FEBS Letters 584 (2010) 3095–3100

journal homepage: www.FEBSLetters.org

Alterations of the Wnt/β-catenin pathway and its target genes for the N- and C-terminal domains of parathyroid hormone-related protein in bone from diabetic mice

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ARTICLE INFO

Article history:
Received 18 March 2010
Revised 20 April 2010
Accepted 20 May 2010
Available online 1 June 2010

Edited by Zhijie Chang

Keywords:
Type 1 diabetes mellitus
Bone formation
PTHrP
Wnt/β-catenin pathway

ABSTRACT

Type 1 diabetes mellitus (T1D) is associated with bone loss. Given that the Wnt/β-catenin pathway is a major regulator of bone accrual, we assessed this pathway in mice with streptozotozin-induced T1D. In diabetic mouse long bones, we found alterations favouring the suppression of this pathway by using PCR arrays and β-catenin immunostaining. Downregulation of sclerostin, an inhibitor of this pathway, also occurred, and related to increased osteocyte apoptosis. Our data show that both N- and C-terminal parathyroid hormone-related peptide fragments might exert osteogenic effects in this setting by targeting several genes of this pathway and increasing β-catenin in osteoblastic cells.

1. Introduction

Type 1 diabetes mellitus (T1D) is usually associated with a decreased bone mass and a higher risk of bone fractures, but the underlying mechanisms remain ill defined [1,2].

The Wnt/β-catenin pathway (Wnt-pahway) is an important modulator of bone formation and repair [3,4]. It is activated upon binding of different Wnt glycoproteins to Frizzled receptors and with low density receptor-like proteins (LRP) 5 and 6 as coreceptors [5]. This is followed by glycogen synthase kinase-3β inactivation by phosphorylation, allowing the stabilization of β-catenin, which then binds to transcription factor 4/lymphoid enhancer-binding factor 1 complex inducing transcription of key osteoblastic genes, namely Runx2 and osteoprotegerin [6,7]. In humans, loss or gain of function mutations in LRP5 leads, respectively, to osteoporosis-pseudoglioma syndrome or high bone mass phenotype; whereas downregulation of Sost/sclerostin, an endogenous inhibitor of the Wnt-pathway, produces sclereostosis and Van Bunchem disease [8].

Parathyroid hormone (PTH) related protein (PTHrP) is an important regulator of osteoblast function [9]. Its N-terminal fragment, sharing homology with PTH, can induce bone anabolic actions through the PTH receptor 1. In addition, the C-terminal 107-139 domain of PTHrP has been shown to display antiresorptive and osteogenic features [10–12]. Recently, both PTHrP domains have been shown to improve bone regeneration after marrow ablation in mice with streptozotocin (STZ)-induced T1D [13,14].

In the present study, we investigated the alterations in the Wnt-pathway induced by T1D, and the putative modulation by the N- and C-terminal PTHrP domains in mice.

2. Materials and methods

2.1. T1D mouse model and treatments

Male CD-1 mice (39 ± 2 g body weight) were treated with STZ (45 μg/g body weight in 50 mM citrate buffer, pH 4.5) or buffer alone (controls) for 5 d. Mice were considered diabetic when blood glucose were ⩾300 mg/dl. Two weeks later, diabetic mice were treated with PTHrP (1–36) or PTHrP (107–139) (100 ng/g/every other day, s.c.) or vehicle (50 mM KCl, pH 4.5) for two weeks. One day after the last injection the animals were sacrificed and femurs and tibiae were collected and assigned to total RNA extraction and histological processing, respectively. The number of

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0014-5793/$36.00
doi:10.1016/j.febslet.2010.05.047

mice per group was 5. No changes in the activity of mice in the different experimental groups was observed during the study. Animal protocols followed ethical guidelines approved by Institutional Animal Care and Use Board at Fundación Jiménez Díaz.

2.2. Dual-energy X-ray absorptiometry (DXA)

Bone mineral density (BMD), bone mineral content (BMC) and fat content of the intact femur were measured in anesthetized control and diabetic mice at the beginning of the study and at the end of treatments by DXA using PIXImus (GE Lunar Corp., Madison, WI) [13].

2.3. PCR array and real time PCR

Total RNA was extracted from the intact femurs with Trizol (Invitrogen, Groningen, The Netherlands). A PCR array comprising up to 82 genes related to the Wnt-pathway (SAbioscience, Frederick, MD) was then carried out. Total RNA from five different mice in each experimental group was pooled (3 μg total) before performing retrotranscription with RT^2 First strand kit (SAbiosciences) followed by PCR array according to manufacturer's instructions. Results were normalized with the less variable house keeping gene available in the array (among 5 possible), ribosomal protein L13a, and a significant ($P < 0.05$) 1.5-fold change over control was the selected criteria for an experimental gene expression change, similarly to other studies of this kind [15]. Results were analyzed with the SAbioscience PCR array tool (http://www.sabiosciences.com/pcr/arrayanalysis.php). To validate the PCR array, real time PCR was done with the same cDNA from the total RNA pool described above, and was repeated three times for each gene, using *Sybr premix ex Taq* (Takara, Otsu, Japan) and the following primers: 5′-CTTCGGCAAGATCGTCAACC-3′, 5′-GCGAAGAT GAACGCTGTTTCT-3′ (Wnt1); 5′-TGGTATGGGCAAGAAAAAGA-3′, 5′-GTGACGCCCAATACCCATTA-3′ [casein kinase 1a1 (Csnk1a1)]; 5′-ATGCTCTTAGCTGAGGTGCCCG-3′, 5′-ATTCCTAGCTGCGGTATCC AGG-3′ [18S rRNA(housekeeping gene)]. These genes were chosen as their expression was altered in at least two experimental groups tested in the PCR array. Gene expression was normalized with that of the housekeeping gene 18S. *Sost* was not included in the PCR array, but it was analyzed by real time PCR, using unlabeled mouse specific primers and TaqMan MGB probes (Assay-by-Design^{SM}, Applied Biosystems, Foster City, CA) in an ABI PRISM 7500 system (Applied Biosystems). Results were expressed in mRNA copy numbers, calculated for each sample using the cycle threshold (Ct) value, and normalized against 18S rRNA as described [10,13].

2.4. Sclerostin and β-catenin immunostaining

Mouse tibiae were fixed in neutral formaldehyde, and subsequently were decalcified with Osteosoft® (Merck, Darmstadt, Germany), dehydrated, and embedded in paraffin. Sclerostin and β-catenin immunostaining were carried out on three different saggital 4-μm sections from each mouse per experimental group. For the former, a goat polyclonal anti-sclerostin antibody (R&D, Minneapolis, MN), at 1:100 dilution, was used following a recently described procedure [12]. For β-catenin immunohistochemistry, antigen retrieval was done by incubation with 1% trypsin (Lonza, Verviers, Belgium) for 30 min at 37 °C. Then, the tissue samples were incubated with blocking solution (4% bovine serum albumin, 6% goat serum) for 1 h, following by incubation with a rabbit polyclonal anti-β-catenin antibody (Abcam, Cambridge, UK), at 1/200 dilution, overnight at 4 °C. Sections were subsequently incubated with Envission-Flex® (Dako, Glostrup, Denmark) and 3,3′-diaminobenzidine as chromogen. Sections were counterstained with haematoxylin (Sigma–Aldrich, St. Louis, MO). Some tissue samples were incubated without the primary antibody as negative controls.

Table 1
Bone mass and fat content in the femur from control and diabetic mice, treated or not with PTHrP (1–36) or PTHrP (107–139).

	Control	T1D	T1D + PTHrP (1–36)	T1D + PTHrP (107–139)
BMD (g/cm^2)	0.087 ± 0.0008	0.079 ± 0.0002*	0.084 ± 0.0002**	0.088 ± 0.0006**
BMC (g)	0.055 ± 0.0012	0.041 ± 0.0008*	0.054 ± 0.001**	0.056 ± 0.003**
% Fat	13.54 ± 0.11	15.22 ± 0.15*	13.53 ± 0.19**	13.11 ± 0.24**

BMD, bone mineral density; BMC, bone mineral content. Values are mean ± S.E.M. of 5 mice per group. *$P < 0.05$ vs control; **$P < 0.05$ vs T1D.

Table 2
Expression of Wnt-Pathway genes by PCR array in the mouse intact femur of every experimental group.

Group	Gene	Function	PCR array Value
T1D vs control	Wif1	Wnt-pathway inhibition	−1.8
	Wisp1	Regulation of growth	−4.8
	Wnt1	Wnt-pathway activation	−2.9
T1D vs T1D + PTHrP(1–36)	Ccnd1	Regulation of cell cycle	−1.9
	Csnk1a1	Protein kinase activity	−3.8
	Fosl1	Transcription regulation	−1.8
	Jun	Transcription factor	−3.0
	Myc	Transcription factor	−1.8
	Tle2	Transcription regulation	1.9
	Wisp1	Regulation of growth	4.0
	Wnt1	Wnt-pathway activation	−7.1
T1D vs T1D + PTHrP (107–139)	Csnk1a1	Protein kinase activity	−1.7
	Cxxc4	Wnt-pathway inhibition	2.2
	Foxn1	Transcription factor	2.0
	Fshβ	Hormone	2.0
	Wnt11	Wnt-pathway activation	2.7

Total RNA from 5 femurs was pooled for cDNA synthesis. Three independent measurements were performed with the same cDNA to obtain the mean in each experimental group.

Total and sclerostin or β-catenin positive osteocytes were counted in 4 to 10 random high power (×200–400) fields per sample in a cortical bone segment between the growth plate and the mid-diaphysis. Osteoblasts positive for β-catenin were counted in 4-6 random ×200-fields per sample in an area subjacent to the growth plate. Stainings were evaluated by three independent observers in a blinded fashion.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Apoptotic osteocytes were also evaluated on 4-μm sections in the same cortical area of the tibia described above by highly sensitive TUNEL staining using the Klenow fragment of the DNA polymerase I (FragEL™ DNA Fragmentation Detection Kit, Calbiochem, Gibbstown, NJ) [16], following the manufacturer's instructions. Sections were counterstained with methyl green. Apoptotic and non-apoptotic osteocytes were counted in 4 different bone samples from each mouse per group.

2.6. Statistics

Results are mean ± S.E.M. Statistical analysis was performed by *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Bone parameters in T1D mice

First, we aimed to confirm that diabetic mice had a decreased bone mass at the time of study. As described in Table 1, femoral BMD was decreased in diabetic mice by regards to controls, consistent with our recent findings in this model [13]. Fat content was significantly increased in the femur of the diabetic mice. Treatment of the latter mice with either N- or C-terminal PTHrP peptides normalized these T1D-related alterations.

3.2. T1D affects expression of Wnt-pathway genes in the mouse femur

We next examined whether diabetic mice would have any alterations in the Wnt-pathway gene expression. We found a significant downregulation of Wif1, Wisp1 and Wnt1 in the femur of these mice (Table 2). PTHrP (1–36) treatment in these mice was shown to significantly reduce the expression of the following genes: the cell cycle regulator cyclin kinase 1; the transcription factors c-Jun and c-Myc; the transcription regulator Fosl1; the serine/threonine kinase Csnk1a1; the cytoskeleton formation protein Dishevelled; and the Wnt-pathway activator Wnt1. On the other hand, this PTHrP peptide upregulated the transcription regulator Tle2 and the member of the CNN familiy of growth factors Wisp1. Treatment with PTHrP (107–139), similarly to the N-terminal PTHrP fragment, downregulated Csnk1a1, but in contrast to the latter fragment, upregulated folicule stimulation hormone (Fshβ), the transcription factor Foxn1, the Wnt-pathway inhibitor Cxxc4, and the Wnt-pathway activator Wnt11. These changes in Wnt1 and Csnk1a1 were confirmed by real time PCR as described in Section 2 (Fig. 1).

3.3. T1D downregulates Sost/sclerostin in the mouse tibia

One of the major regulators of Wnt-pathway is the product of *Sost* gene, sclerostin, which is only expressed in osteocytes [17]. We found that *Sost* expression, as well as the number of total and sclerostin-positive osteocytes, were decreased in the diabetic mouse tibia; and administration of either PTHrP peptide reversed this T1D-related effect (Fig. 2A and B).

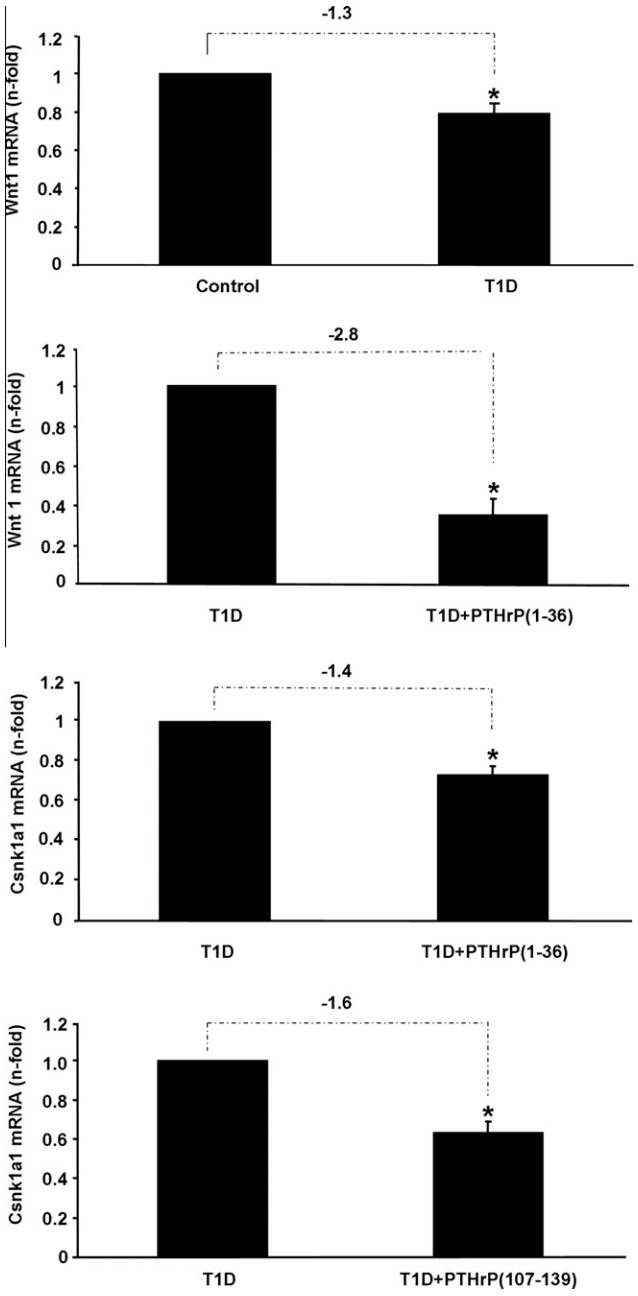

Fig. 1. Changes in Wnt-1 and CsnK1a1 gene expression by real time PCR in the mouse femur of every experimental group as indicated. The numbers above bars represent fold. Results are mean ± S.E.M. (n = 3). *$P < 0.05$ vs control or T1D in each case.

3.4. Osteocyte apoptosis is increased in T1D mice

A diminished number of functional osteoblasts occurs associated with diabetes-related osteopenia [13,18,19]. Given that the number of total osteocytes was decreased in the diabetic mice, we wanted to explore whether this decrease was paralleled with an increased osteocyte apoptosis estimated by TUNEL assay. T1D was associated with an increased number of apoptotic osteocytes in the mouse tibia, but it was significantly lower in the diabetic mice treated with either N- or C-terminal PTHrP peptide (Fig. 3).

3.5. β-catenin is downregulated in the diabetic mouse tibia

We also carried out inmunohistochemistry for β-catenin, the canonical Wnt-pathway final effector, in the diabetic mouse tibia.

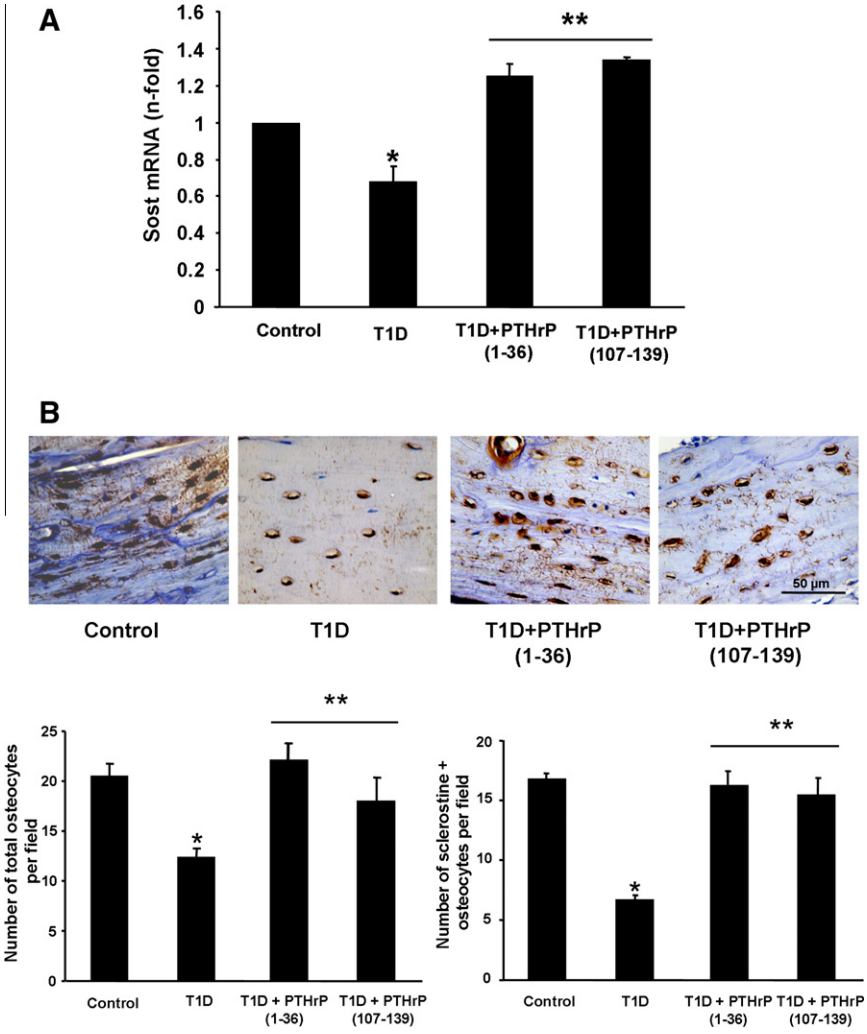

Fig. 2. (A) Changes in *Sost* gene expression by real time PCR in the mouse tibia from diabetic mice, treated or not with each PTHrP peptide. (B) Total and sclerostin-positive osteocytes in the cortical mouse tibia in every experimental group. Representative images are shown. All of the images have equal magnification. Results are mean ± S.E.M. of 4 mice per experimental group. $^{*}P < 0.05$ vs control; $^{**}P < 0.05$ vs T1D.

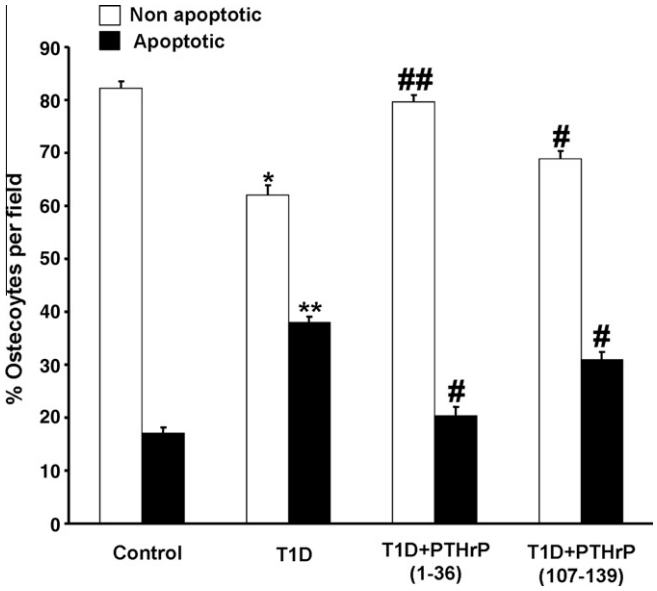

Fig. 3. Osteocyte apoptosis by TUNEL assay in the cortical mouse tibia in every experimental group. Results are mean ± S.E.M. of 4 mice per experimental group. $^{*}P < 0.05$; $^{**}P < 0.01$ vs corresponding control. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs corresponding T1D.

As shown in Fig. 4, β-catenin immunostaining was decreased in both trabecular osteoblasts and osteocytes in the diabetic mice. Interestingly, the decrease in β-catenin positivity in the latter cells was higher than that in the total osteocyte number, indicating that it cannot be accounted for by decreased cell viability (Figs. 2B and 4A). Moreover, the number of osteoblasts showing β-catenin staining in the nucleus was significantly lower in the diabetic mice than in controls. Administration of each N- or C-terminal PTHrP fragment reversed these changes in β-catenin staining levels (Fig. 4B).

4. Discussion

Poorly controlled T1D has been related to the development of bone mass loss [2]. Although we have a poor knowledge about the underlying mechanism of this feature, high glucose has been shown to affect osteoblast viability and function [13]. Our aim here was to provide further insights into the deleterious effects of T1D in bone.

The Wnt-pathway is a major regulator of bone accrual [8]. Analyzing the PCR array results, we found only three altered genes of this pathway in the diabetic mouse femur. Although Wif1, an inhibitor of this pathway [20], was downregulated, the concomitant suppression of Wisp1, a final product of the pathway implicated in growth regulation [21], and of the activator Wnt1, suggests that inactivation of Wnt-pathway might predominate, related to bone loss in diabetic mice.

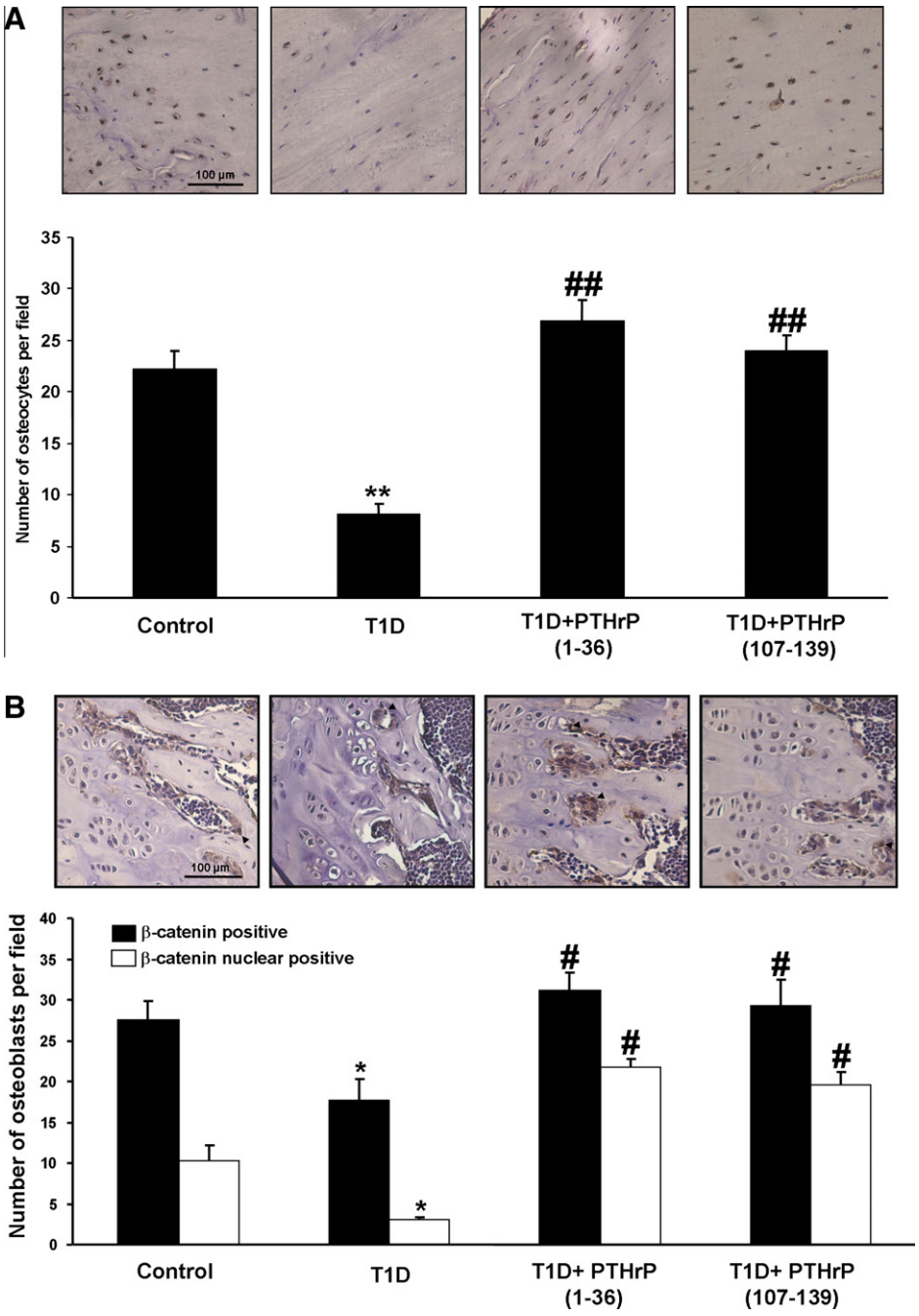

Fig. 4. β-catening staining in osteocytes and osteoblasts. (A) The number of β-catening positive osteocytes per field in the cortical mouse tibia is shown. $^{**}P < 0.01$ vs control; $\#\#P < 0.01$ vs T1D. (B) The number of β-catening positive osteoblasts per field (solid bars) and those presenting nuclear staining (clear bars) in an area subjacent to growth plate in the mouse tibia are shown. Each set of images has equal magnification. Black arrow heads point to osteoblasts with β-catenin nuclear positive staining. $^{*}P < 0.05$ vs control; $\#P < 0.05$ vs T1D. Representative images are shown. Results are mean ± S.E.M. of 4 mice per experimental group.

Both PTHrP peptides tested downregulated Csnk1a1, that together with GSK-3β can phosphorylate β-catenin leading to its degradation [22], in diabetic mice; suggesting that their similar osteogenic effect in these animals might be mediated through downregulation of this enzyme. It is also noteworthy that PTHrP (1–36) administration reversed the observed decrease of Wisp1 expression in the diabetic mice, whereas PTHrP (107–139) increased the expression of another activator of the Wnt-pathway, Wnt11. This PTHrP peptide, also upregulated the levels of final targets of this pathway such as the transcription factor implicated in development Foxn1 [23] and Fshβ [24]. However, these PTHrP peptides induced other changes in the femur of diabetic mice that are apparently contradictory with a putative activation of this pathway. Thus, PTHrP (1–36) upregulated the inhibitor Tle2 [25], and donwregulated cyclin 1 as well as Wnt1, and final targets such as Fosl1 [26], c-Myc [27] and c-Jun [28]. Meanwhile, PTHrP (107–139) treatment upregulated Cxxc4, an inhibitor of Wnt-pathway [29].

We found a downregulation of sclerostin, an inhibitor of the Wnt-pathway, in the T1D mouse tibia, in contrast to what we would have expected. It is unlikely that this might be a consequence of lethargy and decreased activity in these mice, but instead it can be accounted for by a reduction in osteocyte viability. On the other hand, administration of each PTHrP peptide counteracts these

effects of T1D on both *Sost*/sclerostin expression and osteocyte death. This supports the notion that one mechanism whereby these PTHrP peptides might affect bone turnover would involve the osteocyte as a cell target in these mice.

A deleterious effect of T1D on the Wnt-pathway was supported by the finding that total and nuclear β-catenin staining was lower in osteocytes and osteoblasts in the diabetic mouse tibia. Since β-catenin is the major transcription factor involved in the canonical Wnt-pathway, this strongly suggests its inactivation by T1D. Moreover, treatment with either PTHrP peptide induced β-catenin stabilization in the diabetic mice, further supporting their osteogenic action in this setting.

Collectively, these findings show complex effects of both T1D and the PTHrP peptides tested on the Wnt-pathway in the mouse femur. Our data suggest that an inhibition of this pathway occurs associated with T1D-related osteopenia. We identified some Wnt-pathway components which are unaffected by T1D but can be targets for both PTHrP peptides to activate this pathway and thus improve bone formation.

Acknowledgements

We thank A. F. Stewart and A. García-Ocaña (University of Pittsburgh School of Medicine, Pittsburgh, PA) for supplying human PTHrP (1–36) and F. Roncal (Centro Nacional de Biotecnología, Madrid) for human PTHrP (107–139) synthesis. This study was supported by grants from Instituto de Salud Carlos III (ISCIII; PI050117, PI080922, RETICEF RD06/0013/1002 and RD06/0013/1009), Ministerio de Educación y Ciencia (SAF2005-05254), and Fundación de Investigación Médica Mutua Madrileña. L.FdC. and D.L. are predoctoral fellows of Fundación Conchita Rábago. D.L. was the recipient of a travel grant to present part of this work at the 36th European Calcified Tissue Society in Vienna (Austria) on 23–27 May, 2009. S. P-N. is the recipient of a research contract from ISCIII (RETICEF RD06/0013/1002).

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