A Cardioprotective Estrogen Receptor Alpha-BMPR2-Apelin Axis as a Novel Modifier of Right Ventricular Function in Pulmonary Hypertension

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Disclosures

- Speaker Bureau, Bayer
- Scientific Review Committee, Gilead
- Research reagents, Eli Lilly & Company
The critical role of right ventricular (RV) function in pulmonary hypertension (PH)

Features of RV failure in PH

Modulators of RV function of interest to our lab
- 17β-estradiol (E2) and Estrogen receptor (ER)-α
- Apelin
- Bone morphogenetic protein receptor 2 (BMPR2)

Strategies aimed at harnessing these modulators in order to improve RV function
RV function determines survival in pulmonary vascular and cardiac disease

**Pulmonary Arterial Hypertension (PAH)**

- 1. RVEF > 35, PVR < 650 (n = 36)
- 2. RVEF > 35, PVR > 650 (n = 20)
- 3. RVEF < 35, PVR > 650 (n = 13)
- 4. RVEF < 35, PVR < 650 (n = 41)

Van de Veerdonk MC. JACC 2011.

**Systolic Left Heart Failure**

- Normal RV fct
- RV dysfct + pulm HTN

RV failure is common

<table>
<thead>
<tr>
<th>Disease</th>
<th># in US</th>
<th>% PH/RV dysfunction</th>
<th># with PH/RV dysfunction in US</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic HF</td>
<td>5.8 mil</td>
<td>60%</td>
<td>3.8 mil</td>
</tr>
<tr>
<td>Diastolic HF</td>
<td>76 mil</td>
<td>83%</td>
<td>63 mil</td>
</tr>
<tr>
<td>Sleep-disordered breathing</td>
<td>7.6 mil</td>
<td>20%</td>
<td>1.5 mil</td>
</tr>
<tr>
<td>COPD</td>
<td>10 mil</td>
<td>20-40%</td>
<td>2 – 4 mil</td>
</tr>
<tr>
<td>IPF</td>
<td>191,520</td>
<td>28%</td>
<td>53,626</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>67 – 72.4 mil</td>
</tr>
</tbody>
</table>

A need for novel RV-directed treatments

- 3-year survival in PAH despite treatment only 60%
- Better understanding of mechanisms and modulators of RV dysfunction in PAH/PH may lead to development of novel RV-directed therapies
  - NHLBI workshop 2017
- Cannot extrapolate studies from LV

Humbert M. Circulation 2010.
Overview of mechanisms of RV failure development

Cardiomyocyte Stress

- Cardiomyocyte Hypertrophy
- Neurohormones

Extra-cellular Matrix Changes

- ROS/RNS
- Inflammation

Contractile dysfunction
- MHC switch; Ca^{2+} handling; Mitochondropathy

- Ischemia
- Apoptosis

Dilatation & Failure

Bogaard HJ. Chest 2009.
The Sugen/Hypoxia (SuHx) rat model of PAH and RV failure

VEGF receptor 2 antagonist

Su5416 (20 mg/kg sq)

Hypoxia

weeks 1-3

Room air

weeks 4-7

sacrifice

More profound pulmonary vascular remodeling and RV failure than traditional animal models of PH
Hemodynamic and functional alterations in rats with SuHx-PH

![Bar charts showing RV/(LV+S), RVSP, CO, and VO2 max comparisons between Nx and SuHx groups.](image)

#p < 0.05; n=3-5/group
SuHx-induced RV dysfunction is associated with increased cardiomyocyte fibrosis

*\( p < 0.05; n = 4-5/\text{group} \)
Decreased capillary volume in SuHx-induced RV failure
SuHx-induced RV dysfunction is associated with increased pro-apoptotic signaling

Bcl-2/bax

Caspase 3 activity

*p<0.05; n=4-5/group
Mitochondrial dysfunction in SuHx-induced RV failure

*Agglomeration of subsarcolemmal mitochondria*
Mitochondrial dysfunction in SuHx-induced RV failure

* Dysfunctional mitochondrion (loss of cristae)
** Degradation of mitochondria by lysosomes
Interim summary

- PH is common and prognostically important in many highly prevalent diseases (including ESRD)
  - RV function a major determinant of outcomes in PH
- RV failure is characterized by significant hemodynamic, structural and biochemical abnormalities
  - contractile function & exercise capacity
  - structural remodeling
  - capillary rarefaction
  - pro-apoptotic and pro-inflammatory signaling
  - mitochondrial dysfunction
- SuHx is a suitable model to study mechanisms and modulators of RV dysfunction in severe PH
Gender, sex hormones and RV function in pulmonary hypertension
Estrogen paradox in PAH

PAH = sexually dimorphic disease with female predominance

Female PAH patients exhibit better RV function and superior survival

Registry data 1981-2013
Europe (COMPERA)
    UK/Ireland
    Spain
US (REVEAL)
    China
    France
    Scotland
US (NIH)

Observational data
Ventetuolo CE, ERJ 2014
Shapiro S, Chest 2012
Kawut SM, Chest 2009
Olson KM, Circulation 2014
Jacobs W, Chest 2014
Humbert M, Circulation 2010
Benza RL, Circulation 2010

PAH = sexually dimorphic disease with female predominance.
Female PAH patients exhibit better RV function and superior survival.
Estrogen paradox in PAH

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Female PAH patients exhibit better RV function and superior survival

Estrogens detrimental:
- PA remodeling
  - Dean A, Hypertension 2016
  - Wallace E, AJRCCM 2015
  - Wright AF, Cardiovasc Res 2015
  - Mair KM, AJRCCM 2015, AJRCCM 2014
  - White K, Circ 2012
  - Fessel JL, Pulm Circ 2013
  - Austin ED, ERJ 2009

Estrogens protective:
- RV function
  - Lahm T, AJP Lung 2016
  - Frump AL, AJP Lung 2015
  - Liu A, Hypertension 2015
  - Liu A, AJP Heart 2014
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  - Umar S, AJRCCM 2011
  - Ventetuolo CE, AJRCCM 2010
Better survival in women with PAH or PH

Table 3. Multivariable Cox PH Model

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
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<tbody>
<tr>
<td>6MWD</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
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</tr>
<tr>
<td>Female</td>
<td>0.375</td>
</tr>
<tr>
<td>Cardiac output</td>
<td>0.759</td>
</tr>
</tbody>
</table>

n=15,464

Female PAH patients exhibit better RV function than male patients

Kawut SM. Chest 2009.
Higher estradiol levels are associated with better RV systolic function in women using hormone replacement therapy (HRT).

Postmenopausal HRT users:

↑ E2 levels →

↑ RVEF

↓ RVESV

Mechanisms?

Ventetuolo CE. AJRCCM 2011.
Certain PAH patients may require E2 for RV adaptation
1. Does the SuHx model of severe, angioproliferative PH recapitulate sex differences seen in human disease?
2. What is the effect of endogenous sex hormones (in particular, estradiol) on RV function in severe PH?
3. What are the molecular targets of estradiol in the RV?

- Normoxia male controls
- Normoxia female controls
  - Male SuHx
  - Female SuHx
- Female SuHx OVX
- Female SuHx OVX + E2

Room air

Su5416 (20 mg/kg sq)

weeks 1-3

Hypoxia

weeks 4-7

Room air

VO\textsubscript{2} max

Echo RVSP

Sacrifice

Room air

female

E2 75 µg/kg/d (via sq pellets)

male

OVX
Protective effect of female sex hormones on RV function in SuHx rats

Frump AL. AJP Lung 2015.

* p<0.05, *** p<0.001 vs. same sex normox control
°°° p<0.001 vs female SuHx OVX
∧ p<0.05 vs male

Cardiac index

RV wall thickness

n=6-8/group
Estradiol-treated OVX SuHx rats exhibit significant improvement in exercise capacity.

Exercise capacity

Frump AL. AJP Lung 2015.
Estradiol-treated OVX rats exhibit decreased RV pro-apoptotic signaling

RV caspase 3 activity

RV bcl-2/bax

Frump AL. AJP Lung 2015.
Estradiol-treated OVX rats exhibit evidence of decreased RV cytoplasmic glycolysis

Ryan JJ and Archer SL. Circ Res 2014
Estradiol-treated OVX rats exhibit evidence of decreased RV cytoplasmic glycolysis

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>SuHx</th>
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<tbody>
<tr>
<td></td>
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<td>Female</td>
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<td>Female</td>
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<td>Merge</td>
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<td><img src="image21" alt="Image" /></td>
<td><img src="image22" alt="Image" /></td>
</tr>
</tbody>
</table>

Frump AL. AJP Lung 2015.
Estradiol-treated OVX rats exhibit evidence of decreased RV cytoplasmic glycolysis

Ryan JJ and Archer SL. Circ Res 2014

* p<0.05 vs same sex normoxia control
# p<0.05 vs female SuHx

n=4-8/group
Estradiol-treated OVX rats exhibit increased pro-contractile and pro-angiogenic signaling as well as decreased pro-inflammatory signaling

Apelin

MCP-1 (CCL2)

IL-6

Pro-angiogenic
Pro-contractile
Anti-apoptotic
Anti-inflammatory

Andersen C. Pulm Circ 2011.

*, *** p<0.05, p<0.001 vs same sex normoxia control
° p<0.05 vs female SuHx OVX

Frump AL. AJP Lung 2015.
Estrogen receptor (ER) α as a mediator of protective estradiol effects in the RV

Frump AL. AJP Lung 2015.

*p<0.05 vs same sex normoxia control
° p<0.05 vs female SuHx OVX
^^p<0.01 vs male normox
Treatment with ERα (but not ERβ) agonist recapitulates protective estradiol effects in the RV

<table>
<thead>
<tr>
<th></th>
<th>Normox</th>
<th>SuHx</th>
<th>E2</th>
<th>PPT</th>
<th>DPN</th>
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<td><img src="image5.png" alt="Image" /></td>
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<td>Bax</td>
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<td><img src="image9.png" alt="Image" /></td>
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<tr>
<td>Vinculin</td>
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</tbody>
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RV bcl-2/bax

<table>
<thead>
<tr>
<th></th>
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<th>SuHx</th>
<th>E2</th>
<th>PPT</th>
<th>DPN</th>
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</tbody>
</table>

* p<0.05 vs normoxia control
# p<0.05 vs SuHx
$ p<0.05 vs DPN
Estrogen and its receptors in RV failure: Current working model

E2

ERα

Pro-apoptotic signaling (bcl-2/bax, caspase-3)

Pro-inflammatory signaling (IL-1β, MCP-1)

Pro-angiogenic/pro-contractile signaling (apelin)

Cytoplasmic glycolysis (GLUT1)

Oxidative stress (GSH)

↑RV function

↑Exercise capacity

Frump AL. AJP Lung 2015.
Apelin as a target of E2 in the RV
Apelin

- Peptide secreted by endothelial cells

- Vasodilator
- Inotrope
- Pro-angiogenic
- Anti-inflammatory
- Anti-apoptotic

Andersen C. Pulm Circ 2011.
Apelin protects from PH development


Apelin is cardioprotective in the left ventricle

Dalzell JR. J Card Fail 2015.

Chen MM. Circulation 2003.
Apelin increases contractility of isolated RV muscle fibers

What is the role of apelin in RV failure?
How is RV apelin regulated?

E2 → ERα → Apelin → RV cardioprotective effects
Apelin is decreased in RVs from rats with SuHx-PH but not in RVs from chronically hypoxic rats.

Frump AL. AJP Lung 2015.
E2 protects against TNF-α or staurosporine-induced decreases in apelin expression in rat cardiomyoblasts


**H9c2 cells**

![Image of H9c2 cells](image)

Apelin

Vinculin

Control

TNF-α 8 hr

TNF-α 8 hr + E2

17 kDa

128 kDa

Apelin

Vinculin

Control

Stauro 4 hr

Stauro 4 hr + E2

17 kDa

128 kDa

Apelin

Vinculin

Control

Stauro 4 hr

Stauro 4 hr + E2

* p<0.05 vs control, $ p<0.05 vs TNF/Stauro
Apelin is necessary for E2-mediated cytoprotective signaling in cardiomyoblasts

Apelin is necessary for E2-mediated cytoprotective signaling in cardiomyoblasts.
ERα abundance correlates with cardiac output and apelin expression.

**Cardiac Output**

- \( R = 0.41 \)
- \( p < 0.05 \)

**Apelin**

- \( R = 0.46 \)
- \( p < 0.05 \)
ERα is necessary for E2-mediated apelin expression *in vitro*

**Diagram A:**
- E2
- ERα
- Apelin

**Diagram B (ERα):**
- Fold Change vs control ERα/vinculin
- Time treated with E2: 1 hr, 2 hrs, 4 hrs, 16 hrs, 24 hrs

**Diagram C (Apelin):**
- Fold Change vs control apelin/vinculin
- Time treated with E2: 1 hr, 2 hrs, 4 hrs, 16 hrs, 24 hrs

* p<0.05 vs scr control, $ p<0.05 vs si
ERα activation is sufficient to upregulate apelin
*in vitro*

EJO: ERα agonist

* p<0.05 vs vehicle control
E2-mediated upregulation of apelin *in vivo* occurs via ERα.

---

**Rat RV cardiomyocytes**

Negative Control | ERα
---|---
α-actinin
DAPI
Merge

**SuHx-PH Rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RV Apelin (fold change ddCt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
</tr>
<tr>
<td>SuHx</td>
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</tr>
<tr>
<td>SuHx E2</td>
<td>0.8</td>
</tr>
<tr>
<td>SuHx PPT</td>
<td>0.8</td>
</tr>
<tr>
<td>SuHx veh</td>
<td>0.8</td>
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</table>

PPT: ERα agonist

---

**Hypoxic PH Mice**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Apelin</th>
<th>Vinculin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia WT</td>
<td></td>
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<tr>
<td>WT+ E2</td>
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<td></td>
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<tr>
<td>ESR1−/− + E2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR2−/− + E2</td>
<td></td>
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**RV Apelin/Vinculin**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>WT</td>
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</tr>
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* indicates statistical significance compared to control

* indicates statistical significance compared to WT

* indicates statistical significance compared to WT+ E2

* indicates statistical significance compared to ESR1−/− + E2

* indicates statistical significance compared to ESR2−/− + E2
Interim summary and refined hypothesis

E2 → ERα → BMPR2 → Apelin → RV Cardioprotective Effects
Is there a E2-BMPR2-apelin axis in the RV?

- **BMPR2** = Bone morphogenetic protein receptor 2
  - Member of TGFβ superfamily of receptors
  - Pulmonary endothelial cell and smooth muscle cell homeostasis
- Loss-of-function mutations associated with development of hereditary PAH
  - **Role in the RV unknown**
PAH patients with **BMPR2** mutations exhibit worse RV function than non-carriers

Van der Bruggen CE. Circulation 2016.
E2 or ERα agonist PPT upregulates BMPR2 in SuHx RVs

* p<0.05 vs male normox
ERα is necessary for E2 to upregulate BMPR2.
ERα activation is sufficient to increase BMPR2 expression

EJO: ERα agonist

* p<0.05 vs vehicle control
ERα binds to the *BMPR2* promoter
E2 induces formation of a PPARγ/β-catenin complex
BMPR2 is necessary for E2-mediated upregulation of apelin in staurosporine- or TNF-treated cells.
BMPR2 is necessary for E2-mediated cytoprotective signaling in cardiomyoblasts.

- E2
  - ERα
    - BMPR2
      - Apelin
        - Phospho-ERK signaling

* p<0.05 vs scr control,
$ p<0.05$ vs si,
# vs Stauro
Is this E2-mediated pathway active in human RVs?

Summary: *In Vivo And In Vitro Data*
Apelin Expression Correlates With Cardiac Output, ERα, And BMPR2 In Human RVs

- **ER-alpha**
  - Correlation with Cardiac Output: $R = 0.35$
  - Correlation with Protein Absorbance: $R = 0.62$
  - $R^2 = 0.1345$

- **Apelin**
  - Correlation with Cardiac Output: $R = 0.6569$, $p = 0.02$
  - Correlation with Protein Absorbance: $R = 0.7887$, $p = 0.0223$
  - $R^2 = 0.3718$

- **BMPR2**
  - Correlation with Apelin: $R = 0.7887$, $p = 0.0223$
ERα, BMPR2, Apelin And APJ Protein Expression Is Increased In RVs From PAH Patients With Decompensated RV Hypertrophy
Apelin Downstream Signaling Is Altered In Decompensated Human RVs And SuHx Rat RVs
Putting The Human RV Data Into Context

Understanding the mechanism/context of E2-mediated RV-protective effects may lead to new therapeutic interventions targeting the RV in PAH.
Summary and Current Working Model

Cardiomyocyte

E2 → ERα → BMPR2 → Apelin → RV cardioprotective effects

SA1: ↑Bmpr2, ↑apelin

SA2: ↑RV contractile function

SA3: ↑collagenase, ↓lysyl oxidase

↓PA collagen content and cross-linking

↓PA compliance

↑PA-PA coupling and contractile reserve

↑Exercise capacity
Acknowledgments

Funding:
VA Merit Review Award 1I01BX002042-01A2, Catherine and Lowe Berger and Pauline L. Ford Chair, NIH 5T32HL091816-05, CTSI Postdoctoral Fellowship 5TL1TR001107-02 (NIH/NRSA)

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Andrea Frump, PhD
Bakh Yakubov, PhD
Marjorie Albrecht
Amanda Fisher
Todd Cook
Hope Luker
Allison Abel

Sebastien Bonnet, PhD,
Steeve Provencher, MD
(Laval Univ)
Human RV tissue,
Bmpr2 KO

Layton Smith, PhD
(Sanford Burnham Prebys Institute)
Apln, Apinr KO

Naomi Chesler,
PhD (Univ of Wisconsin)
PA stiffness, RV mechanics

Jeff Dodge, PhD
Henry Bryant, PhD
(Lilly)
ER modulators

Kara Goss, MD (Univ of Wisconsin)
Effects of prematurity on PH and RV

Matthias Clauss, PhD
EMAP II in PH

Vinicius de Jesus Perez, PhD
(Univ of Wisconsin)
RV angiogenesis

Xin Sun, PhD (UCSD)
Dustin Rubinstein
(Hypoxia, lung development)

Robert Tepper, MD,
PhD
ER modulators

Beth Brown, PhD
Exercise in PH and RV dysfunction

Jeff Kline, MD
RV function in PE and PH
Generation of ERα knockout rat

Rationale: Lack of good mouse models for study of RV failure

Collaboration with N. Chesler, X. Sun, D. Rubinstein; Univ of Wisconsin
Isolation of primary rat RV cardiomyocytes
Pulmonary Vascular Remodeling Is Abrogated In E2-replete SuHx Rats
E2 increases capillary density in the failing RV

- Normoxia
- SuHx
- SuHx OVX
- SuHx OVX + E2

Number of Capillaries/Field

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Intact</th>
<th>OVX</th>
<th>OVX + E2</th>
</tr>
</thead>
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Lectin, WGA, DAPI

A. Frump
H. Luker
M. Albrecht
E2 increases tube formation in cultured human cardiac endothelial cells

B. Yakubov
E2 increases apelin expression in human cardiac endothelial cells

**Apelin**

E2 (nM) - 10 100

<table>
<thead>
<tr>
<th>E2(nM)</th>
<th>0</th>
<th>10</th>
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<tr>
<td>17kD</td>
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<tr>
<td>42kD</td>
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**β-Actin**

**BMPR2**

E2 (nM) - 10 100

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<th>E2(nM)</th>
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Is there a E2-BMPR2-apelin axis in the RV?

**BMPR2 increases apelin in PAECs**

- **A**
  - hPAEC mRNA
  - APLN mRNA (Rel. to 18S)
  - C, β-catenin, BMPR2 siRNA

- **B**
  - hPAEC protein
  - Apelin (< 16 kDa), Tubulin (< 50 kDa)
  - C, β-catenin, BMPR2 siRNA

**BMPR2 is required for cardiac development**

- **E12.5**
  - Control
    - A, Ao, P
  - Mutant
    - B, Ao, P

- **E14.5**
  - Control
    - C, D, E
  - Mutant
    - F, G

**BMPR2 promoter has an ERE**

- Alastalo TP. JCI 2011.
BMPR2 increases apelin in absence of E2 treatment
Apelin and its receptor are expressed in the RV vasculature

M. Albrecht
Apelin increases tube formation and cell migration in human cardiac endothelial cells

Pyr-Apelin 13 (10 nM; 4h)
Apelin is increased in RVs from steers raised at high altitude that have RV failure.

RNA-seq on RV tissue
RV is embryologically, structurally, physiologically and biochemically distinct from LV.

- Secondary vs. primary heart field
- Thin walled, crescent-shaped
- Muscle mass and stroke work 1/6 of LV

Custom-built to accommodate changes in preload in order to maintain adequate perfusion of pulmonary vasculature:
- RV > LV compliance
- Better adaption to volume overload states
- Afterload sensitivity

Voelkel NF. Circulation 2006.
17β-estradiol (E2) exerts disparate effects on the pulmonary vasculature and RV.

E2 is cardioprotective in the left ventricle


<table>
<thead>
<tr>
<th>ERα</th>
<th>ERβ</th>
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<tbody>
<tr>
<td>Increases left ventricular mass and volume&lt;sup&gt;50&lt;/sup&gt;</td>
<td>Reduces pathological cardiac hypertrophy&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reductions of infarct size after myocardial infarction&lt;sup&gt;50&lt;/sup&gt;</td>
<td>Prevents increases mortality in chronic heart failure&lt;sup&gt;51,52&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cardioprotection against ischemia-reperfusion injury&lt;sup&gt;53-55&lt;/sup&gt;</td>
<td>Cardioprotection against ischemia-reperfusion injury&lt;sup&gt;41&lt;/sup&gt;</td>
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<tr>
<td>Regulates GLUT4 expression&lt;sup&gt;56&lt;/sup&gt;</td>
<td>Regulation of vascular function and blood pressure&lt;sup&gt;57&lt;/sup&gt;</td>
</tr>
<tr>
<td>Regulates cardiac growth&lt;sup&gt;58&lt;/sup&gt;</td>
<td>Modulates sex-specific response of the heart to exercise&lt;sup&gt;59&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Decreases inflammatory response&lt;sup&gt;41&lt;/sup&gt;</td>
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ER indicates estrogen receptor; and GLUT4, glucose transporter type 4.