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A transgenic, mesodermal specific, DKK1 mouse model recapitulates a spectrum of human congenital limb reduction defects

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SUMMARY

Congenital limb reduction defects occurring in isolation of other developmental abnormalities continue to be an important medical problem in which little progress has been made. Herein we generated transgenic mice expressing *Dkk1* in an appendicular mesodermal pattern. *Prx1-Dkk1* mice recapitulate a full spectrum of human congenital limb reduction defects, without other developmental issues, and have normal life-spans. Importantly, a close examination of the inheritance pattern suggests that there is a significant degree of incomplete penetrance as progeny of phenotypically positive or phenotypically negative, but genotypically positive *Prx1-Dkk1* mice, consistently give rise to both phenotypically positive mice and phenotypically normal-appearing mice. Thus, this heterogeneous phenotype is reproducible with each generation regardless of the phenotype of the parents. We further go on to identify that mesenchymal stem cells from *Prx1-Dkk1* mice have limited proliferative ability, but normal differentiation potential which may explain the mechanism for the limb reduction defects observed. We believe *Prx1-Dkk1* mice may prove useful in the future to study the mechanisms underlying the development of congenital limb reduction defects.

Keywords

Dkk1; limb reduction; mesenchymal stem cell

INTRODUCTION

The prevalence of congenital limb reduction defects occurring in the absence of known chromosomal anomalies has been recently reported to be at more than 1 per 1000 births in Europe (Dolk et al., 2010). The consequent morbidity associated with congenital limb reduction defects (mostly surgically uncorrectable (Furniss et al., 2009)) makes elucidating the etiology and mechanisms involved an important issue for both medicine and society. The societal impact of one type of congenital limb reduction defect (namely phocomelia in which hands and feet are attached to abbreviated arms and legs) was sadly felt in the late 1950s when 10,000 children possessing a spectrum of such defects were born worldwide to

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women prescribed the drug thalidomide for “morning sickness” (Franks et al., 2004). Although subsequently removed from the medical market, thalidomide and thalidomide-based analogues have made a clinical resurgence as potent anti-leprosy (Chen et al., 2010; Teo et al., 2002; Teo et al., 2005), anti-inflammatory (Ladizinski et al., 2010) and anti-cancer drugs (Bamias and Dimopoulos, 2005; Cox et al., 2006; Eleutherakis-Papaïakovou et al., 2004). Since newer, more potent thalidomide analogues are now in clinical trials for the latter and other indications, serious safety concerns have been raised (e.g., “black box” warnings (Bwire et al., 2011)). As for most cases of congenital limb reduction defects (and thalidomide-induced phocomelia as well) the underlying mechanism is still remain unclear and is further complicated by the fact that mice are not sensitive to thalidomide’s teratogenicity (Paravar and Lee, 2008; Vargesson, 2009). Thus, given the sporadic nature of congenital limb reduction defects, the lack of a defined genetic component, and our inability to study a thalidomide phenotype in mice (and thus learn from the most consistent environmental exposure that recapitulates one type of congenital limb reduction defect), progress in studying congenital limb reduction defects in mammalian systems has been slow.

Although the mechanism has not been completely elucidated, work in thalidomide-sensitive model systems (i.e., chickens) have implicated the Wnt inhibitor, Dickkopf-1 (Dkk1), as a possible mediator of thalidomide-induced skeletal defects (Knobloch et al., 2008; Knobloch et al., 2007). Work in other vertebrate model systems have demonstrated the importance of Dkk1-regulated Wnt signaling to proper embryologic development particularly of the developing limb. Previous loss-of-function studies in mice which addressed the role of Wnt signaling in limb development have supported the crucial role of Wnt signaling in skeletal development. However, much of these studies either focused on the role of Wnt signaling in the apical ectodermal ridge (AER) where it is difficult to dissociate mesenchymal and ectodermal contributions to the phenotypes observed (Barrow et al., 2003; Soshnikova et al., 2003), or used late promoters resulting in mesenchymal lineage-specific differentiation blocks and lineage switching (Arango et al., 2005; Glass et al., 2005; Hill et al., 2005) without limb defects. The one previously generated transgenic mouse in which the downstream effector of Wnt signaling, β -catenin was specifically deleted in the mesenchyme did show a wide spectrum of shortened forelimb and hindlimbs (Hill et al., 2006). However, the latter mice died shortly after birth for unclear reasons. Thus, a viable transgenic mouse model capable of recapitulating the specific, but full, spectrum of human congenital limb reduction defects is still lacking.

Herein we report on the generation and characterization of the Prx1-Dkk1 transgenic mouse. Transgenic mice expressing Dkk1 under the control of the Prx1 promoter (tissue specific for the appendicular skeleton (Logan et al., 2002)) have a wide spectrum of congenital limb reduction defects without concomitant defects in other organs and have normal longevity. Congenital limb reduction defects range from one limb to four and from mild to severe. Most importantly in terms of recapitulating the sporadic nature of congenital limb reduction defects observed in the human population, progeny of genotypically positive Prx1-Dkk1 mice, consistently give rise to a full spectrum of phenotypically positive mice. Thus, genotypically positive siblings appear quite different, and this heterogeneous phenotype is reproducible with each generation regardless of the phenotype of the parents. We believe Prx1-Dkk1 mice may prove useful in the future to study congenital limb reduction defects either sporadic or drug-induced. Additionally, we identify that mesenchymal stem cells from Prx1-Dkk1 mice have limited proliferative ability, but normal differentiation potential; an observation that may account for the phenotypes observed.

MATERIALS AND METHODS

Generation of Prx1-Dkk1 Transgenic Mice

The murine Dkk1 gene was subcloned from a plasmid kindly provided by S. Millar (U.Penn) containing the cDNA sequence for murine Dkk1 (Andl et al., 2002). The Dkk1 gene was subcloned into an EcoRI site downstream the Prx1 promoter (Martin et al., 2000) kindly provided by C. Tabin (Harvard). The Prx1 enhancer element is flanked 5' with an insulator element derived from the chicken β -globin domain to protect against position effects at the site of transgene integration and 3' by an SV40 polyadenylation signal (Logan et al., 2002). The Dkk1 transgene includes a rabbit β -globin intron for transcript stability as previously described (Saitou et al., 1995). The construct was microinjected into fertilized eggs from a B6 $\times$ B6 cross. Transgenic mice were identified by PCR analysis using the following primers specific for the downstream region of Prx1 and the upstream coding sequence for Dkk1 using genomic DNA isolated from tail biopsies: Prx1 forward primer, 5'-GCACTTTTCTCTCTGTGAT-3', and Dkk1 reverse primer, 5'-GCGTTGGAATTGATGAGA-3'. Founder mice were bred to wild-type B6 mice to generate subsequent generations. The founder line expressing the highest level of Dkk1 was chosen for further study. Differences in morphometric characteristics between Prx1-Dkk1 and wild type mice were assessed by a paired Student's t-test.

Isolation of Bone Marrow-Derived Mesenchymal Stem Cells

Wild-type and Prx1-Dkk1 mice were sacrificed at 8 weeks of age by cervical dislocation in accordance with Columbia University Animal Welfare and IACUC policy under IRB protocol AAAA9669, and cleaned thoroughly with ethanol. The lower limbs were dissected and all connective tissue were carefully removed to expose the femur and tibia. The bone marrow was flushed with a 25-gauge needle into serum media (MEM-alpha with FBS 20%, Penicillin 100 units/mL, Streptomycin 100 μ g/mL; Invitrogen, Carlsbad, CA). A single cell suspension was made by repeated aspiration and flushing of the bone marrow cells through the 25-gauge needle. The cells were allowed to attach onto the surface of a cell culture plate for 72 hours after which the media was replaced with fresh serum media. The cells were further expanded after growth for 14 days.

Skeletal Preparations

Adult mice and pups were sacrificed humanely in accordance with institutional protocols and the mice were skinned and eviscerated (CUMC IRB AAAA1669 PI Matushansky). The adult mice or pups were fixed in 95% ethanol, followed by overnight incubation in acetone at room temperature. The mice were subsequently stained with Alcian blue (Sigma Aldrich, St. Louis, MO) for cartilage followed by tissue maceration in 2% potassium hydroxide until tissues were visibly cleared. The mice were counterstained with Alizarin red (Sigma Aldrich) for bone and cleared with a 1% potassium hydroxide/20% glycerol mixture.

Immunoblot Assay

Wild type and Prx1-Dkk1 MSCs were grown on 6-well plates at a density of 1×10^5 cells/well and the cells were subsequently collected on days 1, 2, 4, and 6. Protein lysates were made using RIPA buffer (Boston BioProducts, Ashland, MA) containing a protease inhibitor cocktail made according to manufacturer's recommendations (Roche). Lysates were denatured by mixing with Laemmli sample buffer (Sigma Aldrich) and heating for 5 minutes at 100°C. Fifty micrograms of protein lysate were run on a denaturing SDS-PAGE gel (Invitrogen) and transferred onto a nitrocellulose membrane. The PARP cleavage product was detected using an antibody against PARP (1:250; Clone C2-10; BD Biosciences, San Jose, CA). A murine β -actin antibody (1:8000; Sigma Aldrich) was used to normalize

samples. For detection of β -catenin, a mouse monoclonal antibody was used (1:500; BDI480 sc-59893; Santa Cruz Biotechnology). An anti-rat or anti-rabbit HRP-conjugated antibody (1:5000; GE Healthcare, Buckinghamshire, UK) was used followed by detection with a chemiluminescence ECL reagent (Denville Scientific, Metuchen, NJ) and exposure onto chemiluminescent film. Quantification was performed using densitometry.

Immunohistochemistry

Skeletal muscle and tissue were dissected from the mice and fixed overnight in a 10% buffered formalin solution, paraffin embedded and sectioned for hematoxylin and eosin staining and immunohistochemistry as previously described by us (Mills et al., 2011). A polyclonal antibody against either Ki67 (rabbit polyclonal AB15580; 1:500) or Dkk-1 (rat monoclonal, R&D Systems, clone 259631, 1:500) dilution were used to detect expression on cut sections of tissue.

Semi-Quantitative RT-PCR

Total RNA was extracted from various tissues and bone marrow-derived mesenchymal stem cells (MSCs) from wild-type and transgenic Prx1-Dkk1 mice. First-strand cDNA generated using the First Stand Invitrogen kit according to manufacturer's instructions were subsequently used to perform semi-quantitative RT-PCR using murine Dkk1 specific primers: forward primer, 5'-TTGACAACTACCAGCCCTACCC-3', and reverse primer, 5'-AGTCTGATGATCGGAGGCAGAC-3'. Mouse GAPDH was used as a normalizer: forward primer, 5'-CGGAGTCAACGGATTTGGTCGTAT-3', and reverse primer, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Differentiation Assays

Wild type and transgenic Prx1-Dkk1 MSCs from 8-week old mice were seeded in 6-well plates at a density of 1×10^5 cells. Cells were subsequently grown in the presence of bone differentiation (dexamethasone 10nM, glycerophosphate 5 mM, L-ascorbic acid 50 μ g/mL, lithium chloride 10 mM; Sigma Aldrich) or fat differentiation media (dexamethasone 0.5 μ M, isobutylmethylxanthine 0.5 μ M, indomethacin 50 μ M; Sigma Aldrich) for 7, 14 and 21 days. Bone differentiation was assessed by Alizarin red (Sigma Aldrich) staining, and fat differentiation was visualized with Oil Red O (Sigma Aldrich) stain. Quantification of Oil-RedO and Alizarin-Red-S was performed using the Adipogenesis Staining Kit (Cayman Chemicals) and Osteogenesis Staining Kit (Millipore), respectively, as described by the manufacturer.

Cellular Viability and Apoptosis Assays

TUNEL was performed on tissue sections using the In Situ Cell Death Detection Kit (Roche). AP⁺ from Roche. Cells derived from wild-type and transgenic Prx1-Dkk1 mice were seeded in triplicate at a density of 1×10^5 cells in 6-well plates and grown as described above. Cells were harvested by trypsinization at days 0, 7, 14 and 21 and cellular proliferation was determined using an MTS assay as previously described by us (Mills et al., 2011). Cell viability determined by trypan blue dye exclusion and calculated by dividing treated well absorbance by wild-type well absorbance

Nuclear β -catenin activity

Reporter assays with pGL3-OT (a β -catenin/TCF-responsive luciferase reporter plasmid) and pGL3-OF (a negative control plasmid) were kindly provided Edward Gelmann (Columbia University) carried out as described previously described (Song and Gelmann, 2008). Briefly, MSCs from wild-type mice and Prx1-Dkk1 mice with mild, moderate, and severe phenotypes (5×10^4 cells per well in 24-well plates) were transfected with 1) TCF4

expression plasmid (10 ng/well), 2) Renilla expression plasmid (5 ng/well) - internal/normalizing control, 3) pOT luciferase reporter plasmid (containing TCF4 binding sites in the promoter upstream of the luciferase gene) (100 ng/well), and 4) varying amounts of beta catenin expression plasmid: 0, 50, and 100 ng/well. The cells were transfected overnight and recovered in serum media for 24 hours prior to assay. Activity was detected using the Dual Luciferase Reporter Assay System (Promega).

RESULTS

Generation and characterization of Prx1-Dkk1 transgenic mice

To assess the effect of in vivo Dkk1 overexpression limited to the appendicular skeleton, we generated an expression vector in which Dkk1 was placed under the control of the Prx1 enhancer element and flanked 5' by an additional insulator element and 3' with the SV40 polyadenylation signal (Logan et al., 2002). We characterized 88 Prx1-Dkk1 mice (as genotypically determined using Prx1-Dkk1 fusion primers validated via Southern analysis). Prx1-Dkk1 mice exhibit severe shortening to near absence of forelimb and/or hindlimbs when compared to their wild type counterparts (Figure 1A). We methodically categorized these deformities using the International Organization for Standardization (ISO)/International Society for Prosthetics and Orthotics (ISPO) classification for human congenital limb deficiencies (1966; Day, 1991; Hall et al., 1962). The ISO/ISPO classification (a) is restricted to skeletal deficiencies; (b) describes deficiencies on anatomical or radiological bases only; and; (c) avoids the use of "melia" derived words due to their lack of precision and international uniform agreement. Furthermore, deficiencies are described as transverse or longitudinal. In the former there is complete normal development up to a specific point past which no skeletal elements are present; while the latter refers to a reduction or absence of elements within the long bones.

As readily observed, all of the skeletal deficiencies observed within our mice were of the longitudinal type as in all cases absence of distal elements as always associated with reduction of proximal elements. Percentages to which individual bones are affected are shown in Figure 1. Although the phenotypes observed are more accurately represented by a spectrum ranging from almost undetectable minor abnormalities of the phalanges to near complete absence of all limbs, for discussion and further experimentation purposes we subdivide the spectrum of phenotypes into three broad categories: mild, moderate, and severe. Mild was defined as defects limited to either the phalanges of either the upper or lower limb; moderate was defined as defects affecting either tibia/fibula or radius/ulna; and severe was defined as defects affecting the humerus or femur. Representative external anatomy, skeletal preparations, and radiological imaging of the three representative types are shown in Figure 1.

As stated in Figure 1, two-thirds of genotypically positive mice showed some degree of forelimb abnormality, while one-third of the mice exhibited hindlimb abnormalities. Additionally, all mice with hindlimb abnormalities had some degree of forelimb anomaly. Generally, mice with forelimb and hindlimb abnormalities tended to exhibit more extreme phenotypes. No abnormalities of the cranial skeleton or viscera were noted.

We were further interested as to whether the congenital defects affected birth weight and length and also whether or not the birth trend (i.e., the relationship to wild-type) would hold throughout development. If the birth trend in term of weight and length would remain constant (to that observed at birth) throughout development this would suggest that these congenital defects do not affect post-natal development. On the other hand if the trend was exacerbated (i.e., a greater relative discrepancy as compared to wild-type than that present at birth) this would suggest that these congenital limb deformities affect postnatal

development. Accordingly we measured weight and length of mice at birth and at 8 weeks of age. We did not observe a correlation (correlation coefficient $R^2=0.2$) between birth length and the spectrum of the phenotype and mouse length as compared to wild-type. This may not be surprising as *Dkk1* expression seems to predominantly affect extremities. There is however some, albeit limited (correlation $R^2=0.5$) between weight and the spectrum of the phenotype. Again, this may not be surprising as this could easily be explained by the absence of extremities. Comparing the birth weights and lengths to those obtained from 8 week old mice clearly show that wild-type mice increase their length 5-fold and their weight 20-fold; while gains in both weight and height of *Prx1-Dkk1* mice are significantly less, and correlate strongly with the severity of the phenotype. Based on the fact that there was not a correlation between birth length and weight with the severity of the phenotype, but that a correlation has developed after 8 weeks indicates that the phenotype does affect postnatal development (Supplemental Figure 1). Yet, despite the deformities and its effect on nutrition, *Prx1-Dkk1* have life spans similar to their wild type counterparts (608.8 days for *Prx1/Dkk1* mice versus 652.2 days for wild type mice; $p=0.59$).

Phenotype Heterogeneity Among Siblings

The wide spectrum of phenotypes among *Prx1-Dkk1* transgenic mice observed from almost normal appearing mice to mice with severely affected and nearly absent limbs despite arising from the same founder, led us to examine the potential outcomes of *Prx1-Dkk1* matings among differently affected mice (Figure 2). Initial matings of a genotypically positive female (●) with a wild-type male (□) results in progeny with a full spectrum of phenotype severity (1: mild; 2: moderate; 3: severe) (Figure 2A). No differences in offspring phenotype were observed with inverse matings (i.e., ■×○) at either early or later generations (e.g., Figure 2B). Subsequent crossings between two genotypically positive, but mildly-affected phenotypic mice (■×●; Figure 2C) yield predominantly genotype positive mice with varying phenotypic severities. As the severity of genotypically positive parents increased (even in a cross between genotypically positive mice), there did not appear to be an increased shift to more severe phenotypes (compare phenotypic spectrum of cross shown in Figure 4D to that of Figure 4A). For crosses in which both parental mice were genotypically positive, all genotypically positive offspring were subsequently back-crossed to wild-type mice and their progeny genotyped in order to establish whether the offspring from crosses in which both mice were genotypically positive were homozygous (+/+) or heterozygous (+/D) for the transgene.

All paired matings were consistent with Mendelian inheritance of the transgene and given the variability of the phenotype suggests an autosomal dominant pattern of inheritance with incomplete penetrance. This stochastic mode of developmental impairment suggests that secondary events need to occur, in a *Dkk1* background, to give rise to the phenotypic defects observed similar to what has previously been described for other transgenic mice carrying *myc* (Stewart et al., 1984) and *ras* (Sinn et al., 1987; Tremblay et al., 1989) transgenes in regards to tumor development.

Dkk1 is preferentially expressed in the limbs of transgenic mice

To determine if ectopic overexpression of *Dkk1* in specified areas persists beyond the embryologic stage and may contribute to the observed phenotype, semi-quantitative RT-PCR was performed on tissues obtained from *Prx1-Dkk1* mice. It has been previously published that *Prx1* expression is mainly limited to the limbs, interlimb flank, latissimus dorsi muscle, craniofacial mesenchyme, and soft-tissues of the eye during embryogenesis (Logan et al Genesis 2002; Cserjesi et al Development 1992) and in skeletal and heart muscle in the adults (Cserjesi et al Development 1992). In agreement with both the predicted tissue specificity of the *Prx1* promoter and the phenotype presented above, RT-PCR

demonstrated a predominant expression pattern of Dkk1 in the skeletal muscle of the limbs and soft tissue of the eye in adult Prx1-Dkk1 mice (Figure 3A). Similar analysis of tissues from adult wild-type mice revealed no expression of Dkk1.

We additionally assayed immunohistochemically for Dkk1 expression in the bone in an attempt to correlate expression to phenotype. As seen in Figure 3B, Dkk1 expression is readily observed in the osteoblasts lining the bones of Prx1-Dkk1 mice (Figure 3Bi) but not in wild-type controls (Figure 3Bii). Additionally, expression of Dkk1 is readily observed in the bone marrow (Figure 3Bi,ii) and skeletal muscle of Prx1-Dkk1 mice, but not wild-type controls (Figure 3Biii,iv).

Since Dkk1 is a potent inhibitor of the Wnt signaling pathway resulting in the downregulation of β -catenin, we assessed the effect of Dkk1 overexpression on β -catenin in skeletal muscle since based on Figure 3Biii, skeletal muscle from Prx1-Dkk1 showed a uniform high level of Dkk1 expression as compared to wild-type controls. Analysis of β -catenin expression in the limb skeletal tissue of Prx1-Dkk1 mice by immunohistochemistry revealed a moderate decrease in β -catenin staining intensity when compared to wild type skeletal tissue (Figure 3C).

Hypothesizing that the Wnt-inhibitory effects, as manifested by a decrease in β -catenin levels, may be more pronounced in the mesenchymal progenitor cells that contribute to limb development and noting (as above) that the certain cellular elements within the bone-marrow of Prx1-Dkk1 mice stained intensely for Dkk1 expression (but not in wild-type controls), we isolated bone marrow-derived mesenchymal stem cells (BM-MSCs) from 8-week old Prx1-Dkk1 (severe phenotype) mice and evaluated the expression levels of β -catenin by Western immunoblot analysis. As seen in Figure 3D there is an almost 80% decrease in total β -catenin in BM-MSCs obtained from Prx1-Dkk1 mice (with severe phenotypes) in comparison to wild type.

To determine if we could establish a direct relationship between the severity of the phenotype and β -catenin nuclear activity, we further assayed for nuclear β -catenin function using a β -catenin/TCF-responsive reporter (pGL3-OT/OF) in BM-MSCs from wild-type and Prx1-Dkk1 mice with either mild, moderate, or severe phenotypes. In Figure 3E endogenous β -catenin nuclear activity is set at a baseline of 1, and the ability of each cell type to increase β -catenin nuclear activity in response to additional β -catenin is assessed. In BM-MSCs from wild-type cells, β -catenin nuclear activity increases proportionally to the amount of exogenous β -catenin (Figure 3E, blue bars). In contrast the ability of BM-MSCs from Prx1-Dkk1 to similarly upregulate β -catenin nuclear activity is significantly reduced in direct correlation to the phenotype (i.e. the more severe phenotype the less β -catenin nuclear activity).

BM-MSCs from Prx1-Dkk1 expression have subtle changes in differentiation potential

Numerous previous publications attest to the fact that in vivo manipulation of the Wnt/ β -catenin pathway affect mesodermal lineage specification by affecting either mesodermal differentiation or proliferation (Matushansky et al., 2008) and see Discussion). Accordingly we sought to assess whether inhibition of differentiation or cessation of proliferation of mesodermal precursors in the adult mouse might contribute to the phenotype observed. In order to determine if Dkk1 overexpression affects differentiation, BM-MSCs from the femurs of 8 week old Prx1-Dkk1 and wild type mice were isolated and grown in the presence and absence of either adipogenic or osteogenic differentiation media. As seen in Figure 4A,B Prx1-Dkk1 BM-MSCs (from severely affected mice) show a slight increased propensity for adipogenic and a slight decreased propensity for osteogenic differentiation as compared to wild type cells as judged by comparable accumulation of terminal markers of

both bone (i.e., calcium mineralization assayed via Alizarin Red S) or fat (i.e., lipid deposition assayed via Oil Red-O). We further quantified both the adipogenic and osteogenic differentiation potential BM-MSCs from mild, moderate, and severely affected Prx1-Dkk1 mice as compared to BM-MSCs from wild-type controls. As seen in Figure 4C, although there does appear to be a direct correlation between the severity of the phenotype and both an increase in adipogenesis and a decrease in osteogenesis, the differences are not statistically significant and thus can only be considered a trend. Overall the effect of Dkk1 expression on the in vitro differentiation potential is mild when compared to the phenotypic abnormalities of the mice. Li et al (Li et al., 2011) recently reported that treatment of adult rats with an antibody to Dkk1 resulted in a significant increase in bone mineral density from increased bone formation (but did not affect bone length). Thus in our mice although a limitation of osteogenic differentiation may underlie a potential concurrent phenotype of these mice (i.e., a decrease in bone thickness/mineralization (data not shown) in a manner analogous to Li et al (Li et al., 2011), we hypothesized that other mechanisms may be more pertinent to the skeletal abnormalities observed.

Bone marrow-derived mesenchymal stem cells from Prx1-Dkk1 mice exhibit increased apoptosis

We further sought to assess if Dkk1 expression may affect proliferation and/or viability of Dkk1 expressing cells. Accordingly we performed immunohistochemistry for Ki67 and TUNEL in similar sections in which Dkk1 expression was noted (Figure 3B) in Prx1-Dkk1 and wild-type mice. No differences were observed in either Ki67 or TUNEL staining (Supplemental Figure 2) in relation to osteoblasts that stained positive for Dkk1. However, we did note that bone marrow cells from Prx1-Dkk1 mice stained more intensely for TUNEL than bone marrow from wild-type cells. Of note the TUNEL stain uses an alkaline phosphatase conjugate to detect TdT activity allowing for IHC-based analysis.

To assess whether the bone marrow cells showing increased TUNEL activity were BM-MSCs that we showed in Figure 3D,E had significantly decreased β -catenin activity, we isolated BM-MSCs from 8 week old wild-type and from Prx1-Dkk1 mice with severe, moderate, and mild phenotypes and performed a direct proliferation assay. As seen in Figure 5B we see a dramatic decrease in cellular proliferation in direct relation to the severity of the animal from which the cells were isolated. Furthermore, a Trypan blue exclusion viability assay (Figure 5C) reveals that there is a direct relationship between the lack of proliferation and the loss of viability and both appear to be a function of the severity of the phenotype of the mice from which the corresponding cells are derived.

To further assess the reason for loss of viability, we performed an apoptotic assay using PARP cleavage and Annexin V, late and early apoptotic markers, respectively comparing MB-MSCs from wild-type and Prx1-Dkk1 (with severe phenotypes). As seen in both Figure 5D&E, Prx1-Dkk1 cells undergo noticeable apoptosis within one week in cell culture. Wild type cells exhibit no evidence of apoptosis under similar conditions. The latter may provide a mechanism underlying the observed limb defects in Prx1-Dkk1 mice.

DISCUSSION

In an attempt to generate viable mice that recapitulate congenital limb defects in a manner that is biologically faithful to the human condition, we generated a transgenic mouse line in which the Wnt inhibitor Dkk1 was expressed under the control of the promoter/enhancer element of the mesodermal specific enhancer element of Prx1 (Logan et al., 2002). As we have described herein, Prx1-Dkk1 mice recapitulate congenital short limb deformities (i.e., without other developmental issues) and have normal life-spans. Importantly, the progeny of phenotypically positive or phenotypically negative, but genotypically positive Prx1-Dkk1

mice, consistently give rise to a spectrum of phenotypes ranging from an almost complete loss of limbs to extremely minor malformations of the phalanges. Mechanistically we show that MSCs from Prx1-Dkk1 mice do not proliferate as well as their wild-type counterparts, but have only subtle deficiencies in differentiation potential. We speculate that this lack of MSC proliferation during development contributes to the congenital short limb defects observed. We additionally show that Wnt/ β -catenin signaling is inhibited in Prx1-Dkk1 mice and that the degree of Wnt/ β -catenin inhibition correlates with the severity of the phenotype.

In addition to the data presented herein, multiple lines of evidence underscore the importance of the Wnt/ β -catenin pathway for mesodermal development differentiation. Perhaps the most pertinent to our work was the conditional deletion of β -catenin using the Prx1-Cre system resulting in complete loss of β -catenin in mouse limb mesenchyme (Hill et al., 2005). In these mice osteoblast precursors lacking β -catenin were blocked in differentiation and developed into chondrocytes and β -catenin activity was necessary and sufficient to repress the differentiation of mesenchymal cells into skeletal precursors (Hill et al., 2005). We speculate that the difference in phenotypes between the mice in which β -catenin was deleted in a Prx1 distribution, and our mice in which Dkk1 is overexpressed in a Prx1 distribution may have to do with complete versus incomplete inactivation of β -catenin activity. Other mouse models harboring either gain-of-function or loss-of-function β -catenin mutations targeted to osteoblasts resulted in high and low bone mass phenotypes, respectively, caused primarily by modification of bone resorption (Glass et al., 2005) without significant effects on skeletal patterning. Finally, ectopic canonical Wnt signaling has been shown to lead to enhanced ossification in mice; conversely, genetic inactivation of β -catenin caused ectopic formation of chondrocytes at the expense of osteoblast differentiation (Day et al., 2005).

Inhibitors of Wnt-signaling have also been extensively studied and appear to be as crucial in regulating mesenchymal development. The dickkopf (Dkk) family encodes secreted proteins and consists of four main members in vertebrates (Dkk1,2,3,4). The founding member of the family, Dkk1, was discovered by its ability to block Wnt signaling during early *Xenopus* embryogenesis and is required for head induction (Glinka et al., 1998). Since then, an overwhelming body of evidence supports the function of Dkk1 in Wnt/ β -catenin signaling, but it has also been suggested that Dkk1 and -2 may have also β -catenin-independent functions. For example, the mesothelioma cell line H28 contains a homozygous deletion of the β -catenin gene and yet Dkk1 overexpression induces apoptosis and growth suppression (Lee et al., 2004). Similarly in HeLa cells, there are no significant changes in either cellular β -catenin localization, expression of Wnt target genes or Tcf-reporter activity after ectopic expression of Dkk1 (Mikheev et al., 2004). The developmental abnormalities observed are generally believed to result from different expression domains of these growth factors and their antagonists, such as Dkk1, create signaling gradients, which pattern the early embryo in a combinatorial fashion and orchestrate regionally specific induction of axial structures, including the head. In the transgene-induced mouse mutant doubleridge, which harbors a hypomorphic Dkk1 allele (Dkk1d) with 10% normal expression levels, head development is normal. However, Dkk1d/- compound heterozygotes on a C57BL/6J genetic background, show a variety of head defects, from hydrocephaly with micrognathia, to loss of anterior head structures and eyes (Cobas et al., 2004), which are still less severe than the truncation of anterior head structures observed in the Dkk1 null mice.

While there is arguably little if any debate about the importance of Wnt signaling to mesodermal/mesenchymal development, there is a fair amount of uncertainty in the field regarding (1) the specific mechanism through which Wnt signaling acts (i.e., does inactivation of β -catenin result in developmental defects because of improper cell adhesion or improper transcriptional regulation (Brembeck et al., 2006; Schambony et al., 2004)); (2)

whether Wnt perturbation results in only Wnt/ β -catenin (canonical Wnt signaling) alterations or whether there are simultaneous perturbations of non-canonical (β -catenin independent) Wnt pathways (Baksh et al., 2007; Baksh and Tuan, 2007) (e.g., Wnt/calcium, Wnt/Rho GTPase, and Wnt/Jnk); (3) the cells directly affected by Wnt perturbations that subsequently account for the phenotype observed (Boland et al., 2004); and (4) the relationship between cell type/stage of differentiation and sensitivity to Wnt manipulations (Foley and Mercola, 2004; Li et al., 2011; Yokoyama et al., 2011). On the latter point Wnt signaling has also been studied directly in MSCs. Early work by Darwin Prockop's laboratory showed that Dkk1 promoted MSC self-renewal and maintained their pluripotentiality (Gregory et al., 2003). In contrast to this work, Rocky Tuan's group at the NIH (Baksh and Tuan, 2007; Boland et al., 2004) showed that Wnt3a (and resultant β -catenin activation) can similarly expand MSCs capable of maintaining their pluripotentiality. The recent identification of β -catenin mutations in some sarcomas (Vijayakumar et al., 2011) would favor Wnt-proliferative properties in MSCs (i.e., sarcoma progenitors) and in some contradiction to our own earlier work in sarcomas in which we have shown that high levels of Dkk1 (and its effect on both canonical and non-canonical Wnt signaling) are tumorigenic for MSCs (Matushansky et al., 2007).

We focused our studies on generation of mesodermal specific (via Prx1 promoter/enhances (Logan et al., 2002)) Dkk1 expressing mice because (1) the Wnt inhibitor Dkk1 has been shown to associate with pro-apoptotic human skeletal disorders and promote skeletal precursor apoptosis in mice (Ko et al., 2010; Wang et al., 2008; Weng et al., 2009); (2) thalidomide upregulates Dkk1 in chick embryos (Knobloch et al., 2007) with subsequent skeletal defects; and (3) increased cell death may underlie thalidomide-induced limb truncations in chick embryos (Knobloch et al., 2008; Therapontos et al., 2009); and (4) it is well known that mice are not sensitive to thalidomide teratogenicity (Franks et al., 2004) but as described above are sensitive to manipulations of the Wnt/ β -catenin pathway.

One interesting finding that has resulted from studying these mice to date is the potential mechanism of Dkk1-mediated inhibition of appropriate musculoskeletal formation. Although a detailed explanation of vertebrate limb development is beyond the scope of this manuscript, suffice it to say, the classical view posits that the vertebrate limb arises from embryonic limb buds in which precursor cells are programmed as a function of their position and time (as a result of cytokine gradients) within specified zones (reviewed extensively in (Allard and Tabin, 2009; Capdevila and Izpisua Belmonte, 2001; Tabin and Wolpert, 2007)) to give rise to specific components of the limb (e.g., either distal or proximal). Disturbances in developmental pathways (e.g., Wnt, hedgehog, FGF) results in "patterning defects" and mis-specification of which cells should give rise to which structures resulting in limb deformities. More recently this classical view of limb development has been questioned and evidence provided that disturbance of embryological developmental pathways may limit either the proliferation or differentiation capacity of skeletal precursors as opposed to reprogramming them to a different fate (Galloway et al., 2009; Mariani and Martin, 2003; Sun et al., 2002; Wolpert et al., 1979).

However, both models presume that the effect on either patterning or proliferation/differentiation is limited to a specific embryological time period. While patterning is by definition an embryological process, proliferation and differentiation of connective tissue precursors is a process that continues throughout the lifespan of the vertebrate organism. As we have reported, connective tissue precursors (i.e., mesenchymal stem cells) isolated from the shortened limbs of adult Prx1-Dkk1 transgenic mice exhibit relatively normal in vitro differentiation capacity, but limited proliferative activity associated with significant apoptosis. The latter observation indicates that Dkk1-mediated apoptosis of skeletal precursor cells is a process that is still on-going in the adult and implies that perhaps early,

in utero and even perinatal intervention aimed at reversing Dkk1 overexpression in certain congenital reduction defects may ameliorate the severity of that defect. It could be argued that since postnatal treatment of growing mice and rats with Dkk1-Ab increases the bone mineral density (due to increased bone formation) but does not change the shape or length of the limb (Li et al., 2011), prenatal Wnt-inhibition may similarly increase bone density without enhancing limb growth. On this note, it should be equally pointed out that Wnt/beta-catenin signaling is not absolutely essential for limb bud regeneration (at least in tadpoles as nerve-derived signals and Wnt/ β -catenin signaling have redundant roles in the initiation of limb regeneration (Yokoyama et al., 2011)) but is necessary for limb growth, suggesting distinct temporal roles for Wnt/ β -catenin signaling in the limb development process.

The commercial availability and current use of neutralizing anti-Dkk1 antibodies and other Wnt-activating strategies in clinical trials for various indications (Fulciniti et al., 2009; Komatsu et al., 2010) makes exploration of its efficacy as a novel treatment agent for congenital limb deformities feasible using our described or similar animal model systems. Of note, while MSCs probably function like embryonic mesenchymal cells in general, a more appropriate in vitro model for the Dkk1 transgenic animal would be embryonic limb bud mesenchyme from these animals. We have attempted to isolate embryonic limb bud mesenchyme. However, although the phenotype is quite obvious even at day e12.5 (data not shown), despite our best effort we have not been able to isolate and expand these cells in vitro for the purposes of performing experiments similar to those described. It is possible that the mesodermal cells derived from the embryonic limb mesenchyme have similar, if not more pronounced, difficulties with proliferation as MSCs derived from Prx1-Dkk1 transgenic mice thus limiting further in vitro analysis. We have also attempted to stain embryonic limb bud mesenchyme for Dkk1, however due to known overlapping expression patterns between Dkk1 (Li et al Bone 2006) and Prx1 (Logan et al Genesis 2002; Cserjesi et al Development 1992) during embryogenesis, we could not discern exogenous Dkk1 from endogenous Dkk1 (data not shown). Thus it is entirely possible that Dkk1 expressed from extra-embryonic limb bud tissue may affect embryonic limb bud development. This is clearly supported by the recent demonstration that Dkk1 expression from osteoblast-precursors (using a collagen promoter) affected hematopoietic stem cells within the bone marrow (Fleming et al., 2008). Although interestingly those mice did not have any reported skeletal developmental defects.

In conclusion, we have generated Prx1-Dkk1 transgenic mice which, based on a synthesis of previously published work and in conjunction with the phocomelia phenotype described, may serve as a biologically faithful mouse model of congenital limb reduction defects observed in people either sporadic or as a result of a known or unknown teratogens. Furthermore, the distinct characteristic of these mice, (i.e. their severe deformities without concomitant impact on longevity) further makes these mice unique tools for the study and development of novel agents and treatment strategies for mitigating or potentially reversing the congenital deformity after birth. The most obvious future work will involve comparing genotypically positive siblings of differing phenotypes to elucidate the differences in Wnt/ β -catenin signaling (and potentially non-canonical Wnt signaling) that may account for the variable phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

1. Prx1-Dkk1 mice recapitulate a spectrum of human congenital limb reduction defects.
2. MSCs from Prx1-Dkk1 mice show a proclivity to apoptosis both in vivo and in vitro.
3. Wnt/ β -catenin inhibition in Prx1-Dkk1 tissues correlate with phenotype severity.

Figure 1. Prx1-Dkk1 transgenic mice demonstrate a spectrum of limb longitudinal defects
(A) General appearance of Prx1-Dkk1 mice and wild-type controls demonstrating a spectrum of forelimb and hindlimb defects. (B) Radiographs showing full skeletal views of both wild-type mice and Prx1-Dkk1 mice displaying a spectrum of forelimb and hindlimb abnormalities. (C, D) Detailed analysis of forelimb (C) and hindlimb (D) abnormalities using the ISO/ISPO classification of congenital limb deficiencies with accompanying external, skeletal preparations, and radiographs of a spectrum of abnormalities. Forelimb and hindlimb deformities were subjectively defined as mild (deformities limited to missing forelimb/hindlimb digits), moderate (loss of nearly all forelimb/hindlimb digits and/or significant truncation or bowing of the distal or proximal limb), and severe (near or total absence of any distal forelimb/hindlimb structures and significant truncation of the proximal limb).

Figure 2. Prx1-Dkk1 matings result in a heterogeneous phenotype regardless of parental phenotypes

(A–D) Representative matings of Prx1-Dkk1 mice with either wild type (A,B) or with other Prx1-Dkk1 mice (C,D). Note no obvious differences were observed in the severity of the phenotype of the (genotypically positive) progeny in relation to either the severity of the parental mice or whether one or both parents were Prx1-Dkk1 transgenic. Genotype: $\blacksquare/\bullet$ = genotype positive male/female; $\square/\circ$: genotype negative male/female. Phenotype: + = mild phenotype; ++ = moderate phenotype; +++ = severe phenotype; F = filial generation.

Figure 3. Dkk1 expression in adult Prx1-Dkk1 mice

(A) Analysis of Dkk1 expression in Prx1-Dkk1 mice (top lane) and compared to wild-type controls (third lane) via RT-PCR. Histone H4 is shown as a loading control. Bands were quantified with Labworks 4.0 software and normalized to H4. The mean of three independent quantifications is shown. Error bars represent SEM. Bars represent relative ratio expression comparing Dkk1 in Prx1-Dkk1 to wild-type after respective normalization to H4. (B) Immunohistochemical analysis of Dkk1 demonstrating expression of Dkk-1 the long bones, bone-marrow and skeletal muscle of Prx1-Dkk1 mice, but not in wild-type controls. (C) Immunohistochemical analysis of β -catenin demonstrating shows a modest decrease in expression in Prx1-Dkk1 limb skeletal muscle compared to wild type control tissues. All images were taken at an original magnification of 100x; scale bar=100um. (D) Immunoblot analysis of β -catenin in bone marrow derived mesenchymal stem cells (BM-MSCs) from 8 week old wild-type mice and Prx1-Dkk1 mice (with severe phenotypes). Quantification was performed using densitometry. The mean of three independent quantifications is shown. Mean quantification of β -catenin was set at 1, and level of β -catenin in Prx1-Dkk1 shown as a relative value. Error bars represent SEM. (E) Quantification of nuclear β -catenin activity (using the β -catenin/TCF-responsive luciferase reporter plasmid pGL3-OT/pGL3-OF) in BM-MSCs from wild-type mice and Prx1-Dkk1 mice with mild, moderate, and severe phenotypes.

Figure 4. Mesenchymal stem cells from Prx1-Dkk1 mice retain differentiation potential
 BM-MSCs from wild-type and Prx1-Dkk1 mice with severe phenotypes shown after 21 days of growth in either control or osteogenic differentiation medium (A); or either control of adipogenic differentiation medium (B) and staining with either Alizarin-Red-S or Oil-Red-O respectively. (C) BM-MSCs from 8 week old wild-type and Prx1-Dkk1 mice with mild, moderate, and severe phenotypes cultured in either adipogenic or osteogenic differentiation medium and degree of differentiation was assessed every 7 days of a 21 day differentiation course using calorimetric assays for either Alizarin-Red-S or Oil-Red-O (see Materials and Methods).

Figure 5. Mesenchymal stem cells from Prx1-Dkk1 mice show an increased rate of apoptosis (A) TUNEL stain of the growth plate from the femurs of wild-type and Prx1-Dkk1 mice with severe phenotypes (i,ii). Higher magnification of blue-boxed area (iii, iv). (B) Proliferation assay (using MTS) of BM-MSCs from 8 week old wild-type and Prx1-Dkk1 mice with mild, moderate, and severe phenotypes. (C) Viability assay (using Trypan blue exclusion) of BM-MSCs from wild-type and Prx1-Dkk1 mice with mild, moderate, and severe phenotypes. Error bars: standard deviation. (D) Immunoblot assay for PARP cleavage showing activity in BM-MSCs derived from Prx1-Dkk1 mice (but not wild-type controls) as early as 6 days of proliferation. (E) Immunofluorescent analysis for Annexin V revealed proportionally increased Annexin V positivity in Prx1-Dkk1-derived BM-MSCs (200X).