Author/s:
Walia, MK;Castillo-Tandazo, W;Mutsaers, AJ;Martin, TJ;Walkley, CR

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Prospects

Murine models of osteosarcoma: a piece of the translational puzzle[1]

Mannu K. Walia[1,4], Wilson Castillo-Tandazo[1,2,4], Anthony J. Mutsaers[3], T. John Martin[1,2] & Carl R Walkley 0000-0002-4784-9031[1,2,*]

[1]St. Vincent's Institute, Fitzroy, Vic, 3065 Australia.
[2]Department of Medicine, St. Vincent's Hospital, University of Melbourne, Fitzroy, Vic, 3065 Australia.
[3]Departments of Biomedical Sciences and Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1, Canada
[4]Co-first authors

*Corresponding author:
Carl Walkley (ORCID: 0000-0002-4784-9031)
St Vincent's Institute
9 Princes St
Fitzroy 3065 Victoria
Australia
T: 61 3 9231 2480
F: 61 3 9416 2676
Email: cwalkley@svi.edu.au

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Abstract
Osteosarcoma (OS) is the most common cancer of bone in children and young adults. Despite extensive research efforts, there has been no significant improvement in patient outcome for many years. An improved understanding of the biology of this cancer and how genes frequently mutated contribute to OS may help improve outcomes for patients. Whilst our knowledge of the mutational burden of OS is approaching saturation, our understanding of how these mutations contribute to OS initiation and maintenance is less clear. Murine models of OS have now been demonstrated to be highly valid recapitulations of human OS. These models were originally based on the frequent disruption of *p53* and *Rb* in familial OS syndromes, which are also common mutations in sporadic OS. They have been applied to significantly improve our understanding about the functions of recurrently mutated genes in disease. The murine models can be used as a platform for preclinical testing and identifying new therapeutic targets, in addition to testing the role of additional mutations *in vivo*. Most recently these models have begun to be used for discovery based approaches and screens, which hold significant promise in furthering our understanding of the genetic and therapeutic sensitivities of OS. In this review, we discuss the mouse models of OS that have been reported in the last 3-5 years and newly identified pathways from these studies. Finally, we discuss the preclinical utilization of the mouse models of OS for identifying and validating actionable targets to improve patient outcome.

Introduction

Osteosarcoma (OS) is the most common cancer of bone, principally impacting teenagers and young adults. A second peak of OS incidence occurs later in life that can be associated with Paget's disease or where OS arises as a second cancer, however how the OS that arises in these two populations compares to that in adolescence has not been defined [Mirabello et al., 2009]. For patients with localised disease 5-year survival rates have remained at 70% for greater than three decades [Janeway et al., 2012]. Despite improvements in imaging and diagnostic technologies, patients with metastatic or relapsed OS presently have a 20-30% 5-yr survival rate. Many subtypes and variants of OS have been described, however, osteoblastic OS is the most common OS subtype clinically (~60%), with fibroblastic and chondroblastic being approximately equally represented at ~15% each. There is no clear association between OS subtype at diagnosis and outcome. Despite being the most common primary cancer of bone, OS is classified as a rare cancer.

Research has so far led to only a limited number of clinically relevant biologic insights, with the improvements in patient outcome arising from the better application of

existing therapies rather than from the introduction of new agents. Current management of OS utilizes combination chemotherapy - doxorubicin and cisplatin +/- methotrexate – and surgery which has improved survival rates to ~70% at 5 years [Janeway et al., 2012; Janeway and Grier, 2010]. Given the age of the majority of patients, these treatment approaches are associated with a significant burden of side-effects, both short and long term. Pre-operative chemotherapy response is the strongest predictor of survival in patients with localized disease. There is an urgent need to discover new therapeutic targets in OS.

Improving outcomes in OS will rely on an improved understanding of both OS biology and the roles that recurrently mutated genes play in tumor initiation and maintenance. Over the last decade, a number of tractable murine models of OS have been established and validated, generated chiefly on the knowledge of human familial cancer syndromes that have elevated rates of OS [Berman et al., 2008; Lin et al., 2009; Molyneux et al., 2010; Ng et al., 2012; Quist et al., 2015; Walkley et al., 2008]. Other reviews have covered this topic in greater detail than will be presented herein [Kansara and Thomas, 2007; Mutsaers and Walkley, 2014; Ng et al., 2012; Uluckan et al., 2015].l, including alternative genes tested and models using orthotopic transplants. More recently knowledge of the mutational burden of OS has greatly expanded as a result of tumour sequencing [Behjati et al., 2017; Chen et al., 2014; Perry et al., 2014]. Unfortunately, the detailed sequencing of the OS genome has not to date presented any immediately actionable pathways allowing direct translation. At the intersection of these approaches, coupled with the establishment of patient derived primary OS xenografts [Chen et al., 2014], a platform of information and reagents has been developed that can be utilised in therapeutic advancement for OS. We will discuss recently developed murine models and how these have been applied and validated to highlight new methods to target OS.

The genetics of human OS

Until recently our knowledge of the genetics of OS was largely derived from the study of familial cancer predisposition syndromes that had elevated rates of OS compared to other cancers and the general population. This has been highly informative, not just in OS, but in understanding genes involved in human cancer more generally. It was identified that patients with Li-Fraumeni syndrome [Li and Fraumeni, 1969; Malkin et al., 1990], hereditary retinoblastoma [Friend et al., 1986; Hansen et al., 1985] and RecQ helicase related disorders, primarily Rothmund-Thomson syndrome [Kitao et al., 1999; Wang et al., 2003], were all at greatly increased risk of OS. This led to the clear association of mutations in *TRP53* (*p53*), *RB1* and *RECQL4* with OS, which for *TRP53* and *RB1* have been experimentally confirmed.

Most recently whole genome sequencing and exome capture have demarcated the mutational burden of conventional human OS [Behjati et al., 2017; Chen et al., 2014; Kovac et al., 2015; Lorenz et al., 2016; Perry et al., 2014; Ribi et al., 2015]. These studies have transformed our understanding of the genetics of OS, with the very high to universal mutation of *TP53* accompanied by recurrent mutation of *RB1*, *ATRX* and *DLG2* in 29-53% of cases [Behjati et al., 2017; Chen et al., 2014; Perry et al., 2014; Ribi et al., 2015]. Mutations within the "p53 pathway" occur in all human cancers [Hanahan and Weinberg, 2011]. The most prevalent mutations in human cancers are point mutations resulting in p53 protein with altered function [Bieging et al., 2014]. In contrast, unique genomic rearrangements and other mutation types in OS frequently result in null alleles of *TP53* [Chen et al., 2014; Ribi et al., 2015]. The reason for this *TP53* mutational preference in osteoblastic cells, the lineage of origin of OS, is not clear. Additional mutations that occur recurrently at low frequency include mutations in the IGF signalling pathway [Behjati et al., 2017]. Other features of OS have been revealed by these studies including a high mutation load as assessed by copy number, structure and nucleotide variants compared to other pediatric tumors [Chen et al., 2015a], the presence of chromothripsis - the generation through a single event of a randomly shattered and reconstructed chromosome [Stephens et al., 2011]; and kataegis - a pattern of localized hypermutation [Nik-Zainal et al., 2012]. The mutational and karyotypic changes reported reflect a fundamentally unstable genome that dramatically reshapes the OS genome and impacts the potential for therapeutic intervention.

Whilst we are now armed with a detailed list of the mutational burden of OS, this has yet to translate into meaningful clinical benefit. In part, this may reflect the lack of specificity of currently available therapies. However, the advent of new technologies such as Crispr or siRNA and the rapid improvements in immunotherapy may yield breakthroughs. In parallel, generating the fundamental knowledge of how the mutations that recurrently arise contribute to OS initiation and maintenance may identify key pathways and nodes that can be exploited to improve outcomes for OS patients. It is as a part of this later endeavor that murine models of human OS hold greatest promise.

Murine models of OS

The generation of murine models of human cancer is an attractive approach to further our understanding of how different mutations or changes in gene expression contribute to tumorigenesis. For osteosarcoma, murine models have been long established based on either the administration of mutagens such as radiation [Martin et al., 1976], from the knock-out of genes associated with familial predisposition to OS (such as $p53^{-/-}$ [Jacks et al., 1994]) or from phenotyping in mutant or transgenic mice [Wang et al., 1995]. However, not all cases of murine/rodent OS are useful in the pursuit of identifying and testing new

therapeutic approaches for human OS. For example, the mutation of *Nf2* in mice led to a relatively high rate of OS, yet humans with neurofibromatosis 2 who have *NF2* mutations do not show elevated rates of OS [McClatchey et al., 1998; Stemmer-Rachamimov et al., 1998], indicating that not all murine models will be useful for this purpose. The generation of high fidelity models recapitulating the mutational pattern and specificity of human OS would be most useful in leveraging these murine cancers to ultimately improving outcomes for human OS.

This became possible with the increasing numbers of mesenchymal and osteoblast targeted Cre expressing mouse strains, coupled with conditional mutant alleles of the key genes implicated in familial OS, principally *p53* and *Rb1* (Figure 1, Table 1). Several groups, including our own, were able to establish high/full penetrant OS models based on the deletion of *p53* and *Rb* in the osteoblast lineage [Berman et al., 2008; Lengner et al., 2006; Lin et al., 2009; Mutsaers et al., 2013; Quist et al., 2015; Walkley et al., 2008]. The models described to date are summarized in Table 1. The genetic basis of these models has been confirmed to accurately reflect sporadic OS based on the subsequent sequencing of the human OS genome. These murine models reproduce the cardinal features of human OS, including histology, site of disease, metastatic preferences, transcriptional program and karyotypic complexity [Mutsaers et al., 2013; Walkley et al., 2008]. Conceptually similar models have been established through the transgenic overexpression of SV40T antigen using the osteocalcin promoter, which acts to sequester p53 and Rb [Molyneux et al., 2010]. The murine models have established the dominance that loss of *p53* exerts on initiating OS, with the evidence indicating that loss of *Rb* co-operates with loss of *p53*. In contrast to the efficiency through which mutation of *p53* induces OS, we did not observe OS induction upon deletion of *Recql4* as is observed in patients with *RECQ4* mutation in Rothmund-Thomson Syndrome [Ng et al., 2015]. This result has been confirmed with an independently generated null allele of *Recql4* [Lu et al., 2015]. Therefore, whilst RTS patients display an elevated rate of OS, the murine models established to date are not recapitulating this finding, suggesting that additional models may be needed to understand the OS predisposition in these patients.

These models have generated several important observations that inform our understanding of the human disease. Firstly, the cell of origin of OS has been a broadly discussed area, yet it has not reached a consensus. The murine models have utilized a range of Cre expressing lines that are active from multi-potential mesenchymal/skeletal progenitors through to late stage mature osteoblasts. The outcome of these studies is that the most efficient and high penetrant generation of OS occurs once *p53* +/- *Rb* are deleted in the early committed osteoblasts (*Osx*-Cre) onward (Figure 1, Table 1). The demonstration that even cells considered mature osteoblasts, marked by expression of *Osteocalcin*-Cre, with limited proliferative capacity could efficiently generate OS, strongly suggests that cells

within the committed osteoblast lineage are the cell population from which OS arises [Quist et al., 2015].

A second conclusion from these models is that the nature of the p53 mutation can influence the subtype of OS that evolves. Through comparing Cre:lox mediated *p53* deletion and shRNA mediated p53 knock-down, we were able to establish models of fibroblastic and osteoblastic OS, respectively [Mutsaers et al., 2013]. This result suggested that the abrupt deletion of p53 immortalized cells rapidly, while in the shRNA model the pre-osteoblasts that express the shRNA were able to continue dividing and undergoing lineage differentiation until the levels of p53 were critically low and immortalization occurred at a more mature cell stage. The cell of origin may impact the clinical subtype observed, with more immature pre-osteoblasts favoring fibroblastic OS and more mature cells favoring osteoblastic OS. As a caveat, the use of *Osteocalcin*-Cre did not yield highly mineralized lesions [Quist et al., 2015], cautioning a direct extrapolation of this observation.

Lastly, the deletion of *Recql4* did not result in the initiation of OS in murine models, including sensitized models with concurrent loss of *p53*. This was unexpected given the precedence from *p53* and *Rb* mutation in similar models, but multiple independent datasets support the failure to generate OS upon osteoblast restricted deletion of *Recql4* [Lu et al., 2015; Ng et al., 2015]. This raised several questions regarding the role of RECQ4 in OS and why RTS patients develop OS at high incidence. Complete null alleles of *Recql4* are not tumor suppressors but this is possibly a role of point mutation or truncating mutations where some protein expression is retained, an interpretation retrospectively consistent with the *RECQ4* mutation pattern in patients [Siitonen et al., 2009]. Another possibility is that Recql4 has distinct functional domains and roles, with the N-terminal region required for general DNA replication through its homology to Sld2, whilst the ATP-dependent helicase region and C-terminal parts of the protein are involved in the tumor suppressive function. This model is supported by data from analysis of hematopoietic cells where we could rescue *Recql4* deficient cells with a mutant form of full length RECQ4 that could not act as an ATP dependent DNA helicase [Smeets et al., 2014]. It is apparent that more nuanced and refined models are required to understand the role of Recql4 in OS. A detailed analysis of the OS that arises in RTS patients would be highly valuable to determine hwether this represents a genetically distinct entity of OS or is similar to sporadic OS, however given the paucity of patients this may be very difficult.

Newly identified pathways: are all of them relevant to human OS?

The establishment of the high-fidelity models of OS has enabled recent studies testing the roles of specific genes and pathways in these models. These studies have

identified several pathways of interest that may be relevant to human OS and progressing new treatment options as well as raising questions about how they relate to human OS.

Newly identified pathways: *Modulation of cAMP related pathways in OS*.

Genome wide association studies (GWAS) in OS have identified changes in cyclic AMP (cAMP) related processes as predisposing to OS. A GWAS defined two OS susceptibility loci in human: the metabotropic glutamate receptor *GRM4* and a region on chromosome 2p25.2 lacking annotated transcripts [Savage et al., 2013]. *GRM4* has a role in cAMP generation. A GWAS in dogs with OS identified variants of *GRIK4*, involved in cAMP pathways [Karlsson et al., 2013]. Using the murine OS model generated by an osteocalcin promoter-driven SV40T/t antigen (MOTO mouse), several tumours had inactivating mutations in a negative regulatory subunit of the cAMP-dependent protein kinase (PKA) complex, *Prkar1a*, which results in activation of the catalytic activity of PKA. One tumour had in this study exhibited amplification of *Prkaca*, the PKA catalytic component [Molyneux et al., 2010].

Osteoblastic cells are highly responsive to cAMP, and major regulators of the osteoblast lineage, such as Parathyroid hormone (PTH) and PTHrP, increase cAMP and activate cAMP-dependent signalling [Suva et al., 1987]. PTHrP/PTH are key regulators of osteoblast and skeletal homeostasis [McCauley and Martin, 2012]. PTHrP and PTH activate their shared receptor, PTHR1, on osteoblasts or OS cells, activating adenylyl cyclase and stimulating cAMP production [Juppner et al., 1991], followed by PKA activation, leading to the transcriptional changes associated with PTH/PTHrP treatment [Gardella and Juppner, 2001; Pioszak and Xu, 2008]. In normal osteoblasts, PTHrP is produced by osteoblastic lineage cells and acts in a paracrine manner upon other osteoblastic cells at different stages of differentiation [Martin, 2005]. Long-term treatment of rats with PTH(1-34) resulted in a high incidence of OS [Vahle et al., 2002]. Elevated expression of PTHR1 is a feature of human and rodent OS [Martin et al., 1976; Yang et al., 2007]. PTHrP is expressed in murine OS subtypes where it acts in an autocrine/paracrine manner [Ho et al., 2015] to activate PTHR1 signalling. Collectively the evidence from multiple species implicates enhanced cAMP-PKA activity in OS.

Using the MOTO mouse, Khokha and colleagues demonstrated that loss of *Prkar1a* rapidly accelerated tumour formation in these mice [Molyneux et al., 2010]. Cells derived from the *Prkar1a* mutant mice exhibited continued signaling downstream of PKA. These mice develop many lesions across the skeleton at a very young age. Interestingly in humans, increased cAMP/PKA activity caused by the missense mutations to *GNAS1* causes fibrous dysplasia of bone [Weinstein et al., 1991]. Inactivating mutations of *PRKAR1A* are associated with Carney complex, an autosomal dominant multiple endocrine neoplasia syndrome [Kirschner et al., 2000], where patients can develop osteochondromyxomas of the

bone but are not noted to develop OS [Carney et al., 2001]. The sequencing of increasing numbers of human OS has not identified recurrent mutations in these specific genes, but has demonstrated an over-representation of mutations in the cAMP pathway, suggesting that the overall deregulation of this signaling cascade may be the critical requirement of mutations in this pathway [Walia et al., 2016; Walkley et al., 2014].

Consistent with a model that the increased activity of the cAMP pathway is required by OS are the results from our analysis of the role of PTHR1 and its ligand PTHrP. PTHR1 expression and responsiveness are a species-conserved feature of OS [Martin et al., 1976; Yang et al., 2007]. Primary cell cultures established from primary and metastatic lesions from the murine fibroblastic and osteoblastic OS models express PTHR1 and display robust induction of cAMP in response to both PTH and PTHrP [Ho et al., 2015]. The knock-down of PTHR1 caused a striking *in vivo* phenotype, with significantly impaired *in vivo* proliferation of the tumor and the acquisition of a mineralized state consistent with the cells having progressed in the differentiation cascade [Ho et al., 2015]. These studies indicate that active signalling through PTHR1 was required to maintain the immature state and proliferation of OS. Our subsequent studies have identified that OS cells require continuous expression of PTHrP, an autocrine ligand made by OS cells and able to act on PTHR1 in OS, with downstream activation of CREB1 [Walia et al., 2016]. OS cells have basally elevated levels of cAMP that are maintained via autocrine PTHrP. Together with reduced expression of negative regulators of the cAMP pathway such as phosphodiesterases, this results in constitutive activation of cAMP in OS. The knock-down of either PTHrP or CREB1 had a major effect on the OS cells, with cells from the fibroblastic OS model demonstrating a significantly reduced proliferative capacity, while cells from the osteoblastic OS model were no longer viable upon knockdown of CREB1 [Walia et al., 2016]. These studies suggested that activation of this signalling pathway is required for maintenance of established OS.

We then used a reductionist system to address the requirement for the cAMP pathway, centred on PTHrP and CREB1 during the initiation of OS. Using primary long bone osteoblasts that could be engineered to become p53 deficient, we observed that these cells reproducibly underwent a hyper-proliferative transformation at approximately 12-14 days after deletion of p53 [Ng et al., 2015; Walia et al., 2016]. The loss of p53 transcriptional activity was accompanied by activation of a cAMP gene signature. This suggested that deregulation of the cAMP pathway is an early event following loss of p53 in osteoblasts. To establish the requirement for this in the manifestations of the p53 deficient state, we knocked down expression of PTHrP or CREB1 immediately prior to deletion of p53. This demonstrated that expression of both PTHrP and CREB1 were necessary for the hyperproliferative transformation of primary osteoblasts in response to mutation of p53. These results, together with the prior related work in the MOTO model and GWAS based

studies, establish that deregulation of the PTHrP - cAMP - PKA - CREB1 pathway is species conserved and required for both initiation and maintenance of OS. Whilst yet to be demonstrated in primary human OS, murine studies demonstrated that osteoblastic OS is more sensitive to reductions in this pathway than fibroblastic OS. Such differential sensitivities between the OS subtypes would warrant further investigation [Walia et al., 2016].

Newly identified pathways: *Is RANK-RANKL a good target?*

Based on their analysis of the MOTO mouse, Khokha and colleagues identified RANK-RANKL signalling as a potential target in OS [Beristain et al., 2012; Chen et al., 2015b], quite apart from its role in osteoclast formation. This pathway is critical in osteoclastogenesis and has been implicated in a number of different cancer types, most prominently breast cancers and cancer associated with bone resorption [Lacey et al., 2012]. This latter action is the basis of a study targeting RANK signalling therapeutically in OS [Gorlick et al., 2013]. In the MOTO mouse model, the effects of RANK-RANKL deletion were compared between whole body deletion (MOTO-*Rankl*$^{-/-}$) and osteoblast vs osteoclast deletion of Rank (MOTO-*Rank* $^{\Delta/\Delta OB}$ and MOTO-*Rank* $^{\Delta/\Delta OC}$ respectively) [Chen et al., 2015b]. It was observed that whole body loss of RANKL potently suppressed tumor formation in the MOTO mice. In contrast, the deletion of RANK in osteoblasts using *Col1*α*1*-Cre did not modify tumor formation in these mice. Strikingly, the deletion of RANK in osteoclasts was proposed to inhibit OS formation in the MOTO background. This was a potentially highly interesting observation and one with translational potential given the development of RANKL inhibitors for clinical application. Unfortunately, the interpretation of the model used to achieve osteoclast specificity warrants caution. The authors used *Mx1*-Cre to undertake these studies and treated the mice with pIpC (for 13 weeks) to induce Cre activity to delete a conditional *Rank* allele. Whilst a good model for broad somatic deletion in the adult mouse, it is difficult to reconcile existing data with the interpretation that the model achieved osteoclast specific RANK deletion. A large body of literature demonstrates the activity of *Mx1*-Cre broadly in hematopoietic cells including hematopoietic stem and progenitors, skeletal stem and progenitor cells (that give rise to osteoblasts and would be forming the OS in these models) and many other tissues in the mouse [Kuhn et al., 1995; Park et al., 2012; Roberts et al., 2002; Walkley et al., 2007; Yilmaz et al., 2006]. The model used and the effects are more consistent with a whole body somatic deletion of *Rank*, rather than the result of osteoclast specificity. Supportive of this is the lack of RANK expression in the tumor cells (mesenchymal derived osteoblast lineage cells) derived from the MOTO-*Rank* $^{\Delta/\Delta OC}$ mice. Additionally, there is conflicting data regarding the expression of RANK and RANKL in

human OS [Branstetter et al., 2015; Lee et al., 2011]. Further work is required to clarify the requirement for RANK in OS cells.

Newly identified pathways: *Notch and OS?*

Work from Lee and colleagues reported that activation of Notch1 could generate OS in mouse models [Tao et al., 2014]. Notch is a member of a family of transmembrane receptors that are important regulators in a range of developmental and tumor pathways. In humans, mutations in *NOTCH* generally target the intracellular domain (termed NICD) and lead to constitutive activity of this pathway. Activation of Notch1 or its ligands leads to alterations in bone homeostasis through proliferative and anti-differentiation mechanisms [Engin et al., 2008; Hilton et al., 2008]. Lee and colleagues crossed the *R26*-LSL-NICD mice to *Col1*α*1*-2.3kB–Cre mice to generate osteoblast specific overexpression of Notch1 in this lineage [Tao et al., 2014]. They observed complete penetrance tumor induction by ~ one year of age. The concurrent deletion of *p53* led to a rapid acceleration of tumor formation and concomitant reduction in survival, in a manner comparable to the effect of loss of one or both *Rb* alleles on a *p53* null osteoblast background [Walkley et al., 2008]. These studies demonstrate that in mice activation of Notch is a potent means to generate tumors of bone. However, the data from the increasingly large numbers of human OS that have been sequenced has not identified recurrent mutations in *NOTCH1*, nor in other components of this signaling cascade [Behjati et al., 2017; Chen et al., 2014; Kovac et al., 2015; Lorenz et al., 2016; Perry et al., 2014; Ribi et al., 2015]. It is presently unclear to what extent this represents a finding that can be leveraged therapeutically against OS or if this represents a murine model of OS with limited translational potential to human OS.

Newly identified pathways: *Interrogating the genome*.

The use of the OS predisposed mice for unbiased gene discovery is a very attractive utilization of these models. These models are potentially powerful discovery platforms and can be used to understand the *in vivo* mutational spectrum that leads to OS. Moriarity and colleagues have done just this, using *in vivo* transposon mediated mutagenesis to identify mutations that co-operate with a *p53* mutation to accelerate OS formation in mice [Moriarity et al., 2015]. Using an *Osx*-Cre LSL-*Trp53*^{R270H} background to mutagenize with a *Sleeping Beauty* transposon system, they analyzed the mutations that occurred in a large number of tumors and metastatic nodules [Moriarity et al., 2015]. A total of 232 recurrent transpositions were identified in the tumors, with the confirmation of the involvement of a number of known pathways including the PI3K-mTOR axis, *Myc* and *Pten* [Chen et al., 2014; Gupte et al., 2015; Perry et al., 2014]. In addition to these, new pathways and targets were identified involving recurrent mutations in the *Sema4d* and *Sema6d*, genes involved in axonal

guidance [Moriarity et al., 2015]. This approach, coupled with newly developed methods involving Crispr mediated genome modulation, provides significant new developments in understanding the genetics of OS. It would be interesting to determine in such a model if there were a fundamental difference between the effects of *p53* hot-spot mutation, as used by Moriarity et al. and as commonly seen in many human cancers, and the impact of a null allele of p53 which is a common mutational preference in OS. It is apparent that the deletion of *p53* using *Osx*-Cre and the introduction of a heterozygous p53 point mutant have significantly different latency *in vivo* [Moriarity et al., 2015; Walkley et al., 2008], with the null allele more rapidly generating disease. Whether the same co-operative events emerged *in vivo* would be an interesting test of the role of how the different p53 mutations influence tumor evolution, in this case in a robust model of human cancer.

Preclinical utilization of murine models of OS

The murine models established based on deletion of *p53* and *Rb1* in the committed osteoblast population demonstrate a high degree of conservation with human OS [Janeway and Walkley, 2010; Mutsaers and Walkley, 2014; Walkley et al., 2008]. The demonstration that these models have conserved chemical/drug sensitivities and genetic vulnerabilities with primary human OS would greatly extend their application. Recent work has begun to provide evidence that this is indeed the case and that the murine OS cells share a similar therapeutic and genetic sensitivity to primary human OS.

Initial studies focused on the testing of specific agents or genetic vulnerabilities. As an example, it was demonstrated that OS cells, both murine and human, are sensitive to the bromodomain (BET) inhibitors [Baker et al., 2015; Lamoureux et al., 2014]. The original rationale for testing these inhibitors arose from the observation in other settings that BRD4 inhibitors inhibited Myc function, and Myc has been strongly linked with OS [Delmore et al., 2011; Shimizu et al., 2010]. A range of structurally diverse BET inhibitors, including JQ1 and I-BET151/I-BET762, demonstrate activity against murine primary fibroblastic and osteoblastic OS and a human PDX derived cell line. In our hands, we did not see an association between the activity of these compounds and a reduction in Myc protein levels, but we did observe reduction in FosL1 levels [Baker et al., 2015]. The activity of JQ1 was confirmed *in vivo*, where it showed potent activity against OS. When tested *in vitro* with standard of care chemotherapy, BET inhibition enhanced the activity of doxorubicin. We further added CDK9 inhibitors together with JQ1, based on the mechanism whereby BRD4 recruits the P-TEFb complex to promoters to alter gene expression [Zippo et al., 2009]. The targeting of both BRD4 and CDK9, with either flavopiridol or dinaciclib, was synergistic and demonstrated efficacy against both murine and human OS [Baker et al., 2015].

More recently multiple groups, including our own, have reported results from genome-wide genetic screens and drug/chemical library screens [Chen et al., 2014; Gupte et al., 2015; Perry et al., 2014]. These initial screens have been conducted to identify drugs/chemicals or genetic susceptibilities in OS cells and, unexpectedly, converged on a common pathway. We conducted a whole genome siRNA and in parallel a selected small molecule inhibitor screen using primary OS cells derived from the *Osx*-Cre $p53^{fl/fl}$ $pRb^{fl/fl}$ model [Gupte et al., 2015; Walkley et al., 2008]. The siRNA screen was performed in the presence and absence of doxorubicin, with the aim of identifying loss of function alleles that killed OS or that modified the response to doxorubicin [Gupte et al., 2015]. From the siRNA screen a number of hits were identified, the most directly interesting from a translational perspective being the PI3K-mTOR axis. We found an enrichment of hits in this pathway in the murine OS cells that was confirmed across independent samples and in human PDX derived OS cells. The same result was achieved using an independent, both technologically and biologically, shRNA loss of representation screen against murine OS derived cell lines [Perry et al., 2014]. Strikingly, the results from our chemical screen of predominantly kinase inhibitors identified activity from a group of PI3K-mTOR inhibitors, with those that inhibited both PI3Kα and mTOR most effective [Gupte et al., 2015]. Further evidence for the tractability of this pathway was provided by Dyer and colleagues, who had been conducting a more extensive chemical screen of primary human OS cells maintained as PDXs [Chen et al., 2014]. In these cells, drugs that targeted the PI3K-mTOR axis were active against OS, and like the mouse, this activity showed a preference for combined inhibitors of PI3Kα and mTOR being most effective [Gupte et al., 2015]. That the murine models and primary human OS demonstrate such a close conserved sensitivity to PI3Kα-mTOR inhibition demonstrated the validity of these models as tools for preclinical discovery and testing. Additional parameters, such as changes in miRNAs [Bhattacharya et al., 2016], can also be demonstrated to show strong conservation between primary murine and primary human OS. These recent studies have further validated the genetically modified mouse models of OS as highly faithful recapitulations of human OS and demonstrated that these can be applied for discovery and testing of new therapeutic approaches.

Conclusions and future directions

Valid murine models are an extremely valuable resource in advancing both our understanding of the genetics of cancer and in the development of new therapies. In the case of rare cancers, such as osteosarcoma, these models are of even greater importance as the ability to test numerous agents simultaneously in humans is limited. The development and validation of the murine models of OS, contributed to by numerous groups, has provided

a new opportunity to explore the genetics and therapeutic targeting of OS. Coupled with the increasing sequence information available from primary human OS, the establishment of patient derived xenografts, and the development of naturally occurring OS and testing of investigational therapeutics in large breed dogs, we are now poised to be able to probe the OS genome at unprecedented depth. The murine models established to date provide a highly flexible and tractable system with which to identify and test new therapeutic approaches for OS.

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Figure Legends

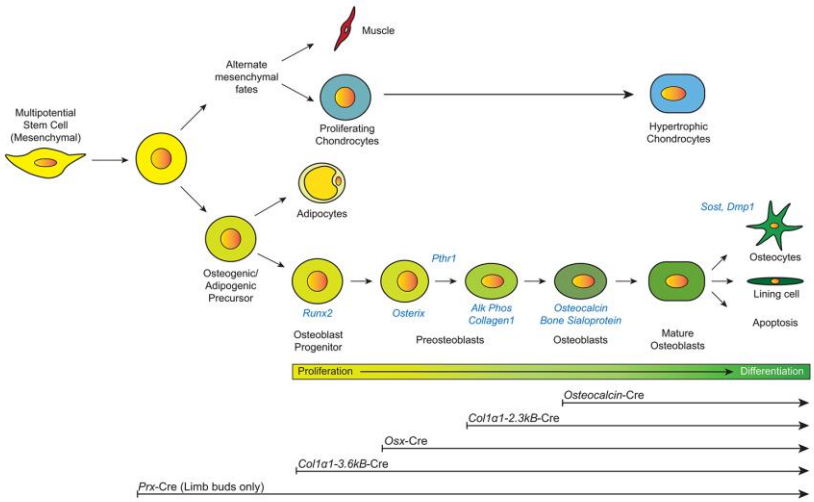

Figure 1. Outline of the cell populations targeted by different Cre expressing mouse models within the osteoblast lineage. Table 1 outlines the results from genetically engineered OS models using these different Cre lines.

Cell lineage	Cre	Gene	OS penetrance	Metastatic disease	Median survival	Mutation in human OS / Familial cancer syndrome	Reference
Mesenchymal / Skeletal progenitors	*Prx1*	*p53$^{fl/fl}$*	61%	Yes (24%)	50 weeks	Yes / Li-Fraumeni syndrome	Lin et al.
		Recql4$^{fl/fl}$	0%			No / Rothmund-Thomson Syndrome	Lu et al.
		p53$^{fl/fl}$ Rb$^{fl/+}$	92%		39 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Calo et al.
		p53$^{fl/fl}$ Rb$^{fl/fl}$	29%		19 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Lin et al.
Pre-Osteoblasts	*Osx1*	*Rb$^{fl/fl}$*	0%			Yes / Hereditary retinoblastoma	Walkley et al.
		p53$^{fl/fl}$	100%	Yes (40%)[a]	~42 weeks	Yes / Li-Fraumeni syndrome	Walkley et al.
		TRE-sh*p53.1224*	100%	Yes (83%)	~ 69 weeks	Yes / Li-Fraumeni syndrome	Mutsaers et al.
		Recql4$^{fl/fl}$	0%			No / Rothmund-Thomson Syndrome	Ng et al.
		p53$^{fl/fl}$ Rb$^{fl/+}$	100%	Yes (3.7%)	~23 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Walkley et al.
		p53$^{fl/fl}$ Rb$^{fl/fl}$	100%	Yes (37%)	~18 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Walkley et al.
		TRE-sh*p53.1224 pRb$^{fl/fl}$*	100%	Yes (85%)	~ 57 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Mutsaers et al.
		p53$^{R270H/+}$ SBmut	75%	Yes (26.4%)	~86 weeks	Yes / Li-Fraumeni syndrome	Moriarity et al.
		SBmut	24.4%		~116 weeks	No/?	Moriarity et al.
	Col1α1-3.6kB	*p53$^{fl/fl}$*	60%		~40 weeks	Yes / Li-Fraumeni syndrome	Lengner et al.
Osteoblasts	*Col1α1*-2.3kB	*p53$^{fl/fl}$*	85%		48 weeks	Yes / Li-Fraumeni syndrome	Lin et al.
	Col1α1-2.3kB	*Notch-ICD*	100%	Yes (40%)	~60 weeks	No	Tao et al.[b]
	Osteocalcin	SV-40 T/t antigen	100%	Yes (90%)	~30 weeks	Sequesters p53 and Rb	Molyneux et al. [c]
		p53$^{fl/fl}$ Rb$^{fl/fl}$	44%		~57 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Quist et al.

Table 1. Summary of penetrance, metastases, associated diseases and latency of described genetically modified murine osteosarcoma models

[a] Small cohort of animals; [b] Notch Intracellular domain; [c] Tumors defined as “Multi-ostotic bone tumors”.

Cell lineage	Cre	Gene	OS penetrance	Metastatic disease	Median survival	Mutation in human OS / Familial cancer syndrome	Reference
Mesenchymal / Skeletal progenitors	*Prx1*	*p53$^{fl/fl}$*	61%	Yes (24%)	50 weeks	Yes / Li-Fraumeni syndrome	Lin et al.
		Recql4$^{fl/fl}$	0%			No / Rothmund-Thomson Syndrome	Lu et al.
		p53$^{fl/fl}$ Rb$^{fl/+}$	92%		39 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Calo et al.
		p53$^{fl/fl}$ Rb$^{fl/fl}$	29%		19 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Lin et al.
Pre-Osteoblasts	*Osx1*	*Rb$^{fl/fl}$*	0%			Yes / Hereditary retinoblastoma	Walkley et al.
		p53$^{fl/fl}$	100%	Yes (40%)[(a)]	~42 weeks	Yes / Li-Fraumeni syndrome	Walkley et al.
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		p53$^{fl/fl}$ Rb$^{fl/fl}$	100%	Yes (37%)	~18 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Walkley et al.
		TRE-sh*p53.1224 pRb$^{fl/fl}$*	100%	Yes (85%)	~ 57 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Mutsaers et al.
		p53$^{R270H/+}$ SBmut	75%	Yes (26.4%)	~86 weeks	Yes / Li-Fraumeni syndrome	Moriarity et al.
		SBmut	24.4%		~116 weeks	No/?	Moriarity et al.
	Col1α1-3.6kB	*p53$^{fl/fl}$*	60%		~40 weeks	Yes / Li-Fraumeni syndrome	Lengner et al.
Osteoblasts	*Col1α1*-2.3kB	*p53$^{fl/fl}$*	85%		48 weeks	Yes / Li-Fraumeni syndrome	Lin et al.
	Col1α1-2.3kB	*Notch-ICD*	100%	Yes (40%)	~60 weeks	No	Tao et al.[(b)]
	Osteocalcin	SV-40 T/t antigen	100%	Yes (90%)	~30 weeks	Sequesters p53 and Rb	Molyneux et al. [(c)]
		p53$^{fl/fl}$ Rb$^{fl/fl}$	44%		~57 weeks	Yes / Li-Fraumeni syndrome, hereditary retinoblastoma	Quist et al.

Table 1. Summary of penetrance, metastases, associated diseases and latency of described genetically modified murine osteosarcoma models

[a] Small cohort of animals; [b] Notch Intracellular domain; [c] Tumors defined as “Multi-ostotic bone tumors”.