeably different type-I collagen ECM than the wildtype fibroblasts, therefore the FKBP65 knockout could effect type-I collagen matrix assembly. As the preliminary results show that the FKBP65 knockout mouse is perinatally lethal and FKBP65-null humans are viable, it is clear that FKBP65 could have an additional role in the mouse.

Conversely, the type-I collagen ECM produced by human wildtype and FKBP65-null patient fibroblasts seem very similar. Again, wildtype, Patient 014 and Patient 381 fibroblasts were plate at equal density and cultured for 6 days with ascorbic acid. At the end of the 6 days, the cells were fixed that the type-I collagen ECM was visualized via immunofluorescence (Figure 11D, 11E and 11F). Even though the humans that the patient cells come from have such a severe bone phenotype, the type-I collagen ECM produced by these fibroblasts

are similar to wildtype.

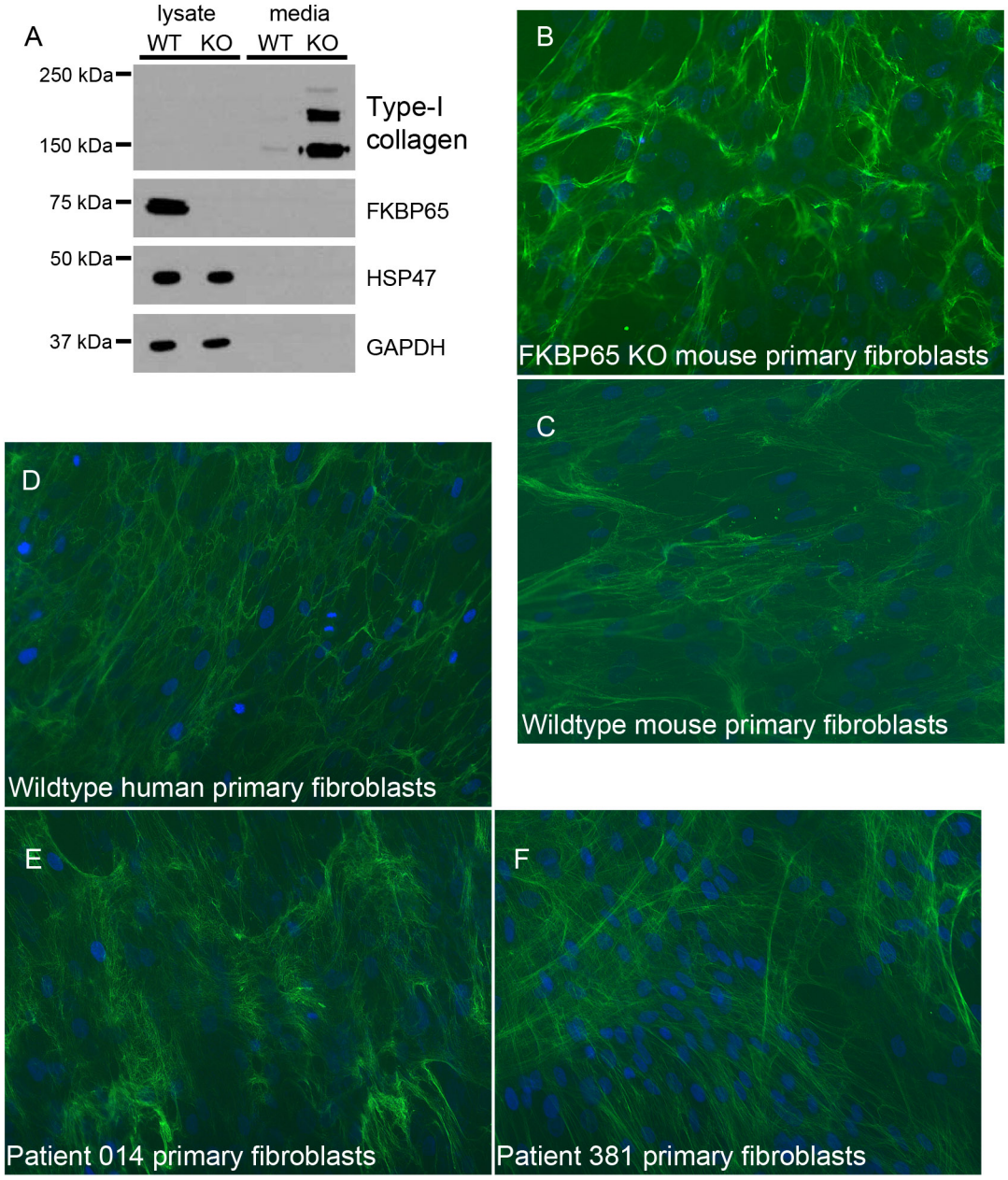


Figure 14 - Observing the effect of FKBP65 disruption on collagen matrix formation via immunofluorescence. (A) Western blot of lysate and media from primary mouse fibroblasts from wildtype mouse and FKBP65 knockout mouse for type-I collagen, FKBP65, HSP47 and GAPDH (loading control). (B) and (C) are the collagen matrices produced by FKBP65 knockout fibroblasts and wildtype fibroblasts after 6 days. Nuclei are visualized via DAPI staining. (D), (E), and (F) are the collagen matrices produced by wildtype human primary fibroblasts, primary fibroblasts from Patient 014 (11 amino acid deletion in *FKBP10*), and primary fibroblasts from Patient 381 (insertion of basepair into *FKBP10*) after 6 days, respectively. Nuclei were visualized by DAPI staining.

Chapter 5: Discussion

The Davis lab generated polyclonal antibody was chosen over the other two commercially available α-FKBP65 antibodies, as it was the most specific for FKBP65 in the widest range of species. The BD Transduction monoclonal FKBP65 antibody seems to be binding something that is the same molecular weight as FKBP65, possibly FKBP60, in the knockout cells where there is no FKBP65 being made. This disqualified it from further use in experiments, as it would be impossible to tell whether it was binding FKBP65 or the other protein. The other commercially available α-FKBP65 antibody from ProteinTech was not sensitive enough for FKBP65 in MC3T3 cells because of the human FKBP65 antigen used to generate it. The Davis lab generated polyclonal α-FKBP65 antibody was sensitive for just FKBP65 in both mice and human tissues and was used in all of the subsequent experiments described.

FKBP65 has been shown to be crucial in proper type-I collagen formation (Alanay et al., 2010; Kelley et al., 2011; Shaheen et al., 2010b; Venturi et al., 2011), but the exact role of FKBP65 still has not been determined. Current publications regard FKBP65 as an ER-resident protein due to the C-terminal HEEL sequence, but my data has shown that FKBP65 can be detected outside the cell and in the ECM via Western blotting and immunohistochemistry. We know that this is not an artifact, as the ER-resident HSP47 does not appear in the media or matrix in the Western blots, indicating that the cells were not lysed at some point in the experiment. Also, FKBP65 is not found in newly formed bone in regions where there is no adjacent osteoblast by immunohistochemistry. This

shows that the FKBP65 detected in the media and matrix is not due to lysate contamination through broken cells in the Western blot, nor due to non-specific binding of antibodies in the immunohistochemistry of bone.

The C-terminal HEEL sequence of FKBP65 is an acceptable modification of the KDEL sequence as it is one of the possible PROSITE motifs for ER-retention ([KRHQSA]-[DENQ]-E-L) (Hulo et al., 2006). Interestingly, in literature there have been several HEEL sequence ER-resident proteins. For example, the CyPB protein in *Aspergillus niger* has a C-terminal sequence that has been proven to keep CyPB inside the ER of the fungus (Derkx & Madrid, 2001), and the luminal binding protein (BiP) equivalent in Douglas fir also has a ER-retention HEEL sequence (Forward & Misra, 2000). Other proteins have been found with putative C-terminal HEEL ER-retention sequences, but they have not been tested to determine the functionality of that sequence (Persson, Rosenquist, & Sommarin, 2002; Tessier, 2000; Xu, Fang, Keirans, & Durden, 2004). Therefore, the assumption that FKBP65 would be an ER-resident is well thought out, and thus, our finding that FKBP65 is secreted is surprising.

There are several possible reasons as to why FKBP65 is secreted and not exclusively an ER-resident protein. My immunofluorescence images showed that FKBP65 is very strongly present in the ER and it is possible that the HEEL-sequence is partially functioning to keep the majority of FKBP65 inside the ER, while a small amount is secreted outside the cell with collagen to function extracellularly. We know that FKBP65 is not just being secreted due to KDEL receptor saturation because of the differences in distribution of FKBP65 in the media when collagen assembly was disrupted using α, α-dipyridyl and because

of the variable FKBP65 secretion over the 12 day MC3T3 differentiation process while type-I collagen appeared consistent. Additionally, if the KDEL receptor pool was saturated then one would expect other KDEL (or KDEL equivalent) containing proteins to be secreted, like HSP47.

It is also possible that FKBP65 is secreted when it is bound to a secreted protein such as type-I collagen. It could be that the C-terminal HEEL sequence is masked from the KDEL receptor in the Golgi when FKBP65 is bound to another protein. This would explain the lack of FKBP65 staining in the later parts of the secretory system in the immunofluorescence experiments as the epitope for the FKBP65 antibody used is the last 14 amino acids of FKBP65 which includes the HEEL sequence.

Apparent in Figure 7A, is the secretion of FKBP65 in the absence of TGF-β, ascorbic acid and β-glycerophosphate. It is interesting to note that the secreted FKBP65 band disappears under non-reducing conditions. It was previously hypothesized that the secretion of FKBP65, in spite of the HEEL C-terminus sequence, was due to the masking of the HEEL sequence when FKBP65 is bound to type-I collagen. However, there is no type-I collagen being secreted, therefore the presence of FKBP65 in the media is puzzling. It is possible that FKBP65 has other binding partners with which it is secreted. Also, FKBP65 may self-associate and be secreted in a complex. Thus, until the direct binding partners of FKBP65 are identified, no conclusive statement can be made. The binding partners of FKBP65 can be determined by performing a co-immunoprecipitation of the FKBP65 in the lysate and media under different

conditions. Any co-precipitated proteins would be identified via mass spectrometry.

It is not unusual for certain ER-resident proteins to be secreted. For example calreticulin, an ER-resident chaperone, can be secreted (Booth & Koch, 1989) and can be found in the human bloodstream (Sueyoshi et al., 1991). The opposite case, where a protein without an ER-retention sequence can be found to function and be sequestered inside the ER, is also possible. CRTAP is one example. CRTAP is a member of the hydroxylation complex that hydroxylates one proline on the proα1(I) chain. A mutation in the gene encoding CRTAP leads to recessive OI, similar to FKBP65, thus CRTAP is crucial in the proper assembly and function of type-I collagen. CRTAP has no ER-retention sequence and no catalytic capabilities, but it is crucial for proper procollagen modifications in the ER. It remains in the ER by binding to the other members of the hydroxylation complex, P3H1 and CyPB, proteins that do have ER-retention signals (Morello et al., 2006). However, CRTAP can also be secreted and has been found outside chondrocytes in cartilage (Castagnola et al., 1997). The function of CRTAP in cartilage has not been determined yet. In the case of FKBP65, it could be possible that either the KDEL receptor which brings ER-resident proteins back into the ER is not catching all of the FKBP65 and some is being secreted into the extracellular environment, or that the HEEL-sequence, which the KDEL receptor would bind to, is being masked due to FKBP65 being in a complex.

Previous unpublished data from our lab showed that a possible binding partner of FKBP65 could be the procollagen inside the cell. Immunoprecipitation

(IP) experiments were performed with two cross-linking chemicals in the preosteoblasts cell line MC3T3-E1. Using reducible and non-reducible crosslinkers it was shown that FKBP65, HSP47 and type-I collagen interact with one another. Specifically, FKBP65 and type-I collagen interact together and HSP47 and type-I collagen interact as well (unpublished). When type-I collagen synthesis was upregulated with TGF-β, or made more efficient with the addition of the cofactor ascorbic acid, FKBP65 showed a corresponding increase in both mRNA stability and protein amount (unpublished). Together, these experiments definitively showed that FKBP65 and type-I collagen interact and are involved in the same pathway *in vitro* (unpublished). In the future, it would be interesting to do IP experiments for FKBP65 that has been secreted into the media to determine if FKBP65 is still bound to collagen in the media.

Also, another potential binding partner for FKBP65 is HSP47. Unpublished work done in the lab of Hans Peter Bächinger has shown that FKBP65 and HSP47 associate with one another *in vitro* (presented at the American Society for Matrix Biology conference, October 2010). A possible scenario is that HSP47 and FKBP65 associate and bind procollagen inside the cell and move through the ER together and then enter the *cis*-Golgi. The pH change between the *cis*-Golgi and the ER causes HSP47 to disassociate and return back to the ER via interactions between its RDEL ER-retention sequence and the KDEL receptor (Thomson & Ananthanarayanan, 2000) and FKBP65 and procollagen continue through the secretory pathway. Any possible functional overlap between FKBP65 and HSP47 is not surprising. Human OI patients with mutations in *FKBP10* (type XI) or

SERPINH1 (type X) have very similar type-I collagen, in that the collagen is more easily destabilized and it is not over modified (Alanay et al., 2010; Christiansen et al., 2010), although, type X OI is much more severe and rarer than type XI OI. Also interesting is that the HSP47 knockout mouse is embryonic lethal due to unstable basement membranes (Nagai et al., 2000), thus the perinatally lethal FKBP65 knockout mice should have their basement membranes investigated as well, to see if FKBP65 is also required for proper assembly of the basal lamina in the mouse.

With regards to the immunofluorescence experiments done, we were not able to show FKBP65 in the later parts of the secretory pathway even when the cells were treated with secretion disrupting drugs. As mentioned above, this could be due to epitope masking. The polyclonal antibody used for this experiment was generated using a 14 amino acid polypeptide from the C-terminal of FKBP65. It is entirely possible that the epitope for the antibody was masked in the later part of the pathways, especially if FKBP65 is disulfide bonded and part of a protein complex. This could also be the same way that FKBP65 avoids being sequestered in the ER by keeping its C-terminal end hidden from the KDEL receptor. It would be interesting to redo this immunofluorescence experiment with the addition of an antigen retrieval step since FKBP65 has been detected when proteins are either denatured, as in the Western blots, or if there is an antigen retrieval step, as in the immunohistochemistry protocol.

It is also possible that FKBP65 cannot be visualized inside the later parts of the secretory pathway in this particular cell type. As mentioned above, CRTAP is another collagen-associated protein that is also secreted and incorporated into

the matrix, but it can only be visualized inside the later parts of the secretory pathway in certain cell types (Morello et al., 2006). FKBP65 could be similar to CRTAP in this respect.

The drugs that we used, monensin and bafilomycin, have been used in other cell types, such as fibroblasts (Satoh et al., 1996) and chondrocytes (Davis & Mecham, 1998), to disrupt the secretory pathway at different points. These drugs cause the secretory proteins to build up in known compartments of the cell. We had hoped that this would force the secreted FKBP65 to be visualized with type-I collagen in the later parts of the secretory pathway. Unfortunately, our results showed that FKBP65 did not take on the same pattern of fluorescence as type-I collagen. A possible reason could be possible that the drugs were not affecting these cells as predicted, since they have not been used on MC3T3 cells before. Also, the lack of co-localization between FKBP65 and the Golgi marker GM130 could be clarified with another Golgi marker and/or repeating these experiments using a confocal microscope rather than a fluorescent light microscope. For even better resolution, double immunogold labeling followed by transmission electron microscopy would help directly visualize the collagen and any FKBP65 possibly bound to it. Immunogold labeling would also be useful to mark any FKBP65 outside the cell that has been incorporated into the matrix made by the MC3T3 cells.

In the published literature pertaining to OI patients with mutations found in *FKBP10*, their fibroblasts show retention of type-I collagen via immunofluorescence (Alanay et al., 2010). However, the Western blot of the mouse primary fibroblasts (both wildtype and with FKBP65-null cells, Figure 11A)

shows that type-I collagen is being readily secreted. The increased presence of type-I collagen in the medium after 24 hours could be also due to impaired assembly of the type-I collagen ECM. In fact, in the human primary fibroblasts, the secretion of type-I collagen is delayed (Alanay et al., 2010). Another possible explanation for this discrepancy is that it is clear that FKBP65 has additional roles in the mouse that are not exclusive to maintain proper type-I collagen synthesis, secretion and assembly. The differences seen between mouse and human fibroblasts could simply be due to differences in the species

The immunofluorescence of the type-I collagen ECM developed by fibroblasts showed that the FKBP65 knockout fibroblasts produced very different matrix when compared to the wildtype. This is something that was seen only in the mouse fibroblasts, as the type-I collagen ECM assembled by the primary human OI skin fibroblasts was very similar to the ECM produced by the wildtype human skin fibroblasts. This is expected, as the human patients have no abnormalities in their skin (Alanay et al., 2010). The human fibroblasts do secrete and form collagen since the FKBP65 mutation doesn't completely eliminate type-I collagen secretion and assembly. Indeed, the human patients are viable, with bone and other connective tissues that contain type-I collagen.

In the human, it is interesting to note that type XI OI is characterized by loose ligaments (Alanay et al., 2010) and the Bruck Syndrome Type 1 (also caused by mutations in *FKBP10)* is characterized by stiffened joints (Shaheen et al., 2011). Clearly, FKBP65 has some role in the tendons and ligaments of joints and this is another area that should be investigated in humans.

The study of FKBP65 inside MC3T3 cells over 14 days showed that FKBP65 secretion peaks on day 6 and then FKBP65 is completely absent in the media during the mineralization phase. Unfortunately, the distribution of FKBP65 in the matrix over this time period was not also observed. A future experiment could be to collect protein from all three compartments (lysate, media and matrix) and then Western blotted. It is currently unknown if the increasing amount of FKBP65 seen in the media is due to increased secretion over time, or decreased incorporation into the matrix over time.

Also interesting would be to determine whether FKBP65 appears in the soluble or the insoluble component of the ECM. This can be determined using lysis buffers containing deoxycholate (DOC) which dissolves the soluble portion of the matrix and leaves the rest insoluble (Kobayashi et al., 2007). The insoluble portion can then be spun out and dissolved directly in the Laemmli buffer, as it is soluble in SDS. If FKBP65 appears in the insoluble portion of the matrix, this would allow for more consistent collection of matrix compared to the method used above. Using different lysis buffers would allow us to determine at which points during the 12 day differentiation process of MC3T3 cells, FKBP65 is being actively incorporated into the matrix. The function of FKBP65 at this point has not been determined. As the catalytic capabilities of FKBP65 are not very strong (Cheung, Bates, & Ananthanarayanan, 2010), it is likely that FKBP65 functions only as a binding chaperone. It could continue to have this function in the matrix where it could be acting as a spacer for the collagen fibers. A possible function of FKBP65 is shown in figure 15.

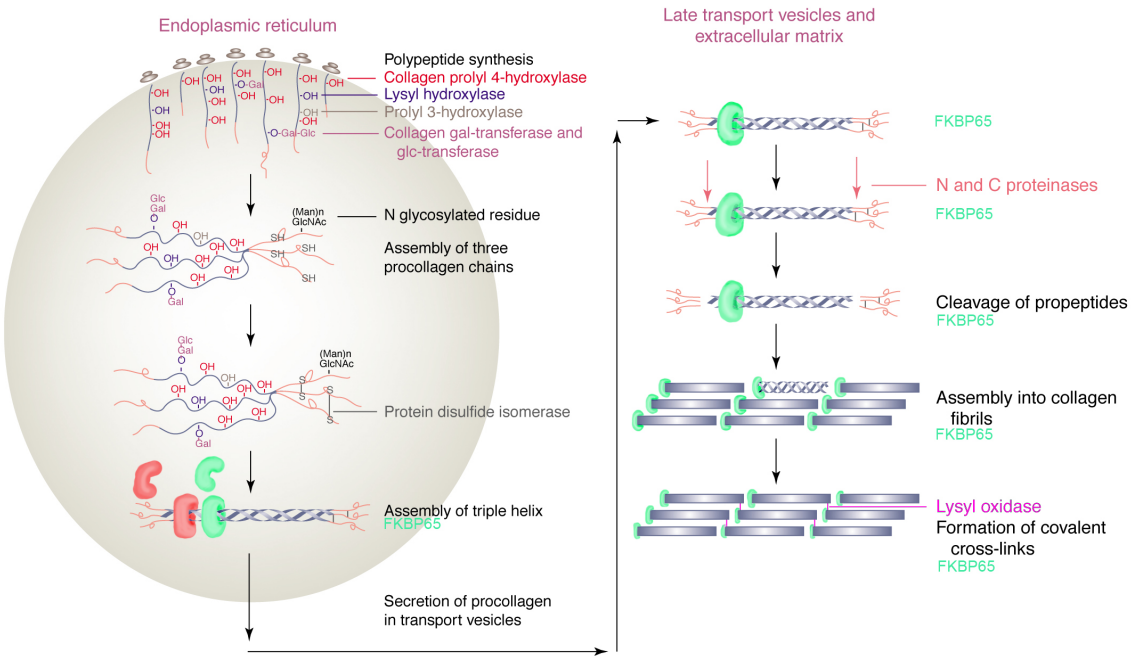


Figure 15 - Summary of type-I collagen synthesis and assembly with hypothesized extracellular function of FKBP65 added. Figure adapted from Myllyharju et al. 2004. With the data in the previous section, the original figure summarizing the synthesis and assembly of type-I collagen can now be modified to add FKBP65 functioning outside the cell. Though most of FKBP65 seems to remain inside the cell in the ER, the actual function of FKBP65 outside the cell is not known and the figure represents a possible function. The presence of FKBP65 is shown in light green, whereas HSP47 is shown in red.

To determine if FKBP65 plays a role in bone formation, a mouse model with bone specific knockout of FKBP65 should be developed. The targeting cassette used to generate the complete KO contains the necessary sites to develop a tissue-specific knockout using Cre-lox technology.

Possible Cre models include the Osx-Cre mouse and the *COL1A1*-Cre mouse. There is no bone-specific Cre-recombinase mouse. There are several candidates that could be used. There is a *COL1A1* Cre-recombinase mice that expresses the recombinase where ever type-I collagen is being expressed (Kim et al., 2004). The Cre-recombinase would proceed to excise the majority of *FKBP10* gene from the genome of these specific genes. The issue with the

COL1A1 Cre-recombinase mouse is that this would not be bone specific. Type-I collagen is heavily present in the ECM of skin and other connective tissues. With such a broad-spectrum knockout of FKBP65, there runs the risk of developing another perinatally lethal knockout. The other option would be the Osx Cre-recombinase mouse (Maes et al., 2007). This option is not without problems too. The Osx Cre mouse may knock the *FKBP10* gene out too late. Even though the Osx knockout mouse doesn't have any bone, the foundations for bone formations have been laid. The Osx Cre-recombinase mouse may knockout the *FKBP10* too late and the mice may not show a bone phenotype. Despite these limitations, both Cre models would likely be informative about the function of FKBP65 in bone formation.

Conclusion

Bone formation is a very complicated process. The organic portion of bone, type-I collagen, requires several crucial proteins to assist the processing and assembly of collagen into the scaffolding that will be mineralized into bone. One of these collagen-associating proteins is FKBP65. Our lab found that FKBP65 was very heavily expressed in developing bone. Immunohistochemistry also showed the presence of FKBP65 both inside and outside osteoblasts in developing bone. FKBP65, which is thought to be an ER-resident protein, was found both inside the cell and in the media and matrix produced by MC3T3 osteoblasts and is disulfide bonded in a complex. This is contrary to literature that says that the C-terminal HEEL sequence of FKBP65 is enough to sequester FKBP65 inside the cell. Also, we showed that the secretion of FKBP65 from

MC3T3 cells is dependent on procollagen production and secretion. We also showed that the distribution of FKBP65 among the lysate, media and matrix is dependent on the folding state of collagen. The FKBP65 knockout mouse appears to be perinatally lethal and the type-I collagen ECM produced by the primary embryonic skin fibroblasts from these mice are different from wildtype. Recent findings have found that OI is not just caused by mutations in the genes encoding the collagen chains, but also in the genes that encode collagen associated proteins. Type XI OI, which is caused by mutations in *FKBP10*, confirmed that FKBP65 is a crucial collagen chaperone. Future research will help clarify the exact role of FKBP65 both inside and outside the cell.

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