Immunohistochemical Characterization of Melanomas in Bigenic Mice Containing Activated Cdk4 and Keratinocytic Specific Ablation of Retinoid-X-Receptor $\alpha$ (RXR$\alpha$) Protein
Melanoma

- The deadliest form of skin cancer
- The 5\textsuperscript{th} most common invasive cancer in Oregon
  - Rank 7\textsuperscript{th} in nation in incident rates
  - Mortality rate 26\% higher than national rate
- No effective treatment for metastatic melanoma
Anatomy of skin

- Largest organ in the body
- 3 main layers:
  - Epidermis
  - Dermis
  - Subcutis (hypodermis)

http://training.seer.cancer.gov/melanoma/anatomy/
Epidermal section of human skin

- Basal layer
- Spinous layer (Squamous cell layer)
- Granular layer
- Cornified layer

Melanocytes to keratinocytes

- Melanocyte manufactures tiny melanin granules
- One melanocyte produces 36 keratinocytes with melanin granules

Retinoids

- Active vitamin A derivative
- Regulate growth and differentiation of cells
- Nuclear receptors binding

Perissi, Nat Rev Mol Cell Biol, 2005
RXRs and Nuclear Receptors

- Retinoic acid receptor ($\alpha, \beta, \gamma$) (DR1, DR2, DR5)
- PPAR ($\alpha, \beta, \gamma$) (DR1)
- TRs ($\alpha, \beta$) (DR4)
- Liver X receptor ($\alpha, \beta$) (DR1)
- Farnesoid X receptor
- Vitamin D receptor (DR3)
- NGFI-B/NURR1 (orphans)
- RXRs ($\alpha, \beta, \gamma$) (DR1)
- Pregnane X receptor
Cyclin-dependent kinase family (CDKs)

- Proteins kinases require cyclin to function
- Involved in cell cycle control
- Target for anti-cancer medication

Cyclin-dependent kinase 4 (CDK4)

- CDK’s inhibitor p16Ink4a is a tumor suppressor protein
- CDK4 is a product of Ink4a locus
  - Involved in G1 phase
  - Point mutation of arginine (R) to cystine (C) (Cdk4^{R24C/R24C}) inhibits p16
    → HYPERACTIVITY in cell cycle
Hypothesis

1. Increased melanocytes proliferation due to ablation of RXRα could be due to deregulated cell cycle control CDK4

2. Loss of RXRα in combination of activated CKD4 increases metastatic progression
Methodology

- Transgenic mice preparation
- Genotyping
- Two-step chemical tumorgenesis
- Collecting tissue samples
- Histological and immunofluorescence analyses
Site-specific ablation of RXRα

- Cre-lox systems to knock-out RXRα in epidermal keratinocytes

**GENOTYPE**

**CONTROL**: K14-Cre\(^{-/-}\)/RXRα\(^{L2/L2}\)

**MUTANTS**: K14-Cre\(^{(tg/-)}\)/RXRα\(^{L2/L2}\)

**EPIDERMIS**

RXRα\(^{L2/L2}\)

RXRα\(^{L-/-}\)

(RXRα\(^{ep/-}\))
Methodology

- Transgenic mice preparation
- Genotyping
- Two-step chemical tumorigenesis
- Collecting tissue samples
- Histological and immunofluorescence analyses
Genotyping

- DNA isolation:
  - Tail samples of 10-day old mice
  - Incubated in Dispase overnight
  - Digested using proteinase K in solution
  - Heated in water bath

- Amplified with semi-quantitative Polymerase Chain Reaction (PCR)

- Gel electrophorosis
Desired genotype groups

- **Control group:**
  - $\text{RXR}_\alpha^{L2/L2}$

- **Single mutated groups: (additional controls)**
  - $\text{RXR}_{\alpha}^{\text{ep-/-}}$
  - $\text{Cdk4}^{R24C/R24C}$ (Knock-in mutation)

- **Bigenic mice:**
  - $\text{RXR}_{\alpha}^{\text{ep-/-}} / \text{Cdk4}^{R24C/R24C}$
Methodology

- Transgenic mice preparation
- Genotyping
- Two-step chemical tumorigenesis
- Collecting tissue samples
- Histological and immunofluorescence analyses
Chemical tumorigenesis

- Initiation, Promotion and Progression model
  - DMBA (7,12-dimethyl-benz[a]anthracene)
    - tumor initiator
    - 50μg/100μg acetone single application
  - TPA (phorbol ester 12-O-tetradecanoylphorbol-13 acetate)
    - tumor promoter
    - 5μg/100μg acetone 2 times/week for 25 weeks
Methodology

- Transgenic mice preparation
- Genotyping
- Two-step chemical tumorigenesis
- Collecting tissue samples
- Histological and immunofluorescence analyses
Tissue samples

- Collect tissue samples and melanocytic tumors
- Fix tissues in 4% paraformaldehyde
- Dehydration process with EtOH
- Embed in paraffin
- Cut into 5μm thick using Microtome
Methodology

- Transgenic mice preparation
- Genotyping
- Two-step chemical tumorigenesis
- Collecting tissue samples
- Histological and immunofluorescence analyses
Histological analyses

- Hematoxylin and Eosin staining (H&E)
- To visualize epidermal thickening and melanocytic growths
Immunohistochemistry (IHC)

- Deparaffinized, rehydration and antigen retrieval
- Melanocytic proliferation:
  - Melanocytic specific marker TRP1 (green)
  - Proliferation marker PCNA (red)
  - Counterstained DAPI (blue)
- Malignant nature of tumors:
  - Cocktail directed against melanoma antigens
  - MART-1 and HMB45
- Vascululization:
  - CD31 antibody
Results

- Increase in tumors size in RXRα<sup>ep-/-</sup> and RXRα<sup>ep-/-/Cdk4<sup>R24C/R24C</sup></sup>
Results

*p < 0.05
** = p < 0.01
*** = p < 0.001
Results

- Significantly higher percent of co-labeling in bigenic mice than Cdk4^{R24C/R24C}
  - 67% vs. 21%
  - P-value < 0.01
Results

- Higher staining for malignant melanocytes in bigenic mice than Cdk4R24C/R24C
- Increase vascularization in melanocytic tumors in bigenic mice than Cdk4R24C/R24C
Conclusion

1. Increased melanocytes proliferation due to ablation of RXRα is due to deregulated cell cycle control CDK4
   - Higher melanocytes proliferation in bigenic mice than individual mutated group

2. Loss of RXRα in combination of activated CKD4 increases metastatic progression
   - Higher maglinant potential in bigenic mice compared to individual mutated group
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Question?