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LRP5 Signaling in Osteosarcomagenesis: a Cautionary Tale of Translation from Cell Lines to Tumors[1,2]

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Abstract

Previous reports document expression of low-density lipoprotein receptor-related protein 5 (LRP5) in osteosarcoma (OS) tissue. Expression of this Wnt receptor correlated with metastatic disease and poor disease-free survival. Forced expression of dominant-negative LRP5 (dnLRP5), which lacks the membrane binding domain of the native protein and therefore functions as a soluble receptor-sponge for Wnt ligands, reduced *in vitro* cellular invasion and *in vivo* xenograft tumor growth for osteosarcoma cell lines. Here, we use a genetically engineered mouse model of osteosarcomagenesis with and without expression of dnLRP5 to assess to what degree tumorigenesis is affected and whether Wnt/β-catenin signaling is circumvented or maintained. Each cohort of mice developed osteosarcoma at a similar ultimate prevalence, but after a slightly increased latency in those also expressing dnLRP5. On histology, there was no difference between groups, despite previous reports that the dnLRP5 osteosarcoma cells specifically undergo a mesenchymal-to-epithelial transition *in vitro*. Finally, immunohistochemistry showed the presence of cytosolic and nuclear β-catenin and nuclear Cyclin D1, markers consistent with preserved Wnt/β-catenin signaling despite constitutive blockade of the cell surface receipt of Wnt signaling ligand. These data suggest that canonical Wnt signaling plays a role in OS progression and that while blockade of singular nodes in signaling pathways can have dramatic effects on individual cell lines, real tumors readily evade such focused attacks.

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Introduction

The most common primary bone malignancy, osteosarcoma (OS) remains a leading cause of cancer death in adolescents and young adults [1]. Current treatment paradigms, which have not changed in 30 years, achieve little better than 50% long-term survival. There remains a great need to understand the underlying mechanisms of tumor progression before more targeted therapies may be realized.

Wnt genes encode a family of highly conserved, secreted proteins, modulating cell fate and cell proliferation during embryonic development and oncogenesis through activation of receptor-mediated signaling pathways. Binding of Wnt ligands to the cell surface receptors frizzled (*FZD*) and low-density lipoprotein receptor related protein 5 (*LRP5*), leads to blockade of a cytoplasmic complex consisting of axin 2 (AXIN2), adenomatous polyposis coli

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(*APC*), and glycogen-synthase-kinase-3-β (*GSK3β*), resulting in hypophosphorylation and stabilization of β-catenin. Cytosolic accumulation and translocation of β-catenin to the nucleus enables formation of complexes with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors to promote the expression of Wnt-responsive genes including c-Myc, cyclin D1, and matrix matalloproteinases [2–4].

Wnt signaling is essential for the differentiation of mature osteoblasts and, consequently, endochondral and membranous bone formation [5–7]. In OS, overexpression of numerous Wnt components including Wnt ligands, FZD, and LRP receptors implicate aberrant Wnt/β-catenin signaling in the development and progression of OS. In 2004, Hoang, et al. utilized reverse transcription polymerase chain reaction (RT-PCR) in four OS cell lines. Of the Wnt related genes, Wnt1 and Wnt3 were expressed in two and three out of four cells lines, respectively. Additionally, various forms of FZD, in addition to LRP5, were expressed by all cell lines [8]. In another study, Chen et al. detected expression of multiple Wnt ligands and receptors in two human OS cell lines. They also, by immunohistochemical staining of 44 human OS samples, identified the presence of Wnt10b in 75% with a trend toward decreased survival in these patients [9].

LRP5, a single-pass transmembrane protein, is required as a co-receptor for canonical Wnt-mediated signaling [10]. Additionally, LRP5 functions as a major regulator of bone homeostasis [11–13]. Human OS metastasis has been linked to LRP5 expression [8]. Loss of LRP5 in transgenic mice markedly reduces mammary tumor formation. In cell culture studies, blockade of LRP5 signaling decreases tumorigenicity of prostate cancer PC-3 cells, and drives a mesenchymal-to-epithelial transition in PC-3 and SaOS-2 cells. Moreover, dominant-negative LRP5 (dnLRP5) caused considerable inhibition of tumor growth, invasion, and metastasis in an *in vivo* xenograft model wherein dnLRP5-transfected 143B cells were injected into a nude mouse [14]. Based on these studies, LRP5 is significantly involved in OS disease progression as reflected in the tendency for tumors expressing this receptor to metastasize.

The interaction between LRP5 and Wnt is implicated in a variety of human diseases such that the aberrant activation of the Wnt/β-catenin pathway is closely associated with a variety of human cancers, skeletal tissue perturbation, and OS [13,15]. The ultimate outcome of Wnt signaling, however, is shaped by those genes whose activity is controlled through β-catenin and TCF. To explore whether dnLRP5 may significantly alter the forward-going process of osteosarcomagenesis, we used a mouse model of OS driven by conditional disruption of tumor suppressor genes.

Materials and Methods

Animals

All experiments were performed with the approval of the institutional animal care and use committee and in accordance with international legal and ethical codes. Mice bearing LoxP-flanked conditional alleles of *Rb1* and *Trp53* were obtained from Jackson Laboratories [16,17]. OsxCreERT2 animals were previously described [18]. All animals were genotyped using published protocols.

The *Hprt-CAGG-LSL-dnLRP5* mice were generated by targeting the mouse *Hprt* locus in R1 embryonic stem cells with a vector containing a CAGG promoter followed by the cDNA for the *dnLRP5* gene separated from the promoter by a floxed stop cassette containing the neomycin resistance gene. After positive and negative selection, clones were screened by PCR for the full-length insertion. A targeted clone was injected into blastocysts. Resultant chimeras were bred and progeny checked for germline transmission *via* tail tip DNA genotyping.

Imaging

Radiographs were obtained of sedated mice using a Kodak Carestream 4000 Pro Fx imaging machine (Carestream Health, Inc., Rochester, NY, USA). Light microscopy was viewed and digitally photomicrographed using an Olympus BX43 microscope and DP26 camera (Olympus America, Center Valley, PA, USA).

Histology

Tissues were harvested post-mortem, fixed in 4% paraformaldehyde overnight, decalcified in 14% EDTA at pH 7.4 for two weeks at 4 degrees, and embedded in paraffin following serial dehydration in ethanol. In addition to standard hematoxylin and eosin (H&E) staining, immunohistochemistry for mouse β-catenin (1:50 dilution, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and Cyclin D1 (1:500 dilution, Santa Cruz Biotechnology) were also performed, using IgG-horse radish peroxidase goat anti-rabbit secondary (1:5000 dilution, sc-2004, Santa Cruz Biotechnology) and counterstained with hematoxylin. All pathology was performed blinded to the sample genotype.

Samples were considered positive if cytoplasmic or nuclear staining was observed. Samples with isolated membrane staining or without staining were considered negative. Stained sections were independently examined by two investigators who were blinded to the clinicopathologic data and LRP5 status of the all samples. Immunohistochemical positivity was scored such that the relevant staining pattern was defined (nuclear or cytoplasmic) followed by applying a systematic random sampling approach for selection of twenty-five separate fields of vision at 20X magnification [19]. In the fields of vision, the percentage of positive cells was assessed followed by grading and scoring positivity of cells as follows: negative (score 0), weakly positive (score 1), positive (score 2), strongly positive (score 3), and very strongly positive (score 4). Each sample was compared to the previously scored sample and, if needed, re-scored to minimize intra observer variance among all samples. A total score was calculated for each sample by averaging the percentage of positive cells and the degree of positivity and subsequently multiplying the average scores.

PCR Detection

PCR was conducted to verify that successful cre-mediated excision of the stop sequence between the CAGG promoter and the dnLRP5 coding sequence occurred, and was maintained throughout the duration of the experiment. Primers were constructed flanking the stop sequence. Tumor DNA was harvested from sacrificed mice using a DNeasy kit. For a negative control, muscle and liver tissue was harvested from *Hprt-CAGG-LSL-dnLRP5* mice that had not been exposed to Tamoxifen. GAPDH primers were used as a loading control. PCR reactions contained 150 μg of DNA per well, and ran for 32 cycles. With successful excision, an amplicon of 172 base pairs was amplified, and visualized on polyacrylamide gel.

Results

Inducing Mouse Osteosarcomagenesis With or Without Wnt Surface Blockade

Cre-LoxP conditional disruption of *Trp53* and *Rb1* has been shown to drive efficient osteosarcomagenesis in genetically

engineered mouse models in which the gene disruptions occur within the osteoblast lineage [20–22]. To generate a highly penetrant control mouse model of OS, we crossed mice bearing conditional alleles of *Rb1* (*Rb1*^{fl}/$_{fl}$) and *Trp53* (*Trp53*^{fl}/$_{fl}$), to mice bearing the *Osterix-CreERT* transgene, a tamoxifen-inducible Cre-recombinase expressed in osteoblast precursors. Osterix is a bone-specific transcription factor required for osteoblast development, and this *Osterix-CreERT* transgene expresses Cre recombinase in a manner that follows that of endogenous *Osterix* [18,23]. To achieve osteoblast lineage-restricted deletion, the *OsxCreERT* transgenic mouse was used to direct Cre expression to committed osteoblast progenitors.

To this background model of genetically driven osteosarcomagenesis was added the conditional expression of dnLRP5. The cDNA for dnLRP5 with a polyA tail was targeted to the *Hprt* locus on the mouse X chromosome, and separated from a *CAGG* promoter with floxed stop (Figure 1*A*). In the presence of Cre-recombinase that conditionally disrupts *Trp53* and *Rb1*, the stop sequence is excised, bringing dnLRP5 into position to be highly expressed by the *CAGG* promoter.

Cell Surface Wnt Signaling Blockade Reduces But does not Abrogate Osteosarcomagenesis

In order to test the relative tumorigenesis with or without blockade of the cell surface receptor signaling of Wnt ligands, cohorts of mice with homozygous *Trp53* and *Rb1* conditional disruption in pre-osteoblasts with or without conditional activation of dnLRP5 expression were generated (Figure 1*B*). Male mice positive for *Hprt-CAGG-LSL-dnLRP5* and female mice homozygous for the allele were used for the experimental group, due to the possibility of X-chromosome inactivation of the targeted allele in half the female cells of heterozygotes. Mice were injected with tamoxifen to enable Cre-mediated recombination at age 4 weeks, and then monitored closely for apparent tumorigenesis or morbidity. Any morbid mice were euthanized and thoroughly checked by radiography and gross dissection for skeletal tumors.

The cohort of mice conditionally expressing dnLRP5 had slightly increased latency to tumorigenesis, compared to the controls lacking this allele (Figure 1*C*). The ultimate prevalence of tumorigenesis was very similar in both groups, suggesting that osteosarcomagenesis was only slightly delayed, but not stopped by the expression of dnLRP5.

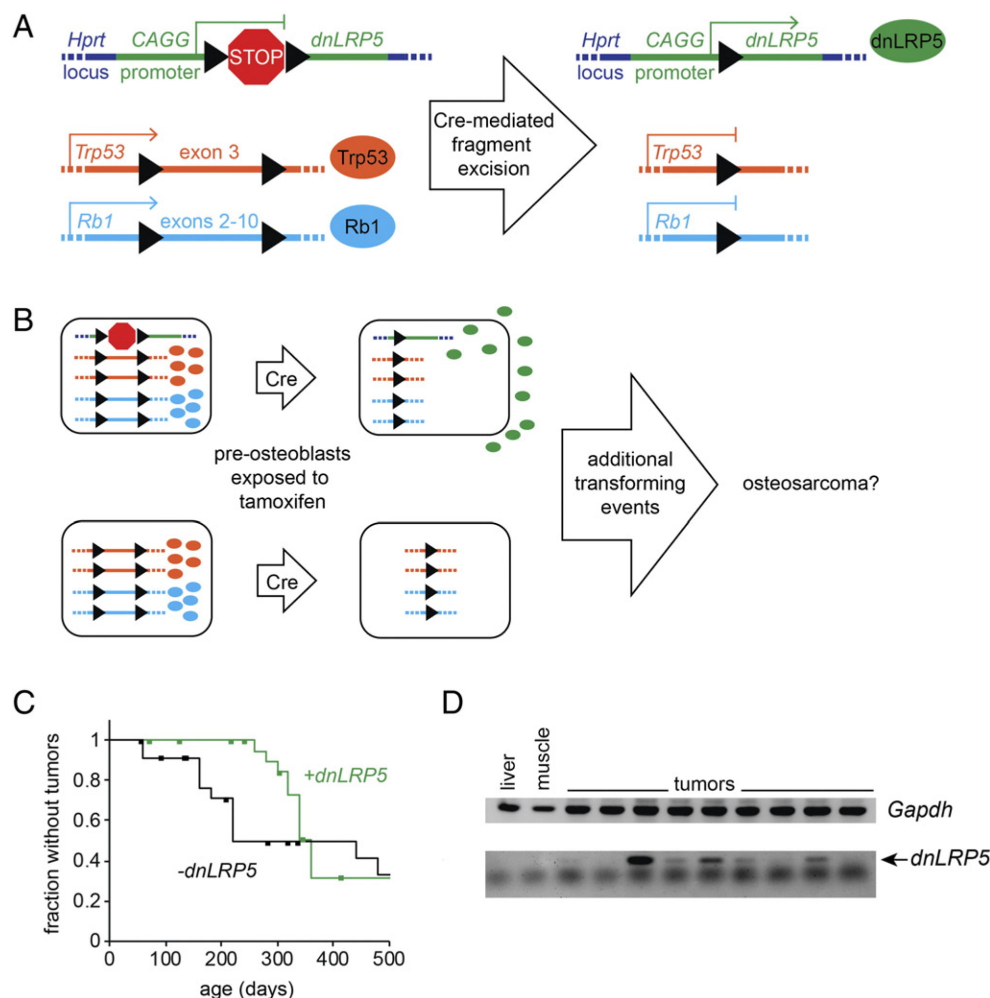

Figure 1. The impact of conditional expression of Wnt cell surface signaling blockade on osteosarcomagenesis. (A) Schematic representing the conditional expression allele of *dnLRP5* targeted to the *Hprt* locus as well as conditionally disrupted alleles of *Trp53* and *Rb1*. (B) Schematic of the experimental design. (C) Kaplan–Meier plot of the fraction of mice without OS in the presence (green line) or absence (black line) of *dnLRP*. (D) PCR gel image demonstrating the presence of the recombined floxed stop amplicon in all but one of the tumors bearing conditional dnLRP5.

PCR was conducted on genomic DNA isolated from tumors that developed in mice bearing conditional dnLRP5 alleles to verify successful excision of the stop sequence between the CAGG promoter and the dnLRP5 gene. Of the tumors selected for PCR testing, all but one demonstrated the presence of the recombined (active) allele, as shown by an amplicon of 172 bp.

Osteosarcomas Formed With or Without Cell Surface Wnt Signaling are Indistinguishable

Radiographs of mice upon reaching morbidity demonstrated tumors of similar appearance between the two groups. Mineralized matrix-producing tumors that destroyed portions of the mouse skeleton were identified almost universally in morbid mice lacking conditional expression of dnLRP5. Tumors of an indistinguishable appearance were identified in mice expressing conditional dnLRP5 (Figure 2*A*).

Histopathologic analysis of H&E stained cross sections of tumors demonstrated a range of osteoblastic differentiation states among tumors in both groups (Figure 2*B*). Each tumor from either group had demonstrably malignant cells producing osteoid matrix somewhere within the central cross section examined, confirming that all fit within the standard diagnosis of osteosarcoma used clinically in humans.

Downstream Wnt Signaling Remains Intact in Osteosarcomas that have Escaped Cell Surface Signal Blockade

Mice conditionally expressing dnLRP5 demonstrated a slightly longer latency to tumorigenesis, suggesting that blocked Wnt signaling impeded osteosarcomagenesis. Immunohistochemistry was used to determine if tumors that developed in spite of dnLRP5 expression arose from bypassing a need for canonical Wnt signaling or from bypassing the cell surface receptor blockade itself. Sections from tumors derived from dnLRP5-expressing and control osteosarcomas were immunostained for β-catenin and Cyclin D1, a known Wnt/β-catenin/TCF/LEF transcriptional target. When scored for each, the two groups of tumors were indistinguishable with regard to staining intensity (Figure 3). This suggests that the cell surface blockade and not the downstream signaling itself is what cells overcame in the process of transformation.

Blockade of Cell Surface Wnt Signaling does not Prevent Metastasis in Osteosarcomagenesis

As noted above, prior work with cell lines suggested that expression of dnLRP5 was sufficient to drive an epithelial cell morphology associated with blunted invasiveness and reduced metastasis. In contrast, the OSs that form *via* circumventing dnLRP5 retain clearly

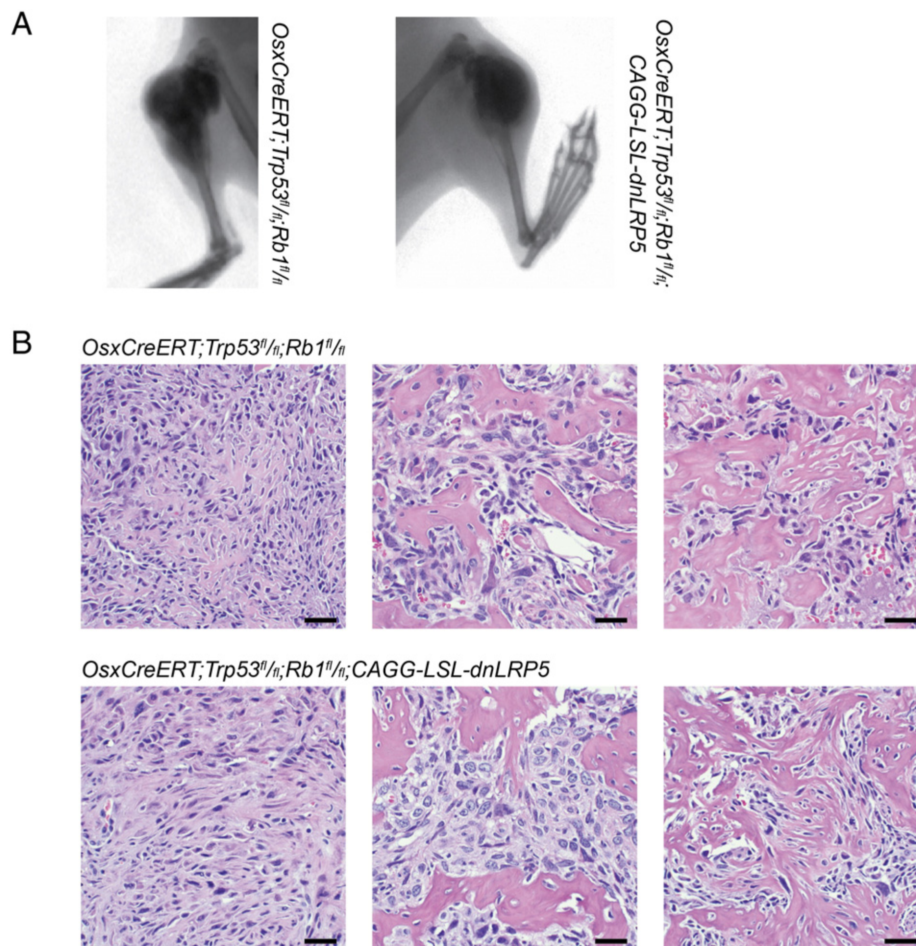

Figure 2. dnLRP5 expression does not change radiographic or histologic features of osteosarcomagenesis. (A) Radiographs of examples of tibia-located osteosarcomas in mice of each genotype. Each radiograph shows a large proximal tibia OS associated with osseous disruption and soft tissue expansion. (B) Representative photomicrographs of H&E histopathology from tumors that arose in control and *dnLRP5* genotypes demonstrate hypercellularity, nuclear pleomorphism, and a range of osteoid matrix production. (Magnification bars are 20 μm in length.)

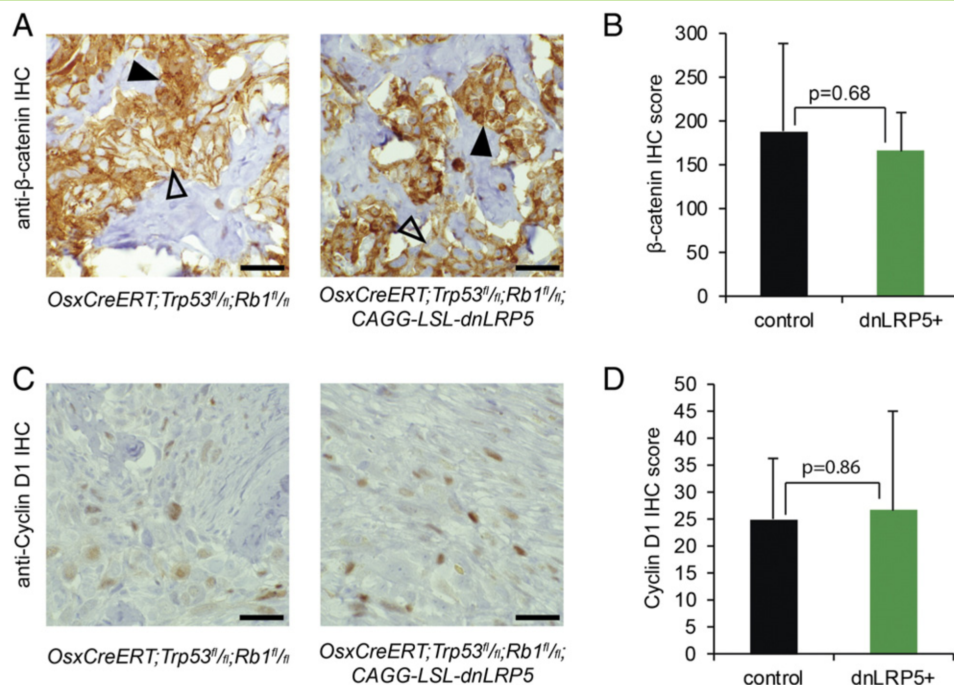

Figure 3. Tumors retain downstream Wnt signaling despite dnLRP5 expression in osteosarcomagenesis. (A) Representative immunohistochemical analysis of β-catenin in osteosarcoma tissue showing positively stained samples in control and dnLRP5 groups. Both cytoplasmic (open arrows) and nuclear (black arrows) staining patterns are evident. (B) Graph demonstrating no difference in the degree of β-catenin detected between groups. (C) Representative photomicrographs of immunohistochemical detection of Cyclin-D1 in osteosarcoma samples in control and dnLRP5 tumors. (D) Graph demonstrating no difference in Cyclin-D1 expression between groups. (Magnification bars are 20 μm in length.)

mesenchymal morphologic characteristics. They included cells of widely varied morphology and nuclear pleomorphism, consistent with a genetically unstable and aggressive malignancy, but nothing that tended toward epithelial differentiation (Figure 4*A*). Further, some of the dnLRP5 expressing mice developed clinically detectable metastasis (Figure 4*B*).

Discussion

Although Wnt signaling in cancer has been extensively studied, its role in osteosarcoma has only recently been investigated. In an osteosarcoma-producing mouse model, we have demonstrated that a block to cell surface Wnt signaling by a soluble, dominant-negative form of the LRP5 receptor impedes, but cannot abrogate osteosarcomagenesis. Although tumors arose in the dnLRP5 group after a slightly increased latency, they arose at a similar ultimate prevalence compared to control animal tumors and had indistinguishable radiographic and histopathologic features. Our data further shows that the osteosarcomas that developed in spite of the block to cell surface Wnt signaling demonstrated preserved Wnt signaling at the nucleus.

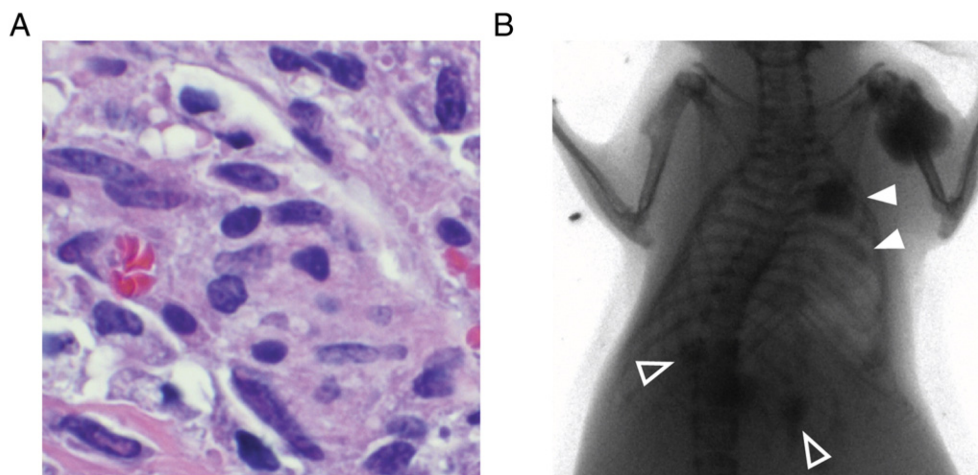

Figure 4. No evidence of mesenchymal-to-epithelial transition in dnLRP5 expressing tumors. High power photomicrograph (A) of a dnLRP5-expressing osteosarcoma sample demonstrating nuclear pleomorphism without evidence of epithelial differentiation. In line with this notion, the radiograph (B) from a dnLRP5-expressing osteosarcoma mouse demonstrates evidence of hepatic (open arrows) and pulmonary (white arrows) metastases. (Width of photomicrograph panel in A is 40 μm.)

To date, several lines of evidence suggest that LRP5 plays an important role in skeletal osteogenesis and sarcomatous transformation [8,12,13,15]. In human OS tissue samples, LRP5 mRNA has been shown to correlate with increased metastatic disease and an inferior disease-free survival. In their orthotopic OS metastasis model in nude mice, Guo et al. showed that expression of dnLRP5 reduced pulmonary metastasis and decreased cellular invasiveness of SaOS-2 cells *in vitro* [14,24].

Previous studies have suggested that blocking Wnt/β-catenin signaling at the level of dnLRP5 in cell lines achieves an apparent mesenchymal-to-epithelial transition. OS cell lines induced to express dnLRP5 showed decreased expression of Pro-MM2, N-cadherin, and Snail [14]. Based on our histological results however, there is no evidence of such a morphologic cellular transition when the tumor develops in spite of cell surface Wnt blockade. The multiplicity of pathways driving mesenchymal differentiation in OSs may not be so readily overturned with inactivation of this pathway from the onset of transformation.

To initiate Wnt/β-catenin signaling, Wnt ligands bind the Frizzled receptor. LRP5 is an identified co-receptor required for transducing extracellular Wnt signaling into an intracellular response [10]. Importantly, the tumors that developed in our osteosarcomagenesis model with blocked cell surface Wnt by dnLRP5 expression did not demonstrate blocked downstream Wnt signaling. They showed similar levels of cytosolic and nuclear β-catenin, and nuclear cyclin D1, by immunohistochemistry in comparison to control tumors lacking dnLRP5. This suggests that tumors have circumvented the cell surface block to Wnt signaling, but not Wnt signaling itself.

Our efforts did not provide the molecular mechanism by which dnLRP5 is circumvented in each tumor, only that it was circumvented, and not merely not recombined into the active allele state in most tumors. The single tumor that displayed no amplified recombined sequence may have developed from a cell that recombined the other alleles, but not the *Hprt* allele. Alternatively, during progression, the tumor cells may have cytogenetically lost the *Hprt* locus altogether. Otherwise, recombined, active alleles were present in all the other tumors assessed. The complexity of Wnt extracellular and membrane components, which consist of at least 19 Wnts, 10 Frizzled receptors, and co-receptors LRP5 and 6, suggest a panoply of escape routes around the blockade by dnLRP5. The tumors may have arisen in a subset of pre-osteoblasts with already upregulated internal Wnt signaling. They may have inactivated the dnLRP5 allele or abundantly up-regulated autocrine Wnt signals. It is also conceivable that transforming cells faced with constitutional blockade of the initiating Wnt/β-catenin signal developed genetic mutations or copy number changes that inactivated the β-catenin destruction complex.

Conclusions

Our data suggest intrinsic difficulty in abrogating Wnt signaling *via* cell surface blockade. Previous experiments in cell lines reported that dnLRP5 mediated a profound suppressive effect on tumor growth and invasiveness. Added to the heterogeneity of osteosarcomagenesis instead, dnLRP5 failed to block OS formation, metastatic disease, and even maintenance of Wnt signaling. Osteosarcomagenesis is likely an adaptive and aggressive transformation process that can overcome blockade of any single node in a complex pathway.

In summary, we demonstrate that OS progression is not inhibited in an *in vivo* model of cell surface Wnt/β-catenin blockade. Additionally, Wnt signaling is maintained resulting in stabilized β-catenin enabling downstream TCF/LEF mediated expression of Wnt-responsive genes.

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