Published in final edited form as:
Curr Top Dev Biol. 2011 ; 94: 1–13. doi:10.1016/B978-0-12-380916-2.00001-2.

Childhood Cancer and Developmental Biology: A Crucial Partnership

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Introduction

Over the past several decades, we have learned a great deal about the molecular, cellular and genetic properties of the most common human malignancies such as breast, colon, lung and prostate cancer. However, less is known about pediatric cancers because they are rare and often form in a complex microenvironment during development. Indeed, it is not uncommon for pediatric cancers to initiate in developing organs characterized by rapid proliferative expansion, growth factor signaling, developmental angiogenesis, programmed cell death, tissue reorganization and cell migration. In addition, the microenvironment is changing as development progresses and this may directly or indirectly impact tumor initiation and growth. Not only is it more difficult to establish the etiology of pediatric cancer because it occurs in the context of development, but it also makes it more difficult to treat. For example, molecular targeted therapies that perturb developmental pathways that are deregulated in the tumor may have devastating effects on normal tissues in a developmental stage and tissue specific manner. Therefore, it is essential to use developmental biology as the foundation for translational research in pediatric cancer. In this chapter, we will use retinoblastoma and retinal development as an example of the importance of developmental biology in translational research and we will draw parallels to other pediatric cancers.

Developmental Biology and Cancer Genetics

At a time when molecular cloning was in its infancy and high throughput genomic sequence analysis was decades away, the research of Eric Wieschaus and Christiane Nusslein-Volhard revolutionized the fields of developmental biology and cancer genetics and ultimately earned a Nobel prize[1]. Their initial goal was to study the mechanisms of cell fate specification and differentiation during development by taking advantage of the tractable genetics of *Drosophila*. Wieschaus and Nusslein-Volhard used phenotype-based mutant screening approaches combined with molecular genetics to link the genetic loci and genes to developmental processes on a large scale. Their results led to the discovery of several of the major developmental pathways that are now widely studied in a variety of invertebrate, vertebrate and mammalian species. Importantly, we now understand that many of these pathways are perturbed in human diseases, including cancer. One of the best examples of a developmental pathway that also contributes to tumorigenesis is the hedgehog pathway (Hh). In *Drosophila*, Hh signaling controls the segmentation pattern and other cell-cell

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signaling events in development[1,2]. In mammals, there are three orthologues of *Drosophilia* hedgehog (Hh): sonic hedgehog, desert hedgehog, and indian hedgehog[3]. There are unique and overlapping functions of these hedgehog family members in mammals. Indeed, hedgehog signaling has been implicated in cell proliferation, cell survival, angiogenesis, and the epithelial-mesenchyme transition that is a hallmark of tumor metastasis[4]. Based on these important functions in normal development, it is not surprising that the hedgehog pathway is deregulated in some forms of pediatric cancer.

Some of the first evidence that aberrant hedgehog signaling contributed to tumorigenesis in humans came from the discovery that patients with Gorlin syndrome had lesions in a gene (*Patched 1*) important for hedgehog signaling[5,6]. Gorlin syndrome is a rare disease characterized by larger body size, developmental and skeletal anomalies, soft tissue fibromas, radiation sensitivity, and predisposition to cancers such as basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma[6-10]. The pleiotropic phenotype of these patients further highlights the important role that hedgehog signaling plays in tissue development and homeostasis. Translating this important discovery into the clinical realm, targeted therapies that can silence aberrant and/or uncontrolled signaling through the hedgehog pathway could be effective for the treatment of pediatric cancers such as medulloblastoma. However, implementing this unique opportunity comes with major challenges. If the hedgehog pathway is silenced using a molecular targeted therapy in children with medulloblastoma, how will this affect all the cells and organs that rely on hedgehog signaling for normal development and homeostasis? Indeed, a recent study of hedgehog inhibitors in juvenile mice showed defects in the development of several organ systems providing critical evidence for the importance of understanding developmental biology in targeting pediatric cancer[11]. Therefore, while the knowledge of this important pathway has moved from bench to bedside in the form of a phase I/II study with hedgehog pathway inhibitors, the trial is limited to older patients who may suffer fewer side effects of targeting the hedgehog pathway (ClinicalTrials.gov Identifier: NCT00939484).

Clinical Features of Retinoblastoma

Retinoblastoma is the most frequent ocular neoplasm that occurs during childhood and the third most common form of cancer in infants after leukemia and neuroblastoma. In the United States approximately 300 children are diagnosed with the disease each year[12]. Internationally, physicians diagnose between 5,000 and 10,000 children with retinoblastoma each year, and the majority of these patients present with advanced stage disease. Retinoblastoma is a disease of infants and young children. This reflects the developmental origin of the tumor. It is often mistakenly assumed that the tumor arises near the time that the disease first presents clinically in the first few years of life. However, it is likely that the *RB1* gene inactivation occurs during DNA replication in proliferating retinal progenitor cells and retinal progenitor cell proliferation occurs only in the fetal retina[13,14]. Consistent with this model, there are reports of premature babies with well-established retinoblastoma[15]. If this is true then the latency from tumor initiation to diagnosis may reflect the time it takes for tumor progression and the accumulation of secondary and tertiary genetic lesions in retinoblastoma.

There are two distinct forms of retinoblastoma that differ in the pattern of *RB1* gene inactivation. A hereditary or germline form of the disease tends to present at earlier age with bilateral disease and multifocal tumors[14]. A non-hereditary form of the disease is typically associated with later onset, is unilateral in presentation and tends to be unifocal. Both forms of disease initiate with *RB1* gene inactivation. One of the unique features of retinoblastoma is that diagnosis of retinoblastoma is often made without pathologic confirmation. Instead, multiple modalities are used to make the diagnosis. A physician performs an exam under

anesthesia during which he/she uses a retinal camera to identify the tumor. An ultrasound of the orbits and MRI of the orbits and brain are also obtained. Information gathered from these studies leads to the diagnosis, after which children are treated according to an individualized plan based on the stage of their disease.

The primary goals of treatment for retinoblastoma are to save life and preserve vision. The principles of treatment depend on the nature of the disease. Multiple factors contribute to the development of a treatment strategy, including tumor locality, presence of an *RB* mutation, potential for vision, and stage of disease. Treatment modalities include surgery, focal therapy, radiotherapy and chemotherapy.

There are many different approaches in the management of retinoblastoma based on the individual, laterality and stage of disease. Patients with advanced unilateral intraocular disease typically undergo enucleation. If the patient has a high-risk tumor then he/she may receive adjuvant chemotherapy. Patients with early low risk unilateral or multifocal retinoblastoma are often offered conservative management with a combination of chemotherapy and focal therapy. These patients are typically treated with 6 – 8 courses of chemotherapy given at 3-week intervals. Their disease is evaluated prior to each course of chemotherapy with an exam performed under anesthesia, measurements of intraocular pressure and an ultrasound of the orbit. While sedated for the studies, these patients will often receive focal therapy and local chemotherapy (subconjunctival administration) if that is part of the treatment protocol. The majority of patients with advanced bilateral disease are treated conservatively with a combination of chemotherapy, focal therapy and enucleation if the tumor progresses.

In the United States, the current survival rate for retinoblastoma is greater than 90% in patients who present with low risk disease[16]. However, in developing countries the survival rate plunges to 50%[17]. In spite of aggressive therapy, the morbidity associated with the disease remains significant. Blindness and enucleation occur in 50% of all patients with advanced bilateral retinoblastoma[18]. Thus further advances must be made to improve the survival rate and quality of life of patients. Preclinical models of retinoblastoma offer the best hope of moving new therapies into clinical trials as for other pediatric cancers.

Mouse Models of Pediatric Cancer

One of the first attempts to model human cancer in the mouse using the same genetic lesion seen in human patients was focused on the *Rb1* gene in retinoblastoma. Children who inherit a defective copy of the *RB1* gene have an increased susceptibility to develop retinoblastoma through inactivation of the second allele. Tyler Jacks and colleagues reasoned that a mouse lacking 1 copy of the *Rb1* gene would similarly have a predisposition to retinoblastoma[19-21]. Interestingly, these mice have perfectly normal retinae and never develop retinoblastoma. Subsequent molecular, cellular and genetic studies demonstrated that the Rb protein plays similar roles in humans and mice and the species specific difference in tumor susceptibility was not due to evolutionary differences in the *Rb1* gene or protein itself[22]. A series of developmental studies focused on retinal progenitor cells in mice and humans revealed that there is a difference in the intrinsic genetic compensation among the Rb family members in human and mouse retinae[22]. In the human retina, the RB1 protein is the major family member expressed in proliferating retinal progenitor cells while in mice there is a complex interplay between Rb1 and p107. When the *Rb1* gene is inactivated in the mouse retina, there is intrinsic compensation by p107 and this prevents retinoblastoma in mice[22]. By inactivating the *Rb* and *p107* genes in the developing mouse retina, researchers were able to model retinoblastoma in mice for the first time[23-25]. This provided unprecedented opportunities to begin to understand the cellular etiology of retinoblastoma using a tractable

experimental system and to begin to develop new therapies for this devastating childhood cancer through preclinical testing programs.

Despite the early setback in the retinoblastoma field with respect to preclinical models, mouse models of cancer have become increasingly important in the field of cancer genetics and translational research. Fundamental advances in our understanding of cancer etiology, tumor progression and therapeutic efficacy have come from murine models of human cancers ranging from prostate and breast cancer to brain tumors and leukemia. Such studies are particularly important for pediatric cancer because the patient population is relatively small and preclinical models are essential for validating the efficacy of new combinations of chemotherapy before initiating clinical trials in children. In addition, animal models of cancer can provide important new insights into the molecular, cellular and genetic mechanisms underlying tumor initiation and progression.

One of the challenges with modeling pediatric cancer in mice is that these tumors initiate in the context of rapidly changing developing tissues where the cells that give rise to the tumors can display an extraordinary degree of plasticity and heterogeneity. This is further complicated by the fact that many tumor suppressor genes and proto-oncogenes play essential roles in regulating cell fate specification and differentiation during development. Specifically, the genetic lesions that contribute to tumor initiation and progression may also alter the intrinsic cell fate specification and differentiation programs in the tumor cells themselves making it very difficult to infer the cell-of-origin for that tumor.

As new chemotherapeutic agents are developed to target particular molecular pathways in cancer cells, the identification of the cell-of-origin becomes increasingly important. For example, if the cell of origin for a pediatric cancer is a progenitor cell, a very different strategy may be employed to target particular pathways in that cell than if it were a highly specialized differentiated cell from the same tissue. This is particularly true for tumors of the central nervous system such as retinoblastoma. Neurons are incredibly diverse and employ a wide variety of cell-type specific death pathways which may be activated under conditions of neuronal stress that are not relevant in other cell types.

Translational Research: Is it Clinically Relevant?

The slow process of translating molecular and genetic discoveries into clinical medicine can be attributed in part to the lack of preclinical models that faithfully recapitulate human disease. In addition, few investigators use those models in ways that recapitulate the schedule, dose, or diagnostic tests that are used clinically. Therefore, it is difficult if not impossible to predict if a new therapeutic approach will be better than the conventional treatment. Indeed, preclinical testing of oncology drugs is widely viewed unfavorably by the pharmaceutical industry because of these limitations. The extremely limited use of preclinical testing programs that have any predictive power for patient outcome creates a reliance on pharmaceutical industry animal studies which focus on toxicity and marginal readouts of biological activity. As we begin to acquire more and more molecular and genetic data on human cancers, mouse models of cancer, human cancer xenografts and cancer cell lines, we will be uniquely positioned to take full advantage of the strengths of each of those reagents while avoiding pitfalls associated with their limitations. As mentioned above, this is important from the perspective of the cell-of-origin for cancer, the differentiated features of the tumors and the microenvironment where they develop must be considered as it relates to normal development. Some of the most important challenges with modeling human cancer in the preclinical setting relate to understanding the developmental milieu where the tumors initiate and progress.

Immortalized Cell Lines

The vast majority of laboratory research on cancer cell biology, molecular and biochemical studies has come from the analysis of immortalized cancer cell lines. Many fundamental and important discoveries have been made from studying cancer cell lines. However, there are obvious limitations, especially related to the developmental microenvironment that is so important for pediatric cancer. Clearly, a clonal population of cells that has been immortalized and selected for growth in culture does not recapitulate the complex 3-dimensional tumor structure or the positive and negative feedback characteristic of the interplay between the normal host cells and tumor cells. Nonetheless, it is remarkable how much has been learned from immortalized cancer cell lines and the degree to which some of these lines recapitulate the primary tumors from which they came. The strength of cell culture systems is their ease of manipulation, their often-rich literature and extensive characterization and their flexibility for use in drug screening and large-scale genomics efforts. Their limitations include genetic perturbations in culture, selection for rare and/or unusual variants that are not representative of the human tumors and the lack of environmental factors and host interactions. For most investigators, cell lines are a useful experimental system that can help to generate hypotheses that are further tested and validated in more biologically relevant *in vivo* models. Unfortunately, we are relatively limited in the number of well-characterized and validated cell lines for pediatric cancers. For example, there are only 2 widely used human retinoblastoma cell lines (Y79 and Weri1) available from the ATCC that were first generated in the 1970's[26]. Pediatric cancer in general and retinoblastoma in particular would benefit from investment in the development of new human cell lines.

Xenograft Models

For over thirty years scientists have been using immunocompromised mice to xenograft primary human tumors. This is a very attractive approach since it allows us to begin to overcome some of the limitations of studying immortalized cell lines in culture. Some of the host-tumor interactions are recapitulated (with the exception of the immune system) and the tumors will often be exposed to many of the complex physiological changes and variations that occur in humans such as changes in hormone levels and other signaling molecules like insulin. Unfortunately, there are still some major limitations to the way in which these models are currently used that are a particular challenge for investigation of pediatric cancers[11,27-35]. Rarely do researchers attempt to recapitulate the developmental environment (stage and organ site) of endogenous tumors. This is due in part to a lack of a detailed understanding of the actual cell of origin or the developmental stage when pediatric tumors first initiate. Most xenograft studies are performed in the flank of adult immunocompromised mice and the tumor response is measured using calipers. The lack of a detailed analysis regarding the developmental environment and the failure to recapitulate that in the xenograft models poses a major challenge for interpreting any therapeutic data for such studies such as the data generated from the Pediatric Preclinical Cancer Testing Program. Retinoblastoma is an excellent example of this concept. It is well known that retinoblastomas do not grow in the flank of immunocompromised mice, however they engraft with virtually 100% efficiency in the vitreous of the eye of immunocompromised mice (Flores-Otero and Dyer, unpublished). Moreover, cells can be injected into the eyes of newborn pups and thereby recapitulate the developmental milieu of human retinoblastomas[36].

Genetically Engineered Mouse Models

As mentioned above, there is a long history of efforts to model retinoblastoma in mice. Developmental biology played an essential role in helping us understand why *Rb+/−* mice do not develop retinoblastoma and these studies have been extended to begin to model secondary genetic mutations in this cancer. In 2004, three research groups generated the first knockout mouse models of retinoblastoma by conditionally inactivating Rb^{Lox} in the developing retinae of *p107*-deficient mice[23-25]. Each of these strains mirrored the histopathologic features of human retinoblastoma and created unique opportunities to integrate studies on mouse models into human cancer research.

The newly developed knockout mouse models provided a key tool to study genetic pathways important for retinoblastoma progression. It was discovered that *Chx10-Cre;*$Rb^{Lox/-}$*;*$p107^{-/-}$*;*$p53^{Lox/-}$ mice develop bilateral aggressive retinoblastoma with ~95% penetrance[23-25]. This finding is in contrast to *Chx10-Cre;*$Rb^{Lox/-}$*;*$p107^{-/-}$ mice that develop unilateral, minimally invasive retinoblastoma with ~50% penetrance[25]. These results suggested that the p53 pathway suppresses retinoblastoma in mice. However, the *p53* gene is intact in human retinoblastoma[37,38]. Subsequent molecular genetic analyses revealed an amplified *MDMX* gene in 65%-70% of human cases, and amplified *MDM2* in 10% of cases[36]. This results in a functionally silent p53 pathway. Mouse models of retinoblastoma, retinoblastoma cell lines, human fetal retinae, and primary retinoblastoma tumors from enucleated eyes were used to confirm that amplification of *MDMX* indeed suppresses *p53*-mediated cell death in *RB1*-deficient retinoblasts, thereby promoting clonal expansion of the tumors[36] and the ectopic expression of MDMX has now been successfully modeled in retinoblastoma in combination with *Rb;p107* inactivation.

Genetically engineered mouse models provide several advantages over cultured immortalized cell lines or xenograft studies. The genetic lesions can be engineered to initiate in the presumed cell-of-origin in vivo and the tumors progress through similar stages as those seen in patients. That is, tumors initiate from focal hyperplasia, progress to malignant tumor foci and eventually expand and metastasize. If the timing of the genetically engineered oncogenic lesions is properly modeled after human cancer development, then such mice may prove to be very accurate models of human disease. However, even with the best genetic manipulations in mouse models there are some limitations.

First, most conditional inactivation approaches are relatively crude with respect to the cells where the genetic lesion occurs. For example, *Nestin-cre* has been used to inactivate the *Rb* gene in the developing mouse retina but it also inactivates the *Rb* gene throughout much of the CNS[24]. Second, mouse tumors do not metastasize as often as human tumors. It is not clear if this is due to a fundamental difference between the two species or if we have simply failed to recapitulate the appropriate genetic lesions that drive metastasis in mice. Third, there are many cases where the initiating genetic lesion is followed by a series of secondary genetic lesions that contribute to tumorigenesis. It is possible that those secondary lesions are not the same in mouse models as in human tumors and this could have a profound impact on how the tumors respond to therapy in preclinical trials. Finally, there may be physiological differences that make it difficult or impossible to perform clinically relevant preclinical trials in mice. For example, some chemotherapeutic drugs (i.e. cisplatin) are not tolerated at the same dose and schedule as in humans making it difficult to directly compare across species.

If we consider the strengths and limitations of each of the experimental models it may be most prudent to integrate all of these systems while keeping in mind the critical limitations of each approach. That is, the strengths of one system may help to complement the

limitation of another system and by integrating across experimental paradigms we can hope to gain the most insight into the efficacy of new therapeutic agents. However, even with the most accurate preclinical testing programs using pharmacokinetic guided dosing and delivery of drugs, the experimental paradigms are not valuable unless there is predictive power in the clinic. For example, if studies in cell culture provide a perfect correlate to identify agents that will be effective in the clinic, then the more extensive studies in mice would not be needed. Clearly, cell culture models are not sufficient as there are many examples of drugs that are very effective in culture but fail to show efficacy in animals or in humans. One example for retinoblastoma is vincristine. This is the most toxic agent used to date to treat retinoblastoma cells in culture but it has little if any effect in animal models of retinoblastoma and its contribution to clinical management of the disease is limited at best[39]. We do not yet know if orthotopic xenografts or genetic mouse models will have greater or equal predictive power for retinoblastoma. The best way to establish predictive power is to compare a new combination of therapy to a standard of care for that disease and then perform clinical trials to determine if the new drug combination is also better in the patients. A positive correlation between the animal models and the clinical outcome would provide the essential predictive power needed to justify the use of a particular preclinical model. It is reasonable to assume that the preclinical models which recapitulate the developmental milieu, the genetic lesions and the focal nature of the tumors will be the best models for testing new combinations in preparation for clinical trials.

Comprehensive Preclinical Trials

Even with the best models, most preclinical testing programs use metrics for tumor response that are unrelated to the criteria used in clinical trials. For example, in flank xenografts calipers are used to measure tumor response[11,27-35]. We propose that there are several advantages to using the same diagnostic test and functional assessments that are used in the clinic. In this way, the data from the preclinical testing will more closely parallel the data from clinical trials.

For retinoblastoma, the tumors are diagnosed with a digital retinal camera. Once enrolled, a mouse undergoes baseline studies, including optometry to measure visual acuity and tonometry to measure intraocular pressure. Additionally, each mouse has a baseline complete blood count with differential (CBCD) obtained by facial vein blood draw. Chemotherapy is started after all baseline tests have been performed. Similar to pediatric dosing, the study includes six courses of chemotherapy given on a 21-day cycle. The AUC guided doses are based on human and mouse pharmacokinetic studies. Each mouse has surveillance monitoring every 3 weeks prior to the next course of chemotherapy to track tumor growth, intraocular pressure, visual acuity and blood counts. If a mouse experiences tumor progression, an enucleation is performed. Any mouse that requires enucleation has an ultrasound of the orbits and an MRI of the orbits and brain performed prior to the surgery. Bilateral or unilateral enucleation can be performed as needed. Once removed, the eye is fixed in 4% paraformaldehyde by immersion, embedded in paraffin, and sectioned (5 μm) through the optic nerve for hematoxylin and eosin staining and histopathologic analysis. Following recovery, the mouse continues chemotherapy, diagnostic imaging, and functional assessments until it reaches one year of age and completes the study.

Ultimately, multiple chemotherapeutic regimens can be tested in such standardized preclinical trials and compared to the standard of care. The goal is to determine which therapeutic regimen in the mouse is more effective and carries fewer side effects than the prevailing standard of care and then translate this to the clinical setting. Additionally, targeted and/or novel agents directed at a specific molecular pathway could be investigated. As discussed above, it is very important to test the toxicity in juvenile animals as the

ongoing development of some organ systems may lead to different toxicities than seen in adult populations.

Summary

Developmental biology and cancer biology are inseparable when we consider the development and progression of pediatric cancer. A thorough understanding of the cell-of-origin and the unique developmental microenvironment where tumors form is essential for understanding tumor emergence and for identifying key developmental pathways that may provide valuable targets for therapy. A deep understanding of developmental biology is also important for modeling preclinical studies. The best animal models will recapitulate the developmental environment where the tumors form. Moreover, effective use of targeted therapies will require a deep understanding of developmental processes in a variety of tissues. Therefore, pediatric cancer in general and retinoblastoma in particular presents some distinct challenges for translational research. However, unique opportunities exist. We understand a great deal about the genetics of retinoblastoma and the long tradition of clinical trials has resulted in over 90% of pediatric cancer patients enrolling on clinical studies. This allows researchers and clinicians to directly compare therapy. If we develop preclinical testing programs with proven predictive power, we will have an unprecedented opportunity over the next decade to effectively impact both retinoblastoma and pediatric cancer treatment.

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