The Koala and its Retroviruses:
Implications for Sustainability and Survival

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Molecular Characterization of Koala Retroviruses Isolated from Koalas *(Phascolarctos cinereus)* Reared in Japanese Zoos

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Abstract. In northern Australia most koalas *(Phascolarctos cinereus)* are infected with the gammaretrovirus known as koala retrovirus (KoRV). KoRV is believed to be currently endogenizing into its host. Koalas were first introduced into three Japanese zoos in 1984 and now about 50 koalas are held in eight zoos. In 2007 KoRV was isolated from koalas reared in Japanese zoos, and, for the first time, an infectious molecular clone termed pKoRV522 was constructed. Using the molecular clone and KoRV isolates, we revealed the budding mechanism of KoRV and genomic diversity of KoRVs isolated from Japanese koalas. We found that KoRV utilizes the multivesicular body-sorting pathway. We also discovered a novel KoRV subgroup, named KoRV-J, which utilizes thiamine transport protein 1 as an entry receptor. The original KoRV, which utilizes Pit-1 as an entry receptor, is now named KoRV-A. In two Queensland koalas examined, the copy numbers of KoRV-J was less than 1 copy per cell and varied in tissues. These data, at least in these two koalas, suggest that KoRV-J is an exogenous retrovirus not an endogenous retrovirus.

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Endogenous retroviruses (ERVs), occupy about 8 to 13 percent of mammalian genomes. Most ERVs are defective due to genomic mutations and deletions. However, some ERVs retain functionality and contribute to host physiological processes, exemplified by the human syncytins in placentation (Feschotte & Gilberet, 2012). In this regard, ERVs are believed to play a role in the evolution of mammals, yet the process of endogenization of retroviruses, resulting in the establishment of ERVs, has not been elucidated. The koala retrovirus (KoRV), found in koalas *(Phascolarctos cinereus)*, is a gammaretrovirus which is believed to be currently endogenizing into its host, thus providing us with a rare opportunity to investigate the mechanisms involved in retrovirus endogenization (Stoye, 2006; Tarlinton *et al*., 2006). Genetically and phylogenetically, KoRV is closely related to gibbon ape leukemia virus (GALV) which is an exogenous gammaretrovirus and induces leukemia/lymphoma in gibbons (Delassus *et al*., 1989). In addition, KoRV shares the viral receptor (Pit-1, a phosphate transporter) with GALV when it infects cells (Oliveira *et al*., 2007).

In addition to benefits provided by ERVs, there are also negative consequences of harboring them in the host genome. Indeed, increased levels of KoRV infection in koalas have been associated with several diseases. For instance, koalas suffer from leukemia and lymphoma with a rate of 3–5% in the wild and an even higher rate of up to 60 % in some captive colonies (Canfield *et al*., 1988; Hanger *et al*., 2000). Tarlinton et al. reported that, using quantitative real-time reverse transcriptase (RT)-PCR, KoRV RNA levels in plasma were significantly increased in koalas suffering from leukemia or lymphoma when compared with

healthy koalas (Tarlinton *et al*., 2005). These observations suggest that KoRV is linked to oncogenesis in koalas, making the study of this virus important for understanding its pathogenesis.

Construction and characterization of an infectious clone of KoRV

To date, studies on KoRV infection have been limited due to the lack of a replication-competent molecular clone. Quite recently, we succeeded in constructing an infectious molecular clone of KoRV, termed pKoRV522 (Shojima *et al*., 2013a). It is known that gammaretroviruses bud from the plasma membrane of infected cells. Gag proteins of many retroviruses include short peptide motifs required for virus budding, termed L-domain motifs. To date, three types of L-domain motifs (PT/SAP, PPXY, and YXXL) have been identified. It was reported that the disruption of the PPXY motif in the L-domain of KoRV was involved in the attenuation of KoRV in the process of endogenization into the host (Oliveira *et al*., 2007). Although pKoRV522 has the same mutation in this motif, the virus derived from the pKoRV522 replicated efficiently in human embryonic kidney (HEK) 293T cells, reaching a maximum titer of 10^6 focus-forming units/ml (Shojima *et al*., 2013a). By comparing the Gag sequences of KoRV and GALV, we found an additional intact PPXY motif 18 bp downstream of the PSAP motif in KoRV. By virus budding assays, mutations in the PSAP motif did not affect KoRV budding, whereas mutations in the novel PPXY motif had a significant impact on KoRV budding (Shojima *et al*., 2013a). Therefore, the second PPXY motif is considered to be the major L-domain sequence for the KoRV budding while the first PPXY is dispensable (Shojima *et al*., 2013a).

It has been reported that the PPxY motif interacts with the WW domains of the cellular Nedd4-like E3 ubiquitin ligases (Martin-Serrano *et al*., 2005; Yasuda *et al*., 2002). These host factors are the cellular proteins involved in the multivesicular body (MVB) sorting pathway. The interaction of a viral L-domain with Nedd4-like E3 ubiquitin ligases is essential for the virus budding, and budding of the retroviruses possessing L-domains and MVB vesicle formation might be analogous processes. To further analyze the molecular mechanism of KoRV budding, we examined the involvement of Nedd4-like E3 ubiquitin ligases on the KoRV budding. Consequently, we demonstrated that WWP2 or WWP2-like E3 ubiquitin ligases, possessing the WW domain closely related to WWP2 and Vps4A/B, are involved in KoRV budding (Shimode *et al*., 2013). These data suggest that KoRV Gag recruits the cellular endosomal sorting complex required for transport (ESCRT) machinery through the interaction of the PPPY L-domain with the WW domain(s) of WWP2 and progeny virions are released from cells by utilizing the MVB sorting pathway.

Genomic diversity of KoRVs isolated from Japanese koalas

From 2007 to 2009, we conducted a survey of KoRV infection in koalas in Japanese zoos and succeeded in isolating KoRVs. We identified 4 genotypes whose receptor binding sites are different with each other. By using pseudotype viruses harboring these subgroups, we found that two subtypes (named A and J) infect human cell lines. KoRV-A is similar in nucleotide sequences to the original KoRV clone, termed *pcindy*. The subtype A pseudotype virus shares the receptor with GALV and feline leukemia virus (FeLV) subgroup B (FeLV-B) and utilizes human Pit-1 molecule as a viral entry receptor. The subtype J pseudotype virus utilizes thiamine transport protein 1 (THTR1) to infect human cells as described in the next section. All koalas which are positive for KoRV provirus had KoRV-A in common and many koalas harbor additional subtypes. The long terminal repeat (LTR) of KoRV-J has three tandem repeats in the enhancer region (unpublished data). The promoter activity of LTR of KoRV-J was stronger than that of KoRV-A LTR in HEK293 cells (Shimode *et al*., 2014). The pathological differences in distinct subtypes have not been identified because most Queensland koalas in Japanese zoos are infected with a combination of several subtypes.

Characterization of KoRV-J and prevalence of KoRV-J in koalas in Japanese zoos

Phylogenetic analysis of *env* using the maximum likelihood approach revealed that KoRV isolates and GALV clustered together, but they were distinct from the cluster that consists of FeLVs, murine leukemia viruses (MLVs) and porcine endogenous retroviruses (PERVs) (Fig. 1). KoRVs and GALVs are distantly related to PERVs. Similarities of the Env amino acids among the KoRV-A isolates were shown to be high, and the degree of diversity between KoRV-A and KoRV-J was less than those of FeLV and PERV subgroups.

To further characterize the receptor usage of KoRV-J, we conducted a receptor interference assay using six gammaretroviruses which utilize different receptors, namely, FeLV-A, -B, and -C, RD-114 virus, xenotropic murine leukemia virus (X-MLV) and A-MLV. We found that *lacZ*(KoRV-J) pseudotype virus interfered with FeLV-A on FEA cells (feline fibroblast). The receptor for FeLV-A is known to be thiamine transport protein 1 (THTR1). *lacZ*(KoRV-J) and *lacZ*(FeLV-A) infected *Mus dunni* tail fibroblast (MDTF) cells expressing human THTR1 (hTHTR1), but not naïve MDTF cells. These data indicate that KoRV-J utilizes THTR1 as a receptor (Shojima *et al*., 2013b).

To investigate the prevalence of KoRV subtypes in koalas reared in Japanese zoos, in 2007 to 2009, we collected heparinized blood samples of 40 northern koalas (Queensland, New South Wales and hybrids of Queensland and New South Wales koalas) and 11 southern (Victorian) koalas from 9 zoological parks in Japan, and performed differential PCR analysis using subgroup-specific primer sets. KoRV-A was detected in all northern koalas tested and 4 out of 11 Victorian koalas (Shojima *et al*., 2013b), consistent with previous reports that KoRV had endogenized in koalas in northern Australia. In contrast, KoRV-J was detected in 67.5 % of northern koalas, but not in southern (Victorian) koalas (Shojima *et al*., 2013b). These data indicate that the prevalence of KoRV-J is more limited than KoRV-A, and the invasion of KoRV-J into the koala population may have occurred more recently than KoRV-A.

To determine whether KoRV-J is exogenous or endogenous, we determined the copy numbers of each subgroup in the genomes of different tissues in the individual animals. Copy numbers of each subgroup in tissues of two Queensland koalas (KoRV-A positive, KoRV-J positive) were measured by quantitative real-time PCR. Approximately 3–6 copies of KoRV-A per cell were present in the tissues tested (Shojima *et al*., 2013b). In contrast to the relatively constant copy numbers of KoRV-A among tissues, the copy numbers of KoRV-J were less than 1 copy per cell and varied in tissues in both koalas (Shojima *et al*., 2013b). These data suggest that KoRV-J is not an ERV, at least in these two koalas.

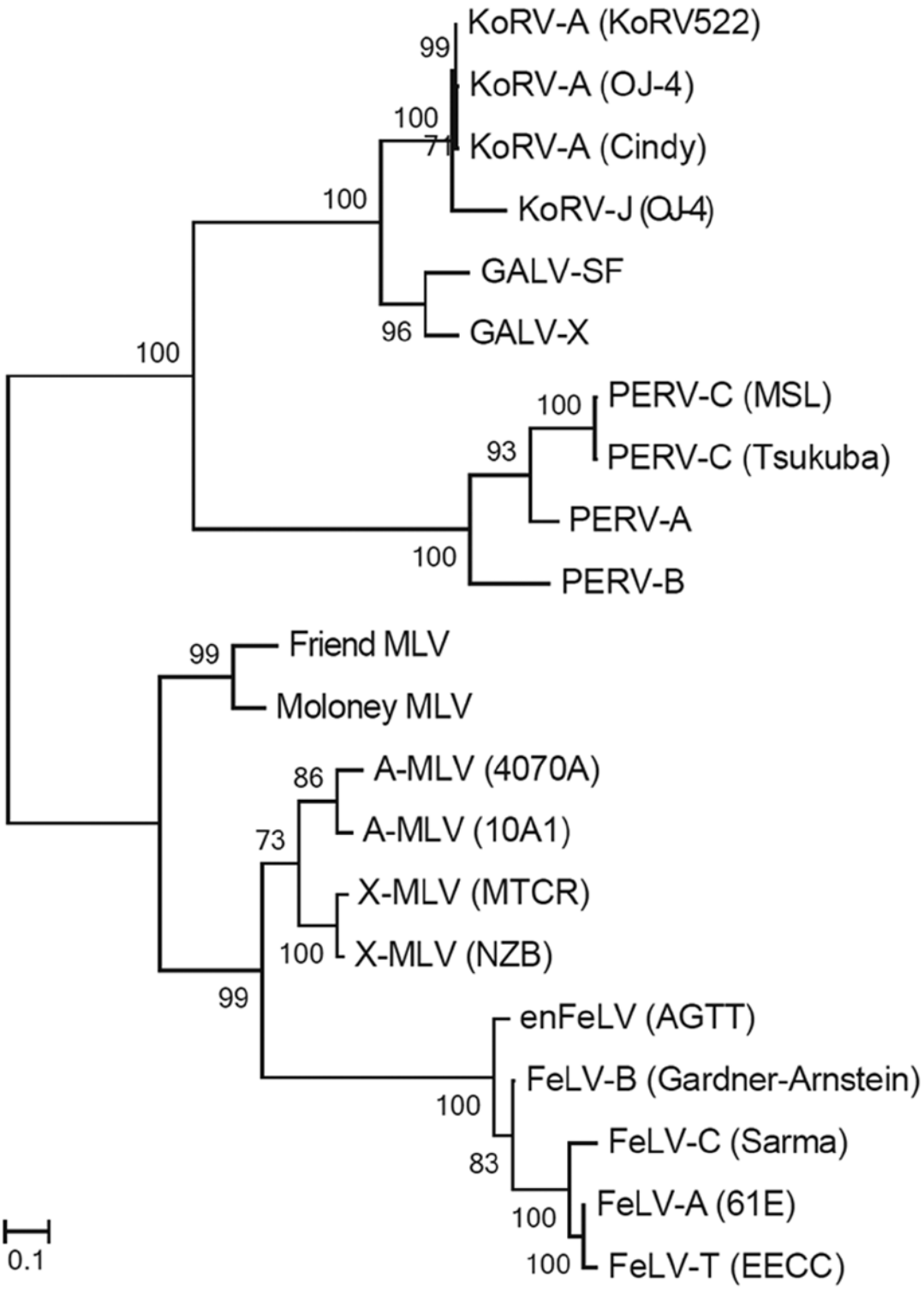

Figure 1. Maximum likelihood tree of the entire amino-acid sequences of *env* genes of KoRV isolates and other gammaretroviruses. Numbers at the nodes indicate percent of rapid bootstrap values (1000 replicates). Amino acid sequences used for the analyses were retrieved from the GenBank database. Abbreviations: A-MLV, amphotropic MLV; X-MLV, xenotropic MLV; enFeLV, endogenous FeLV.

It is plausible that KoRV-J-infected northern koala(s) were introduced into Japanese zoos rather than the virus being derived from other animals within the facilities, especially given that koalas are kept separately from other animals except humans. The origin of KoRV-J is unknown at present. The low amino acid similarity on the surface of Env was not simply caused by nucleotide insertions and/or deletions. Furthermore, it is unlikely that KoRV-J was generated from KoRV-A due to an accumulation of nucleotide mutations. KoRV-J could have been prevalent in an unknown host species in Australia that infected a population of northern koalas quite recently. It is also possible that KoRV-J may be the result of a recombination event between KoRV-A and another KoRV-related gammaretrovirus. Thus far, we have been unable to find any KoRV-J variable region A-like sequences in the NCBI nr/nt database, meaning that further studies are needed to elucidate the origin of the virus. Different receptor usage of KoRV subtypes may explain the wide range of diseases seen in koalas.

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