



β -Catenin mediates glucose-dependent insulinotropic polypeptide increases in lysyl oxidase expression in osteoblasts[☆]

Eileen J. Daley¹, Philip C. Trackman^{*,2}

Boston University Henry M Goldman School of Dental Medicine, Department of Translational Dental Medicine, 700 Albany Street, Boston, MA 02118, United States of America

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ABSTRACT

Osteoblast lysyl oxidase (LOX) is a strongly up-regulated mRNA and protein by the glucose-dependent insulinotropic polypeptide (GIP). LOX is critically required for collagen maturation, and was shown to be dramatically down-regulated in a mouse model of type 1 diabetes, consistent with known low collagen cross-linking and poor bone quality in diabetic bone disease in humans and in mouse models. GIP is a gastric hormone released by the gut upon consumption of nutrients, which then stimulates insulin release from β -cells in the pancreas. GIP is directly anabolic to osteoblasts and to bone, while gut-derived dopamine attenuates effects of GIP on osteoblast anabolic pathways, including LOX expression. GIP-stimulation of LOX expression was shown to be dependent on increased cAMP levels and protein kinase A activity, consistent with the fact that GIP receptors are G protein coupled receptors. Downstream signaling events resulting in increased LOX expression remain, however, unexplored. Here we provide evidence for β -catenin mediation of signaling from GIP to increase LOX expression. Moreover, we have identified a TCF/LEF element in the *Lox* promoter that is required for GIP-upregulation of LOX. These findings will be of importance in designing potential therapeutic approaches to address deficient LOX production in diabetic bone disease by pointing to the importance of exploring strategies to stimulate β -catenin signaling in osteoblasts under diabetic conditions as potential therapeutic strategies.

1. Introduction

Incretins are a class of gastric hormones released by intestinal enteroendocrine cells in response to the consumption of macronutrients, and are now well established as important contributors to glucose control in healthy and diabetic individuals (Drucker, 2006; Willard et al., 2020; Kim and Egan, 2008). The two major incretins, GLP-1 and GIP, facilitate postprandial insulin secretion from pancreatic β -cells to enhance the insulinotropic response to elevated blood glucose, a phenomenon known as the “incretin effect” (Drucker, 2006; Kim and Egan, 2008). Consequently, both incretins have been exploited therapeutically and developed as potential drug treatments for diabetes mellitus (Willard et al., 2020; Baggio and Drucker, 2020). These treatments, along with others, have led to major improvements in diabetes care and decreased mortality from diabetic complications (Meier, 2012; Zheng

et al., 2018; Gilbert and Pratley, 2020). However, effective glucose control, while clearly important for diabetes treatment, does not prevent some of the downstream, secondary effects of diabetes, including diabetic bone disease, a low bone formation osteopenia (McCabe et al., 2011; Palermo et al., 2017; Sellmeyer et al., 2016). This observation, combined with the ever increasing incidence of diabetes, necessitates a greater understanding of the intersection of systemic energy metabolism with bone turnover.

Receptors for GIP are expressed on both osteoblasts and osteocytes, and GIP has anabolic effects on bone (Bollag et al., 2000; Mieczkowska et al., 2015; Xie et al., 2007). Interestingly, GIP receptor (GIPR) KO mice have a similar bone phenotype to that observed in diabetes, i.e. defects in organic matrix and bone quality, but with minimal or no loss of mineral matrix parameters (Tsukiyama et al., 2006; Gaudin-Audrain et al., 2013; Mieczkowska et al., 2013). This prompted our laboratory to

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^{*} Corresponding author at: Forsyth Institute, Department of Applied Oral Sciences, 250 First Street, Cambridge, MA 02118, United States of America.
E-mail address: ptrackman@forsyth.org (P.C. Trackman).

¹ Present address: Massachusetts General Hospital and Harvard Medical School, Endocrine Unit, Massachusetts General Hospital, 50 Blossom Street Boston, MA, USA 02114.

² Present address: Forsyth Institute, Department of Applied Oral Sciences, 250 First Street, Cambridge, MA 02142.

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investigate the effects of GIP on extracellular matrix enzymes in the context of diabetes, specifically the collagen crosslinking enzyme lysyl oxidase (LOX). The importance of LOX in maintaining the bone collagen framework and consequently bone integrity has been well documented (Paschalis et al., 2011; McNerny et al., 2015; Saito and Marumo, 2010), and defects in enzymatic cross-linking have been observed in diabetic bone (Saito and Marumo, 2010; Saito et al., 2014; Saito et al., 2006). GIP directly upregulates LOX mRNA and protein in osteoblasts (Mieczkowska et al., 2015; Daley et al., 2019), and LOX expression is heavily downregulated in osteoblasts derived from a streptozotocin (STZ)-induced type I diabetes (T1DM) mouse model (Daley et al., 2019). Given the loss of responsiveness to GIP in the pancreas and other tissues in diabetes (Nauck et al., 1993; Krarup et al., 1987; Killion et al., 2020), we contend that impaired GIP signaling in osteoblasts results in decreased LOX expression and defects in the organic matrix of diabetic bone, which can contribute to increased known increased fracture risk in type 1 and type 2 diabetes (McCabe et al., 2011; Vestergaard, 2007; Ferrari et al., 2018) due to deficient LOX-dependent cross-linking [19, 21] and supported by our initial study in a type 1 diabetes mouse model (Daley et al., 2019).

Due to the potential importance of this GIP mediated LOX pathway in diabetic bone disease, it is important to understand the specific signaling mechanisms responsible for the upregulation of LOX in osteoblasts. The receptor for GIP is a class B G-protein coupled receptor, and in both pancreatic β -cells and osteoblasts has been shown to signal through a $G_s\alpha$ /cAMP/PKA mediated pathway (Drucker, 2006; Daley et al., 2019; Trümper et al., 2002). Our previous results show that LOX mRNA as well as LOX protein are significantly increased in osteoblasts in response to GIP treatment dependent on cAMP and PKA, suggesting that GIP regulation of LOX occurs subsequently at the transcription level (Daley et al., 2019). However, the mechanism by which this occurs remains unknown. The canonical WNT pathway activates β -catenin signaling in osteoblasts and osteoblast precursor cells, and is essential for osteoblast differentiation and proliferation (Monroe et al., 2012). Several recent studies have shown that WNT/ β -catenin signaling is impaired in osteoblasts in T1DM rodent models, resulting in decreased osteoblast activity (Chen et al., 2018; Yee et al., 2016). β -catenin promotes the transcription of target genes by complexing with TCF/LEF transcription factors (Liu and Habener, 2008), and our laboratory has previously reported that there are functional TCF/LEF cis-elements in the lysyl oxidase promoter (Khosravi et al., 2014). Based on these observations, we investigated whether GIP increases LOX expression in osteoblasts in a pathway that involves β -catenin/TCF/LEF mediated transcription downstream of GIP-induced cAMP levels, and that this pathway is impaired under diabetic conditions.

2. Methods

2.1. Cell culture

For initial signaling studies to elucidate the GIP signaling pathway in osteoblasts, the calvaria pre-osteoblast cell line MC3T3-E1-E1 was used (ATCC CRL-2593). Cells were cultured in α -MEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were replated in 6 well plates at a density of 1×10^6 cells per well.

2.2. LOX promoter studies

The 2.5 kbp mouse lysyl oxidase promoter DNA extending from -2,073 bp upstream from the translation start site to position +434 bp in pGL4.10 luciferase reporter construct (Promega cat# E6651) was employed. Site directed mutagenesis was performed using the Quick-Change II XL Site-Direct Mutagenesis Kit (Genomics Agilent cat#200521) to mutate three TCF/LEF conserved consensus sequences (Table 1) within the lysyl oxidase promoter. TCF/LEF binding elements were selected based on in silico analyses of putative element locations and conservation

Table 1

Wildtype and Mutant Sequences of TCF/LEF Elements in the Lysyl Oxidase Promoter.

| Mutated Site | Wildtype sequence | Mutant sequence |
|------------------------|-------------------|-----------------|
| Site #1: -913 to -906 | AAGCTTTGTTCCC | AAGCTTTGGCCCC |
| Site#2: -1321 to -1328 | CACCTTTGTTTAG | CACCTTTGGCTAG |
| Site#3: -1392 to -1385 | TTTCTTTGAATTT | TTTCTTTGGCTTT |

between species using PROMO v.3 and TRANSFAC 7.0 software (http://a.lggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3 and <http://www.gene-regulation.com/pub/databases.html#transfac>), all as previously described (Khosravi et al., 2014). FuGene-6 reagent (Roche catalogue # 11914443001) was used to transiently transfect MC3T3-E1 cells with the wildtype non-mutated lysyl oxidase promoter firefly-luciferase construct and the TCF/LEF mutated lysyl oxidase promoter firefly-luciferase constructs. Cells were co-transfected with the renilla luciferase thymidine kinase luciferase reporter construct as a normalization control for the degree of transfection (pRL-TK, Promega catalogue #E2241). DNA:FuGene-6 ratio was 1:3 (0.33 μ g DNA/1 μ L FuGene-6 per well of a 24-well plate to a final volume of 300 μ l per well). Cells were incubated with the DNA:FuGene-6 mixture in serum- and antibiotic-free media for 6 h. Cells were then fed with fresh medium, and after 24 h medium was replaced with serum-free medium containing 0.1% BSA. Cells were then treated with 10 nM GIP (determined to be optimal) for 4 h. Cells were lysed, and firefly and renilla luciferase activities were separately measured using a Dual Luciferase Reporter Assay System (Promega catalogue #E1910). The data were first normalized by calculating the ratio of firefly to renilla luciferase activity then presented as a fold change between GIP treated and control cells as a function of *Lox* promoter construct.

2.3. TOP/FLASH assays

To confirm TCF/LEF involvement in the GIP/LOX signaling pathway, a TOP/FLASH assay was performed (Qiagen Signal Reporter Assay Kit, TCF/LEF, catalogue #CCS-018 L). MC3T3-E1 cells were transfected with either a positive control, which is a constitutively active TCF/LEF luciferase reporter construct, a negative control, which is a mutated (inactive) TCF/LEF luciferase reporter construct, or an inducible TCF/LEF luciferase reporter construct. Cells were co-transfected with each of these constructs, respectively, with the same renilla luciferase reporter construct as in the *Lox* promoter studies described above to normalize for transfection efficiency. Cells were then treated with GIP for 4 h and luciferase activity measured and reported as a ratio of firefly to renilla luciferase activity. GIP-dependent increase in normalized firefly luciferase activity in intact TCF/LEF construct-transfected cells compared to mutated TCF/LEF construct-transfected cells would support GIP-dependent activation of TCF/LEF transcriptional activity.

2.4. β -catenin knockdown

To investigate β -catenin involvement in the GIP/LOX pathway, β -catenin expression was knocked down in MC3T3-E1 cells using a β -catenin shRNA construct (Sigma Aldrich, catalogue# SHCLND). Cells were transfected with the shRNA construct plasmid using FuGene6 as previously described (Khosravi et al., 2014). Cells were also transfected with an empty plasmid as well as a non-target shRNA construct against GFP as controls. Cells were grown to confluence for 24 h and then changed to medium containing 4 μ g/mL puromycin to select for transfected cells. After two days, cells were transfected with the previously described wild-type LOX promoter luciferase reporter construct, as well as the renilla luciferase reporter construct. Cells were then serum starved overnight and treated with GIP for 4 h. The data were first normalized by calculating the ratio of firefly to renilla luciferase activity then presented as a fold change between GIP treated and control cells.

Protein was also isolated by direct lysis of cells in protein sample buffer and analyzed by Western blot with a β -catenin (Santa Cruz catalogue #7963) and LOX antibody (Abcam, catalogue #Ab31238) to examine their respective protein levels.

2.5. Statistical analysis

All signaling experiments were run in triplicate and were performed three times. One way ANOVA with Tukey's post-hoc test for specific differences in between groups was employed for all analyses involving multiple groups, and student's *t*-test employed for all other analyses. Significance was declared as $p < 0.05$. Data are presented as means \pm standard deviation.

3. Results

3.1. β -catenin knockdown osteoblasts have decreased LOX protein expression

Our previous work on GIP signaling in osteoblasts reveals that, as in pancreatic β -cells, GIP signaling is mediated through the "classical" signaling pathway for class B GPCRs, whereby $G_s\alpha$ stimulates adenylyl cyclase to make cAMP that activates PKA, which phosphorylates a number of downstream targets. Inhibition at any step in this pathway prevents upregulation of LOX by GIP in osteoblasts (Daley et al., 2019). Although GIP treatment of osteoblasts activates CREB as expected (Daley et al., 2019; Mabileau et al., 2018), in silico analysis using PROMO v.3 and TRANSFAC 7.0 software revealed no CREB *cis*-elements in the 2.4 kbp regulatory region of the mouse and human *Lox* promoter. Some studies have shown cross-talk between the cAMP/PKA and WNT pathways in osteoblasts (Weivoda et al., 2016; Ramaswamy et al., 2017; Zhang et al., 2014), and PKA can phosphorylate β -catenin at Ser675, preventing its ubiquitination and subsequent degradation, allowing β -catenin to accumulate in the cytoplasm (Zhang et al., 2014; Chiang et al., 2012). We therefore sought to examine the role of β -catenin in GIP stimulation of LOX in osteoblasts. To do this, MC3T3-E1 calvaria osteoblasts were transfected with a shRNA β -catenin knockdown plasmid, or empty and non-target shRNA control plasmid. The western blot in Fig. 1A confirms that β -catenin protein levels in these cells is significantly reduced. These same cell lysates were then probed for LOX protein and results show that β -catenin knockdown cells express very little LOX protein (Fig. 1B), suggesting the involvement of β -catenin in LOX regulation in osteoblasts.

GIP stimulated increase in LOX transcriptional activity is dependent on β -catenin in osteoblasts.

To determine the involvement of β -catenin in GIP mediated LOX regulation, MC3T3-E1 cells were co-transfected with the same β -catenin

shRNA knockdown plasmid, as well as a luciferase reporter construct containing the LOX promoter. Our previous work showed that GIP treatment of osteoblasts results in a profound increase in LOX RNA transcripts (Daley et al., 2019). This is corroborated by the results in Fig. 2, which show that osteoblasts transfected with an empty control (no β -catenin knockdown) plasmid show a ~ 1.75 fold increase in LOX promoter activity in response to 10 nM GIP. Interestingly, in cells where β -catenin has been knocked down, there is a significant decrease in GIP stimulated LOX promoter activity compared to the non-target and empty vector controls. Taken together, these results point to a β -catenin dependent mechanism for GIP regulation of LOX.

3.2. GIP treatment activates TCF/LEF promoter elements

The canonical WNT pathway culminates in nuclear translocation of

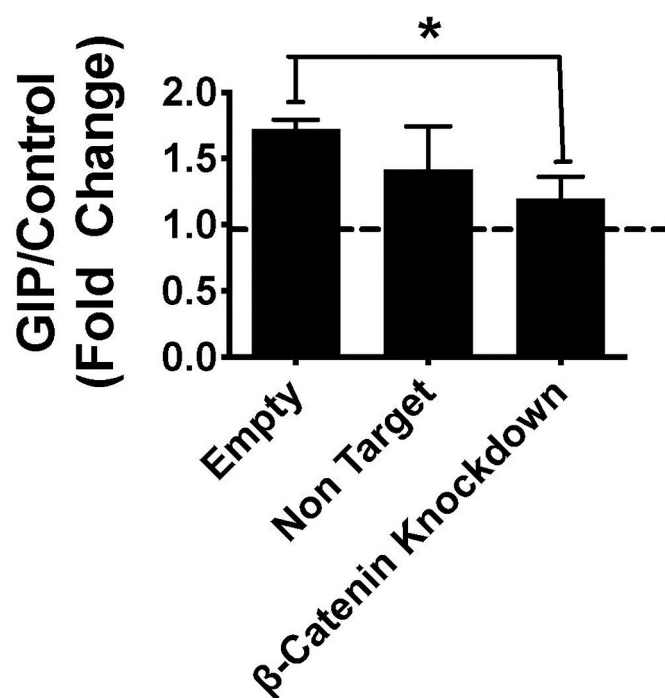


Fig. 2. β -Catenin shRNA inhibits GIP stimulated LOX promoter activity. Fold change in GIP-induced transcriptional activity compared to non-treated transfected cells. Data are pooled from three independent experiments. Data are means \pm SE, * $p < 0.05$ by One-way ANOVA and Tukey's test for multiple comparisons.

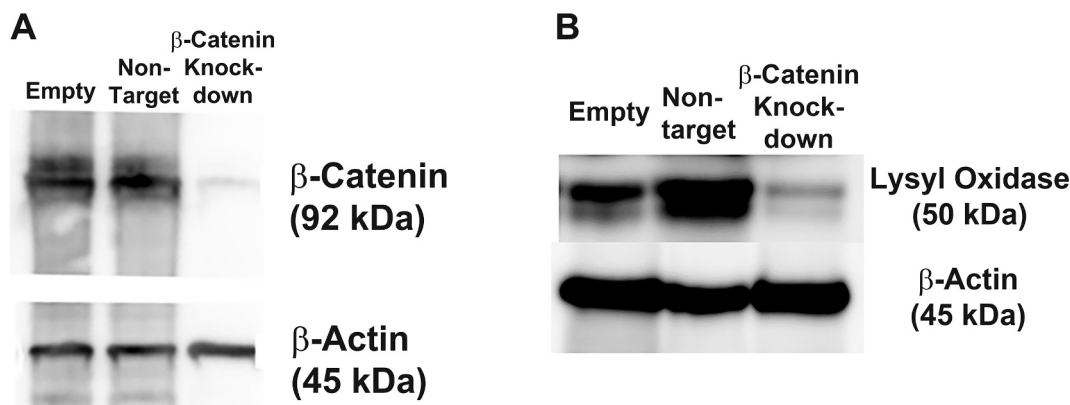


Fig. 1. β -Catenin shRNA knockdown in MC3T3 osteoblasts (A) express low levels of β -catenin resulting in (B) low LOX protein expression. Western blot analysis for β -catenin and LOX protein in β -catenin knockdown cell layer lysates from shRNA transfected MC3T3 cells, as described in Methods.

accumulated cytoplasmic β -catenin and co-activation of TCF/LEF transcription factors, which bind to corresponding TCF/LEF elements in target promoters (Monroe et al., 2012). To determine if GIP can activate TCF/LEF promoter element responses, a TOP-FLASH assay was implemented in MC3T3-E1 osteoblasts. Cells were transfected with either an inducible TCF/LEF luciferase reporter construct containing TCF/LEF responsive promoter elements, a constitutively active positive control construct, or a mutated negative control construct. In response to GIP treatment, inducible TCF/LEF promoter elements show a significant increase ($p < 0.001$) in transcriptional activity compared to untreated control cells, while there was no change in transcriptional activity in cells expressing the negative control mutated TCF/LEF promoter elements (Fig. 3). This confirms that GIP is able to activate TCF/LEF mediated transcription.

3.2.1. GIP-induced increases in LOX promoter activity depend on a TCF/LEF promoter element

Our laboratory has previously reported that there are putative TCF/LEF cis elements in the lysyl oxidase promoter, and has created and implemented mutated TCF/LEF element LOX promoter constructs to study the role of each of these TCF/LEF sites on LOX promoter activity (Khosravi et al., 2014) (Table 1). These constructs or wild type control promoter constructs were transfected into MC3T3-E1 cells and LOX promoter activity was measured using a luciferase reporter in response to GIP treatment. As with Fig. 2, Fig. 4 shows ~ 1.75 fold increase in LOX transcriptional activity in response 10 nM GIP treatment. However, this induction was not observed in cells that were transfected with a construct containing a mutation in a TCF/LEF conserved consensus sequence located -913 bp upstream of the transcriptional start site in the LOX promoter (site 1 TCF mutant), or in any co-transfection of this construct with other mutated constructs. Mutations in site 2 and site 3 TCF/LEF promoter elements had no effect on GIP stimulated LOX promoter activity. These data support the conclusion that the GIP signaling pathway intersects with the WNT signaling pathway in osteoblasts, culminating in the activation of a conserved TCF/LEF responsive element in the LOX promoter to increase LOX levels in these cells.

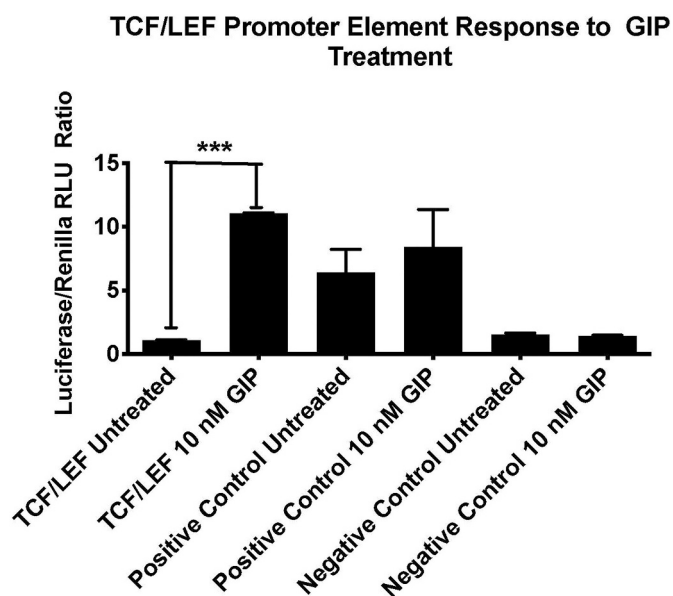


Fig. 3. TOP/FLASH assay for TCF/LEF promoter element response to GIP treatment in MC3T3 osteoblasts. TCF/LEF promoter elements show an increase in transcriptional activity in response to GIP treatment. Data are represented as a ratio of luciferase to renilla RLUs and are pooled from three independent experiments. Data are means \pm SE, *** $p < 0.001$ by One-way ANOVA followed by Tukey's test for multiple comparisons.

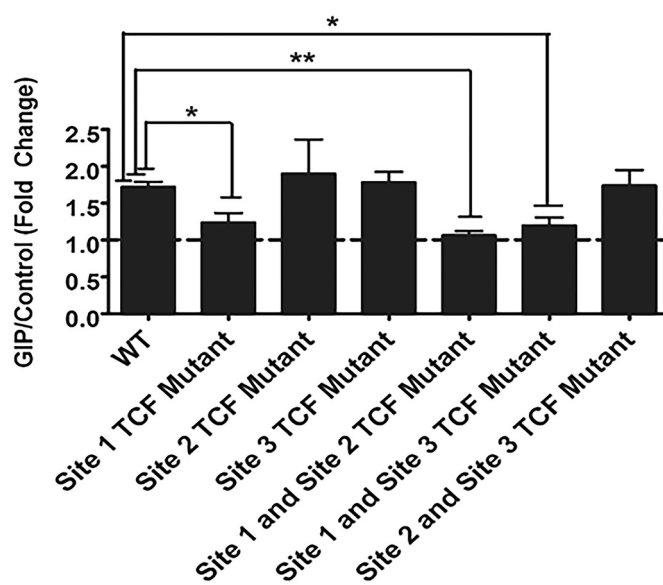


Fig. 4. Luciferase activity from the LOX promoter constructs in response to GIP treatment in MC3T3 cells. Data identify a cis-element for GIP regulation of lysyl oxidase located -913 to -906 upstream of the *Lox* transcription start site. The fold change of lysyl oxidase transcriptional activity of wild-type and mutated reporter constructs in response to GIP. Data are pooled from three independent experiments. Data are means \pm SE, * $p < 0.05$, ** $p < 0.01$ by One-way ANOVA followed by Tukey's test for multiple comparisons.

4. Discussion

It was originally thought that the sole function of GIP was to stimulate insulin secretion by targeting the pancreas. Now, however, the role of GIP in regulating metabolism in non-pancreatic tissues is greatly appreciated (Bollag et al., 2000; Xie et al., 2007; Yamada et al., 2016). We have previously reported that GIP upregulates lysyl oxidase in osteoblasts via the $G_s\alpha$ /cAMP/PKA pathway (Daley et al., 2019), and now have further elucidated the downstream effects in this pathway by revealing intersection with β -catenin dependent transcription in committed osteoblasts. This report is also the first to show a potential cis-element for GIP regulation of lysyl oxidase located -913 to -906 bp upstream of the LOX transcriptional start site. Based on these results, we propose a novel pathway for GIP regulation of lysyl oxidase in which 1) GIP binds to its receptor causing dissociation of the $G_s\alpha$ subunit and subsequent activation of adenylyl cyclase and cAMP production 2) cAMP activates PKA, which then phosphorylates β -catenin, preventing its ubiquitination and allowing its cytoplasmic accumulation 3) β -catenin translocates to the nucleus, forming a complex with TCF/LEF transcription factors that bind to TCF/LEF inducible cis-elements in the LOX promoter, resulting in increased transcription from the LOX promoter (Fig. 5).

Lysyl oxidase like-1 (LOXL1) is the second most abundant paralogue expressed by osteoblasts of the lysyl oxidase family, and like LOX (but unlike LOXL2, LOXL3 and LOXL4) LOXL1 is downregulated in osteoblasts in a model of type 1 diabetes, and up-regulated in vitro by GIP (Daley et al., 2019). In silico analysis of the LOXL1 promoter region revealed a cluster of five TCF/LEF elements within 1.5 kbp of the transcription start site, similar to the 3 TCF/LEF elements found within the 1.5 kbp upstream region of the *Lox* promoter (Fig. S1 and (Khosravi et al., 2014)). Although we did not analyze the functionality of these elements in the LOXL1 promoter, it is quite possible that β -catenin acting in concert with TCF/LEF transcription factors may mediate GIP regulation of both LOX and LOXL1 in osteoblasts. LOXL1 has been primarily linked to cross-linking of elastin in vascular and other elastin rich-tissues, however, LOXL1 knockout mice have a bone phenotype mostly in females that points to the relevance of LOXL1 regulation as a

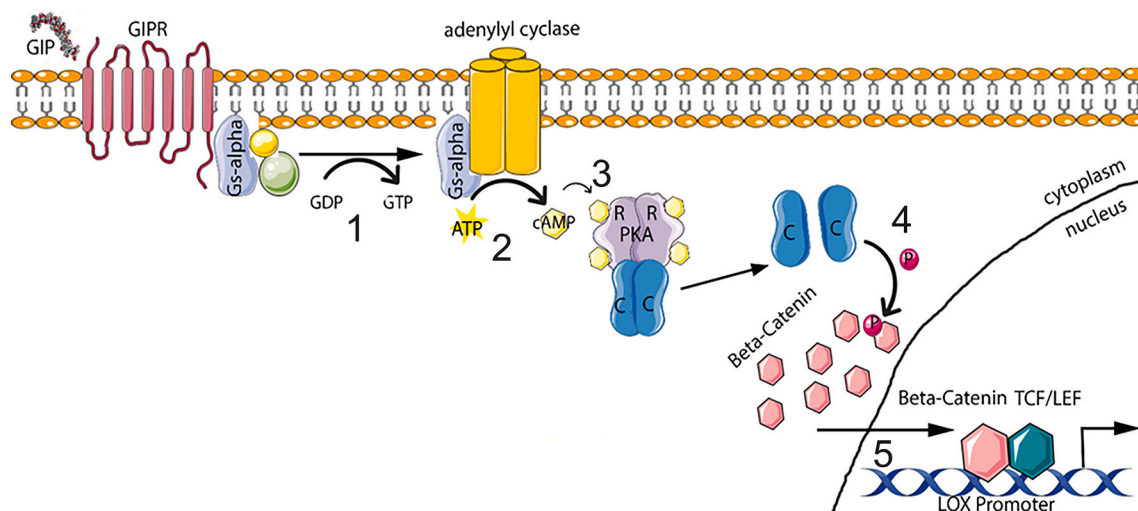


Fig. 5. Scheme summarizing the pathway for GIP-stimulated LOX expression in osteoblasts. 1) GIP binds to its receptor (a GPCR) causing the dissociation of G α subunit 2) G α stimulates adenylyl cyclase to make cAMP from ATP 3) cAMP binds to the regulatory subunits of PKA (R), causing activation of the catalytic subunits (C). 4) PKA catalytic subunit (C) phosphorylates β -catenin at Ser675, preventing ubiquitination and allowing cytoplasmic accumulation 5) β -catenin translocates to the nucleus, forming a complex with TCF/LEF transcription factors that bind to TCF/LEF inducible cis-elements in the LOX promoter, resulting in increased transcription from the LOX promoter.

contributor to bone structure (Alsofi et al., 2016).

Previous work done by our group reveals that the canonical WNT pathway ligand Wnt3a can upregulate lysyl oxidase in pluripotent mesenchymal precursor cells, despite the inability of these cells to produce any appreciable matrix (Khosravi et al., 2014). This led to the novel discovery that lysyl oxidase is required for the proliferation of progenitor cells, and may be responsible for creating a reservoir of osteoblast precursors that can later differentiate into mature osteoblasts (Khosravi et al., 2014). Our current study complements these results by showing that committed osteoblasts also utilize signaling intermediates in common with the WNT pathway, albeit in response to GIP, to upregulate lysyl oxidase. This later-stage up-regulation of LOX is more likely related to its classic role in crosslinking and stabilizing the collagen matrix framework of bone, consistent with its increased expression observed in differentiating osteoblast cultures (Hong et al., 2004). These studies emphasize the importance of β -catenin and TCF/LEF elements regulation of LOX in mesenchymal bone cells in different combinations at multiple stages of bone cell differentiation.

It is important to note that the canonical Wnt pathway was previously found to upregulate LOX in progenitor cells but not in committed osteoblasts through the TCF/LEF site at -1321 to -1328 upstream of the translation start site (Khosravi et al., 2014), while the -913 to -906 site was found to mediate β -catenin effects here in committed osteoblast MC3T3 cells. This finding suggests that β -catenin is activated by different mechanisms in progenitor compared to committed osteoblasts, and is consistent with the lack of effect of Wnt3a to stimulate LOX transcription in committed osteoblasts compared to mesenchymal progenitor cells. These differences point to the existence of a central and critical role for β -catenin activation for the expression of LOX by independent up-stream stimuli, and point to potential therapeutic approaches that may independently stimulate β -catenin activation in osteoblasts under diabetic conditions. For example, PKA can directly stabilize β -catenin by phosphorylation at Ser675 as in the present study (Fig. 5) and (Zhang et al., 2014), or alternatively AKT can lead to phosphorylation of GSK3 β and inhibit its subsequent phosphorylation of β -catenin (Pan et al., 2019). Both pathways lead to stabilization and nuclear localization of β -catenin and increased β -catenin nuclear co-activating transcriptional activity. Additional signaling pathways have also been implicated as being activated by GIP in osteoblasts including p38, CREB, AMPka2 and STAT (Mabilleau et al., 2018). As noted, the LOX promoter lacks CREB cis-elements in the proximal promoter region,

but the additional signaling pathways regulating LOX and LOXL1 in response to GIP cannot be excluded at this time. It is also possible that some GIP effects on bone can be indirect, since GIP has been localized to a variety of other tissues, as has GIP receptor (Mabilleau et al., 2018), but direct effects of GIP on osteoblasts and implications for bone health is clearly important and requires additional investigation.

Several recent studies have shown the dysregulation of β -catenin signaling in many tissues in diabetes, including in osteoblasts (Yee et al., 2016; Chiang et al., 2012; Welters and Kulkarni, 2008; Jin, 2008). Reduced WNT/ β -catenin signaling leads to impaired osteoblast activity in several mouse models of diabetes, including the streptozotocin (STZ)-induced T1DM mouse model used in our previous work (Daley et al., 2019; Chen et al., 2018; Yee et al., 2016). Previous studies have shown that diabetic bone abnormalities resulting from T1DM more severely affect the trabecular rather than the cortical bone (Daley et al., 2019; Shah et al., 2018; Jiang and Xia, 2018), and trabecular bone is known to be more readily affected by changes in β -catenin (Li et al., 2017). In a recent study by Chen et al., T1DM was induced by STZ injection in mice expressing constitutively activated β -catenin, in an effort to reverse the bone loss seen in T1DM mice. Interestingly, the increase in bone mass observed in mice with activated β -catenin occurred only in the trabecular bone (Chen et al., 2018). In a similar study by Yee et al. using an antibody against the WNT antagonist sclerostin in a STZ-T1DM mouse model, antibody treatment also increased bone mass compared to untreated controls; however bone mineral density did not increase, and even decreased in the cortical bone (Yee et al., 2016). Taken together with our previous results showing a dramatic decrease in LOX expression in diabetic bone (Daley et al., 2019), and the current study results showing decreased LOX expression in β -catenin knockdown osteoblasts, it is very likely that the bone abnormalities in T1DM are partially due to a defect in matrix synthesis as a result of impaired β -catenin regulation of LOX.

This study reveals an important link between the decreased responsiveness to GIP and the impaired β -catenin signaling seen in osteoblasts in diabetic conditions. Previous studies have shown that incretins signal through β -catenin and activate TCF7L2 transcription factors to increase pancreatic β -cell proliferation (Liu and Habener, 2008), so their utilization of the same pathway in osteoblasts is not entirely surprising. Despite the appealing benefits of GIP for the treatment of diabetic bone disease, its therapeutic potential is controversial because of tissue desensitization to GIP (Nauck et al., 1993; Killion et al.,

2020), or anti-incretins that may be elevated in diabetes (Chaudhry et al., 2016). Our previous results demonstrate that this attenuated GIP activity in osteoblasts could be due to increased circulating dopamine in diabetes, since dopamine was shown to antagonize GIP stimulated increases in cAMP and LOX expression in osteoblasts (Daley et al., 2019). Treatment of mice with amisulpride, a dopamine receptor inhibitor, provided partial rescue of the bone phenotype in STZ induced T1DM mice, but not a complete reversal (Daley et al., 2019). Based on the results presented here, it is reasonable to conclude that the combination of amisulpride with a β -catenin stabilization strategy could enhance the anabolic effects of amisulpride on bone by leading to increased LOX synthesis and a greater improvement in the diabetic bone defect improvements observed with amisulpride alone. Such strategy could include Wnt-independent activation of GSK3 β , possibly by Ras-mediated pathways converging on AKT and GSK3 β (Pan et al., 2019; Chiaradonna et al., 2008). The unique connection presented here between incretin effects and β -catenin signaling in the context of diabetic osteopenia could aid in the development of possible new potential treatment strategies for this debilitating disease.

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Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of competing interest

The authors declare no financial or any other conflicts of interest related to the initiation or completion of this work. This is also indicated on the title page of the manuscript.

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