The Koala and its Retroviruses:
Implications for Sustainability and Survival

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How does Koala Retrovirus (KoRV) Induce Disease at the Genomic Level?

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Abstract. This manuscript summarizes the break-out session held on how does koala retrovirus (KoRV) induce disease at the genomic level at the *Koala Conservation Workshop: The koala and its retroviruses: implications for sustainability and survival* held at San Diego Zoo, April 17–18, 2013. The goals of this break-out session were to review current knowledge in this area and identify studies required to fill important gaps. KoRV is a gammaretrovirus with close similarity to MLV and FeLV, well-characterized pathogens of the laboratory mouse and the domestic cat. The parallel wth FeLV is particularly striking as cats harbor related endogenous retroviruses that share receptor specificity with endogenous KoRV. Also, transmission and pathogenesis of FeLV in its natural host is well understood and the virus is routinely controlled by measures that include vaccines. Alternative models for the roles of endogenous and exogenous KoRV in disease were discussed and prospective studies required to test these hypotheses were outlined.

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Introduction

What do we know? Most koala populations contain integrated KoRV-A. It appears that a subpopulation of koalas e.g. on Kangaroo Island (KI) may be free of KoRV, although available data based on PCR and hybridization analysis with KoRV-specific probes cannot be regarded as a definitive negative. The prevalence of disease appears to correlate with the copy number of KoRV, high in Queensland and low in other areas such as KI. Southern blot analysis of integrated KoRV from "high copy number" Queensland koalas reveals a similar pattern across tissues suggesting that most or all KoRV copies are germ-line rather than somatically acquired.

The length of time KoRV has been in the koala population is unclear but the recovery of integrated KoRV from koala skins in museum collections suggests that the infection may be older than previously supposed. However, the remarkably high copy number in some koala populations suggests that expansion of endogenous KoRV sequences may be more recent.

Analysis of lymphomas from captive koalas in US zoos has revealed the presence of variant KoRV with altered host range (KoRV-B, C) due to mutational changes in the viral *env* gene. They also show duplications of the core enhancer sequences in the viral LTR. Similar changes have been observed previously in murine and feline gammaretroviruses and are associated with increased replication in lymphoid tissues and leukemogenicity. These features suggest that KoRV induces lymphoma by an insertional mutagenesis mechanism similar to other gammaretroviruses. There is a further remarkable parallel between KoRV and feline leukemia virus (FeLV). Endogenous FeLV-related sequences, which are ancient (c. 6 million years) and invariably replication defective, encode an envelope protein that binds Pit-1, like KoRV-A. The prevalent infectious form of FeLV, FeLV-A, utilizes THTR1, like KoRV-B. FeLV-A recombines with endogenous FeLV to generate FeLV-B, and such recombinant viruses are more common in leukemic cats. However, the KoRV variants appear to arise by limited mutations from KoRV-A, presenting a challenge to the development of simple molecular typing and detection methods such as those used to analyse *de novo* integrated MLV and FeLV on a complex background of related endogenous viruses.

Gaps in knowledge

1 If it could be shown that KoRV is capable of infecting somatic cells and induces lymphoma by insertional mutagenesis, this would firmly establish its role in disease and argue in favour of measures to limit transmission and dissemination. However, although the parallels with other gammaretroviruses are persuasive, direct evidence is lacking. Lack of koala genome sequence data is another significant constraint.

2 Based on existing data, three main scenarios are possible:

- 2.1 Endogenous KoRV (KoRV-A) is unable to re-enter somatic cells due to defectiveness or interference barriers, with KoRV-related disease arising due to superinfection with horizontally transmitted forms such as KoRV-B (the FeLV model)
- 2.2 Endogenous KoRV is capable of replication, leading to evolution of more pathogenic forms within an individual animal (the Akv model).
- 2.3 An intermediate situation where KoRV-B and other variants arise occasionally by mutation and are then transmitted to koalas in contact, either horizontally or via milk to offspring.

It will be important to distinguish between these possibilities as they have significantly different implications for disease prevention and control.

3 Another deficiency is the lack of information on immune responses to KoRV. This is important to establish whether control by vaccination will be feasible. Specifically :

- 3.1 Do apparently KoRV-negative koalas make immune responses due to exposure to infected animals?
- 3.2 Does expression of germ-line KoRV-A lead to immune tolerance and susceptibility to *de novo* infection with more pathogenic strains (e.g., KoRV-B)?

4 Innate/intrinsic immunity to KoRV has not yet been examined.

Major questions to be addressed

Analysis of *de novo* integrations and somatic mutations in lymphomas of KoRV-infected koalas will require the collection of uninvolved tissue as well as tumor at post-mortem. Disease arising in zoos offers the best prospect of obtaining fresh port-mortem tissues and should be prioritized. There are problems with adopting methods used in the mouse, as high copy numbers of endogenous KoRV may obscure *de novo* integrations, and the precise genomic location is unlikely to be clear in the absence of koala genome sequence. A more accessible though indirect method of testing the insertional mutagenesis hypothesis would be to look by Southern blot analysis for rearrangements in the homologues of known lymphoma target genes that are common to other gammaretroviruses across species (e.g., *Myc, Gfi1, Pim1, Myb*). Probes derived from conserved coding sequences of the genes should be first tested for their ability to detect unique sequences in the koala genome by blot analysis. PCR amplification of specific exons could also be used to generate higher specificity. If rearrangements are found, further restriction enzyme digests and/or direct PCR amplification and sequencing could then be used to demonstrate the presence and location of newly integrated KoRV.

1 Tumour typing is limited due to relative lack of surface phenotype markers. Demonstration that tumours are clonal expansions of T or B-cells could be achieved using conserved probes from TCR or IgH loci. Again, cloning of conserved exons from koala orthologues should be straightforward.

2 There is a need to characterize KoRV isolates further and establish whether KoRV-B/J or other variants are essential for disease development. This will require virus isolation from healthy and diseased animals and analysis of tropism. It is known that KoRV-A and B can infect and replicate in permissive human cells (e.g., HEK293) but the possibility that other *env* variants may be unable to replicate in these cells should also be considered. Development of primary fibroblast cultures from koalas would be advantageous if this can be achieved (e.g., from non-viable joeys).

3 Sequence analysis of KoRV-B/J variants from multiple sources may give clues to the frequency of occurrence and transmission e.g., geographical localization of unique signature sequences of variants would indicate local transmission rather than de novo generation.

4 Analysis of multiple tissues from postmortem samples of koalas with lymphoma or other diseases would indicate whether variant KoRV are present in germ-line or somatically acquired.

5 A neutralization test for KoRV would be helpful for analysis of specific immune responses. A suitable assay could be generated using pseudotype viruses (lacZ/GFP). Western blot analyses would complement these studies, but will require anti-koala Ig.

6 The unusual germ-line amplification of KoRV-A in Queensland koalas is of potential significance. The possibility that koalas are deficient in restriction factors that confer innate or intrinsic immunity to retroviral spread (e.g., the APOBEC family) could be investigated by cloning and functional analysis of koala orthologues of this and other relevant gene families. It would be important to determine whether e.g. southern koala populations are more intrinsically resistant to KoRV despite their relative lack of reproductive fitness.

Resources required:

- Well annotated KoRV isolates from healthy and diseased koalas.
- Control and tumour tissues from diseased animals.
- Specific probes for likely target genes for insertional mutagenesis and koala TCR/Ig.
- Koala fibroblast cultures (if feasible) to examine KoRV growth properties in natural host cells.
- Sera from koalas apparently lacking KoRV and KoRV positive controls.
- DNA from divergent koala populations for restriction factor cloning and analysis.

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