# CREATION OF KNOCK-OUT MOUSE MODELS BY TRANSGENESIS TO UNDERSTAND HUMAN PHYSIOLOGY AND DISEASES

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## ABSTRACT

The anatomy, genetics and physiology of mouse model is very close to higher mammals including human, thus mouse is a rational and relevant model for biomedical research. The rapid advancement in bio-informatics and transgenic technology enable fine mapping and precise engineering of mouse genome.

Loss or gain of gene function is the fundamental mechanism in biological process as well as in various human anomalies and diseases. Therefore, we exploit transgenic approaches to create knock-out or knock-in mouse models to understand the function of homology gene in human (patho)-physiology.

Candidate genes were selected based on experimental and bio-informatics based evidences. Gene targeting vectors were constructed; genes of interest were targeted in mouse embryonic stem (ES) cell line. Chimera mice were generated from targeted ES cell clones and bred to establish knock-out or knock-in mice for gene of interest. Phenotypic (survival/behavioral, histological and/or molecular) analysis was conducted in knock-out mice and wild-type/heterozygous controls. Animal breeding, tissue harvesting and analysis were performed according to the National Advisory Committee for Laboratory Animal Research (NACLAR) and the Institutional Animal Care and Use Committee (IACUC).

Subtle or severe phenotypes are correlated with the gene-expression pattern and molecular function of a specific gene of interest. Homeo-domain genes Emx/Otx are involved in early patterning of embryonic brain, knock-out or knock-in of these genes caused regionalization defects in the brain. Zo2/Tjp2 a scaffolding proteins associated with tight junction is required in early embryogenesis and targeted inactivation caused embryonic lethality at embryonic day 6.5 (E6.5). Testis specific cold-shock-domain gene (Msy4) is important in spermatogenesis and inactivation of these gene caused male infertility in mice. A transcriptional co-activator Taz/Wwtr1, highly expressed in kidney and lungs, is a candidate gene for polycystic kidney diseases and pulmonary emphysema. Aberrant expression of Taz is associated with non-small cell lung cancer (NSCLC).

Knock-out and knock-in mice are powerful tools to understand the genetic link in biological process and diseases, thus serves as rational pre-clinical model for the therapeutic target discovery and validation for many diseases.

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## INTRODUCTION

The loss or gain of gene or gene-product function is the fundamental mechanism of physiological and disease process in living organisms. Therefore the genetic animal models (e.g., transgenic, knock-out and knock-in) provide rationale insights into role of gene in physiological andpathological conditions in animal as well as in human.

The recent advances in biotechnology had revolutionized the methods of gene manipulation in mammals (e.g., mouse and rat). The transgenesis by zygotic pronuclear DNA microinjection,[1] embryonic stem (ES) cell technology,[2-3] evidence of homologous recombination based targeting in mammalian cells[4] and later the development of homologous recombination based targeting of any gene of interest by using gene-specific targeting vector[5-6] were the major breakthroughs that enable generation of transgenic, knock-out or knock-in mice. The whole span of technologies had been successfully applied to generate first knock-out animal model in 1989[7] and the Nobel Prize in Physiology or Medicine 2007 was awarded jointly to Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies "for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells".

Further completion of whole genome sequencing of human and mouse[8-9] ease the fine mapping of human gene as well as search for homologous disease relevant genes in other animal genomes including rodents. Whole genome sequence analysis and annotation revealed that diseases relevant gene are 90% conserved in human and rodents.[10] Therefore, gene manipulation technologies in mice is very relevant to understand the role and function of human genome to lead the discovery of molecular targets in biology, diseases and drug development.[11]

We've established the gene manipulation facility at the National University of Singapore; the facility serves life science industries including academia, hospital and biotech by generating novel mouse models for basic and preclinical studies in collaborations. Further we serve germplasm cryopreservation of mouse model, rederivation of mouse lines from cryopreserved or critical source of germplasm. We are also dedicated for the development of innovative and high throughput technology in mouse genetics. Here interesting preliminary phenotypes of two mouse lines, generated by gene targeting in ES cells, are presented.

## MATERIAL AND METHODS

The primary material for gene targeting is the isolation of locus specific DNA material by screening A lambda-phage DNA[12] or bacterial artificial chromosome (BAC) libraries for whole mouse genome. However, the mouse BAC clone containing the genomic DNA for gene of interest is available from BACPAC resource (https://bacpac.chori.org/). The desired clone was further confirmed by gene specific PCR. The exon-intron mapping was identified using sequence information from database (http://genome.ucsc.edu/ ). To generate a knock-out, a critical exon was disrupted or for knock-in external gene/reported cassette were placed in-frame to the start codon of targeted locus together with positive selection cassette (PGK-gb2-Neo) using conventional recombinant DNA technology or Red/ET based BAC homologous recombineering

system from Gene Bridge, Germany (http://www.genebridges.com/).Targeting vector containing the homology arms (each of 4-3 kb) flanking the target exon or reporter were cloned and then finally cloned into vector either harboring diphtheria toxin-A or thymidine kinase (a negative selection cassette).[13]

The W4/129S6 mouse embryonic stem (ES) cells were cultured using standard protocol. The targeting vector with positive-negative selection marker was electroporated into ES cells; cells were selected against geneticin (200ug/ml) for 7 days. The total of 96-192 isolated (undifferentiated) clones were picked-up for long-PCR screening for the homologous recombination. Clones identified by PCR were further confirmed by secondary screening using Southern blotting hybridization or q-PCR technique.

ES cell cloneswere further grown and chimera were be generated using "ES cell injection into blastocyst" technique following standard protocol.[14] The prospective male chimeras with higher percentage (70-90%) of coat-color contribution from ES cells were further mated with wild-type females to establish F1-heterozygous breeders, which werethen mated to establish homozygous breeders if required and possible.

The preliminary phenotypes in heterozygous and homozygous offspring were analyzed using classic histological (eosin-hematoxylin), immuno-histochemical and anti-sense RNA in-situ hybridization techniques. Animal breeding, tissue harvesting and analysis were performed according to the National Advisory Committee for Laboratory Animal Research (NACLAR) and the Institutional Animal Care and Use Committee (IACUC).

## RESULTS

We had generated more than 10 animal models for loss/gain of gene function using gene targeting technology. The phenotypes were subtle in some mouse models as well as severe in other models, while often correlated with the gene-expression pattern, molecular function and evolution of a specific gene of interest, thus we conclude that gene targeting is a reliable approach for genetic manipulation in mouse model often with predictable phenotypes.

For instance, homeo-domain genes Emx1/2 and Otx1/2 maintain nested expression pattern in neuro-ectoderm during early patterning of embryonic brain development at 9.5 days post coitum (dpc), the Emx2 gene maintains its expression boundary within the telencephalon in the forebrain (comprise of telencephalon and diencephalon)[15] and the loss of Emx1/2 genes was associated with defect in histological boundaries and neurogenesis inthe telencephalon.[16] Further to understand the role of Emx2 gene in the establishment of brain compartment, Emx2 gene was over expressed (knock-in) under the regulation of Otx2 locus using gene targeting approach, hence Emx2gene expression was forcefully extended beyond the telencephalic region and overlapped with the expression region of Otx2 locus which directed the Emx2 gene expression covering the telencephalon (Te), diencephalon (Di) and mesencephalon (Mes) regions; maintain a boundary at the mesencephalon and metencephalon (Met) junction (isthmus, Is)shown in (Fig 1).

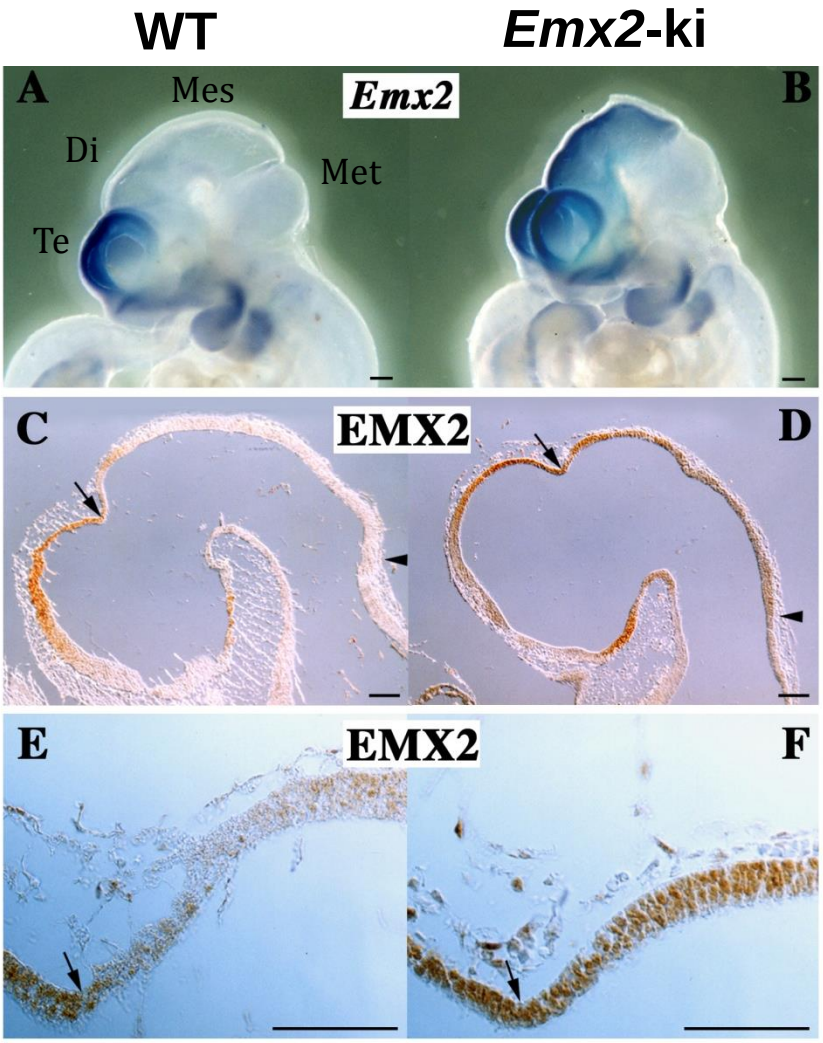


**Figure 1:** Over expression of *Emx2* gene under *Otx2* locus: *Emx2* antisense RNA in-situ hybridization showed normal *Emx2* gene expression in telencephalon of whole-mount embryonic brain at 10.5 dpc (A), whereasextended *Emx2* expression along the telencephalon, diencephalon and mesencephalon in Emx2-ki brain (B). Immuno-staining of sagittal section of brain tissue at 10.5 dpc embryos using Emx2 antibody confirm the expected over-expressed Emx2 protein in Emx2-ki brain (D ) & magnified image (F) corresponding to the RNA expression in (B).

The over expression of Emx2caused reduced brain size in $Otx2^{+/Emx2}$ knock-in mice with compare to wild type (WT) littermates. Histological analysis confirmed that lossof fore-midbrain junction (region 4 in Fig 2A) was the main cause of smaller brain size in $Otx2^{+/Emx2}$ (Emx2-ki) mice and the corresponding posterior commissure structure originating from this boundary region was not visible at the mid-sagittal section of the (Emx2-ki) brain at 10.5 dpc (Fig 2F).

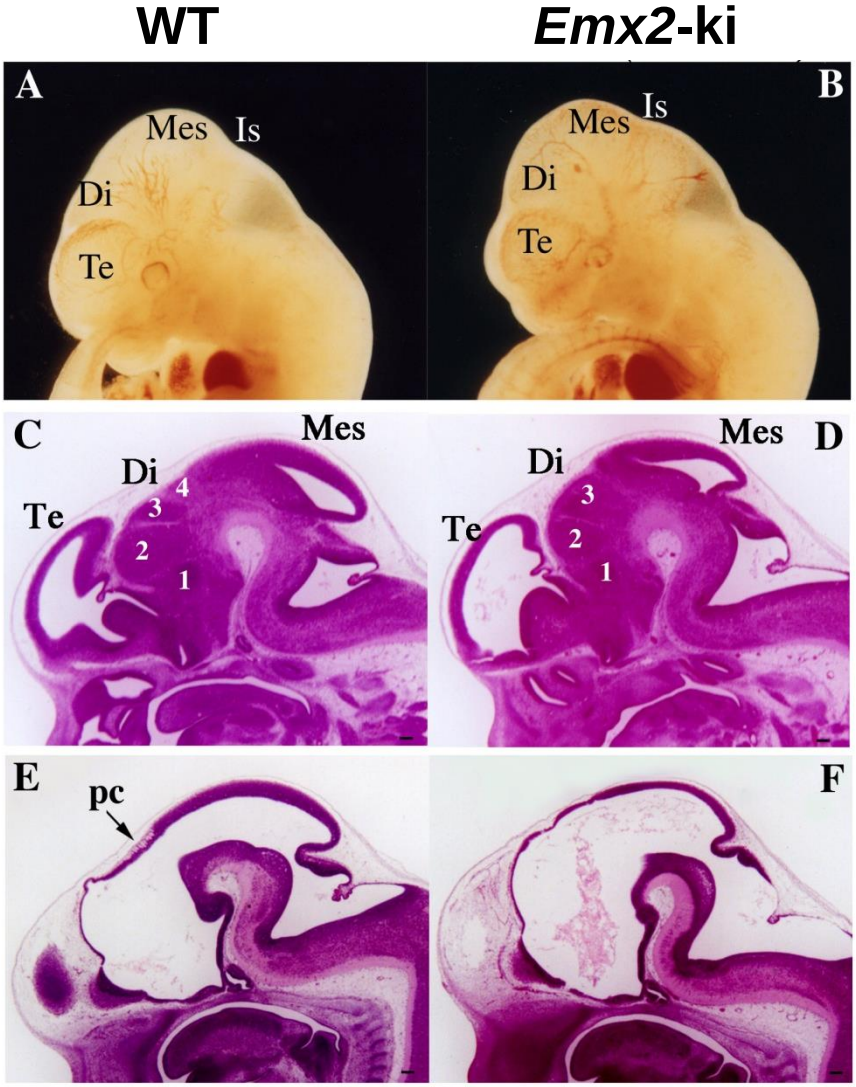


**Figure 2:** Anatomical analysis of brain structure in *Otx2*$^{+/Em2}$ knock-in mice: Whole brain size at 10.5 dpc (A&B) shows that overall brain size is smaller in *Otx2*$^{+/Emx2}$ knock-in mice. The hematoxylin and eosin staining of 12.dpc brain (B-F), where lateral sagittal section shows that brain structure (marked by 4) corresponding to fore-mid brain junction is lost in *Otx2*$^{+/Emx2}$ knock-in mice (D), further mid-sagittal section shows that posterior commissure (PC, shown by arrow in wild type mice) that derived from the fore-mid brain junction is not morphologically distinct in *Otx2*$^{+/Emx2}$ knock-in mice.

Together with earlier findings this data strongly suggest that the restricted expression pattern of Emx2is essential for the proper compartmentation of central nervous system (CNS) during early neurogenesis. The mutual transcriptional gene repression mechanism, yet to be understood clearly, are believed to play role to maintain restricted expression pattern of developmentally important genes (e.g., homeo-domain genes Emx1/2 and Otx1/2)

We also showed that Zo2/Tjp2 scaffolding proteins associated with tight junction is required in early embryogenesis and targeted inactivation caused embryonic lethality at 6.5 dpc (data not shown). Testis specific cold-shock-domain gene (Msy4) is important in spermatogenesis and inactivation of these gene caused infertility in male mice (data not shown).

Recently, we had targeted a transcriptional co-activator Taz/Wwtr1which interacts with many transcription factorsincluding mesenchymal stem cell (MSC) factors (e.g., Runx2, Pparγ).[17-18] Further Taz has been identified as component of hippo-pathway[19] which play a critical role in controlling organ size by regulating cell proliferation, apoptosis, survival and differentiation. Tazis normally expressed in the lungs, liver, kidney and skeletal muscle; Taz knock-out ($Taz^{-/-}$) mice survive, however, 50% of knock-out mice died by the age of weaning at 21 days of postnatal period. Gross anatomical analysis of Taz knock-out mice showed pale, fluid filled enlarged kidney, further histological analysis of kidney identified cystic lesion within the corticomedullary junction of kidney (Fig 3).

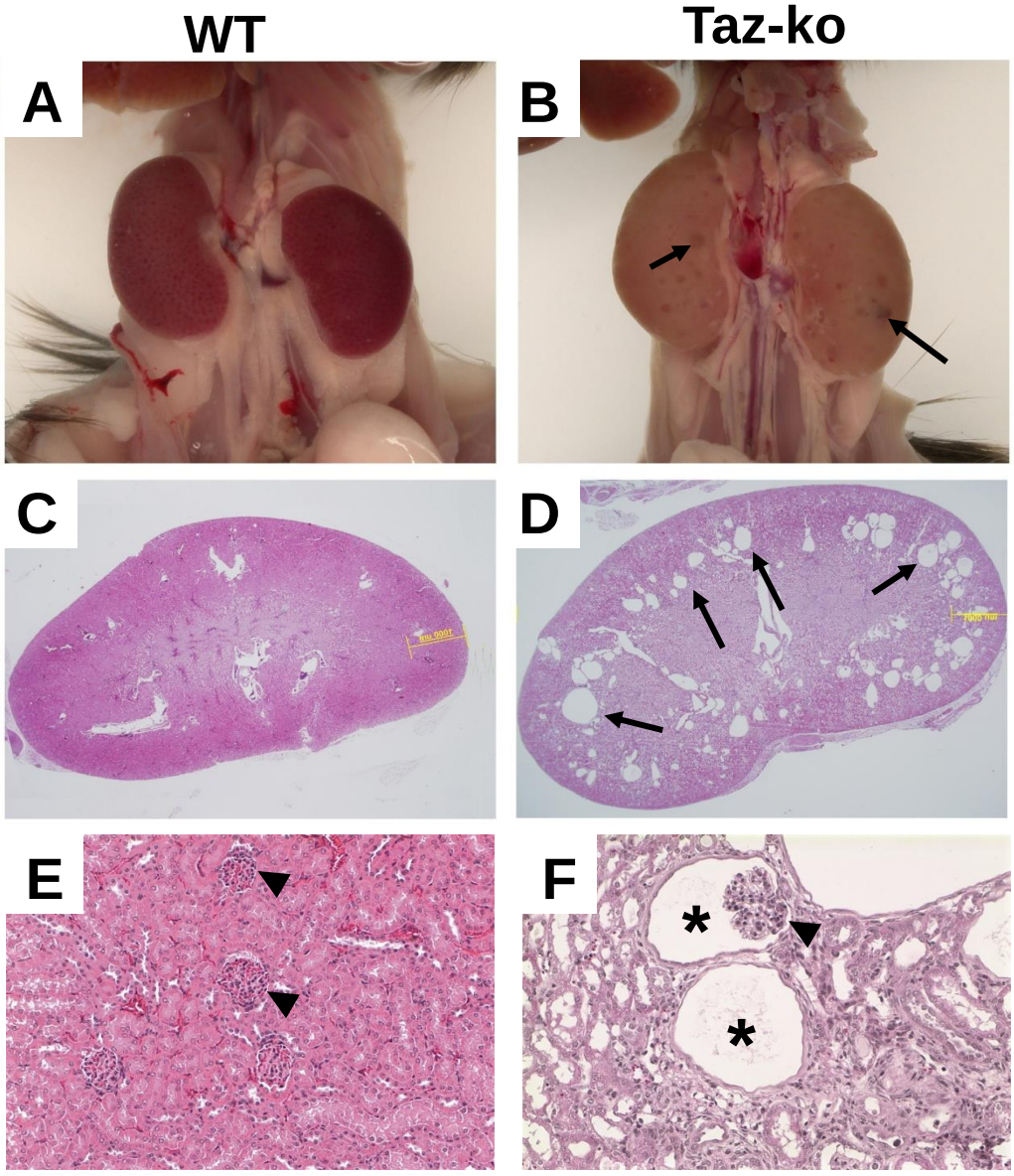

**Figure 3:** Anatomical analysis of kidney structure in Taz knock-out mice: Kidneys of a 3- to 4-month-old Wild type and Taz knock-out mice are shown through the abdominal cavity after removal of obstructing organs. Note a larger size and pale appearance of the kidneys and the fluid filled lesions on the renal capsules of the Taz knock-out animal (B). Renal histology (C-F), hematoxylin and eosin staining of longitudinal kidney sections from an 8-week-old wild type and Tazknock-out mice, showing numerous cysts in the cortico-medullary region of the Tazknock-out kidney (arrows in D). The cysts are predominantly origination from glomerula of Tazknock-out kidney (marked by *), arrow heads shows the glomerular tufts.

First signs of histological anomalies in Taz-/- kidneys were apparent at 15.5 dpc (Fig 4D) as dilations of the Bowman's space between visceral podocytes and the parietal cell layer of the Bowman's capsule and tubular dilations were apparent by 16.5 dpc.

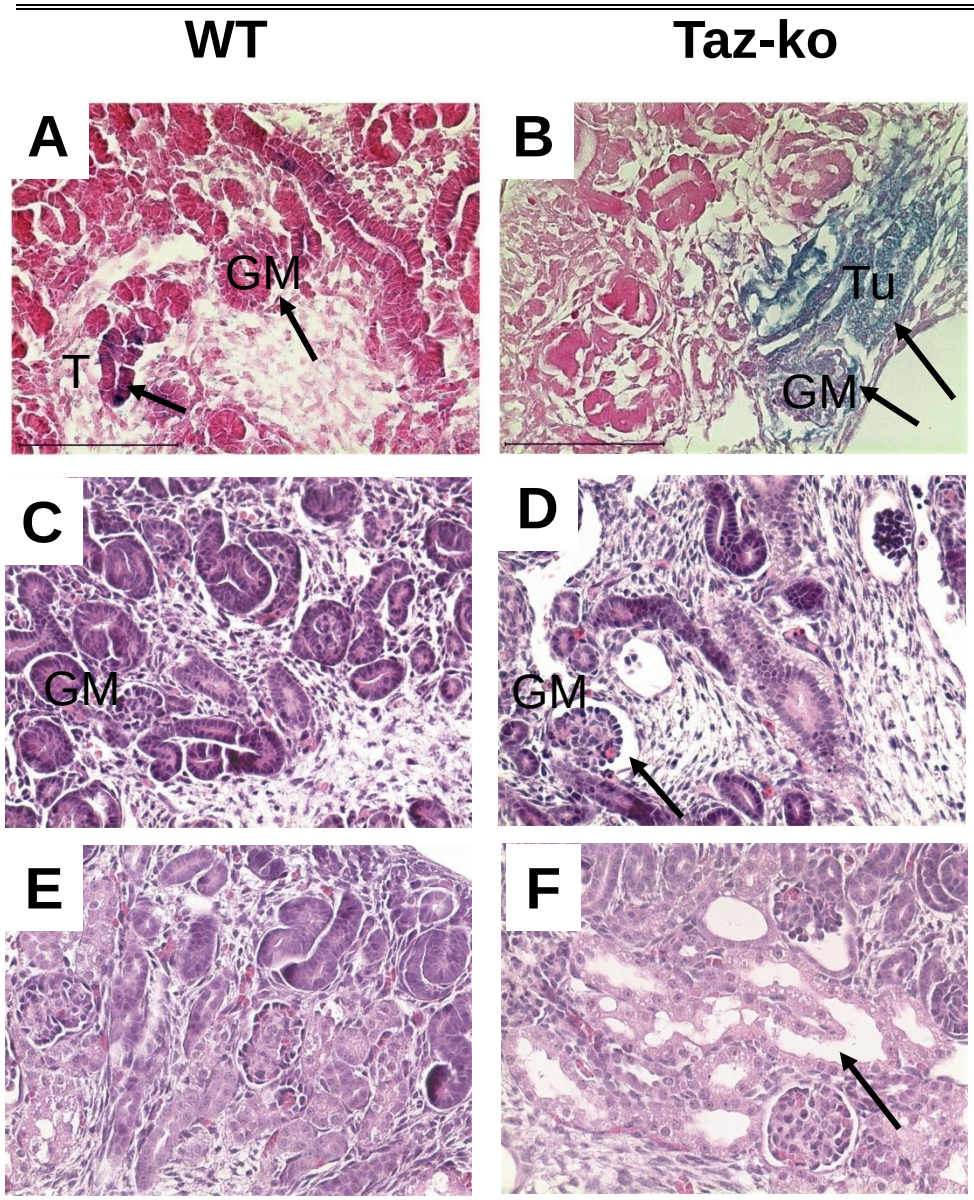

**Figure 4**: Analysis of embryonic kidney phenotypes in Taz knock-out mice: No anomaly was identified in the Taz knockout embryonic kidney at 14.5 dpc (B), whereas glomerular dilation (D) was detected at 15.5 dpc; progressively dilation in both glomerula and tubule were identified at 16.5 dpc (F).

Glomerular morphology gradually degenerated, resulting in cysts with enlarged Bowman's space and atrophy of tufts. Cyst number and size increased with age, with the largest cysts present in the juxtamedullary region. Pathological changes in Taz-/- kidneys included parietal and tubular basement membrane thickening, thinning and folding tubular dilations and atrophy and interstitial fibrosis and mild inflammation (data not shown).

To determine the impact of cyst formation on renal function, blood urea nitrogen and creatinine levels in the serum of 4-6 months mice were determined (data not shown); blood urea nitrogen was two-fold higher in Taz-/- mice relative to heterozygote or WT animals, indicative of end-stage renal diseases (ESRD) and consistent with the reduced lifespan of 10-12 months of the KO mice. Creatinine levels were normal and blood glucose levels slightly elevated (data not shown).

Further molecular analysis of nephron structure confirmed that cysts are predominantly originating from the glomerular origin (66%), collecting duct (11%) and other parts of nephron. Interestingly Taz is highly expressed in the normal kidney, predominantly found in the glomerular and collecting duct region (data not shown). The earliest expression of coincides with the maturation of nephron during development at 14.5 dpc and the formation of cyst in the Taz-knock-out kidney was first identified at 15.5 dpc. So far no link has been identified for Taz with cystic kidney diseases (polycystic kidney disease, PKD).Since family linkage analysis suggests many additional loci associated with PKD that yet to be identified (F. Hildebrandt, pers. commun.), thus Taz is potential novel candidate gene for polycystic kidney diseases; however, still more genetic targets need to be identified for the complete understanding molecular pathology of PKD. We also identified that histological lesion in lung (data not shown) similar to the pulmonary emphysema, an obstructive pulmonary diseases (COPD), a major health concern of mortality and morbidity specially in low- and middle-income countries where behavior of tobacco smoking and incidence of air pollution are not under control. The world health organization (WHO) predicts that COPD will become the third leading cause of death worldwide by 2030. However, to establish Taz as a potential target in emphysema, we further plan to generate conditional Taz knockout mouse model, where Taz would be conditionally inactivated in mice using inducible cre-loxP recombination in-vivo[20] at certain age in adulthood rather than constitutive knock-out (the mice presented in this manuscript) from the beginning of embryonic life.

We also showed that Taz is aberrantly expressed non-small cell lung cancer (NSCLC) cell line (data not shown) and tumor tissue samples (Fig 5). Thus Taz is a candidate moleculeto target multiple anomalies.

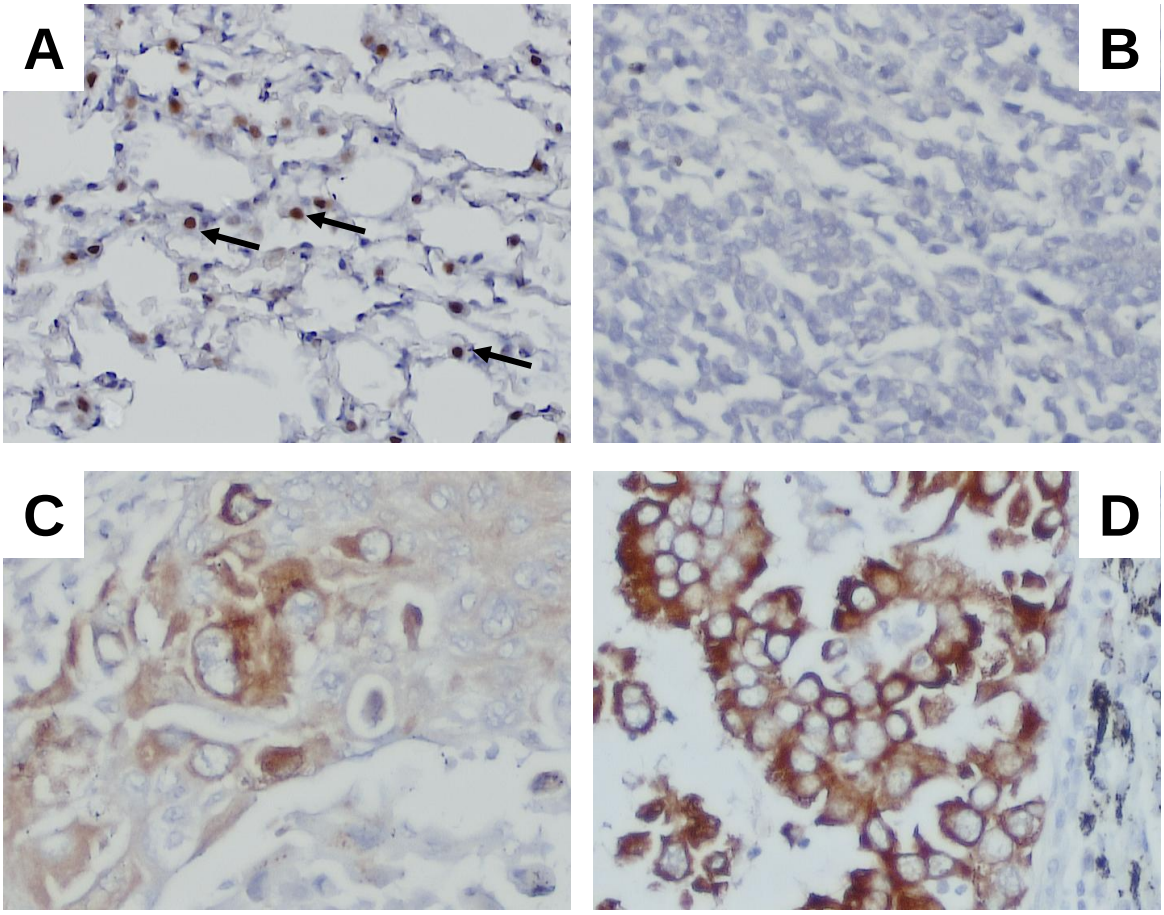


**Figure-5**: Analysis of Taz expression by immune-histochemical staining of lung tumor tissue sections: Taz expression in normal lung tissue is detected in cell nucleus (arrows in A). Taz expression is either lost in small-cell lung cancer (B) or aberrantly expressed in non-small-cell lung cancer tissue sections (C-D).

## DISCUSSIONS

Several breakthroughs within the last decades including development of transgenic and gene targeting technologies, discovery of embryonic stem (ES) cells,[1-7] completion human[8] and mouse[9] genome sequence in 2000 and 2001 respectively have revolutionized strategies for discovery of molecular targets in biology, diseases and drug development.

Mouse is a premier animal model with significant genome homology with human.[21] The number of exons in both species is almost equal (245,200), existence of a very similar number of protein-coding genes (~25,000 in the mouse and ~24,200 in human)., about 90% of the mouse and human genomes can be partitioned into regions of conserved synteny, reflecting the structural

organization of the chromosome in the common ancestor. About 99% of mouse genes have a homolog in the human genome.

The anatomical, behavioral; and physiological similarities of mouse model with human made this model to be accepted as one of the premier animal model in life scienceindustries. Establishment of various inbreed mouse strain (e.g., C57BL/6, 129/S, FVB/N, C3H) with genetic homogeneity facilitate life science investigators to use them as refined genetic models. The continued supply of inbreed mouse strain by Jackson Laboratory, Taconic Farm, Charls-River, Harlan and their subsidiaries made this animal most available to study human physiology and diseases in genetic mouse model. Engineered animal facility, decent husbandry techniques, short span of reproductive cycles and well-studied staging of life-span made this model very popular in reproductive, pediatric, orthopedics, cardiovascular, geriatric, neuro-psychiatric and cancer research.

Large national and international initiatives, Knockout Mouse Project (https://www.komp.org/); International Knockout Mouse Consortium (https://www.mousephenotype.org/martsearch_ikmc_project/about/eucomm) for the mouse model generation and repository were recently established. Large pharmas, Novartis, Deltagen Takeda, Lexicon, Genentech and other pharmasstrengthen their R&D by the establishing dedicated mouse genetic facility, where knock-out and knock-in mouse models serve as unique platform for novel drug targets discovery and validation.[22]

In the post-genomic era, the discoveries and insights of DNA sequence corresponding to non-coding RNA (ncRNA); their role in gene regulation and pathology of diseases are major emerging challenge in academia and biotech industries. Application of mouse geneticsplatform for targeting DNA sequence corresponding to ncRNA would be instrumental to solve the mystery of many more new ncRNAs and to establish their link with patho-physiology, thus application of mouse genetics to study the ncRNAs pose great promise for the development of future medicine to overcome complex health problems (e.g., neurodegenerative disease, cancer and metabolic disease).[23-24]

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