The Sox2 promoter-driven CD63-GFP transgenic rat model allows tracking neural stem cell-derived extracellular vesicles

Aya Yoshimura1,2,3, Naoki Adachi2,4, Hitomi Matsuno2, Masaki Kawamata5, Yusuke Yoshioka5, Hisae Kikuchi6, Haruki Odaka2,7,8, Tadahiro Numakawa2,8, Hiroshi Kunugi2, Takahiro Ochiya5* & Yoshitaka Tamai1,9

1. Division of Laboratory Animals Resources, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan
2. Department of Mental Disorder Research, National Institute of Neuroscience, NCNP, Tokyo, Japan
3. Education and Research Facility of Animal Models for Human Diseases, Center for Research Promotion and Support, Fujita Health University, Aichi, Japan
4. Department of Biomedical Chemistry, School of Science and Technology, Kwansei Gakuin University, Sanda, Hyogo, Japan
5. Division of Molecular and Cellular medicine, National Cancer Center Research Institute (NCC), Tokyo, Japan
6. Department of Degenerative Neurological Diseases, National Institute of Neuroscience, NCNP, Tokyo, Japan
7. Department of Life Science and Medical Bioscience, School of Advanced Science and Engineering, Waseda University, Tokyo, Japan
8. Department of Cell Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan
9. chromocenter Inc., Kobe, Japan

*Corresponding author:
Takahiro Ochiya
Division of Molecular and Cellular medicine, National Cancer Center Research Institute (NCC), 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan.
E-mail: tochiya@ncc.go.jp
Telephone number: +81-3-3542-2511 (ext. 4800)
Fax number: +81-3-5565-07
KEY WORDS:
Model animals,
Extracellular vesicles,
CD63,
Neural stem cells,

Summary statement:
We generated a novel transgenic rat model expressing CD63-GFP driven by Sox2 promoter, which enables tracking and analysing neural stem cell-derived extracellular vesicles.

ABSTRACT
Extracellular vesicles (EVs) can modulate microenvironments by transferring biomolecules including RNAs and proteins derived from releasing cells to target cells. To understand the molecular mechanisms maintaining the neural stem cell (NSC) niche through EVs, a new transgenic (Tg) rat strain that can release human CD63-GFP expressed EVs from the NSCs was established. Human CD63-GFP expression was controlled under the rat Sox2 promoter (Sox2/human CD63-GFP), and it was expressed in undifferentiated foetal brains. GFP signals were specifically observed in in vitro cultured NSCs obtained from embryonic brains of the Tg rats. We also demonstrate that embryonic NSC (eNSC)-derived EVs were labelled by human CD63-GFP. Furthermore, when we examined the transfer of EVs, eNSC-derived EVs were found to be incorporated into astrocytes and eNSCs, thus implying an EV-mediated communication between different cell types around NSCs. This novel Sox2/human CD63-GFP Tg rat strain should provide resources to analyse the cell-to-cell communications via EVs in NSC microenvironments.
INTRODUCTION

Neural stem cells (NSCs) persist and generate new neurons in the adult mammalian brain as well as in the foetal developing brain. While neurogenesis during prenatal development is responsible for brain growth, adult neurogenesis contributes to learning and memory throughout life (Deng et al., 2010). Furthermore, recent studies have suggested that decreased adult neurogenesis is involved in neuropsychiatric diseases such as epilepsy and depression (Braun and Jessberger, 2014). To continue neuronal production, NSCs are present mainly in two regions, the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) in the lateral wall of the lateral ventricles in the adult brain, and NSC proliferation and differentiation are regulated via extracellular stimulation from the stem cell niche (Riquelme et al., 2008). Therefore, research has focused on understanding the molecular mechanisms maintaining the NSC microenvironment.

Extracellular vesicles (EVs) are important microenvironmental factors (Fujita et al., 2015). Many different cell types in the body release EVs into the extracellular environment; they contain lipids, proteins, mRNAs and microRNAs (miRNAs) originated from releasing cells, and these biomolecules are transferred to target cells via the uptake of EVs. A number of cancer studies have reported that EVs mediate communication within the tumour microenvironment, contributing to cancer progression (Hu et al., 2015) and metastasis (Peinado et al., 2012; Tominaga et al., 2015). In the central nervous system (CNS), neurons, astrocytes, oligodendrocytes and microglia have been reported to secrete EVs, which influence synaptic plasticity and the immune system. For example, neurons release EVs carrying the GluR2/3 subunits of AMPA receptors depending on enhanced glutamatergic activity (Lachenal et al., 2011), and astrocytes shed EVs carrying Hsp70 in response to heat stress to protect neurons (Taylor et al., 2007). Moreover, EVs have been shown to remove obsolete proteins from cells (Danzer et al., 2012; Rajendran et al., 2006). Although these studies have suggested EVs’ function to maintain CNS homeostasis, whether EVs have influences on shaping and maintaining the niche around NSCs and how EVs move between cells in the NSC region remain unclear. Generally, most in vitro and in vivo assays so far use EVs isolated from cultured donor cells and imaging are performed using cell membrane-tracking reagents; such as PKH dyes, or transfection of a fluorescent-tagged EV marker proteins to examine the transfer of EV contents into the recipient cells (Grapp et al., 2013; Koumangoya et al., 2011; Suetsugu et al., 2013). However, behaviours of EVs in cultured cells may not reflect that of EVs released in vivo. To address this challenge, we generated a new transgenic (Tg) rat model expressing fluorescent-tagged EVs in the NSC region.
EVs are classified into diverse types depending on their membrane origin, either the endosomal or plasma membrane, and are called exosomes and microvesicles, respectively (Raposo and Stoorvogel, 2013). Exosomes are vesicles, approximately 40–100 nm in diameter, produced from late endosomes/multivesicular bodies (MVBs) formed by invagination and budding of early endosomes, whereas microvesicles are larger EVs (up to ~1000 nm in diameter) generated by outward budding of the plasma membrane. The CD63 (lysosomal-associated membrane protein 3) is an EV marker, in particular an exosome marker, because it is highly enriched in late endosomes via an intracellular pathway from the trans-Golgi network, or via endocytosis from the cell surface (Pols and Klumperman, 2009). A fluorescent-tagged CD63 gene has been transfected into cultured cell lines, and in vivo in Drosophila melanogaster, to detect EV release from donor cells and EV transfer into recipient cells (Corrigan et al., 2014; Gross et al., 2012; Koumangoya et al., 2011; Suetsugu et al., 2013; Sung et al., 2015). In addition, we previously generated a Tg rat strain expressing human CD63-copGFP under the control of the ubiquitous CAG promoter (CAG/human CD63-GFP) and showing human CD63-GFP labelled EVs in body fluids (Yoshimura et al., 2016b). In this study, we constructed a human CD63-copGFP gene regulated by the Sox2 promoter (Sox2/human CD63-GFP). The transcription factor gene Sox2 is expressed in the NSCs of both embryonic and adult brains, and is required for the maintenance of NSCs (Ferri et al., 2004). Therefore, it is supposed that Sox2/human CD63-GFP rats have GFP-labelled EVs in NSCs.

Here, we demonstrated that exogenous human CD63-GFP expression was detected in the NSCs of the Tg rats and that the human CD63-GFP labels were detected in embryonic NSC (eNSC)-derived EVs in recipient cells in vitro.

RESULTS AND DISCUSSION
Sox2/human CD63-GFP Tg rats express GFP in diverse tissues including the developing brain

To investigate communication via EVs in specific regions of the brain, we generated a Sox2/human CD63-GFP Tg rat strain using rat embryonic stem cells (rESCs). The vector encoding human CD63-GFP under control of the proximal 6.65-kb rat Sox2 promoter was transfected into rESCs (Fig. 1A); several rESC colonies showed GFP fluorescence because SOX2 is essential for maintaining self-renewal in ESCs. Three rESC lines (No. 6, No. 10 and No. 22) indicated a bright and stable fluorescence at the passages shown in Fig. 1B and Fig. S1, and therefore, these lines were used to produce chimaeras. Two rESC lines (No. 6 and No. 22) and nine rats (two males and seven females) showed coat colour chimaerism resulting from the injection of GFP rESCs into blastocysts. As the rESC line used in this study was established
from female blastocysts of Wistar rats (Kawamata and Ochiya, 2010), the seven chimaeric females were bred to Wistar males. One female chimaeric rat originated from No. 6 rESC line produced a GFP-positive male, thus indicating the transgene’s successful germline transmission and it was named as Wistar-esTgN(Sox2/CD63-GFP)3NCCRI strain.

The GFP fluorescence was observed in the telencephalon and along the spinal cord of the E14 foetal rats (Fig. 1C), indicating that the Sox2 promoter fragment contains regulatory elements for region-specific expression. Similar expression patterns were observed in previous studies using the regulatory elements of the Sox2 promoter in mice (Kang and Hebert, 2012; Zappone et al., 2000). SOX2 expression was down-regulated in the developing cerebral cortex (Fig. 1D). Consistent with its expression pattern, exogenous human CD63 and copGFP exhibited reduced expression in the cerebral cortex of postnatal rats (Fig. 1D). In contrast, the expression of endogenous rat CD63 was increased depending on the development of the cerebral cortex. In the developing telencephalon at E16, immunohistological analysis showed SOX2 expression along the ventricular zone (VZ) (Fig. 2A). A punctate distribution of GFP was observed in the SOX2-positive region of the Tg telencephalon, but not in the Wt (Fig. 2B, Fig. S2). The GFP signals were also observed in SOX2-negative region, implying the possibility of EV transfer in the physiological condition. In the adult brain of Tg rats, the GFP fluorescent signals were also detected in some SOX2-positive cells in the SVZ (Fig. S3, arrows). Unexpectedly, the intense GFP signals were distributed along the blood vessels indicated by lectin immunoreactivity (Fig. S3). These GFP signals seemed to be localized at the feet of astrocytes contacting with the blood vessel (Fig. S3, arrowheads). These images indicated that endothelial cells and/or pericytes that form the blood-brain barrier with astrocytes contain human CD63-GFP. Considering that serum EVs do not carry detectable levels of human CD63-GFP (see Fig. 4B), GFP signals around the blood vessels are likely attributable to human CD63-GFP expressed in these cells or uptaken and accumulated GFP-labelled EVs from other SOX2-expressing cells although further detailed studies are required. Furthermore, the adult hippocampus of Tg rats showed a normal distribution of the NeuN-positive neurons (Fig. S4).

Human CD63-GFP expression was detected in other new-born tissues as well as in the brain (Fig. 3A, B). A high level of GFP fluorescence was observed in the skin and heart, and especially in the kidney and stomach (Fig. 3A viii’). Furthermore, hair follicles in the whiskers (Fig. 3A ii’, arrow) but not on the body (Fig. 3A iii’, arrows) expressed GFP fluorescence, while the pancreas showed a weaker fluorescence. Slight signals were detected in the thymus and spleen by western blot analysis with anti-human CD63 and copGFP antibody, whereas GFP fluorescence was not observed in these tissues. SOX2 signals in western blotting were detected in brain tissues and the stomach, but not in the kidney, in which very high levels of GFP fluorescence and human CD63 expression were present (Fig. 3A, B). Previous studies also
reported expression of Sox2 in the stomach and other tissues in mice and human (Arnold et al., 2011; Cimpean et al., 2011; Driskell et al., 2009; Kang and Hebert, 2012; Raghoebir et al., 2012; Rickstina et al., 2009). However, they did not mention the expression of Sox2 in the kidney. Considering that high level of human CD63 mRNA expression was also observed in the kidney of the Tg rat (Fig. S5), it was likely that the human CD63-GFP protein originated from the kidney itself, not from other tissues-derived EVs accumulated in the kidney. Therefore, human CD63-GFP expressed in the kidney of our Tg rats because the kidney-specific regulatory element would be present in the 6.65-kb fragment or in the inserted chromosomal region of the Sox2/human CD63-GFP vector.

We previously generated another Tg rat strain (CAG/human CD63-GFP), resulting in expression throughout the body (Yoshimura et al., 2016b). Although the CAG/human CD63-GFP Tg rats showed embryonic lethality in males and premature death in females, The Sox2/human CD63-GFP Tg rats in this study showed no lethality or infertility in either sex. However, the Sox2/human CD63-GFP Tg rats exhibited frequent water drinking behaviour and a large amount of urine output, with no significant difference between male and female individuals. A previous report by other group found that CD63-deficient mice displayed an increased urine output (Schröder et al., 2009), and that the transfection of CD63-FLAG in Cos7 cells showed the internalisation of endocytosed gastric-type H,K-ATPase β-subunit (HKβ) into CD63-positive vesicles to prevent the recycling of HKβ to the cell surface (Duffield et al., 2003). These results suggested that CD63 in the kidney might strongly affect the functions of other membrane proteins such as potassium channels that are involved in epithelial electrolyte and water transport. Importantly, CAG/human CD63-GFP Tg rats in previous study did not show such a phenomenon even though the kidney expressed very high levels of human CD63-GFP (Yoshimura et al., 2016b). This is likely resulted from the different human CD63-GFP expression patterns depending on the cell types via the control of different promoters.

**Characterisation of EVs isolated from serum of human CD63-GFP Tg rats**

The Tg rats in this study showed specific human CD63-GFP transgene expression in tissues/cells driven by the Sox2 promoter. It is essential to identify the releasing cells of EVs circulating in the body to reveal the transfer pathway in the body. To examine GFP-labelled EVs that were released into blood, we collected EVs from the serum of Wt, Sox2/human CD63-GFP and CAG/human CD63-GFP Tg rats using ultracentrifugation. NanoSight analysis showed that approximately 50 to 100-nm sized vesicles were contained in the serum of all three rat genotypes (Fig. 4A). To assess expression of the EV markers and exogenous human CD63-GFP, isolated pellets were examined by western blot analysis (Fig. 4B). All samples showed the presence of the EV markers rat CD63 and flotillin-1. The EV pellets from CAG/human
CD63-GFP Tg rats were further characterised by the presence of human CD63 and copGFP, whereas these exogenous proteins were not detected in the EVs from Wt and Sox2/human CD63-GFP Tg rats. This is explained by region-restricted expression of the transgene in Sox2/human CD63-GFP Tg rats compared with CAG/human CD63-GFP Tg rats (Yoshimura et al., 2016b), or alternatively the tissues/cells expressing Sox2/human CD63-GFP may not be the principal donor cells of EVs circulating in the blood because hematopoietic tissues in the transgenic rats, especially bone-marrow cells (Fig. S6), expressed less amount of human CD63 and copGFP than that of CAG/human CD63-GFP Tg rats.

Expression of Sox2/CD63-GFP in differentiated eNSCs

To investigate the expression of human CD63-GFP driven by the Sox2 promoter in eNSCs and differentiated neural and glial cells, we used an in vitro model for eNSC differentiation (Fig. 5A). Rat eNSCs obtained from the telencephalon at E14 formed neurospheres in the presence of EGF and bFGF. Dissociated neurospheres were passaged twice, and cells were then induced to differentiate on pre-coated tissue culture dishes in EGF- and bFGF-free medium. After seven days of differentiation, eNSCs developed a neuronal morphology with long and branched neurites or radial glial morphology. In the Tg neurospheres, all spheres expressed GFP (Fig. 5B, Fig. S7). Western blot analysis confirmed up-regulation of neuron-specific protein (TUJ1), synaptic proteins (syntaxin 1 and GluR1), astrocyte-specific protein (GFAP) and oligodendrocyte-specific protein (CNPase) during in vitro differentiation of Tg eNSCs, similar to Wt eNSCs (Fig. 5C). Decreased expression of the NSC marker SOX2 was observed after differentiation, and the expression of human CD63 and copGFP in Tg samples was reduced in accord with the SOX2 expression. The expression of endogenous rat CD63 was increased after differentiation with a similar expression pattern in the developing cerebral cortex. These results indicated that eNSCs of the rat model differentiated normally and expression of the transgene was well regulated by the Sox2 promoter. This is also supported by the normal distribution of the NeuN-positive cells in the hippocampus of the adult brain (Fig. S4).

We observed the expression of human CD63-GFP during differentiation of eNSCs using immunostaining (Fig. 5D, left panels, Fig. S8). GFP signals were detected close to nuclei in Tg cells that were positive for the stem cell markers nestin and SOX2 at day 0, when the cells were in an undifferentiated state. The GFP fluorescence was co-localised with human CD63 and endogenous rat CD63. Five days after differentiation, GFP signals were no longer detected in differentiated cells, including neural cells (MAP2), oligodendrocytes (CNPase) and astrocytes (GFAP) (Fig. 5D, right panels, arrows). These results clearly showed that human CD63-GFP was expressed specifically in undifferentiated NSCs, and the expression of GFP was reduced after differentiation. Wt eNSCs lost SOX2 expression during the in vitro differentiation process.
and became to express differentiation markers at day 5 (Fig. S9A, B) such as MAP2 (neuron: Fig. S9B, upper, arrows) and CNPase (immature and mature oligodendrocytes: Fig. S9B, lower, arrowheads and arrows, respectively). In contrast, GFAP-positive cells showed SOX2 expression (Fig. S9C, arrows). It is known that SOX2 plays a role in proliferating cells including not only NSCs but also proliferating astrocytes in the developing cerebral cortex (Bani-Yaghoub et al., 2006). However, GFP was not visible in the differentiated astrocytes from the eNSCs of Tg rats at day 5 because they were thought to be lack of proliferation. To activate the proliferation of astrocytes, we maintained the differentiated cells in astrocyte medium for six-ten days after differentiation. When GFAP-positive cells were dramatically proliferated, and expanded on the bottom of dishes (Fig. S9D), GFP fluorescence was detected in GFAP-positive cells of Tg rats (Fig. S9D, arrows). These results suggested that the differentiated astrocytes resumed human CD63-GFP expression associated with proliferation.

Taken together, we demonstrated in in vitro culture that Sox2/human CD63-GFP Tg rats showed GFP signals specific to NSCs and proliferating astrocytes from embryonic brains. When EVs are secreted into the extracellular space for cytophysiological functions such as cell movement (Sung et al., 2015) and intercellular contact with adjacent recipient cells (Cicero et al., 2015), secretion sites of EVs and MVB polarisation in donor cells could be indicated by using GFP or other fluorescent-tagged-CD63 markers. In Drosophila development, the accumulation of secreted EVs and MVBs sorting of Wnt proteins in the imaginal disc can also be shown by using a CD63-GFP marker, demonstrating that the EVs are transporters of Wnt proteins that act as morphogens (Gross et al., 2012). The Sox2/human CD63-GFP Tg rats will be useful to find unknown regulatory functions of EVs associated with microenvironment of NSCs or proliferating astrocytes by assessing the behaviours of the visualised MVBs and EVs.

**EVs from eNSCs transfer to eNSCs and astrocytes in vitro**

We next analysed the transmission of EVs from eNSCs to recipient cells in vitro. EVs were prepared from the conditioned media of neurospheres cultured for four days (Fig. 6A). Isolated EV pellets showed a size distribution with peaks between 50 and 100 nm (Fig. 6B). The particles that were imaged by electron microscopy (EM) were observed to be mainly between 50 and 150 nm in size and showed typical cup-shaped structures (Fig. 6C). The EV markers rat CD63 and flotillin-1 were detected by western blotting in both WT and Tg eNSCs-derived EVs while human CD63 and copGFP were expressed only in Tg eNSCs-derived EVs (Fig. 6D).

We next examined whether the collected EVs could be incorporated into eNSCs and astrocytes. The eNSCs as recipient cells were cultured in proliferation medium on pre-coated 24-well plates until cells adhered on the bottom (Fig. 6A). The collected EVs were incubated with eNSCs in vitro for eight hours (Fig. 6E). Wt EVs were pre-labelled with the fluorescent lipid dye PKH26
We observed fluorescent signals in the cells, and PKH67-labelled Wt EVs showed highly intense fluorescent signals around nuclei in nestin-positive cells (Fig. 6E). When Tg EVs were stained with PKH26, the internalisation of PKH26-labelled Tg EVs also showed highly intense fluorescent signals around nuclei (Fig. 6E). Human CD63 immunofluorescence was clearly detected in the cells incubated with Tg EVs but not those incubated with Wt EVs (Fig. 6E). We next determined the incorporation of EVs into different cell types. We prepared astrocytes from the cerebral cortex of new-born rats at P1. Wt and Tg EVs were taken up by GFAP-positive cells after eleven hours (Fig. 6F). To confirm the EVs transfer between cells in more physiological condition, we performed co-culture of Tg neurospheres and Wt astrocytes using cell culture inserts with 1.0 μm pores for four days (Fig. 7A). Both the GFP signals and human CD63 immunoreactivity were observed in Wt astrocytes co-cultured with Tg neurospheres whereas they were not detected in the astrocytes cultured with Wt neurospheres (Fig. 7B).

Thus far, studies of EVs in the CNS have demonstrated novel neuron-neuron, glia-glia and neuron-glia intercellular communication via EVs. It has been reported that neuronal EVs were transferred into astrocytes and that neuronal EV-derived miRNA-124a, the least expressed miRNA in astrocytes, could regulate the expression of the astroglial glutamate transporter GLT1 (Morel et al., 2013). In contrast, oligodendroglial EVs were mainly taken up into neurons and microglia (Fitzner et al., 2010, Frühbeis et al., 2013). However, the functions of NSC-derived EVs including eNSCs are poorly understood. This study found that EVs released from eNSCs were transferred into astrocytes. NSC-derived EVs might be involved in the maintenance of self-renewal of NSCs because their regeneration ability by the transfer of stem cell-derived mRNA was reported in a study of other stem cell-derived EVs (Katsman et al., 2012); EVs from a human NSC line shuttled miRNA-1246, which is known to play a role in regulating cell growth (Stevanato et al., 2016). It is also possible that NSC-derived EVs activates some signalling pathways associated with neurogenesis in astrocytes because NSCs and astrocytes in the adult hippocampus have a close relationship to promote neurogenesis. The Wnt3a protein released from astrocytes stimulates target genes in NSCs through Wnt signalling, and regulates neuronal differentiation (Kuwabara et al., 2009). Although more work is required to examine whether EVs released from the NSCs of adult brains are also taken up into astrocytes, future experiments will elucidate the function of NSC-derived EVs in astrocytes by miRNA analysis to find the most abundant miRNAs in the EVs and their potential targets in astrocytes.

Interestingly, our Tg rats showed GFP expression in the hair follicles after birth. Li et al. (2003) reported that exogenous GFP expression driven by the promoter for nestin which is a marker for neural progenitor cells was observed in stem cells of hair follicles. In nestin-driven GFP (ND-GFP) mice, it was also revealed that ND-GFP expressing cells in the whisker follicle
have a role in the growth of the follicle sensory nerve (Mii et al., 2013). In addition, nestin-expressing cells in the hair follicle could potentially differentiate into neurons and cardiac muscle cells (Amoh et al., 2005, Yashiro et al., 2015).

EVs has become the focus of extensive investigations as an important communication factor between transplanted stem cells and host tissues in stem cell therapies (Barile et al., 2014; Katsuda et al., 2013). Cossetti et al. (2014) reported that EVs derived from NSC exposed to inflammatory cytokines carried interferon-γ (IFN-γ) bound to IFN-γ receptor 1 on its surface, and then activated the STAT1 signalling pathway in target cells in vitro, implying functional communication between transplanted NSCs and the host immune system via EVs. Our animal model may potentially provide a visual approach to study the EV-dependent communication in the transplanted tissues in stem cell therapies.

We demonstrated here that GFP-labelled EVs released by NSCs were incorporated into astrocytes in vitro although further studies regarding the in vivo transfer of EVs in the Sox2/human CD63-GFP Tg rats are needed. Recent technological advances in in vivo imaging have made it possible to monitor the real-time release of EVs from transplanted human tumor cells in the mammary glands of mice (Zomer et al., 2015). With such methods, our Tg rats with GFP labels on EVs will provide significant advantages for studying the molecular processes of releasing EVs from NSCs and receiving by other cell types both in vitro and in vivo, especially in the developing brain.

**MATERIALS AND METHODS**

**Animals**

All animal experiments were conducted in accordance with the institutional guidelines of the Animal Ethics Committee for the care and use of animals, and all experiment were carried out in accord with the approved guidelines of the National Institute of Neuroscience, National Centre of Neurology and Psychiatry and National Cancer Centre Research Institute, Japan.
Production of Sox2/human CD63-GFP Tg rats

Wistar rESCs were used to generate Tg rats. The rESCs were derived from cell lines established by Kawamata and Ochiya (2010). The rESCs were maintained on mitomycin-C treated neomycin-resistant mouse embryonic fibroblasts (MEFs) (Millipore, MA, USA) in YPAC medium, as described previously (Kawamata and Ochiya, 2010). DMEM GlutaMAX (Life Technologies, CA, USA) with 20% FBS (ES Cell Qualified Fetal Bovine Serum; Life Technologies), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, MO, USA), 1% MEM non-essential amino acids solution (Life Technologies), 1% antibiotic-antimycotic solution (Life Technologies), and the following four inhibitors: 10 μM Y-27632 (Wako, Tokyo, Japan), 1 μM PD0325901 (Axon Medchem, Groningen, The Netherlands), 0.5 μM A-83-01 (Tocris, Bristol, UK) and 3 μM CHIR99021 (Axon Medchem).

The Sox2 promoter fragment used in this study consists of the rat Sox2 5’ flanking sequences (6,647-bp) obtained from the genomic DNA of Wistar rats by PCR (Forward primer; 5’ agegggectcc tgtgcaac aga 3’, Reverse primer; 5’ tatttcege cagatctccg egc 3’) using KOD Plus Neo (Toyobo, Osaka, Japan). The 6.65-kb Sox2 5’ flanking sequences includes 5.6-kb BglII fragment which is homologous region with the mouse Sox2 promoter (D’Amour and Gage, 2003). A DNA fragment encoding human CD63-copGFP (human CD63-GFP) from a pCT-CMV-CD63-GFP vector (System Biosciences, CA, USA) and the Sox2 promoter were subcloned into a pECFP-1 plasmid (Clontech, Shiga, Japan). The Sox2/human CD63-GFP plasmid was linearised by digestion with Sall and was then transfected into Wistar rESCs using a Mouse ES Cell Nucleofector Kit (Lonza, Basel, Switzerland) and an Amaxa Nucleofector as described previously (Kawamata and Ochiya, 2010). The rESCs were seeded onto mitomycin-C treated neomycin-resistant MEFs in YPAC medium with 2% Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C in 5% CO2, and one day later, 0.2 mg/mL G418 (Sigma-Aldrich) was added to the culture medium for the selection of transfected cells. The rESC colonies showing GFP fluorescence were selected and amplified.

Rat embryos were prepared using the protocol described previously (Kawamata and Ochiya, 2010). Blastocysts were collected from LEA × LEA or Wistar × LEA matings 4.5 days post-coitus (dpc). Approximately 12 rESCs were injected into each blastocyst. The injected blastocysts were surgically transferred into the uterine horns of pseudopregnant Wistar females at 3.5 dpc. Chimaeric rats were confirmed by coat colour chimaerism. The potential of rESCs for germline transmission was examined by the coat colour of F1 rats resulting from mating with Wistar rats, and the inheritance in the offspring was assessed by GFP fluorescence and PCR analysis of genomic DNA isolated from ear snips.
Primary eNSC culture and differentiation

All eNSCs were obtained from telencephalon tissues of embryonic-stage day 14 (E14) Wistar wild-type (Wt) and Tg (Wistar-esTgN(Sox2/CD63-GFP)3NCCRI) rats. Isolated eNSCs were cultured on non-coated tissue culture plastic as neurospheres in KBM neural stem cell proliferation medium (Kohjin Bio, Saitama, Japan) with 0.2% KBM supplement containing EGF and bFGF (Kohjin Bio) at 37°C in 5% CO₂, as described previously (Yoshimura et al., 2016a). After three-four days, neurospheres were dissociated using trypsin (Sigma-Aldrich) and passaged. Differentiation was carried out by seeding on tissue culture dishes pre-coated with 0.2% polyethyleneimine (PEI) (Wako). Differentiation medium consisted of KBM containing 2% B-27 serum-free supplement containing vitamin A (Life Technologies) without EGF and bFGF. Dissociated cells were plated at a density of 1.8\times10^6 cells per 35-mm culture dish (Becton Dickinson). For immunocytochemistry, 1.4\times10^5 cells/0.2 mL were reseeded on pre-coated glass-bottom dishes (Matsunami, Osaka, Japan). The culture medium was changed three-four days after plating, and differentiation proceeded for seven days. To promote the proliferation of differentiated astroglial cells, culture medium was replaced with astroglial culture medium (described in the following section) at six days after differentiation.

Primary astrocyte culture

Primary astrocytes were prepared from the cerebral cortex of Wistar rats on postnatal day 1 (P1) as described previously (Numakawa et al., 2011). Dissociated astroglial cells were plated onto non-coated tissue culture flask and cultured in the presence of 0.1 μg/mL mouse recombinant EGF (PeproTech, NJ, USA) in an MEM (Life Technologies)-based growth medium containing 5% FBS (Biological Industries, Beit Haemek, Israel), 20 mM glucose, 25 mM NaHCO₃ and 0.5 mM glutamine at 37°C in 5% CO₂. Cells were maintained in the astroglial culture medium until EV transfer analysis, and the culture medium was changed once a week.

Immunoblotting

Protein lysis buffer composition comprised 1% SDS, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride and 2 mM Na₃VO₄. Samples of tissues and cells were sonicated on ice, and then lysates were cleared by centrifugation. The lysates were dissolved in SDS sample buffer without 2-mercaptoethanol (Wako) after determination of protein concentration using a Pierce BCA Protein Assay Kit (Life Technologies).
After being blocked with 5% skimmed milk, membranes were probed with specific primary antibodies to CNPase (1:1000; ab6319, Abcam, Cambridge, UK), copGFP (1:1000; AB501, Evrogen, Moscow, Russia), flotillin-1 (1:500; 610820, Becton Dickinson), GFAP (1:1000; AB5804, Millipore), GluR1 (1:1000; AB1504, Millipore), human CD63 (1:250; 556019, Becton Dickinson), rat CD63 (1:250; MCA4754GA, AbD Serotec, San Jose, CA, USA), SOX2 (1:1000; ab97959, Abcam), syntaxin 1 (1:1000; S0664, Sigma-Aldrich) and TUJ1 (1:1000; MMS-435P, Berkeley Antibody Company, Berkeley, CA, USA) followed by incubation with peroxidase-conjugated anti-mouse IgG (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) or rabbit IgG (1:1000; Rockland, Limerick, PA, USA) secondary antibody. β-actin (1:5000; A5441, Sigma-Aldrich) was used as a loading control. Signals were detected using chemiluminescent reagents (ImmunoStar; Wako).

**Immunocytochemistry**

Cultured cells were fixed with 4% PFA in PBS for 15 minutes following a pre-treatment with 2% PFA in the culture medium for 30 minutes at room temperature. After being washed, cells were incubated in PBS containing 0.2% Triton X-100 (Sigma-Aldrich) and 10% FBS with primary antibodies at 4°C overnight. Primary antibodies specific to the following proteins were used: CNPase (mouse IgG1 1:250; ab6319, Abcam), GFAP (rabbit IgG 1:1000; ab7260, Abcam), human CD63 (mouse IgG1 1:200; 556019, Becton Dickinson), MAP2 (mouse IgG1 1:200; M1406, Sigma-Aldrich), nestin (mouse IgG2a, 1:200; MAB2736, R & D systems, MN, USA), rat CD63 (mouse IgG1 1:200; MCA4754GA, AbD Serotec) and SOX2 (rabbit IgG 1:200; ab97959, Abcam). After cells were stained with Alexa Fluor 546 mouse IgG1 (1:2000; Life Technologies), Alexa Fluor 546 mouse IgG2a (1:2000; Life Technologies), Alexa Fluor 546 rabbit IgG (1:2000; Life Technologies), Alexa Fluor 488 mouse IgG1 (1:2000; Life Technologies) and Alexa Fluor 488 rabbit IgG (1:200; Life Technologies), they were observed through a BIOREVO BZ-9000 (Keyence, Osaka, Japan) fluorescence microscope. Hoechst 33342 was used to stain cell nuclei.
**Immunohistochemistry**

Head samples were collected from Wt and Tg rats at E16, and they were fixed with 4% PFA overnight at 4°C. They were washed in PBS, and then cryoprotected in 30% sucrose. After freeze in O.C.T. compound, the frozen brains were sectioned using a cryostat into 10 µm slices. Primary antibody for SOX2 (1:200; GTX101507, GeneTex, LA, USA) were diluted in blocking solution (0.25% Triton and 2% BSA in PBS) or (0.05% saponin and 2% BSA in PBS). Note, using saponin gave better results to minimize quenching of GFP signals. All images were captured using a FV1000 confocal microscope (OLYMPUS, Tokyo, Japan).

**Characterisation of EVs isolated from serum and eNSC culture medium**

Blood samples were collected from Wt and two Tg adult female rats (Wistar-esTgN(Sox2/CD63-GFP)3NCCRI and Wistar-esTgN(CAG/CD63-GFP)3NCCRI) (Yoshimura et al., 2016b). Blood was centrifuged at 1,400 rpm for 10 minutes. The collected serum was further centrifuged twice at 10,000 g for 15 minutes to remove blood cells and debris. The resultant supernatant was transferred to ultracentrifuge tubes, and then EVs were isolated by ultracentrifugation at 35,000 rpm for 70 minutes at 4°C using a Beckman SW41Ti rotor (Beckman, Fullerton, CA, USA). The pellets were washed with PBS and ultracentrifuged again.

Culture media were sampled from the eNSC culture prepared from Wt and Tg (Wistar-esTgN(Sox2/CD63-GFP)3NCCRI) rats. Rat eNSCs, which were passaged once, were cultured in proliferation medium for four days on non-coated tissue culture flasks. To isolate EVs, culture supernatant was centrifuged at 1,400 rpm for 10 minutes and then filtered through a 0.22-µm membrane filter (Millipore) to remove cellular debris. EVs were collected by ultracentrifugation at 35,000 rpm for 70 minutes at 4°C and washed with PBS. The collected EV samples were diluted 100-fold with PBS for the analysis. Analysis of the size distribution of EVs was carried out with the Nanosight LM10HS (NanoSight, Amesbury, UK), as described previously (Yoshioka et al., 2013). For EM observation, eNSC-derived EVs were plated on collodion-carbon-coated grids and negatively stained with uranyl acetate. The images were captured using a transmission EM (Tecnai Spirit; FEI, OR, USA).

**Analysis of EV transfer in vitro**

Isolated EVs from eNSC cultures of Wt or Tg (Wistar-esTgN(Sox2/CD63-GFP)3NCCRI) rats were visualised using a PKH26 or PKH67 fluorescence labelling kit (Sigma-Aldrich). Rat eNSCs were reseeded on coated 24-well glass-bottom plates in proliferation medium, and astrocytes prepared from rat cerebral cortex were reseeded on non-coated 24-well glass-bottom plates in astroglial culture medium. The EVs were incubated with the eNSCs or astrocytes on...
the 24-well plates at 37°C in 5% CO₂. On washing with PBS, the cultured cells were fixed with 4% PFA. Anti-human CD63 antibody was used to detect the EVs from Tg eNSCs in the recipient cells. Internalisation of EVs was captured by using a BIORÉVO BZ-9000 fluorescence microscope.

In the co-culture system, Wt astrocytes were plated onto micro cover glass (Matsunami) pre-coated with PEI, and they were incubated in the bottom of 24-well plates. Cell culture insert with 1.0 μm pores (Falcon; Corning, NY, USA) was set on each well. Tg or Wt neurospheres were put into the insert and cultured in KBM medium with 0.2% KBM supplement at 37°C in 5% CO₂. After four days of co-culture, the Wt astrocytes were fixed with 4% PFA for 20 minutes. For immunocytochemistry, anti-human CD63 and GFAP antibody were used. TrueBlack Lipofuscin Autofluorescence Quencher (Biotium, CA, USA) was used after immunostaining to quench lipofuscin autofluorescence in astrocytes. The images were captured using an Axiovert 200 fluorescence microscopy (Carl Zeiss, Oberkochem, Germany).

Acknowledgements
We thank Dr. Nobuyoshi Kosaka for expert advice on analysis of extracellular vesicles.

Competing interests
The authors declare no competing financial interests.

Author contributions
A.Y., M.K., Y.T. and T.O. designed the study. A.Y. performed the experimental work, analysed the data and wrote the manuscripts. N.A. provided technical assistance on the culture and helpful discussion. H.M performed the histological experiments. M.K. assisted in the generation of Tg rats using ES cells. Y.Y. provided technical assistance and helpful discussion on the analysis of extracellular vesicles. H.Kikuchi performed the electron microscopy experiments. H.O. provided technical assistance on the culture. T.N. and H.Kunugi provided helpful comments. The manuscript was finalized by T.O.
Funding

This work was supported by grants from the Grant-in-Aid for Young Scientists (B) [grant number 26830061 to A.Y.] and the Grant-in-Aid for Challenging Exploratory Research [grant number 16K14601 to A.Y.] in the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant in Aid for the Japan Science and Technology Agency (JST) through the Center of Open Innovation Network for Smart Health (COINS) initiated by the Council for Science and Technology Policy, and a Grant in Aid for the Japan Agency for Medical Research and Development (A-MED) through the Basic Science and Platform Technology Program for Innovative Biological Medicine, and Takeda Science Foundation (N.A.).
References


Fig. 1. Generation of Tg rats by transfection of Sox2/human CD63-GFP gene into rESCs.

(A) The localisation of Sox2 promoter including two BglII sites (left) and structure of the transgene construction (right). (B) Photomicrograph of No. 6 rESC line transfected with the Sox2/human CD63-GFP gene. The rESCs expressed GFP ubiquitously. Scale bar: 300 μm. (C) E14 rats obtained by mating a Tg (Wistar-esTgN(Sox2/CD63-GFP)3NCCRI) male rat with a Wt female rat. The foetus not expressing GFP is a non-Tg littermate (i). The Tg foetus showed a high level of GFP fluorescence in the spinal cord (ii, arrowhead) and telencephalon (ii, arrow, iii and iv). Scale bar: 5 mm. (D) Western blot analysis for SOX2, endogenous rat CD63, exogenous human CD63 and copGFP during rat brain development. The lysates were collected from the telencephalon at E14 and the cerebral cortex at E19, P4 and P10 (n = 3). β-actin was used as a
loading control. Error bars represent standard deviations. (*P<0.001 versus E14, one-way ANOVA with Bonferroni’s post-hoc test).
Fig. 2. Immunohistological analysis of the telencephalon of Tg rat embryo. (A) Low and (B) high magnification of coronal sections of the telencephalon demonstrating immunoreactivity of SOX2 and GFP fluorescence. (B) Tg rat telencephalon images showed GFP signals (green) not only around SOX2-positive cells (red) of the VZ but also in SOX2-negative cells (blue), as shown in the magnified view (white square region). No GFP signals was observed in the Wt telencephalon. Scale bar: 100 μm.
Fig. 3. Human CD63-GFP expression in the major organs of new-born Wistar-esTgN(Sox2/CD63-GFP)3NCCRI Tg rats. (A) Pictures from Tg offspring at P2 (ii and iii: merged, i'-viii': GFP). The hair roots in the whiskers (ii and ii', arrows), but not in the body (iii and iii', arrows), expressed GFP fluorescence. The skin, brain, heart, kidney and stomach showed strong fluorescent signals, and pancreas showed a weaker fluorescence. Scale bar: 5 mm. (B) Expression of endogenous rat CD63, exogenous human CD63, copGFP and SOX2 in the tissue lysates from GFP-negative (-) and GFP-positive (+) offspring at P4. Abbreviations: ctx, cortex; cbl, cerebellum; hip, hippocampus.
Fig. 4. Identification and characterisation of the EVs isolated from the serum of Wt and two Tg rats (Wistar-esTgN(Sox2/CD63-GFP)3NCCRI and Wistar-esTgN(CAG/CD63-GFP)3NCCRI). (A) Size distribution of the EVs collected by ultracentrifugation was determined using the NanoSight system. (B) Western blot analysis of the EVs for flotillin-1, rat CD63, human CD63 and copGFP (n = 2).
Fig. 5. Human CD63-GFP expression and localisation during differentiation of eNSCs obtained from the telencephalon of Tg rats at E14. (A) Schematic representation of the protocol for eNSC proliferation and differentiation in vitro. (B) GFP-positive neurospheres from Tg rats. Scale bar: 50 μm. (C) Protein expression profiles during differentiation of eNSCs. Markers for neurons (TUJ1), astrocytes (GFAP), oligodendrocytes (CNPase), neural stem cells (SOX2) and presynaptic (syntaxin 1) and postsynaptic (GluR1) proteins as well as rat CD63 and human CD63-GFP were examined in Wt and Tg rat cells. The experiment was performed one time. (D) GFP expression in the cultured Tg rat cells at day 0 (left) and five days after differentiation (right). Immunostaining indicated the co-localisation of GFP with human CD63 and with rat CD63-positive signals around nuclei (blue) in the nestin- and SOX2-positive undifferentiated cells at day 0. At day 5, no GFP signal was seen in the MAP2-positive, CNPase-positive and GFAP-positive differentiated cells (arrows). All the experiments except (C) were repeated at least two times using different cultures. Scale bar: 20 μm.
Fig. 6. Transfer of eNSC-derived EVs into recipient cells in vitro. (A) Schematic representation of the protocol for collection of EVs from the conditioned medium of eNSCs. The eNSCs as recipient cells were cultured on pre-coated dishes in proliferation medium. (B) Size distribution of the eNSC-derived EVs obtained from the conditioned medium of Wt and Tg rat cells by the NanoSight system. The experiments were performed four times. (C) Electron microscopic images of the EVs. Scale bar: 200 nm. The experiment was performed one time. (D) Western blot analysis showed the EV markers flotillin-1 and endogenous rat CD63 expression in the isolated EVs as well as exogenous human CD63-GFP in the Tg EVs. The experiment was performed one time. (E) The Wt EVs were pre-labelled with PKH26 (red) or PKH67 (green), and Tg EVs were labelled with PKH26. Wt eNSCs were incubated with the EVs for eight hours. Furthermore, the EVs from Tg cells were detected using anti-human CD63 (red) after having been transferred into eNSCs. The cells were immunostained with antibodies against nestin (red), showing PKH67 signals around nuclei (blue). The experiments were performed two times. (F) The Wt and Tg EVs were incubated with primary astrocytes of the cerebral cortex of Wt rats. Wt EVs were labelled with PKH67, whereas Tg EVs were detected by immunostaining of human CD63 (red). Astrocytes were recognised by the antibody against GFAP. The experiment was performed one time. Scale bar: 20 μm.
Fig. 7. In vitro transfer of neurosphere-derived EVs to astrocytes in the co-culture system.
(A) A schematic illustration of the co-culture protocol. Wt astrocytes as recipient cells were cultured on pre-coated cover glass on the bottom of wells, and then they were cultured with Tg or Wt neurospheres put into the culture insert with 1.0 μm pores for four days. (B) EVs derived from Tg neurospheres in the Wt astrocytes were detected by GFP (green) and immunoreactivity of human CD63 (red). Astrocytes were stained with an antibody against GFAP (cyan). Nuclei were visualised by DAPI (blue). All the experiments were duplicated in different plates using sister cultures. Scale bar: 50 μm.
Fig. S1. Transfection of Sox2/human CD63-GFP gene into rESCs. Photomicrographs of two transfected rESC lines (No. 10 and No. 22) with ubiquitous expression of GFP. Scale bar: 300 µm.
Fig. S2. GFP expression in the telencephalon of Tg rat embryo. Coronal sections of the Wt and Tg telencephalon. GFP signals were clearly observed around the lateral ventricle (LV) of the telencephalon in Tg rat. Scale bar: 100 µm.
Fig. S3. GFP expression in the adult Tg rat cortex. About 7 month-old Wt and Tg female rats were perfused with PBS and 4% PFA, and the removed brains were postfixed in 4% PFA overnight at 4°C. The fixed brains were sectioned using a microslicer into 50 µm slices. Neural stem cells and astrocytes were identified by immunostaining with antibodies against SOX2 (GeneTex) and GFAP (G3893, Sigma-Aldrich), respectively. The blood vessels were stained with lectin antibody (B-1175, Vector Laboratories, CA, USA). Nuclei were stained with Hoechst 33342 (blue). Scale bar: 25 µm.
Fig. S4. Distribution of NeuN-positive neurons in the hippocampus of adult Wt and Tg rats. The sagittal sections of the hippocampus from 7 month-old Wt and Tg female rats. Neural cells were identified by an antibody against NeuN (ab177487, Abcam). Nuclei were stained with Hoechst 33342 (blue). Scale bar: 100 µm.
Fig. S5. Quantitative PCR analysis of rat CD63 and human CD63 mRNA expression levels in the major organs of newborn Wt and Wistar-esTgN(Sox2/CD63-GFP)3NCCRI Tg rats. The RNA samples were purified from a Wt (postnatal day 1) and a Tg (postnatal day 2) rats by RNeasy Mini kit (QIAGEN, Venlo, Netherlands). Complementary DNAs (cDNAs) for mRNAs were generated using SuperScript VILO cDNA synthesis kit (Life Technologies). The cDNAs were subjected to StepOnePlus Real Time PCR System (Applied Biosystems, MA, USA) with TaqMan Gene Expression Assays; rat CD63 (ID: Rn01529322) and human CD63 (ID: Hs01041238). The relative expression levels of mRNAs were normalized to gapdh (ID: Rn01775763) mRNA levels and calculated by the ΔΔCt method. A high level of human CD63 was detected in the hippocampus, stomach and especially kidney of the Tg rats but not in any tissues of Wt rats. Abbreviations: ctx, cortex; cbl, cerebellum; hip, hippocampus.
Fig. S6. Human CD63-GFP expression in the bone-marrow cells isolated from Wt and two Tg rats (Wistar-esTgN(Sox2/CD63-GFP)3NCCRI and Wistar-esTgN(CAG/CD63-GFP)3NCCRI). Bone-marrow cells were obtained from the femurs of adult female rats. Cells were collected in RPMI1640 medium (Life Technologies) and then centrifuged at 1,400 rpm for 5 minutes. The pellets were washed with the medium and PBS. The cell lysates were analysed by Western blot analysis for endogenous rat CD63, exogenous human CD63 and copGFP. The signals of human CD63 and copGFP were barely detected in the bone-marrow cells from Sox2/CD63-GFP rats, whereas these signals were strongly detected in that of CAG/CD63-GFP rats.
Fig. S7. GFP-positive neurospheres obtained from Tg rats at E14. The wide-field images of Fig. 5B. The neurospheres shown in Fig. 5B are indicated by white square. Scale bar: 50 µm.
Fig. S8. GFP expression in cultured Tg eNSCs at day 0. The wide-field images of Fig. 5D. GFP signals were detected in the SOX2-positive cells. The images shown in Fig. 5D are indicated by white square. Scale bar: 100 µm.
Fig. S9. Differentiated astrocytes maintained SOX2 expression and GFP expression was resumed as astrocytes proliferated. (A-D) Immunostaining of the rat eNSCs during differentiation with antibodies against SOX2, nestin, MAP2, CNPase and GFAP. (A) Nestin-positive cells showed SOX2 expression. (B) MAP2-positive and CNPase-positive cells showed reduced SOX2 expression (arrows). SOX2 expression was maintained in the cells expressing immature CNPase signals (arrowheads). (C) In contrast, GFAP-positive cells showed SOX2-positive nuclei (arrows). (D) After the culture medium was replaced with astrocyte medium on day 6, GFAP-positive cells dramatically proliferated and GFP fluorescence was detected in the GFAP-positive cells of Tg rats (arrows). Nuclei were stained with Hoechst 33342 (blue). Scale bar: 25 µm.