TRANSFERRABLE HOMEOPROTEINS
IN CANCER PROGRESSION AND NEURONAL SURVIVAL

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Homeoproteins are transcription factors that contain homeodomain.

Homeodomain (HD) is folded into helix-turn-helix structure that three helixes constitute (Abate-Shen, 2002).

Homeoproteins orchestrate developmental processes.

Homeogenes comprising HOX clusters determine anterior-posterior axis (Abate-Shen, 2002).

Mutations in Antp gene replace antennae with legs (Klugs and Cummings 2000).
Homeoprotein is localized not only in the nucleus but also in the cytoplasm.

Homeoprotein BP1 in breast cancer cells

Homeoprotein regulates protein translation.

eIF4E-binding consensus is identified in Engrailed, Emx2, HoxA5, Otx2, and Proline-rich homeoprotein (Nedelec, et al., 2004, Topisirovic, et al., 2005).
Homeoproteins transfer between cells

*Homeoprotein is secreted from and internalized into cells.*

**GFP-Engrailed** fusion protein that is expressed in COS-7 cells, center pointed with outlined arrowhead, is transferred to co-cultured embryonic neurons, indicated by filled-in arrowheads (Maizel, et al., 2002).

Secretion of engrailed protein does not depend on signal sequence and internalization occurs independently to endocytosis (Brunet, et al., 2007).

Secretion of homeoprotein is dependent on Δ1 sequence that lies in Nuclear Export Signal (NES) and internalization is on Penetratin sequence in the third helix (Brunet, et al., 2007).

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Hydrophobic residues in Penetratin interact with acyl chain of phospholipid and positive residues pull phosphate group into hydrophobic regions of cell membrane (Zhang and Smith, 2005).

Increased hydrophobicity of Penetratin-cargo complex induced transient membrane instability and allows cell-impermeable chromatin dye, Sytoxi-orange (Dupont, et al., 2007).

Hydrophobic residues (WF) in Penetratin sequence is crucial for internalization of homeoproteins.

Site-directed mutations on WF motif sabotaged internalization capability of homeoproteins.
Non-cell autonomous functions of homeoproteins

Homeoproteins function as morphogens that define compartments during development.

The mid-hindbrain boundary (MHB) is formed by the reciprocal interaction between two homeoproteins, Otx2 and Gbx2 (Simeone, 2000).

Blocking the transfer of homeoprotein Pax6 with anti-Pax6 antibody induces eye maldevelopment (Lesaffre, et al., 2007).

A compartmentalization model. homeoprotein A and B transfer to neighboring cells and activate expression of themselves respectively. The reciprocal interaction between the homeoproteins halts their territory expansion (Joliot and Prochiantz, 1997).
Neutralize secreted oncogenic homeoproteins to prevent cancer progression
Internalize homeoproteins that function as tumor suppressor into cancer cells

Anti-BP1, HoxB7, HoxC13...antibody targeting onco-homeoproteins

Treat exogenous Tumor-suppressor homeoproteins, e.g. HoxA5
Non-cell autonomous functions of homeoproteins

Homeoprotein Engrailed guide axonal growth.

Engrailed protein is internalized into retinal axons and regulates local translation (Brunet, et al., 2005).

Axons from temporal retina are repelled by Engrailed proteins and comparably, nasal axons are attracted by Engrailed (Brunet, et al., 2005).

Homeoprotein Engrailed confers neuroprotection.

Progressive death of mesencephalic dopaminergic (mDA) neurons is induced in En1 +/- heterozygote mice (Sonnier et al., 2005).

Infusion of exogenous Engrailed protein protects mDA from progressive death (Sonnier et al., 2005).
Transferrable homeoproteins may be evolved before the division of animalia and plantae.

Plant homeoprotein Knotted-1 is secreted from HEK cells and internalized into embryonic rat neurons. The white arrow at the center points HEK cells expressing Knotted-1 and arrow heads point rat neurons (Tassetto, et al., 2005).

Transferrable homeoproteins had existed before animals and plants evolved.

Were homeoproteins were penetrable from the evolutionary beginning?

Scenario 1. Some homeoproteins were evolved to be penetrable during evolution.

Scenario 2. Different peptides had been loaded on penetrable homeodomains and each were evolved to individual homeoproteins.
Non-cell autonomous functions of homeoproteins

Homeoproteins deliver various functions in non-cell autonomous manner.

- In morphogenesis
- In compartmentalization
- In axonal guidance
- In cortical plasticity
- In neuroprotection
- In cancer progression
BP1 is Secreted from Cancer Cells but Not Normal Cells in Breast and Prostate.
Internalization of endogenous homeoprotein BP1

BP1 is internalized into breast cells.
Internalized BP1 stimulates cell growth in a non-cell autonomous manner.
Internalized BP1 stimulates upregulation of oncogenes.

<table>
<thead>
<tr>
<th>GENE</th>
<th>CHAMBER</th>
<th>10X CM</th>
<th>rpBP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF 10A</td>
<td>MCF-7</td>
<td>MCF-7</td>
</tr>
<tr>
<td>BCL2</td>
<td>N.C.</td>
<td>1.4 ± 0.07</td>
<td>1.8 ± 0.06</td>
</tr>
<tr>
<td>TWIST</td>
<td>3.6 ± 0.01</td>
<td>2.0 ± 0.07</td>
<td>2.6 ± 0.18</td>
</tr>
<tr>
<td>MET</td>
<td>1.6 ± 0.09</td>
<td>1.4 ± 0.08</td>
<td>1.6 ± 0.08</td>
</tr>
<tr>
<td>BP1</td>
<td>2.8 ± 0.03</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
</tbody>
</table>
Anti-BP1 antibody kills breast and prostate cancer cells.

**Breast Cells**

- **MCF-7**
- **MDA-MB-231**

**Prostate Cells**

- **DU145**
- **MCF 10A**
- **H16N2**
- **RWPE-1**

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Anti-BP1 antibody kills breast and prostate cancer cells.

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Non-cell autonomous function of homeoproteins

Homeoproteins are secreted out of prostate and breast cancer cells.

Secreted Engrailed protein is detected in urine from prostate cancer patients (Morgan, et al., 2009).

Homeoprotein BP1 is secreted from breast cancer cells but not from immortalized breast cells (Rheey, et al., 2008).

Homeoprotein BP1 is internalized into breast cancer cells and stimulates cell growth.

BP1 protein is internalized into breast cancer cells (MCF7)(Rheey, et al., 2009).

Exogenous BP1 protein stimulates growth of breast cancer cells (MCF7) and immortalized breast cell (Rheey, et al., 2009).
1. pBP1 is Secreted from Cancer Cells but Not Normal Cells of Breast and Prostate.

2. Internalized pBP1 Exhibits Mitogenic Activity and Changes Gene Expression.

3. Anti-pBP1 Treatment Induced Cell Death of Cancer Cells but Not Normal Cells of Breast and Prostate
Homeoprotein EN2 is secreted from prostate cancer cells.

Secreted Engrailed protein is detected in urine from prostate cancer patients (Morgan, et al., 2009).

Secreted Engrailed protein is detected in conditioned media from prostate cancer cells. (Morgan, et al., 2009).
Non-cell autonomous functions of homeoproteins

Homeoproteins deliver various functions in non-cell autonomous manner.

In morphogenesis
In compartmentalization
In axonal guidance
In cortical plasticity
In neuroprotection
In cancer progression
Non-cell autonomous functions of homeoproteins

Homeoprotein Otx2 controls cortical plasticity and critical period onset.

Cortical plasticity allows monocular occlusion to shift ocular dominance in critical period due to cortical plasticity. However, the plasticity is abolished by deficiency of Otx2 (Sugiyama, et al., 2008).

Infusion of Otx2 to visual cortex accelerates critical period onset, whereas depletion of Otx2 delays (Sugiyama, et al., 2008).

Homeoprotein Otx2 cell-autonomously prevents death of mDA neurons.

Knock down of Otx2 reduces survival of TH-positive cells in primary ventral mesencephalic culture (Sugiyama, et al., 2008).
Homeoprotein Otx2 controls cortical plasticity and critical period onset.

Infusion of Otx2 to visual cortex accelerates critical period onset, whereas depletion of Otx2 delays (Sugiyama, et al., 2008).
Homeoprotein Engrailed functions as a survival factor.

Infusion of exogenous Engrailed protein protects mDA from progressive death caused by haplodeficiency of Engrailed (Sonnier et al., 2005).
### Homeoproteins confer neuroprotection in a non-cell autonomous manner.

<table>
<thead>
<tr>
<th>Engrailed</th>
<th>Otx2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functions as survival factor for developing mDA neurons in a <strong>cell autonomous</strong> manner</td>
<td>Functions as survival factor for VTA mDA neurons in a <strong>cell autonomous</strong> manner</td>
</tr>
<tr>
<td>Transfers between cells</td>
<td>Transfers between cells</td>
</tr>
<tr>
<td>Functions as survival factor for SNpcmDA in a <strong>non-cell autonomous</strong> manner</td>
<td>Functions as survival factor for ? in a <strong>non-cell autonomous</strong> manner</td>
</tr>
</tbody>
</table>
Otx2 and retina

Otx2 expressed in photoreceptors and bipolar cells are transferred to RGCs.
Exogenous Otx2 protein promoted RGC survival in culture of adult mouse retinal cells.

Dissociated adult mouse retina was cultured in Neurobasal A media supplemented with Asp, Glu, Gln, and B27.

Exogenous Otx2 was treated simultaneously as plating on Day 0.

RGCs are identified with NF200 staining and counted along the diameter of coverslip.

Single administration of Otx2 increased RGC survival for as long as 18 days.
Promoted RGC survival by Otx2 was detected as early as 24h after the treatment \textit{in vitro}.

RGCs expressing NF200 are semi-automatically counted along the diameter of coverslip.

Otx2 also increased survival of Thy1-expressing RGCs \textit{in vitro}.

Retina from Thy1-CFP transgenic mice was dissociated for the culture.

Thy1 is specifically expressed in RGCs and CFP expression is under control of Thy1 promoter. Thus, CFP-expression can be RGC index in Thy1-CFP mice.

CFP-expressing RGCs were semi-automatically counted along the diameter of coverslip.
Exogenous Otx2 protein promoted immunopurified adult rat RGC survival.

Adult rat RGCs were immunopurified against RGC-specific marker Thy1 expressed on cell surface.

Otx2 protein or neutralized Otx2 by anti-Otx2 antibody was added at the same time as plating.

Live RGCs were identified by calcein staining and manually counted along the diameter.

Otx2 increased survival of immunopurified adult rat RGCs in a dose-dependent manner. Anti-Otx2 antibody abolished the effect.
Glaucoma is caused by RGC death and, not always, but often associated with ocular hypertension.
Glaucoma is caused by RGC death highly associated with ocular hypertension.

Impeded drainage of aqueous humor in anterior chamber elevates IOP and subsequently induces RGC death.

**Open-angle Glaucoma (IOP <18)**

**Closed-angle Glaucoma (IOP >21)**

However, glaucoma is not always associated with elevated IOP.

Increased level of excitatory amino acids was observed in glaucoma patients and monkey glaucoma models (Dreyer, et al., 1996).
**Rodent models for glaucoma**

**Manipulation of Episceral vein**
- Injection of hypertonic saline
- Episceral vein cauterization
  - Progressive RGC death is associated with IOP elevation
  - Takes 6 months to observe RGC death and IOP elevation.
  - Technical efficient (relatively easy and 3 min / mouse)
  - Relatively acute effect (12 hrs for NMDA)
  - IOP elevation lasts 1 week / treatment.
  - Instrument costs high.
  - Time-demanding experiment.

**Optic nerve crush / transection**
- Progressive RGC death occurs around 2 weeks.
- Technical difficulty and no ocular hypertension is induced.
- RGC death varies with experiments.

**DBA/2J mouse**
- Progressive RGC death is associated with IOP elevation
- Takes 6 months to observe RGC death and IOP elevation.

**Excitotoxicity model**
- Technical efficient (relatively easy and 3 min / mouse)
  and acute effect (12 hrs for NMDA)
- Experiment costs relatively less
- Excitotoxic RGCs death is not primary cause of glaucoma
Intraocular injection of NMDA killed RGCs.

Intraocular injection of 1mM NMDA

Dissection of retina

Control eye

Injected eye

Thy1-CFP mouse

Flat-mount retina

Count CFP-positive cells in retina

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Intraocular injection of NMDA killed RGCs.

1 mM NMDA reduced number of CFP-positive RGCs.
Intraocular injection of NMDA killed RGCs.

Intraocular injection of 1mM NMDA

Dissection of retina

Control eye

Injected eye

RNA extraction

qRT-PCR against RGC marker Brn3a or other retinal cell markers
Intraocular injection of NMDA killed RGCs.

Intraocular injection of 1mM NMDA reduced Brn3a mRNA level 55% after 4 days. NMDA excitotoxicity kills specifically RGCs in our model.

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**Graph:**

- **Brn3A:** RGC
- **Islet A:** BP
- **Synt A:** H
- **mGluR6:** BP
- **Recov:** BP
- **Rhod:** PR

Relative mRNA level vs. mRNA level in non-injected eye

**Legend:**

- RGC: Retinal Ganglion Cell
- A: Amacrine cell
- BP: Bipolar cell
- H: Horizontal cell
- PR: Photoreceptor

Note: The graph shows the relative mRNA levels for various cell types in the retina. The Brn3A mRNA level in RGCs is significantly reduced (55%) compared to the non-injected eye.
Otx2 was internalized into retinal cells in vivo.

154ng Otx2-FITC was injected and eyes were fixed 6 hrs after the injection.

Otx2-FITC was internalized into all retinal cells and internalized Otx2-FITC was localized in nucleus.
Intraocular injection of NMDA killed RGCs.

Intraocular injection of 1mM NMDA or 30ng Otx2

Dissection of retina

Control eye

Injected eye

Thy1-CFP mouse

1μl

4 days

Flat-mount retina

Count CFP-positive cells in retina

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Otx2 protected RGC against NMDA excitotoxicity.

30ng Otx2 was injected with 1mM NMDA.

[Images of fluorescence microscopy showing the effects of Otx2 and NMDA on cell counts]
Intraocular injection of NMDA killed RGCs.

Intraocular injection of 1mM NMDA or 30ng Otx2

Dissection of retina

Control eye

Injected eye

RNA extraction

qRT-PCR against RGC marker Brn3a or other retinal cell markers

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Otx2 protected RGC against NMDA excitotoxicity.

Otx2 completely prevents decrease in Brn3a mRNA level against NMDA.

Otx2 did not regulate expression level of Brn3a.

30ng Otx2 provided full protection for RGCs against NMDA excitotoxicity.

Otx2 protein protected RGCs for up to 3 weeks.
Otx2 protects RGC against NMDA excitotoxicity.

Neutralizing Otx2 by anti-Otx2 antibody abolished the protective effect on RGC.

30 ng Otx2 was pre-absorbed with equimolar amount of anti-Otx2 antibody before the injection.

36ng and 144ng Otx2 injection produced the same full protection on RGC as 30ng Otx2.
Otx2 protects vision against NMDA excitotoxicity.

Functional aspect of protective effects on RGCs by Otx2 was assessed by optomotor test.

Left eye enucleation

Right eye injection

1μl

Day 0

Day 4

Optomotor test
Otx2 protects vision against NMDA excitotoxicity.

Otx2 protects vision loss caused by NMDA excitotoxicity.

Day 0       4 days post injection

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of CCW turns</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
</tr>
<tr>
<td>A NMDA</td>
<td>2</td>
</tr>
<tr>
<td>B NMDA + Otx2</td>
<td>1</td>
</tr>
</tbody>
</table>

P < 0.002
P < 0.05
Internalization-deficient Otx2 YL mutant

Hydrophobic residues WF is crucial for internalization of homeoprotein.

Internalization-deficient homeoprotein mutants

En2  *- - - - TAFTAEQLQRLKAEPQTNRYLTERQRQRSLAQLSLNE---SQIKIKWFKQNKRKKKK---
Otx2 *-RRERTTFTRAQLDVEALEFALFKAQTRPYDIFMREEVALKINLE---SRVQVFKNRRAKC---

En2 SR  *- - - - TAFTAEQLQRLKAEPQTNRYLTERQRQRSLAQLSLNE---SQIKIWerQQNKRKKKK---
Otx2 YL *-RRERTTFTRAQLDVEALEFALFKAQTRPYDIFMREEVALKINLE---SRVQVLKNRRAKC---
Otx2 YL mutant is not internalized into retinal cells.

Otx2 YL mutant does not promote survival of RGCs after stress.
Conclusion

Otx2 protein acts directly on injured adult RGCs and promote their survival in a dose-dependent manner \textit{in vitro}.

Otx2 protein protects adult RGCs and vision loss against NMDA excitotoxicity.

Survival-promoting effect of Otx2 protein on RGCs is dependent on internalization of Otx2 protein.

In sum: Otx2 function as a survival factor for adult RGCs in a non-cell autonomous manner.
Otx2\textsuperscript{+/GFP} mice have diminished visual acuity.

Haplo-deficiency of Otx2 induces diminished visual acuity.

Optomotor

2.5 months

\begin{figure}
\centering
\includegraphics[width=\textwidth]{optomotor_2.5_months}
\caption{Optomotor performance of Wt and Otx2\textsuperscript{+/GFP} mice at 2.5 months.}
\end{figure}

4 months

\begin{figure}
\centering
\includegraphics[width=\textwidth]{optomotor_4_months}
\caption{Optomotor performance of Wt and Otx2\textsuperscript{+/GFP} mice at 4 months.}
\end{figure}

\textit{p} < 0.05 (Mann-Whitney U test)
ON- and OFF-retinal circuitry

ON-bipolar cells (BPs) are depolarized and OFF-BPs are hyperpolarized on light onset.

ON-BPs synapse with ON-RGCs and OFF-BPs with OFF-RGCs.

On light onset, photoreceptors are hyperpolarized and level of glutamate decreases in synapse.

In ON-BPs, mGluR6 allows influx of cations and induces depolarization.

In OFF-BPs, iGluR allows influx of cations and induces hyperpolarization.

ON-BPs synapse with ON-RGCs in sublamina a of IPL and OFF-BPs with OFF-RGCs in sublamina b of IPL.
Rod and cone retinal circuitry

**Cone Circuitry**

**ON-pathway**
Photon -> (-) Cone -> (+) ON cone bipolar cells -> (+) ON RGCs

**OFF-pathway**
Photon -> (-) Cone -> (-) OFF cone bipolar cells -> (-) OFF RGCs

**Rod Circuitry**

**ON-pathway**
Photon -> (-) Rod -> (+) Rod Bipolar cells -> (+)All Amacrine cells -(Gap junction) -> (+) ON cone bipolar cells -> (+) ON RGCs

**OFF-pathway**
Photon -> (-) Rod -> (+) Rod Bipolar cells -> (+)All Amacrine cells -(Glycinergic synapse) -> (-) OFF cone bipolar cells -> (-) OFF RGCs

(Sharpe and Stockmak, et al., 1999)
Electroretinogram (ERG)

Photocurrents elicited in retina generates mass electric-response detected as Electroretinogram (ERG).

a-wave is generated by activity of photoreceptor cells activity.

b-wave reflects ON-bipolar cells’ activity (Miura, et al., 2009).
Otx2^{+/GFP} mice have retinal dysfunction.

Haplo-deficiency of Otx2 induces reduction of b-wave amplitude in ERG response.

Otx2^{+/GFP} has dysfunction in ON-bipolar cells or fewer number of ON-bipolar cells.
Otx2+/GFP may have fewer rod bipolar cells, a type of ON-bipolar cells.

Ratio
Otx2+/GFP
Wtmice

γ-actin (HK)  Brn3A (RGC)  Thy1 (RGC)  γ-synuclein (RGC)  PKC (Rods/bipolar)  Syntaxin (Amacrine/Horizontal)  Rhodopsin (Rods)

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

OFF-bipolar  ON-bipolar

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Inner nuclear layer of Otx2+/GFP mice
Otx2+/GFP mice have thinner inner nuclear layer.

In the figure, the inner nuclear layer (INL) and outer nuclear layer (Outer Nuclear Layer, ONL) thicknesses are compared between wild type (Wt) and mutant (Wt+/-) conditions. The INL thickness is significantly thinner in the mutant mice compared to the wild type, with a p-value of less than 0.005. The ONL thickness is also shown, but there is no significant difference between the two conditions.
**Inner nuclear layer of Otx2+/GFP mice**

Otx2+/GFP mice have fewer cells in inner nuclear layer.
Rod bipolar cells in Otx2\(^{+/GFP}\) mice may have fewer rod bipolar cells.
Development and types of bipolar cells

Development of retina and retinal cells in a post-natal period

Development of bipolar cells peaks by P3 and completes by P11 (Cepko, et al., 1996).

Cone bipolar cells emerge earlier than rod bipolar cells (Morrow, et al., 2008).

Development and subtypes of bipolar cells

Cone bipolar cells are divided into 9 subclasses depending on axonal projection. Type 1 through 4 are OFF bipolar cells and type 5 through 9 are ON bipolar cell. All rod bipolar cells are ON bipolar cells (Ghosh, et al., 2004).
Haplo-deficiency of Otx2 protein induces diminished visual acuity in mouse.

Haplo-deficiency of Otx2 protein may develop fewer mature bipolar cells in mouse.