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### BAC Libraries: Precious Resources for Marsupial and Monotreme Comparative Genomics

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#### 1. Introduction

Over the past decade, the construction of Bacterial Artificial Chromosome (BAC) libraries has revolutionized gene mapping in marsupials and monotremes, and has been invaluable for genome sequencing, either for sequencing target regions or as part of whole genome sequencing projects, making it possible to include representatives from these two major groups of mammals in comparative genomics studies. Marsupials and monotremes bridge the gap in vertebrate phylogeny between reptile-mammal divergence 310 million years ago and the radiation of eutherian (placental) mammals 105 million years ago (Fig. 1). The inclusion of these interesting species in such studies has provided great insight and often surprising findings regarding gene and genome evolution. In this chapter, I will review the important role BACs have played in marsupial and monotreme comparative genomics studies.



Fig. 1. Amniote phylogeny showing the relationship between 'model' monotreme and marsupial species used in comparative genomic studies.

#### 1.1 Monotreme BAC libraries

Monotremes are the most basal lineage of mammals (Fig. 1), diverging from therian mammals (marsupials and eutherians) around 166 million years ago (mya) (Bininda-Emonds et al., 2007). Like all other mammals, they suckle their young and possess fur, but their oviparous mode of reproduction and their rather unique sex chromosome system are two features of most interest to comparative genomicists. BAC libraries have been made for two of the five extant species of monotremes, the platypus (*Ornithorhynchus anatinus*) and the short-beaked echidna (*Tachyglossus aculeatus*). These species last shared a common ancestor approximately 70 mya. The platypus genome, consisting of 21 pairs of autosomes and 10 pairs of sex chromosomes, has been sequenced (Warren et al., 2008) and a male and a female BAC library constructed (see Table 1). Similarly, the echidna genome has nine sex chromosomes and 27 pairs of autosomes, with a male BAC library available for this species (Table 1).

Species	Library Name	Sex	Average insert size (kb)	Number of Clones
Platypus	CHORI_236	Female	147	327,485
Platypus	Oa_Bb	Male	143	230,400
Short-beaked echidna	Ta_Ba	Male	145	210,048

Table 1. Available monotreme BAC libraries

#### **1.2 Marsupial BAC libraries**

Marsupials, a diverse group of mammals with over 300 extant species found in the Americas and Australasia, diverged from eutherian mammals approximately 147 mya (Bininda-Emonds et al., 2007) (Fig. 1). They are renowned for their mode of reproduction, giving birth to altricial young that usually develop in a pouch. Three species of marsupials were chosen as 'model' species for genetics and genomics studies 20 years ago: the grey short-tailed South American opossum (Monodelphis domestica) representing the Family Didelphidae, the tammar wallaby (Macropus eugenii) from the kangaroo family Macropodidae and the fat-tailed dunnart (Sminthopsis macroura) as a member of the speciose Family Dasyuridae (Hope & Cooper, 1990). The opossum, the first marsupial to have its genome sequenced (Mikkelsen et al., 2007), is considered a laboratory marsupial and has been used as a biomedical model for studying healing of spinal cord injuries and ultraviolet (UV) radiation induced melanoma (Samollow, 2006). The tammar wallaby has also recently had its genome sequence (Renfree et al., 2011) and has been extensively used for research into genetics, reproduction and physiology. Although there have been a few studies carried out on the fat-tailed dunnart, the recent emergence of the fatal devil facial tumour disease (DFTD) has led to the Tasmanian devil replacing it as the model dasyurid, with many resources being made available, including genome (Miller et al., 2011) and transcriptome sequence (Murchison et al., 2010). These model species represent three distantly related marsupial orders, with comparisons between these species being valuable for discerning the features that are shared among marsupials and those that are specific to certain lineages. BAC libraries have been made for all four species mentioned above and are summarized in Table 2. The three current model species will herein be referred to simply as opossum, wallaby and devil.

In addition to the model species, BAC libraries have also been constructed for the Virginia opossum (*Didelphis virginiana*), another member of the Family Didelphidae and the Northern brown bandicoot (*Isoodon macrourus*) (Table 2) from the Family Peramelidae. The phylogenetic position of the bandicoots, located at the base of the Australian marsupial radiation, and some of their more unique features make them interesting animals to study (Deakin, 2010). They possess the most invasive placentas among marsupials, with an allantoic placenta more like that found in eutherians, which would make them a valuable species in which to study genomic imprinting. They also deal with dosage compensation in an unusual way by eliminating one sex chromosome in somatic cells (Hayman & Martin, 1965; Johnston et al., 2002).

Species	Library Name	Sex	Average insert size (kb)	Number of Clones
Didelphis virginiana	LBNL-3	Female	170	148,162
Isoodon macrourus	IM	Male	125	
Macropus eugenii	ME_KBa	Male	166	239,616
Macropus eugenii	Me_VIA	Male	108	55,000
Monodelphis domestica	VMRC-6	Male	155	276,480
Monodelphis domestica	VMRC-18	Female	175	364,800
Sarcophilus harissii	VMRC-49	Male	140	258,048
Sarcophilus harissii	VMRC_50	Male	140	165,888
Sminthopsis macroura	RZPD688	Male	60	110,592

Table 2. Marsupial BAC libraries

#### 2. BACs used for gene mapping and sequencing of target regions

Prior to the availability of BAC libraries for marsupials and monotremes, gene mapping by fluorescence in situ hybridization (FISH) was an arduous task, which relied on the isolation of the gene of interest from a lambda phage genomic library. The construction of BAC libraries for the species listed above has facilitated the mapping of many marsupial and monotreme genes by FISH. Initially, PCR products were used to screen these BAC libraries for genes of interest but more recently overgo probes (overlapping oligonucleotides) have proven to be the method of choice, permitting the isolation of many genes from one screening, thereby facilitating the rapid construction of gene maps. Likewise, before the availability of genome sequence, isolating and sequencing BACs containing genes of interest proved a very useful method for obtaining sequence from particular regions of interest. In some cases, even after whole genome sequencing had been performed, it proved necessary to take this targeted approach. These mapped or sequenced BACs have led to a number of important findings, with examples of those having had a significant impact on previously held theories reviewed here. Examples include the determination of the origins of monotreme and marsupial sex chromosomes, the evolution of regions imprinted in eutherian mammals, the unique arrangement of the Major Histocompatibility Complex (MHC) in the tammar wallaby and the evolution of the

 $\alpha$ - and  $\beta$ -globin gene clusters. BACs have played a vital role in many more studies using gene mapping and/or target region sequencing than can be included in detail in this review and hence, other studies that have utilized BACs for these purposes are listed in Table 3. This is not an exhaustive list but an indication of the breadth of studies in which BACs have played a role.

Species	Genes or Region	Purpose	Reference
Echidna and Platypus	SOX 3	Mapping	(Wallis et al., 2007b)
Opossum (M.domestica)	Immunoglobulins	Mapping	(Deakin et al., 2006a)
Opossum (M.domestica)	T cell receptors	Mapping	(Deakin et al., 2006b)
Platypus	DMRT cluster	Sequencing	(El-Mogharbel et al., 2007)
Platypus	Defensins	Mapping	(Whittington et al., 2008)
Platypus	SOX9 and SOX10	Mapping	(Wallis et al., 2007a)
Platypus	Sex determination pathway genes	Mapping	(Grafodatskaya et al., 2007)
Dunnart	LYL1	Sequencing	(Chapman et al., 2003)
Tammar wallaby	Prion protein gene	Sequencing	(Premzl et al., 2005)
Tammar wallaby	Immunologulins & T cell receptors	Mapping	(Sanderson et al., 2009)
Tammar wallaby	Mucins & Lysozyme	Mapping	(Edwards et al., 2011)
Tammar wallaby	SLC16A2	Sequencing	(Koina et al., 2005)
Tammar wallaby	BRCA1	Mapping	(Wakefield & Alsop, 2006)
Tammar wallaby	Cone visual pigments	Sequencing	(Wakefield et al., 2008)

Table 3. Studies in marsupial and monotreme comparative genomics that relied on BAC clones.

#### 2.1 Origins of marsupial and monotreme sex chromosomes

Determining the evolutionary origins of marsupial and monotreme sex chromosomes was the driving force behind much of the gene mapping conducted in these species. The earliest gene mapping work showed that at least some genes found on the human X chromosome were also on the X in marsupials, resulting in the hypothesis that the X chromosome of these two mammalian groups had a common origin. Gene mapping using heterologous probes and radioactive in situ hybridization (RISH) supported the extension of this hypothesis to include monotremes. However, it was only when BAC clones became available for gene mapping that the true evolutionary history of sex chromosomes in these species was revealed.

#### 2.1.1 The marsupial X chromosome

Like humans, marsupial females have two X chromosomes whereas their male counterparts have a X and a small Y chromosome, meaning that they require a mechanism to compensate for the difference in dosage of X-borne genes between females and males. Several decades ago, it was shown that several X-linked genes in human were also located on the X in marsupials and one X chromosome was inactivated in somatic cells to achieve dosage compensation. However, even in these early studies, striking differences in the characteristics of X inactivation in eutherians and marsupials were evident. Marsupials were found to preferentially silence the paternally derived X chromosome rather than subscribing to the random X inactivation mechanism characteristic of eutherian mammals. This inactivation was found to be incomplete, with some expression observed in some tissues from the inactive X and thus, appeared to be leakier than the stable inactivation observed in their eutherian counterparts (reviewed in Cooper et al., 1993). Therefore, there was a great interest in investigating the marsupial X chromosome and X inactivation in greater detail, a task in which marsupial BAC libraries have been indispensable.

The first step towards gaining a deeper understanding of X inactivation in marsupials was determining the gene content of the marsupial X chromosome. Early gene mapping studies showed that not all genes located on the human X chromosome were present on the X in marsupials. This was supported by cross-species chromosome painting which showed that the human X chromosome could be divided into two regions; one being a region conserved on the X chromosome both in marsupials and human, referred to as the X conserved region (XCR), and a region added to the X chromosome in the eutherian lineage - the X added region (XAR) (Glas et al., 1999; Wilcox et al., 1996). This added region corresponded to most of the short arm of the human X chromosome.

Progress in determining the boundaries of the XCR and XAR was slow until the release of the opossum genome assembly, which revealed this boundary in this species and pathed the way for detailed gene mapping in a second species, the tammar wallaby. Wallaby specific overgos were designed for human X-borne genes from sequence generated by the genome sequencing project and used to screen the wallaby BAC library in large pools. BACs for these genes were mapped to wallaby chromosomes using FISH. Genes from the XAR mapped to chromosome 5 (52 genes) and the XCR genes mapped to the X chromosome (47 genes). This mapping data enabled comparisons in gene order to be made between wallaby, opossum and human, revealing a surprising level of rearrangement on the X chromosome between these species (Deakin et al., 2008b).

One region that was of particular interest for comparative gene mapping in marsupials, given the differences in X inactivation between marsupials and eutherians, was the X inactivation center (XIC) located within the XCR on the human X chromosome. This region contains the *XIST* (X inactive specific transcript) gene, a master regulatory non-coding RNA transcribed from the inactive X, and a number of other non-coding RNAs that play an important role in X inactivation (reviewed in Avner & Heard, 2001). The *XIST* gene is poorly conserved between eutherian species (Chureau et al., 2002; Duret et al., 2006; Hendrich et al.,

1993; Nesterova et al., 2001). Sequence similarity searches failed to identify any sequence with homology to *XIST*. As a consequence, a BAC-based approach was taken to determine whether *XIST* was present in marsupials.

Three independent research teams used similar BAC-based approaches to determine the location of genes flanking the eutherian XIC locus on marsupial chromosomes. Shevchenko et al (2007) isolated BACs containing XIST-flanking genes as well as other genes from the XCR in two opossum species (M.domestica and D.virginiana). FISH-mapping of these BACs in both species revealed an evolutionary breakpoint between XIST-flanking genes. Likewise, Davidow et al (2007) and Hore et al (2007) mapped BACs identified to contain XISTflanking genes from BAC-end sequence data generated as part of the opossum genome project and mapped them to different regions of the M.domestica X chromosome. Further sequence searches around these flanking genes failed to identify an orthologue of XIST (Davidow et al., 2007; Duret et al., 2006) and it was concluded that the XIST gene is absent in marsupials (Davidow et al., 2007; Hore et al., 2007). This conclusion was further supported by mapping of *XIST*-flanking genes to opposite ends of the tammar wallaby X chromosome (Deakin et al., 2008b). Hence, marsupial X inactivation is not under the control of XIST but then this raised more questions regarding marsupial X inactivation. Is there is a marsupial specific X inactivation centre? To answer this question, a more detailed investigation of the status of inactivation of marsupial X-borne genes was required.

Fortunately, the BACs isolated for mapping genes to the tammar wallaby X chromosome could be used construct an 'activity map' of the tammar wallaby X chromosome, where the inactivation status of X-borne genes at different locations along the X was determined. By using RNA-FISH, a technique that detects the nascent transcript, it was possible to determine the inactivation status of an X-borne gene within individual nuclei. The large insert size of BAC clones makes them ideal for hybridization and detection of the nascent transcript. Al Nadaf et al (2010) determined the inactivation status of 32 X-borne genes. As was suggested by earlier studies using isozymes, X inactivation in marsupials is incomplete. Every gene tested showed a percentage (5 – 68%) of cells with expression from both X chromosomes. This activity map of the wallaby X chromosome demonstrated no relationship between location on the X chromosome and extent of inactivation, suggesting that there is no polar spread of inactivation from a marsupial-specific inactivation center (Al Nadaf et al., 2010).

Although there are still many questions to be answered concerning marsupial X chromosome inactivation, BAC clones have proven to be extremely valuable resources for these studies and have resulted in the rapid advance of knowledge in this field. Further work is already underway to construct activity maps of genes in other species, using BACs from the opossum and the devil. Including a further species, the bandicoot (*I. macrourus*) would be particularly interesting as this species has an extreme version of X inactivation where they eliminate one sex chromosome (either a X in females or the Y in males) from somatic cells. The availability of a BAC library for this species makes it possible that this research could be carried out in the future.

#### 2.1.2 Gene content of the marsupial Y chromosome

Although gene poor, the Y chromosome has an exceptionally important function, being responsible for sex determination and other functions in male sex and reproduction. A comparison of the chimpanzee and human Y chromosomes demonstrates the rapid evolution of the Y chromosome (Hughes et al., 2010). Extending this comparison to include marsupials would provide even further insight into the evolution of this remarkable chromosome. Orthologues of several eutherian Y-borne genes were mapped to the Y chromosome of marsupials but it was of more interest to see if there were novel genes found on the marsupial Y, which could be revealed by sequencing a marsupial Y chromosome.

Sequencing of the highly repetitive Y chromosome is extremely difficult by shot-gun sequencing. A BAC-based approach is seen as the best option to obtain well-assembled sequence. A novel method has been used to obtain Y specific BAC clones in the wallaby, in which the Y chromosome was isolated by flow sorting or manual microdissection and used to probe a wallaby BAC library and create a sub-library enriched with Y-specific BAC clones (Sankovic et al., 2006). Sequencing of two of these clones resulted in the identification of novel genes on the Y chromosome, *HUWE1Y* and *PHF6Y* (Sankovic et al., 2005). These genes are not on the Y chromosome of eutherians but do have a homologue on the X chromosome. It is hoped that more of these Y-specific BACs will be sequenced in the future to enable the evolutionary history of the therian Y chromosome to be unraveled.

#### 2.1.3 Gene content of the platypus sex chromosomes

Monotremes, like other mammals, have male heteromorphic sex chromosomes, but their sex chromosome system is somewhat complex. Female platypuses have five different pairs of X chromosomes and their male counterparts have five X and five Y chromosomes that form a multivalent translocation chain during male meiosis (Grutzner et al., 2004). Similarly, the echidna (T. aculeatus) has five X chromosomes in females, and five X and four Y chromosomes in males (Rens et al., 2007). Early gene mapping studies using RISH with several heterologous probes suggested that at least one monotreme X chromosome shared homology with the therian X (Spencer et al., 1991; Watson et al., 1992; Watson et al., 1990). Subsequent mapping of BAC clones containing XIST-flanking genes indicated that at least some therian X-borne genes had an autosomal location in the platypus (Hore et al., 2007). The sequencing of the platypus genome made it possible to more thoroughly investigate the gene content of all platypus X chromosomes. By FISH-mapping BACs end-sequenced as part of the genome project, it became evident that, in contrast to the original gene mapping data, the platypus X chromosomes share no homology the therian X. Instead, at least some of the X chromosomes share homology with the chicken Z. Genes from the XCR were located on platypus chromosome 6 (Veyrunes et al., 2008). Furthermore, mapping of platypus X chromosome BACs onto male chromosomes identified the pseudoautosomal regions on the platypus Y chromosomes, providing the first glimpse into the gene content of the platypus Ys. Finding a lack of homology between monotreme and therian X chromosomes had a major impact on our understanding of the timing of therian sex chromosome evolution and provided surprising insight into the ancestral amniote sex determination system, which may have resembled the ZW system observed in birds (Waters & Marshall Graves, 2009).

The complicated sex chromosome system of monotremes makes determining the sequence of platypus Y chromosomes especially interesting. Since only a female platypus was sequenced as part of the genome project, no Y-specific sequence was obtained (Warren et al., 2008). Kortschak et al (2009) isolated and sequenced six Y-specific platypus BAC clones. The gene content of these BACs has not been reported but a detailed analysis of the repeat

content has shown a bias towards the insertion of young SINE and LINE elements and segmental duplications (Kortschak et al., 2009). As some differences in gene content between platypus and echidna X chromosomes have been identified, a comparison of the gene and repeat content of their Y chromosomes could provide important insight into the evolution of this complicatied sex chromosome system. Undoubtedly, a BAC-based approach will continue to be the best strategy for obtaining Y-specific sequence.

The unexpected finding of no homology between monotreme and therian sex chromosomes begged the question as to how monotremes achieved dosage compensation. BAC clones were instrumental in determining the expression status of platypus X-borne genes in RNA-FISH experiments. Genes on platypus X chromosomes were monoallelically expressed in approximately 50% of cells and were biallelically expressed in the remainder, and so it appeared that the platypus employs a very leaky form of X inactivation for dosage compensation (Deakin et al., 2008a). This stochastic transcriptional regulation resembled the leaky inactivation of X-borne genes in the wallaby (Al Nadaf et al., 2010), suggesting that despite different origins of the X chromosome in monotremes and marsupials, their X inactivation mechanisms may have evolved from an ancient stochastic monoallelic expression mechanism that has subsequently independently evolved in the three major mammalian lineages (Deakin et al., 2008a, 2009).

In an attempt to further characterize features of the platypus X inactivation system, BAC clones were used to examine replication timing and X chromosome condensation, two features common to X inactivation in therian mammals. Replication timing of X-borne genes was determined by hybridizing fluorescently labeled BACs to interphase nuclei and counting the number of nuclei with asynchronous replication represented by double dots over one homologue of the gene of interest and a single dot over the other. These dot assays revealed asynchronous replication of some regions on the X chromosomes, namely those not shared on the Y (Ho et al., 2009). Condensation status of three platypus X chromosomes was determined by hybridizing two BACs mapped to opposite ends of the chromosome and measuring the distance between the two signals on the two X chromosome condensation. Consequently, chromosome condensation may not play a significant role in platypus dosage compensation (Ho et al., 2009). It would be interesting to perform these same experiments in echidna for comparative purposes. Since an echidna BAC library is available, it is hoped that this data will be obtained in the future and such a comparison made.

#### 2.2 Evolution of genomic imprinting

Most autosomal genes in diploid organisms are expressed from both the maternal and paternal copies at equal levels. However, there are roughly 80 exceptional genes in eutherian mammals that are monoallelically expressed in a parent of origin fashion. The silent allele is marked (imprinted) by epigenetic features, such as CpG methylation and histone modifications. The evolution of a genomic imprinting mechanism appears counterintuitive since surely it would be more advantageous to have two expressed copies of a gene to protect the individual against deleterious mutations occurring in one copy. Consequently, genomic imprinting raises many questions regarding the how and why genomic imprinting evolved, although there appears to be some link between the evolution of viviparity and genomic imprinting (Hore et al., 2007).

By examining the orthologues of eutherian imprinted genes in marsupials and monotremes, it becomes possible to begin addressing some the questions regarding the evolution of genomic imprinting. Gene mapping with BAC probes and BAC clone sequencing have contributed greatly to research in this area. Below are just a few examples where the use of BAC clones has proven critical for tracing the evolutionary history of imprinted loci. Even in the fairly well covered opossum genome sequence, it has been necessary to sequence BAC clones spanning regions of interest in order to fill gaps in the genome assembly. Major conclusions drawn from these studies propose that imprinting arose independently at each imprinted locus and that the acquisition of imprinting involved changes to the genomic landscape of the imprinted region.

#### 2.2.1 Analysis of the *IGF2/H19* locus

The IGF2 imprinted locus has been extensively characterized in humans and mice, and was the first gene reported to be subject to genomic imprinting in marsupials (but not monotremes) (O'Neill et al., 2000). Elucidating the mechanism by which this is achieved was the subject of a number of subsequent studies. Sequence comparisons between the nonimprinted IGF2 locus of platypus and the imprinted locus of marsupial and eutherian mammals were made in an attempt to identify potential sequence elements required for imprinting of this locus. A platypus BAC clone containing the IGF2 gene was fully sequenced and compared to opossum, mouse and human. This study failed to identify any sites of differential methylation in intragenic regions but did uncover strong association of imprinting with both a lack of short interspersed transposable elements (SINEs) and an intragenic conserved inverted repeat (Weidman et al., 2004). Isolation of an opossum BAC clone (Lawton et al., 2007) and more extensive interrogation of the locus, identified a differentially methylated region (Lawton et al., 2008). This BAC clone was used in RNA-FISH experiments to show that demethylation of this differentially methylated region results in biallelic expression of IGF2 (Lawton et al., 2008). Therefore, differential DNA methylation does indeed play a role in IGF2 imprinting in marsupials.

In humans, *H19* is a maternally expressed long non-coding RNA located near the *IGF2* locus. While protein coding genes in this region were easily identified from genome sequence, the low level of sequence conservation typical of non-coding RNAs made the identification of *H19* more challenging. Three wallaby BACs spanning the the *IGF2/H19* locus were isolated by screening the library with probes designed from all available vertebrate sequences for genes within the region (Smits et al., 2008). Sensitive sequence similarity searches of the sequence obtained from these BAC clones identified a putative *H19* transcript with 51% identity to human *H19*. This sequence was found to be absent from the opossum genome assembly and hence, a BAC clone containing the opossum *H19* orthologue was isolated and sequenced. Like eutherians, *H19* is maternally expressed in marsupials (Smits et al., 2008).

#### 2.2.2 Assembly of the Prader-Willi/Angelman's syndrome locus

Mutations in imprinted genes on human chromosome 15q11-q13 are responsible for the neurological disorders Prader-Willi and Angelman's syndrome. Imprinting of genes in this region is controlled by an imprinting control region (ICR) located within the Prader-Willi/Angelman's syndrome domain (Kantor et al., 2004). The ICR is flanked by the

paternally expressed SNRPN gene and maternally expressed UBE3A. A cross-species comparison of the arrangement of these two genes across vertebrates uncovered an unexpected finding. A wallaby BAC clone containing the SNRPN gene mapped to wallaby chromosome 1, whereas the BAC containing the UBE3A localized to the short arm of chromosome 5. Furthermore, a fully sequenced platypus BAC clone containing UBE3A identified the gene adjacent to be CNGA3, a human chromosome 2 gene (Rapkins et al., 2006). Subsequent analysis of the chicken, zebrafish and opossum genome sequence assemblies unequivocally showed this to be the ancestral arrangement, with UBE3A adjacent to CNG3A while SRNPN is located elsewhere in the genome. Both UBE3A and SRNPN were found to be biallelic expressed in marsupials and monotremes. It appears that the other imprinted genes found in this region in eutherians do not exist in marsupials and originated from RNA copies of genes located in other parts of the genome. Rapkins et al (2006) concluded that these genes only became subject to genomic imprinting when the region was assembled in the eutherian lineage. This study also provided the first evidence that genomic imprinting was acquired by different loci at different times during mammalian evolution.

#### 2.2.3 Evolution of the Callipyge imprinted locus

The Callipyge locus, so named after a muscle trait observed in sheep, contains a cluster of three paternally expressed genes (DIO3, DLK1, RTL1). In order to carry out a comprehensive analysis of this locus, seven platypus and 13 wallaby overlapping BAC clones were fully sequenced and assembled into a single contig for each species (Edwards et al., 2008). Comparative genome analysis revealed that the genomic landscape of this locus has undergone a number of changes during mammalian evolution. In marsupials, the locus is twice the size of the orthologous region in eutherians as a result of an accumulation of LINE1 repeats. In addition, there has been selection against SINE repeats in eutherians along with an increase in GC and CpG island content. Over 140 evolutionary conserved regions were found by phylogenetic footprinting but none of these regions corresponded to the imprint control element identified in eutherians. These findings were consistent with the absence of imprinted expression for this locus both in monotremes and marsupials. Similar to the situation described above for the Prader-Willi/Angelman locus, it appears that a retrotransposition event resulted in the formation of a novel gene in eutherians and it was suggested that this may have been the driving force behind the evolution of imprinting at this locus (Edwards et al., 2008).

#### 2.3 Major Histocompatibility Complex

One the most studied regions of the vertebrate genome is the Major Histocompatibility Complex (MHC), a region central to the vertebrate immune response. In humans, the MHC is a large, gene dense region, spanning 3.6Mb and containing 224 genes divided into three regions; Class I, II and III (MHC Sequencing Consortium, 1999). Classes I and II encode genes involved in endogenous and exogenous antigen presentation respectively. Class III contains immune genes, involved in the inflammatory, complement and heat-shock responses, as well as a number of non-immune genes. This organization is in stark contrast to the chicken MHC consisting of only 19 genes within a 92kb region (Kaufman et al., 1999), making it difficult to establish the evolutionary history of the MHC. The position of marsupials and monotremes in vertebrate phylogeny ideally situates them to bridge the gap

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between chicken and eutherian mammal divergence and trace the evolutionary history of this important region. BAC clones have, once again, played an essential role in the study of the MHC organization and sequencing in marsupials and monotremes.

#### 2.3.1 The opossum MHC

The opossum MHC was the first multi-megabase region to be annotated for the opossum genome project. Annotation of this region was performed on preliminary genome assemblies MonDom1 and MonDom2. The MHC region in MonDom1 was distributed across five sequence scaffolds. Previous mapping localized MHC Class I genes to different locations on opossum chromosome 2, with genes *UB* and *UC* located at the telomeric end of the short arm (Belov et al., 2006) and *UA1* located near the centromere on the long of arm (Gouin et al., 2006). Thus, it was imperative that this assembly of MHC scaffolds was accurately determined to establish whether the separation of these genes was the result of a chromosomal rearrangement or a transposition event. This was achieved by isolating BAC clones corresponding to the ends of the MHC scaffolds. All BACs from these scaffolds, with the exception of one containing *UB* and *UC*, mapped to the centromeric region of chromosome 2. As a result of this information, the MHC was assembled into a single scaffold in the MonDom2 assembly (Belov et al., 2006). Furthermore, mapping of BAC clones from the genes at either end of this large scaffold enabled the orientation of the MHC on the chromosomes to be determined.

The complete annotation of this region provided the necessary information required to start piecing together the changes which have occurred throughout vertebrate evolution. In contrast to the chicken, the MHC of the opossum spans almost 4Mb and contains at least 140 genes, making it similar in size and complexity to the human MHC (Belov et al., 2006). However, the opossum has a very different gene organization with Class I and II genes found interspersed rather than separated by the Class III region as they are in eutherian mammals. This organization is similar to that of other vertebrates, such a shark and frog, suggesting that the marsupial organization is similar to that of the vertebrate ancestor and the eutherian organization is derived.

#### 2.3.2 Mapping and sequencing of the wallaby MHC

The opossum and wallaby are distantly related species, having diverged from a common ancestor around 60 – 80 mya, making a comparison of these two species similar to the informative human-mouse comparison. Unlike the opossum, the wallaby genome was only lightly sequenced (Renfree et al., 2011), leaving many gaps in the genome assembly. If detailed comparative analysis was to be carried out on the MHC, an alternative approach was required.

Initial comparative analysis of these two MHCs was carried out using gene mapping. BACs containing MHC genes from all three Classes were isolated from a tammar wallaby BAC library. These clones were FISH-mapped to wallaby chromosomes with startling results. All Class II and Class III genes, as well as MHC flanking genes, mapped to the expected location on chromosome 2. Surprisingly, all of the MHC Class I BACs mapped to locations on every chromosome except chromosome 2 and the sex chromosomes (Deakin et al., 2007). This unexpected and unprecedented result made a more thorough analysis of these genes critical. As a result, a concerted effort was made to sequence the entire

tammar wallaby MHC, including the 'core' MHC located on chromosome 2 and many of the dispersed Class I genes found elsewhere in the genome. A BAC-based approach was taken, with the idea of constructing a BAC-contig across the core MHC, as well as sequencing the dispersed Class I genes.

After finding Class I genes dispersed across the genome, a thorough screening of the wallaby BAC library was performed in order to isolate as many Class I genes as possible. As a result four additional BAC clones containing Class I genes were isolated, with FISH-mapping of these BACs localizing these genes to the core MHC region on chromosome 2 (Siddle et al., 2009). Complete sequencing of these BACs identified six Class I genes within the core MHC, which were interspersed with antigen processing genes and a Class II gene. Sequencing of ten BACs mapping outside this region identified nine Class I genes with open reading frames. In depth sequence analysis of these BACs revealed a tendency for Kangaroo Endogenous Retroviral Element (KERV) to flank these dispersed Class I genes, suggesting that this element may be implicated in the movement of these genes to regions outside the core MHC (Siddle et al., 2009).

A BAC contig across the core MHC on wallaby chromosome 2 was constructed for sequencing purposes (Siddle et al., 2011). Unfortunately, despite extensive library screening with overgo probes designed from BAC end sequence, a single contig spanning the entire region was not obtained. Instead, the isolated BACs assembled into nine contigs and three 'orphaned' BACs. The order of these contigs and orphaned BACs was determined using BAC clones as probes for FISH on metaphase chromosome spreads and interphase nuclei. The resulting 4.7Mb sequence contained 129 predicted genes from all three MHC Classes. A comparison of the gene arrangement between wallaby, opossum and other vertebrates indicated that the wallaby MHC has a novel MHC gene arrangement, even within the core MHC. The wallaby Class II genes have undergone an expansion, residing in two clusters either side of the Class III region. Once again, KERV sequences are prominent in this region and may have contributed to the overall genomic instability of the wallaby MHC region (Siddle et al., 2011).

#### 2.3.3 The MHC in monotremes

Although the platypus genome has been sequenced, the high GC and repeat content hampered this sequencing effort, leaving the assembly with many more gaps than other mammalian genomes sequenced to a six-fold depth by Sanger sequencing (Warren et al., 2008). As a result, complete annotation of the platypus MHC as a region was impossible because MHC genes were found on many sequence contigs and/or scaffolds. However, three BAC clones were completely sequenced and mapped to platypus chromosomes (Dohm et al., 2007). One of these BACs, localized to chromosome 3, only contained a processed class I pseudogene. Of the remaining two BACs, one contained two Class I genes and two Class II genes as well as antigen processing genes, while the other contained mainly Class III genes. The most surprising result came from FISH-mapping, which revealed that platypus MHC is not contiguous and maps to the pseudoautosomal region of two pairs of sex chromsomes. The Class I and II genes were located on  $X_3/Y_3$  and the Class III region on X<sub>4</sub>/Y<sub>4</sub>. Subsequent FISH-mapping of BACs containing these same genes in the echidna demonstrated that this separation of the MHC onto two different pairs of sex chromosomes was a common feature for monotremes. Monotremes are the only mammals known to date to have the MHC reside on sex chromosomes (Dohm et al., 2007).

#### 2.4 Tracing the evolutionary history of globin genes

Haemoglobin is essential for oxygen transportation in vertebrates. The haemoglobin molecule is encoded by members of the  $\alpha$ - and  $\beta$ -globin gene clusters. These gene clusters were presumed to have arisen from a single globin gene that duplicated to form a combined  $\alpha$ - and  $\beta$ -globin gene cluster as is seen in amphibians (Jeffreys et al., 1980). It was proposed that either a fission event or a chromosome duplication event, followed by independent evolution of the duplicate copies, gave rise to the separate  $\alpha$ - and  $\beta$ -globin gene clusters observed in amniotes (Jeffreys et al., 1980). Determining the gene content of the marsupial and monotreme globin gene clusters has had a tremendous impact in this field. This work was facilitated by sequencing and mapping of BACs containing globin genes.

The discovery of a novel  $\beta$ -like globin gene called *HBW* residing adjacent to the wallaby  $\alpha$ globin cluster provided support for the chromosome duplication hypothesis (Wheeler et al., 2004). Further support was provided when BAC clones from the dunnart (*S.macroura*) spanning the separate  $\alpha$ - and  $\beta$ -globin gene clusters were sequenced and it was found that, like the wallaby, the *HBW* was adjacent to the  $\alpha$ -globin cluster (De Leo et al., 2005). The next obvious step in testing the chromosome duplication hypothesis was to determine the organization of the platypus  $\alpha$ - and  $\beta$ -globin gene clusters. The fragmented nature of the platypus genome meant that a BAC-based approach was required to obtain a more complete sequence of the alpha and beta globin gene clusters in this species (Patel et al., 2008). Analysis of the sequence obtained from these BAC clones was instrumental in the formation of a new hypothesis for the evolution of these gene clusters.

The platypus  $\alpha$ -globin cluster also contained a copy of *HBW*, which taken on its own would support the chromosome duplication hypothesis. However, an examination of the genes flanking the two clusters revealed that the combined  $\alpha/\beta$ -globin cluster in amphibians was flanked by the same genes as the  $\alpha$ -globin cluster in all amniotes, whereas the  $\beta$ -globin cluster in amniotes was surrounded by olfactory receptors. This led to a hypothesis where the  $\alpha$ -globin cluster in amniotes was proposed to correspond to the original  $\alpha/\beta$ -globin cluster present in other vertebrates. The  $\beta$ -globin cluster was proposed to have evolved after a copy of the original  $\beta$ -globin gene (*HBW*) was transposed into an array of olfactory receptors (Patel et al., 2008).

#### 3. Anchoring marsupial and monotreme genome assemblies

Genome sequence data on its own is an extremely valuable resource but it is also equally as important to know how the genome fits together. BACs have played an essential role in anchoring marsupial and monotreme sequence to chromosomes. Different approaches have been taken that have utilized BACs to improve genome assemblies, with the strategy employed dependent the quality of the genome assembly.

The opossum and platypus genome projects employed BACs in a similar fashion. BAC-end sequencing was used to assist in connecting sequence contigs into scaffolds (Mikkelsen et al., 2007; Warren et al., 2008). Scaffolds were anchored and oriented on chromosomes by FISH-mapping BACs from ends of sequence scaffolds (Duke et al., 2007; Warren et al., 2008). For the opossum genome, the mapping of 381 BACs resulted in 97% of the genome being assigned to chromosomes (Duke et al., 2007). The more fragmented nature of the platypus

genome assembly made it more difficult to anchor each scaffold but FISH-mapping of BACs assigned 198 scaffolds, corresponding to approximately 20% of the genome, to chromosomes (Warren et al., 2008).

Anchoring of the even more fragmented wallaby and devil genome assemblies required a different approach. A novel approach was developed to anchor the low-coverage wallaby genome sequence to chromosomes. A cytogenetic map of the genome was constructed by mapping BACs containing genes from the ends of human-opossum conserved gene blocks. This strategy was first trialed on tammar wallaby chromosome 5 (Deakin et al., 2008b) and later applied to the entire genome (Renfree et al., 2011). A virtual map of the wallaby genome was made by extrapolating from the content of these mapped conserved blocks from the opossum genome assembly, thereby allowing the location of each gene on tammar wallaby chromosomes to be predicted (Wang et al., 2011a). A similar approach is being used to construct a map of the devil genome, which has been sequenced entirely by next generation sequencing (Miller et al., 2011).

#### 4. BACs and marsupial linkage maps

Linkage (genetic) maps are a useful resource as they provide information not only on the order of genetic markers on a chromosome but on the location and frequencies of crossover events. Such maps are even more valuable if the maps are anchored to chromosomes and integrated with available genome assembly and/or cytogenetic mapping data. Linkage maps have been constructed for two marsupial species, opossum (Samollow et al., 2007) and the wallaby (Wang et al., 2011b). BACs containing markers at the ends of linkage groups have been used to cytogenetically assign these groups to chromosomes and determine the genome coverage of the linkage maps (Samollow et al., 2007; Wang et al., 2011b). The opossum linkage map was integrated with the genome assembly and cytogenetic map by FISH-mapping 34 BAC clones from the ends of linkage groups (Duke et al., 2007; Samollow et al., 2007). A sophisticated approach was used in the marker selection for construction of the wallaby linkage map to facilitate the integration of cytogenetic and linkage map data. Three strategies were developed to fill gaps in the 1st generation linkage map (Zenger et al., 2002) using information from BACs. The first strategy involved identifying microsatellites in BACs that had been previously assigned to chromosomes by FISH. The second strategy identified microsatellites within BAC end sequences and the third used the wallaby genome sequence to identify microsatellite markers near BACs that had been mapped by FISH. This resulted in a linkage map that could easily be incorporated with the physical map data to generate an integrated map (Wang et al., 2011a, 2011b). Information from the integrated map has been used to improve and anchor the tammar wallaby genome assembly (Renfree et al., 2011).

#### 5. Conclusions

Our understanding of marsupial and monotreme genomes has been greatly advanced due in large part to the availability of BAC libraries for several key species. With the emergence of Devil Facial Tumour Disease (DFTD), a transmissible cancer threatening the extinction of this species in the wild within the next 25 years (McCallum et al., 2007), many marsupial researchers are are focusing their efforts on characterization of this devastating disease. BAC libraries are playing a play a major role in this work, building on the strategies developed

for other species described here to rapidly gain as much information as possible on the normal and DFTD tumour genomes. In addition, genome sequencing of other marsupial and monotremes species is currently underway using next generation sequencing technology and it is anticipated that BAC libraries will continue to be a very precious resource for comparative genomic studies in these species.

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#### **Bacterial Artificial Chromosomes**

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This book focuses on the numerous applications of Bacterial Artificial Chromosomes (BACs) in a variety of studies. The topics reviewed range from using BAC libraries as resources for marsupial and monotreme gene mapping and comparative genomic studies, to using BACs as vehicles for maintaining the large infectious DNA genomes of viruses. The large size of the insert DNA in BACs and the ease of engineering mutations in that DNA within the bacterial host, allowed manipulating the BAC-viral DNA of Varicella-Zoster Virus. Other reviews include the maintenance and suitable expression of foreign genes from a Baculovirus genome, including protein complexes, from the BAC-viral DNA and generating vaccines from BAC-viral DNA genomes of Marek's disease virus. Production of multi-purpose BAC clones in the novel Bacillus subtilis host is described, along with chapters that illustrate the use of BAC transgenic animals to address important issues of gene regulation in vertebrates, such as functionally identifying novel cis-acting distal gene regulatory sequences.

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