Molecular Basis For Potential Therapeutic Targets in Uterine Leiomyoma

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Endometrium

Myometrium

**Myofibroblastic Transformation**

**Normal State**

ECM Dysregulation

Excessive Production → Reduced Degradation → Aberrant Synthesis

**Disease State (Leiomyoma)**

Aberrant Mechano-transduction

Integrin β1 → RhoA

RhoA Kinase (ROCK)

MicroRNA: ECM/tissue remodeling & collagen gene targets

- miRNA 200c
  - ↑ FBLN5
  - ↑ TIMP2
- miRNA 29b
  - ↑ COL1A1
  - ↑ COL3A1

Collagen Components

- TGFβ3
  - ↑ Collagen 1A1
  - ↑ Fibronectin
  - ↑ CTGF
  - ↑ Versican 0,1

**Signaling Pathways (Profibrotic)**

- ↓ SMAD7
- ↑ SMAD2
- ↑ SMAD3
- TGFβ3

- ↑ PDGF
- 17β Estradiol
- Progesterone pathway

**ECM Cell-Cell Interaction**

- ↓ Dermatopontin, ↑ Integrin β1

**Tissue Turnover:** ECM resorption/degradation

- ↑ MMP2, ↓ MMP11
- TGFβ3
- TGFβ3
- ↑ MMP2, ↑ MMP9
- Relaxin
- Relaxin
- ↑ Collagen
- Proliferation
- MAPK

**FGF 1&2**
Potential molecular targets

- GFs and interaction with their receptors
  - hyperactivated cell signaling pathways
  - dysregulated ECM deposition and destruction

- differential/aberrant miRNA expression
GFs implicated in the development and progression of leiomyoma

EGF, HB-EGF, IGF, acidic FGF, basic FGF, TGF\(\alpha\), TGF\(\beta\), VEGF, PDGF, CTGF, etc..
Transforming Growth Factor $\beta$ (TGF$\beta$)

- a dimeric polypeptide
- contribute to proliferation and differentiation
- 3 isoforms exist, TGF$\beta$1-3
- TGF$\beta$3 is the most abundant of the isoforms found in reproductive tissues
- high levels of TGF$\beta$3 in LM relative to Myo
TGFβ3 increases the expression of ECM components

- collagen
- fibronectin
- versican
- fibromodulin
Co-factor Smad

Smad 2/3

Smad 4

P

Smad 2/3

Smad 4

P

Co-factor Smad 2/3

Smad 4

P

Cell proliferation

Differentiation

Apoptosis

Angiogenesis

Canonical TGF signaling

TGFβ
Cell proliferation, Differentiation, Apoptosis, Angiogenesis

Non Canonical TGF Signaling

TGFβ

PTEN

PIPK2

PIPK3

p110

p85

PD

K

AKT

P

mTOR

SK6

RAF

RAF

MEK

MAP

K

JAK

STAT
Impact of TGFβ3 treatment on hMC and LMC
TGF-βRI Inhibition in Eker Rats

SB-525334  ALK5/type I TGF-bR kinase inhibitor

Significantly decreased tumor incidence and multiplicity, and reduced the size of the mesenchymal tumors

Laping et al. 2007, Clin Can Res
Epidermal Growth Factor (EGF)

LMW polypeptide
growth, proliferation and differentiation

Controversy related to its expression in Myo Vs. LMC

EGF induces proliferation in LMC

Activation of Ras/MEK/ MAPK signaling in LMC

EGFRi and MEKi inhibit proliferation
Differential effects of EGF on hSMC and hLMC

Ren et al. (Fertility and Sterility, 2011)
Fibroblast Growth Factor (FGF)

Basic FGF
- overexpressed in LMC relative to SMC
- promotes proliferation
- promotes angiogenesis
- stimulator of collagen and GAGs biosynthesis
- multiple receptors

FGF receptor-1
- abnormally regulated in the endometrium of women with LM
FGF

PTEN

PIPK2

PIPK3

p110

p85

FGFR

RA

S

RAS

RAF

MEK

MAPK

AKT

mTOR

SK6

Cell proliferation
Survival
Angiogenesis
Many Common targets activated by a number of GFs

- BKM 120
- GDC -0941
- BYL- 719
- MK2206
- Selective mTORi
- PF-04691502
- Everolimus
- Ridoforlimus

- SK6

- p110
- p85

- PTEN

- PIP
- PIP

- RAS

- RAF

- MEK

- MAPK

Cell proliferation
Survival
Angiogenesis

Pan mTORi
AZD 8055
Rapamycin
INK128

AZ 6244
PD0325901

BKM 120
GDC -0941
BYL- 719
MK2206
SK6

p110
p85
PTEN
PIP
PIP

RAS
RAF
MEK
MAPK

Cell proliferation
Survival
Angiogenesis
Selective vs Pan inhibitors

TORC1/2 inhibitor

TORC1 inhibitor
Many Common targets activated by a number of GFs

**PTEN**

**PI3K**

**p110**

**p85**

**AKT**

**TORC1**

**TORC2**

**SK6**

**RAS**

**RAF**

**MEK**

**MAPK**

**Cell proliferation**

**Survival**

**Angiogenesis**

Selective mTORi

PF-04691502

Everolimus

Ridoforlimus

Pan mTORi

AZD 8055

Rapamycin

INK128
Extrapolation potential of current findings

**In vitro models**
- Long term established cell lines (immortalized)
- Short term cultured primary lines
- Mono layer vs. 3D cultures
- Serum vs. non serum

**In vivo models**
- Immunocompromised mice hosting primary human LM tissue or cells
- Humanized mouse models
- Genetically modified mouse models
- Ecker rat model
Target Population

Utilizing specific cell signaling inhibitors may prove challenging.

Impact on non-leiomyoma cells
Toxicities (single agent or in combination)
Durability
Reproductive considerations
MicroRNAs (miRNA)

- Novel class of stable small RNAs (20-24 nt)
- > 1000 miRNAs in human genome
- Target approximately 60% of mammalian genes
- Role in gene silencing
- Post transcriptional regulation (multiple targets*)
- Emerging role as biomarker in various diseases
  - Aberrant expression in fibroids compared to myometrium
  - Potential biomarker: natural history and clinical presentation
  - Possible future therapeutic target
Transcription of miRNA gene leads to the production of Pri-miRNA. Subsequently, Drosha and DGCR8 catalyze the cleavage of Pri-miRNA, yielding Pre-miRNA. Pre-miRNA is then exported from the nucleus to the cytoplasm through the action of Exportin 5. In the cytoplasm, Dicer cleaves the pre-miRNA into a mature miRNA:miRNA duplex. This duplex is separated by a helicase, allowing mature miRNA to be incorporated into the miRNA RISC complex. The RISC complex then targets specific messenger RNA (mRNA) molecules via sequence complementarity, leading to translational inhibition or degradation of the target mRNA.
Clinical miRNA Applications in Human Disease

Murine amyotrophic lateral sclerosis (ALS, Lou Gehrig’s disease)
- SOD1-G93A mice
- Increase of miR 206 with disease progression
- Aberrant miR 206 expression in presymptomatic stage
- ALS patient cohort with elevated CSF miR 206
  
  Toivonen JM et. al., PloS one 2014

Murine neuroblastoma orthotopic model
- miR 380-5p overexpression-poor clinical prognosis
- *In vivo* delivery of miR 380-5p inhibitor
  - Upregulation of p53
  - Reduced tumor volume
  - Induction of apoptosis

  Swarbick A et. al., Nat Med 2010

Translational application of miRNA in chronic HCV
- First reported miRNA human trial
- Phase 2a multicenter RCT
- miR 122 inhibitor (Miravirsen™)
- Dose dependent HCV genotype 1 VL reduction
  - Janssen HL et. al., NEJM 2013

Diagnostic Biomarker

Predictive Biomarker

Therapeutic Target

Diagnostic Biomarker

Predictive Biomarker

Therapeutic Target
miR-21  miR-206  miR-1  miR-133  miR 200  miR-18a
miR-206  miR-221  miR-192  miR-17-92  miR-18a

Let-7  miR-206  Let-7f  miR-378
miR-20a  miR-27a  miR-27b  miR-26a
miR-222  miR-221  miR-291  miR-20a
miR-192  miR-24-1  miR-222  miR 200
miR-15b  miR-199a  miR-16  miR-106

Cyr61  TSP-1  IL-11  TGF-β  E2F1  c-myc  GM-CSF
CTGF  IL-11  HMGA1  HMGA2  MyoD  Myt-1  CDK9  Caspase
Ras GTPase-activating protein

VEGF  TSP-1  c-kit  eNOS  endothelin
FGF  HIF  adrenomeullin  CTGF

ECM accumulation  Cell cycle progression / hypertrophy  Angiogenesis  Inflammation

Tissue fibrosis  Leiomyoma Growth

Adapted from Luo et al. 2008
<table>
<thead>
<tr>
<th>miRNA expression (fibroid vs myometrium)</th>
<th>Predicted target genes</th>
<th>Target genes validated in fibroids</th>
<th>Proposed function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>miRNA 34a</strong></td>
<td><strong>GAS1, CDK4, CDK6, E2F3</strong></td>
<td>---</td>
<td>Cellular proliferation, anti-apoptosis</td>
<td>Marsh et al. (2008) Ma et al. (2013) Wang et al. (2007)</td>
</tr>
<tr>
<td><strong>Let-7 family</strong></td>
<td><strong>HMGA2, TSC1, IMP-1, IMP-3, IRS2</strong></td>
<td><strong>HMGA2</strong></td>
<td>Cellular proliferation</td>
<td>Peng et al. (2008) Klemke et al. (2010) Wang et al. (2007)</td>
</tr>
<tr>
<td><strong>miR200 family</strong></td>
<td><strong>ZEB1/ZEB2, TIMP2, FBLN5, VEGFA, TUBB, CYP1B1, CTBP2, MAF, BCL2, CITED2, LASS6, PHF21A, TSC22D1, ATXN1, JUN, NFLB</strong></td>
<td><strong>ZEB1/ZEB2, TIMP2, FBLN5, VEGFA, TUBB, CYP1B1, CTBP2</strong></td>
<td>Cellular transition, angiogenesis, matrix remodeling</td>
<td>Chuang et al. (2012) Zavadil et al. (2010)</td>
</tr>
<tr>
<td><strong>miRNA 93/106B</strong></td>
<td><strong>F3, IL8, CTGF, PAI-1</strong></td>
<td><strong>F3, IL8, CTGF, PAI-1</strong></td>
<td>Inflammation and tissue turnover</td>
<td>Chuang et al. (2012)</td>
</tr>
<tr>
<td><strong>miRNA 30a</strong></td>
<td><strong>FLAGL2, RAR, SMARCD2, SLC29A3, HLF, MAP2K5, TNXA</strong></td>
<td>---</td>
<td>Oncogene</td>
<td>Wang et al. (2007) Zavadil et al. (2010)</td>
</tr>
<tr>
<td><strong>miRNA 23b</strong></td>
<td><strong>PPARG, HIVEP2, GATA2, FOSB</strong></td>
<td>---</td>
<td>Oncogene</td>
<td>Wang et al. (2007) Zavadil et al. (2010)</td>
</tr>
<tr>
<td><strong>miRNA 27a</strong></td>
<td><strong>TSC1, RXR, RAR, IGF1</strong></td>
<td>---</td>
<td>Tumor suppressor</td>
<td>Zavadil et al. (2010)</td>
</tr>
<tr>
<td><strong>miRNA 29B</strong></td>
<td><strong>TRAF4, COL1A2</strong></td>
<td>---</td>
<td>Inflammation, extracellular matrix</td>
<td>Wang et al. (2007) Zavadil et al. (2010)</td>
</tr>
<tr>
<td><strong>miRNA 197</strong></td>
<td><strong>RNPC1, TNRC5, ESR1, GLUR2, PI3K, IGFBP3, GRIP1</strong></td>
<td>---</td>
<td>Tumor suppressor</td>
<td>Wang et al. (2007) Zavadil et al. (2010)</td>
</tr>
<tr>
<td><strong>miRNA 212</strong></td>
<td><strong>COL1A1, E2F5, HB-EGF, CDKN1A</strong></td>
<td>---</td>
<td>Extracellular matrix</td>
<td>Wang et al. (2007)</td>
</tr>
</tbody>
</table>

Adapted from Karmon et al. Hum Reprod Update 2014
Biologic Function

Cellular phenotype following miR 200c gain of function (GOF)

Adapted from Chuang T. et al. Endocrine Related Cancer 2012
miRNA Expression Signatures
Myometrium vs. Fibroids

- 360 miRNAs detected per sample
- 149/742 common miRNA detected in all serum samples

Fibroids: 2-5 cm
1 cm from outer capsule
Myometrium 2 cm from fibroid
Processed within 30 min

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<table>
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<tbody>
<tr>
<td></td>
<td>37 fibroids</td>
<td>15 myometrium</td>
</tr>
<tr>
<td>CC:</td>
<td>25 fibroids</td>
<td>12 myometrium</td>
</tr>
<tr>
<td>AA:</td>
<td>12 fibroids</td>
<td>3 myometrium</td>
</tr>
</tbody>
</table>

Figure 2. Graphical illustration of the number of microRNAs detected. On average, 360 microRNA were detected per sample.
Combined Population
Myometrium (N=15) vs. Fibroid (N=37)

Significant Clustering for Myometrium vs Fibroids

108 differentially expressed fibroid miRNA species (myometrium control)

<table>
<thead>
<tr>
<th>miRNA species</th>
<th>Expression (relative to control myometrium)</th>
<th>Gene /Signaling Pathway Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-21-5p</td>
<td>↑</td>
<td>SMAD2, TGFβ1, TGFβR1, TGFβR2 VEGF-A, REST, PTEN,</td>
</tr>
<tr>
<td>hsa-miR-34a-5p</td>
<td>↑</td>
<td>Notch-1, GAS1, HMGB1, PI3/AKT</td>
</tr>
<tr>
<td>hsa-miR-335-5p</td>
<td>↑</td>
<td>Rb, TGFβ1</td>
</tr>
<tr>
<td>hsa-miR-15b-5p</td>
<td>↑</td>
<td>RECK, SMAD7, TGIF2</td>
</tr>
<tr>
<td>hsa-miR-140-5p</td>
<td>↓</td>
<td>VEGF-A, SMAD3, MMP13, COL1A1</td>
</tr>
<tr>
<td>hsa-miR-142-3p</td>
<td>↓</td>
<td>TGFβR1, VEGF-A, IL10</td>
</tr>
<tr>
<td>hsa-miR-29c-5p</td>
<td>↑</td>
<td>mTOR, VEGF-A, PI3K/AKT</td>
</tr>
<tr>
<td>hsa-miR-126-5p</td>
<td>↓</td>
<td>TGFβR2, VEGF-A, PECAM,1 CASP3</td>
</tr>
<tr>
<td>hsa-miR-150-5p</td>
<td>↓</td>
<td>BCL2,HMG2A,IL6, FGF7</td>
</tr>
<tr>
<td>hsa-miR-193a-5p</td>
<td>↓</td>
<td>TGFβ1, TNF, IFNG</td>
</tr>
</tbody>
</table>
Functional Studies

Lentiviral-mimic-infection (hCMV promoter, GFP reporter)

Missense miR (NC) Control

miR 21 mimic

Immortalized myometrial and fibroid cell line (Catherino et al. [NIH/NICHD])

? Impact of ↑ miR 21 and 15 b

Normal State

Myometrium

Disease State (Leiomyomata)
Identification and confirmation of differential myometrial/leiomyoma miRNA expression

Prediction and confirmation of miRNA gene targets and gene products

In vitro miRNA functional studies

Matched primary myometrial & leiomyoma cells

Anti-miRNAs

miRNA mimics

Immortalized myometrial & leiomyoma cell lines

In vivo miRNA functional studies

Assessment of miRNA “gain and/or loss of function”:

- miRNA gene targets and protein expression
- Fibroid relevant endpoints: proliferation, apoptosis, angiogenesis, ECM deposition, tissue turnover, target oncogene expression
- Recapitulation or regression of fibroid cellular phenotype

Confirmation of miRNA biologic function

Development of miRNA biomarkers

miRNA targeted therapeutics
Challenges in miR strategies as therapeutics

- Applicability of miRNA gene targets from cancer biology to fibroid biology
- Multiplicity of miRNA gene targets and signaling pathways
- Emerging understanding of sex steroid/miRNA interactions
- Translation from *in vitro* loss/gain of miRNA function to clinical phenotype
- Delivery systems
- Diagnostic and predictive biomarker applications
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