The impact of osteoblastic differentiation on osteosarcomagenesis in the mouse

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Abstract

Osteosarcomas remain an enigmatic group of malignancies that share in common the presence of transformed cells producing osteoid matrix, even if these cells comprise a minority of the tumor volume. The differentiation state of osteosarcomas has therefore become a topic of interest and challenge to those who study this disease. In order to test how the cell of origin contributes to the final state of differentiation in the transformed cells, we compared the relative tumorigenicity of Cre-*LoxP* conditional disruption of the cell cycle checkpoint tumor suppressor genes *Trp53* and *Rb1* using *Prx1-Cre*, *Collagen-1α1-Cre,* and *Osteocalcin-Cre* to transform undifferentiated mesenchyme, pre-osteoblasts, and mature osteoblasts, respectively. The *Prx1* and *Col1α1* lineages developed tumors with nearly complete penetrance, as anticipated. Osteosarcomas also developed in 44 percent of *Oc-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$* mice. We confirmed using EdU click chemistry that the *Oc-Cre* lineage includes very few actively cycling cells. By assessing radiographic mineralization and histologic osteoid production, the differentiation state of tumors did not correlate with the differentiation state of the lineage of origin. Some of the osteocalcin-lineage-derived osteosarcomas were among the least osteoblastic. Osteocalcin immunohistochemistry in tumors correlated well with expression of DNA methyl transferases, suggesting that silencing of these epigenetic regulators may influence the final differentiation state of an osteosarcoma. Transformation of differentiated, minimally proliferative osteoblasts is possible, but may require such an epigenetic reprogramming that the tumors no longer resemble their differentiated origins.

Keywords

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Introduction

The recognition of stem-like characteristics among cancer initiating cells has reinvigorated the debated relationship between differentiation and oncogenesis[1, 2]. There is no doubt that many malignant cells exhibit differentiation that is blunted relative to their host tissue to some degree[3], but any single tumor may also include cells at varied states of differentiation[4].

Much of the related experimental work in solid tumors has been performed using carcinogenesis in epithelial tissues as a model, in which differentiation states of potential originating cells will map to specific locations in a crypt or gland[5]. Osteosarcomagenesis provides another useful model, in that the gamut of osteoblast differentiation is well delineated by marker gene expression profiles[6, 7] and osteosarcomas bear incomplete osteoblast differentiation characteristics[8-10].

Osteosarcoma is the most common primary malignancy of bone and is a leading cause of cancer death among adolescents and young adults[11-13]. The diagnostic entity of osteosarcoma includes neoplasms with a range of morphologies and cellular characteristics[14]. Histopathologically, the single diagnostic feature that all osteosarcomas share in common is the presence of malignant cells producing osteoid matrix[13]. It is the presence of such cells--not their predominance--that renders the diagnosis of osteosarcoma. The malignant-appearing cells producing osteoid matrix may comprise a large or small portion of the overall tissue volume in a given tumor. In addition, osteosarcomas may be composed of osteoblastic, chondroblastic, fibroblastic, and/or telangiectatic (vascular spaces) tissue types[15-17].

Osteosarcomas have been evaluated for their expression of osteoblast differentiation marker genes[18]. These marker gene profiles locate most osteosarcomas on the range of osteoblast differentiation at a point near pre-osteoblasts, expressing some lineage-committed markers, but often lacking expression of the markers of terminal differentiation and cell cycle exit, such as osteocalcin[18-20]. Given this observation, as well as the widely variable differentiation state of the bulk of neoplastic cells in osteosarcomas, many have debated whether the cell of origin or the transforming events themselves contribute more to the final differentiation state of the tumor[14]. The loss of *p53* or *RB1*, each associated with osteosarcoma, has been shown to impact osteoblast differentiation powerfully in ways that are likely independent from their tumor suppressor roles, the former actually moving undifferentiated mesenchyme toward the osteoblast lineage[19-23].

A number of investigators have induced osteosarcomas in mice by disrupting cell cycle checkpoint tumor suppressor genes at early stages of osteoblast differentiation[24-27]. Prior work has indicated that some measure of reversed differentiation (or de-differentiation) is possible during osteosarcomagenesis, in that the tumor cells often mimic less differentiated osteoblasts than the cells of origin[28]. What is not clear is how far along osteoblast differentiation this principle can be extended. One prior study drove SV40 T antigen by elements of the osteocalcin promoter in a transgene[29]. This suggested that osteosarcomagenesis initiated late in differentiation is possible, but did not permit precise localization of the initiation state along the course of differentiation as osteocalcin promoter

elements can lead to variable expression along that course. This recommends initiation from a better known cell of origin using well-characterized drivers. Further, we do not yet know how the differentiation state of the cell of origin impacts the final differentiation state of the tumor cells, for which head-to-head comparisons are needed.

Results and Discussion

For a head-to-head comparison, we compared the relative tumorigenicity of conditional disruption of the cell cycle checkpoint tumor suppressor genes *Trp53* and *Rb1* using osteoblast-related promoters for Cre-recombinase in the mouse at different stages along the course of osteoblast differentiation (Fig.1A). To transform relatively undifferentiated mesenchyme, we used *Prx1-Cre*[30]. For the pre-osteoblast stage we used *Col1a1-Cre*[31]. While another Cre-recombinase driven by a portion of the *Col1a1* promoter was previously used to induce osteosarcomas by conditional *Trp53* disruption[22], that particular transgene has been noted to display "leaky" expression in earlier stages of mesenchymal differentiation[32]. Therefore, the prior osteosarcoma model may have derived either from earlier, leaky expression or from pre-osteoblast expression of Cre leading to Cre-mediated *Trp53* disruption. The particular *Col1a1-Cre* selected for our experiments has been shown to be more specific to the pre-osteoblast and osteoblast stages of differentiation[31, 33]. To disrupt the terminally differentiated osteoblasts, we used *osteocalcin-Cre* (*Oc-Cre*)[34]. While transgenes with portions of the osteocalcin promoter have led to expression broadly crossing the range of osteoblast differentiation, this particular transgene has been shown to have expression limited to committed, mature, terminally differentiated osteoblasts and osteocytes[34].

For this study, mice with *loxP*-flanked, conditional alleles of *Trp53* and *Rb1* were crossed to produce offspring with homozygosity for each allele. Similar to prior studies, the strong majority of *Prx1-Cre*;*Rb1$^{fl}/_{fl}$*;*Trp53$^{fl}/_{fl}$* mice developed tumors[30]. Distinct from the prior report, nearly all mice in our cohort developed sarcomas involving the skeleton directly, detected by plain radiography and gross dissection; a few had soft-tissue sarcomas as well or instead (Supplemental Table 1). We limited our additional experiments to the tumors involving the skeleton.

Col1a1-Cre;*Rb1$^{fl}/$*;*Trp53$^{fl}/_{fl}$* mice also developed osteosarcomas efficiently. This was not surprising, as prior Cre-recombinase transgenes driven by the *Col1a1* promoter and effecting *Trp53* loss have led to osteosarcomagenesis[22]. Nonetheless, the more specific and more differentiated state of the early osteoblasts expressing this particular *Col1a1*-Cre suggested that this later stage of differentiation is also capable of transformation.

Much more surprising was osteosarcomagenesis arising from *Oc-Cre*;*Rb1$^{fl}/_{fl}$*;*Trp53$^{fl}/_{fl}$* mice. The expression of *osteocalcin* itself is considered to begin simultaneously with cell cycle exit. We therefore had not anticipated that disrupted cell cycle checkpoint regulation would have any impact in *osteocalcin*-expressing osteoblasts. However, regardless of the initial differentiation state of the cell, osteosarcomagenesis was inducible via *Trp53* and *Rb1* homozygous ablation, as evidenced by skeletally destructive neoplasms that

histopathologically displayed the presence of malignant cells producing osteoid matrix arising from all three lineages (Fig.1B).

The total number of *Prx1-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$*, *Col1a1-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$*, and *Oc-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$* mice observed for osteosarcomagenesis was 23, 32, and 25, respectively. *Prx1-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$* and *Col1a1-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$* mice developed tumors with nearly complete penetrance (Fig.1C). Osteosarcomas developed in 44 percent of *Oc-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$* mice. A Kaplan-Meier curve measuring survival to osteosarcomagenesis was generated and showed a similar latency to osteosarcomagenesis in each lineage (Fig.1D). However, many *Oc-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$* mice died or became morbid without forming tumors (noted as censored). The lower incidence, but similar latency to osteosarcomagenesis in the Oc-Cre lineage suggests that these cells may have a higher hurtle to pass with secondary pertubations, but that the opportunity to accrue such secondary hits is limited. As this transgene is expressed sometime near cell cycle exit, it may be that cells must have already accrued any necessary additional hits prior to the controlled disruption of *Trp53* and *Rb1*.

In order to confirm that expression of *Oc-Cre* follows cessation of proliferation, we crossed mice bearing each Cre-recombinase transgene to a tandem dimer Tomato (*tdTomato*) conditional reporter line. We then harvested embryos 2 days following reporter initiation of expression during embryogenesis, embryonic day 11.5 for the *Prx1-Cre* lineage, embryonic day 17.5 for the *Col1a1-Cre* lineage, and post-natal day 1 for the *Oc-Cre* lineage. Four hours prior to harvest, EdU was administered by intraperitoneal injection to label actively cycling cells (Fig.2A). This experiment identified that less than 2 percent of the *Oc-Cre* lineage were actively synthesizing DNA, confirming the very late differentiation state and predominantly non-proliferating statusof these cells (Fig.2B). These results were consistent with previous studies that identified *osteocalcin* and this particular Cre-recombinase transgene as a marker of terminal differentiation and cell cycle exit[18-20]. Importantly, these results show that osteosarcomagenesis followed loss of function in tumor suppressor genes *Trp53* and *Rb1* even after osteoblasts are fully mature and minimally proliferative.

The possibility that all *Oc-Cre*-induced tumors arose from this tiny sub-population of proliferative osteoblasts is challenged by the relatively high incidence of tumors from this lineage (44 percent). While lower than the incidence from the *Prx1-Cre* and *Col1a1-Cre* lineages, some mice from which had more than one tumor, the *Oc-Cre*-induced incidence was not even close to proportional to the actively proliferative number of cells in the less differentiated lineages. This suggests that slowing of proliferation or even exit from cell cycle might not protect against transformation.

In order to quantify the osteoblastic differentiation state of the skeletal tumors derived from tumor suppressor silencing in the *Prx1-Cre*, *Col1a1-Cre*, and *Oc-Cre* lineages, we assessed tumors with radiographs obtained at the time of euthanasia for morbidity. The number of *Prx1-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$*, *Col1a1-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$*, and *Oc-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$* tumors assessed via radiographs was 48, 48, and 18, respectively. Each skeleton-associated tumor was classified according to mineralization density relative to the femoral diaphysis by an evaluator unaware of the host animal's genotype. Tumors were classified on a scale of 1

to 4, with class 1 having no radiographically apparent mineralization, class 2 having mineralization present, but at a density less than the femoral diaphysis, class 3 having mineralization density similar to the femoral diaphysis, and class 4 having more dense mineralization than the femoral diaphysis (Fig.3A). Transformation in each of the three lineages led to a wide range of mineralization states in the resultant tumors (Fig.3B). We concluded that the differentiation state of the cell of origin did not correlate with mineralization in the resultant tumor. Surprisingly, some tumors derived from the *Oc-Cre* lineage had little to no mineralization, despite the robust mineralizing capacity of the cells from which they derived.

In order to quantify the osteoblastic differentiation state of tumors by another means, tumors were harvested, fixed, embedded in paraffin and sectioned. Mid-cross-sections were obtained and stained with hematoxylin and eosin for standard histopathology. An observer blinded to the genotype of the host mouse measured the cross sectional area represented by each classed production of osteoid matrix, 1 through 4, with class 1 areas showing no osteoid matrix, class 2 areas showing scant osteoid matrix, class three areas showing equivalent osteoid matrix and cellularity, and class 4 showing more dense osteoid matrix production than cellularity (Fig.3C). The number of *Prx1-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$*, *Col1a1-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$*, and *Oc-Cre*;*Rb1^{fl}/$_{fl}$*;*Trp53^{fl}/$_{fl}$* tumors assessed via H&E was 8, 9, and 6, respectively. Each tumor's class, based on mid-cross-sections of tumors, was determined via a weighted average of various classes throughout the tumor and their associated cross-sectional areas measured using ImageJ software. Regardless of lineage genotype, tumors demonstrated a range in the amount of osteoid matrix present (Fig.3D). Importantly, the matrix production in tumors did not correlate with the initial differentiation state of the cell of origin. In fact, there were more minimal-matrix-producing tumors arising from the *Oc-Cre* lineage than from the *Prx1-Cre* lineage.

The wide variation in the mineralization and matrix production in osteosarcomas arising from the Oc-Cre lineage suggested that some degree of de-differentiation had taken place during or after transformation. To investigate this further, we performed immunofluorescence against Cre-recombinase in some low- and high-matrix producing *Oc-Cre*-induced tumors. The low-matrix Oc-Cre induced tumors no longer demonstrated *Oc-Cre* expression from its differentiation-associated promoter, but their high-matrix counterparts retained Cre expression (Fig.4A).

This confirmed loss of expression from a differentiation-associated transgene suggested that some genetic or epigenetic re-set during transformation had altered the differentiation state of the cell. Because DNA methylation has an established role in osteoblast differentiation, we next tested the expression of three DNA methyl transferases (Dnmt1, Dnmt3a, Dnmt3b) as well as osteocalcin in the tumors, each by immunohistochemistry (Fig.4B). Tumors were ranked by two blinded investigators for each stain. Osteocalcin ranking did not correlate whatsoever with lineage of origin (Fig.4C), but correlated loosely with matrix production ranking, as expected (Fig.4D). Osteocalcin ranking also correlated strongly with the mean Dnmt immunohistochemical ranking for each tumor. This suggests that one potential route toward epigenetic resetting of a tumor's differentiation state may come through silencing or loss of DNA methyl transferases.

We report that conditional disruption of *Trp53* and *Rb1* using osteoblast-related promotors for Cre-recombinase expression in the mouse proved to be sufficient for osteosarcomagenesis in not only mesenchymal pluripotent progenitor cells and pre-osteoblasts but also in mature osteoblasts that had largely stopped proliferating. Furthermore, the final differentiation state of tumors did not correlate with the differentiation state of the lineage from which they originated, but did correlate with expression levels of DNA methyl transferases, suggesting that epigenetic re-setting had taken place during or after transformation in the de-differentiated tumors.

It is important to note that while the findings of this study suggest that cell cycle checkpoint loss can lead to transformation even in almost non-proliferative cells, the same may or may not be mechanistically happening in the generation of clinically-relevant human osteosarcomas. In mice we can only test sufficiency for transforming genetic events, not necessity. Understanding the cells of origin that enable transformation may help us understand some of the intrinsic vulnerabilities of the cancer cells that our therapies may target and exploit. In converse, they may also elucidate inherent strengths of the cells that will be unlikely to be overcome with biological therapies. The generation of an indiscernibly different range of osteosarcomas at only a slightly lower incidence when beginning from a terminally differentiated osteoblast population versus a proliferative stem-like progenitor population argues that osteosarcomagenesis can include a significant re-set of differentiation. That such is possible suggests that recent attempts at differentiation therapy[35, 36] would have to overcome powerful de-differentiation programs set in motion during tumor initiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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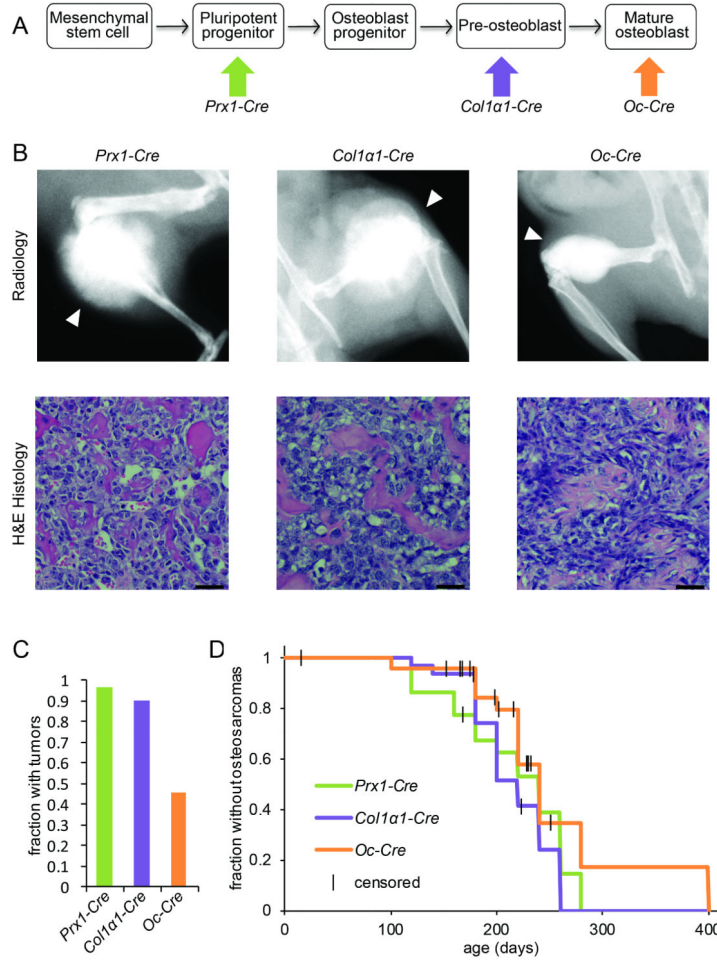
Figure 1. Osteosarcomagenesis can be induced by conditional tumor suppressor disruption across the range of osteoblast differentiation

(A) Schematic representing the differentiation course from mesenchymal stem cell to mature osteoblast with the expression point for each Cre-recombinase promoter plotted. (B) Representative radiographs and hematoxylin and eosin (H&E) histology photomicrographs from the osteosarcoma-mimicking skeletal tumors that arise following homozygous conditional disruption of floxed alleles of *Trp53* and *Rb1* induced by *Prx1-Cre*, *Col1α1-Cre*, or *Oc-Cre*. (C) Fraction of mice in each lineage that developed skeletal osteosarcoma-like tumors from a denominator sample size of 23, 32, and 25 mice for *Prx1-Cre*, *Col1α1-Cre*, and *Oc-Cre*, respectively. (D) Kaplan-Meier plots of the same cohorts presenting the relative latency to osteosarcomagenesis; censored animals reached morbidity requiring euthanasia without the development of a skeleton-associated tumor.
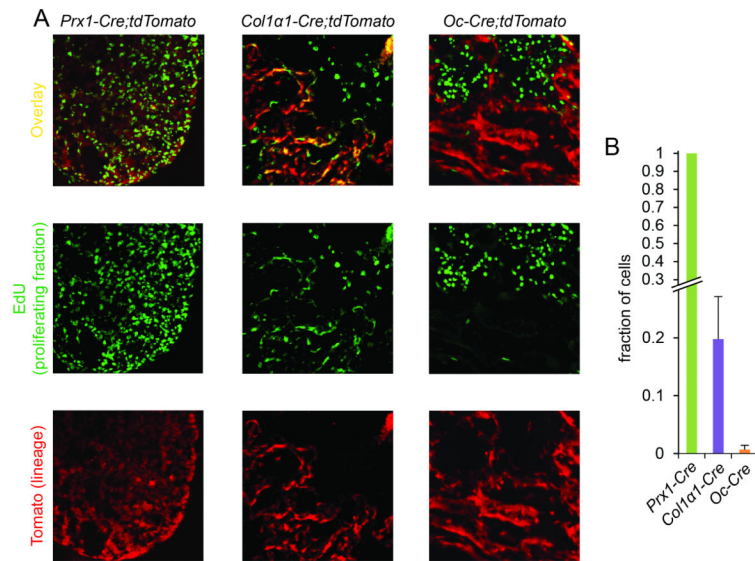
Figure 2. Active cell cycling among distinct osteoblast differentiation-state lineages
(A) Fluorescence photomicrographs of limb tissues from mice bearing a conditional
tdTomato linage marker (red) activated by *Prx1-Cre*, *Col1α1-Cre*, or *Oc-Cre*, collected two
days after the reported expression of each and 4 hours after intraperitoneal administration of
50 mg/kg EdU. Cells in active S-phase during that four hour period were detected by EdU
Click-iT chemistry detection kit (Life Technologies) on 8μm frozen sections obtained after
embedding in OCT (Tissue-Tek, Sakura Finetek). (B) Fraction of cells in each *tdTomato*-
fluorescent lineage that co-stained with EdU as evidence of active cell cycling based off of 3
Prx1-Cre, 6 *Col1α1-Cre*, and 16 *Oc-Cre* tissue samples analyzed with ImageJ software.
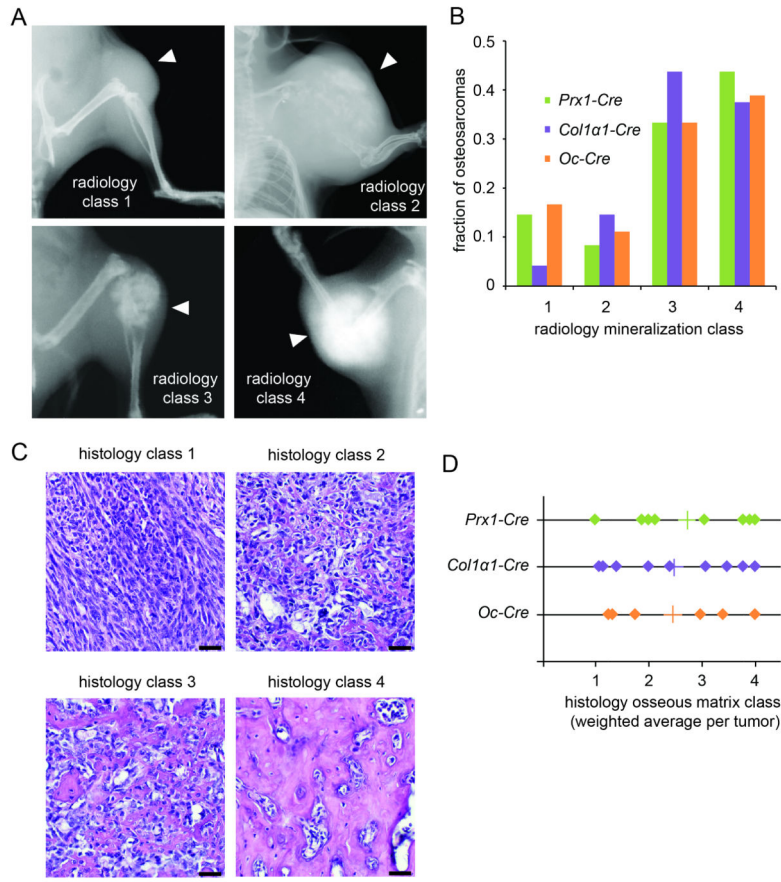
Figure 3. Radiographic mineralization and osteoid matrix production do not correlate with the differentiation state of an osteosarcoma's cell of origin

(A) Representative radiographs for each class of differentiation (1 through 4) as assessed by tumor mineralization. Lower and higher classes associated with lower and higher mineralized osseous matrix relative to the femoral diaphysis, respectively, in the tumors. (B) Distribution of radiographic mineralization classes among 48 $Prx1\text{-}Cre$;$Rb1^{fl}/_{fl}$;$Trp53^{fl}/_{fl}$, 48 $Col1\alpha1\text{-}Cre$;$Rb1^{fl}/_{fl}$;$Trp53^{fl}/_{fl}$, and 18 $Oc\text{-}Cre$;$Rb1^{fl}/_{fl}$;$Trp53^{fl}/_{fl}$ tumors. (C) Representative hematoxylin and eosin (H&E) photomicrographs of sectioned tumors demonstrating areas with the range of classes (1 through 4) of osteoid matrix production within tumors. Lower and higher classed areas associated with lower and higher production of osteoid matrix, respectively, in the tumors. (D) Distribution (and mean for each group, denoted by the +) of osteoid matrix production classes among skeletal tumors arising in 8 $Prx1\text{-}Cre$;$Rb1^{fl}/_{fl}$;$Trp53^{fl}/_{fl}$, 9 $Col1\alpha1\text{-}Cre$;$Rb1^{fl}/_{fl}$;$Trp53^{fl}/_{fl}$, and 6 $Oc\text{-}Cre$;$Rb1^{fl}/_{fl}$;$Trp53^{fl}/_{fl}$ mice. Tumor class, based on mid-cross-sections of tumors, was determined via an average of each class weighted by its associated cross-sectional area measured by ImageJ.
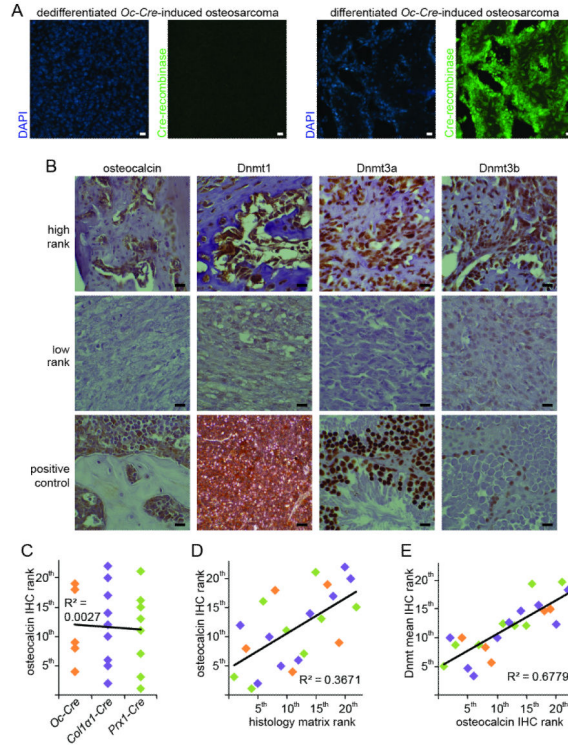
Figure 4. Dedifferentiation occurs during osteosarcomagenesis and correlates with silencing of epigenetic regulators

(A) Immunofluorescence photomicrographs of representative low matrix- and high matrix-producing osteosarcomas from *Oc-Cre*;*Rb1*^{fl}/$_{fl}$;*Trp53*^{fl}/$_{fl}$ mice demonstrating loss (left) and retention (right) of Cre-recombinase, following 10mM sodium citrate boiling antigen retrieval for 15 minutes, staining with an anti-Cre primary antibody (Covance, 1:3000) followed by an Alexa 488 secondary antibody (Jackson, 1:500). (B) Representative photomicrographs from immunohistochemical stains with anti-osteocalcin (Abcam, 1:500 dilution, following 20 minute protease 2 treatment, but no other antigen retrieval), anti-Dnmt1 (LifeSpan Biosciences, 1:200 dilution), anti-Dnmt3a (Novus Biologicals, 1:50 dilution), and anti-Dnmt3b (Novus Biologicals, 1:100 dilution) antibodies, applied each for 2 hours at 37°C, following 1-hour pH8.0 cell conditioning 1 (Ventana Medical Systems) antigen retrieval, using the BenchMark Ultra automated immunostainer (Ventana Medical Systems) with a biotinylated secondary antibody, DAB detection (IView) and hematoxylin counterstain. (C) Osteocalcin immunohistochemical ranking by 2 investigators blinded to sample identification, charted against Cre-initiation group, demonstrating no correlation. (D) Chart of osteocalcin rank by osteoid matrix production rank, demonstrating modest correlation. (E) Mean Dnmt immunohistochemical rank, averaged from individual Dnmt1, Dntm3a, and Dnmt3b ranks, charted by osteocalcin rank. (All magnification bars = 10μm)