Angiomodulin is required for cardiogenesis of embryonic stem cells and is maintained by a feedback loop network of p63 and Activin-A

Zohar Wolchinsky\textsuperscript{a,1}, Shoham Shivtiel\textsuperscript{a,1}, Evelyn Nathalie Kouwenhoven\textsuperscript{b}, Daria Putin\textsuperscript{a}, Eli Sprecher\textsuperscript{c}, Huiqing Zhou\textsuperscript{b,d}, Matthieu Rouleau\textsuperscript{e,f}, Daniel Aberdam\textsuperscript{a,g,h,*}

\textsuperscript{a} INSERTECH, Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel
\textsuperscript{b} Department of Human Genetics, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centrum, The Netherlands
\textsuperscript{c} Department of Dermatology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel
\textsuperscript{d} Department of Molecular Developmental Biology, Radboud University Nijmegen, The Netherlands
\textsuperscript{e} Laboratoire de PhysioMédecine Moléculaire, CNRS FRE-3472, Nice, France
\textsuperscript{f} University of Nice-Sophia Antipolis, France
\textsuperscript{g} INSERM U976, Paris, France
\textsuperscript{h} University of Paris Diderot, France

Received 16 June 2013; received in revised form 23 September 2013; accepted 27 September 2013
Available online 6 October 2013

Abstract The transcription factor p63, member of the p53 gene family, encodes for two main isoforms, TAp63 and ΔNp63 with distinct functions on epithelial homeostasis and cancer. Recently, we discovered that TAp63 is essential for in vitro cardiogenesis and heart development in vivo. TAp63 is expressed by embryonic endoderm and acts on cardiac progenitors by a cell-non-autonomous manner. In the present study, we search for cardiogenic secreted factors that could be regulated by TAp63 and, by ChIP-seq analysis, identified Angiomodulin (AGM), also named IGFBP7 or IGFBP-rP1. We demonstrate that AGM is necessary for cardiac commitment of embryonic stem cells (ESCs) and its regulation depends on TAp63 isoform. TAp63 directly activates both AGM and Activin-A during ESC cardiogenesis while these secreted factors modulate TAp63 gene expression by a

☆ Author Contributions: Z.W. and S.S.: conception and design, collection and assembly of data, data analysis and interpretation, final approval of manuscript; E.N.K., D.P., H.Z.: collection and assembly of data, final approval of manuscript; M.R.: collection and assembly of data, data analysis and interpretation, final approval of manuscript; D.A.: conception and design, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript.

* Corresponding author at: INSERM U976, Hôpital St-Louis, 1 Av. Vellefaux, 75010 Paris, France.
E-mail address: daniel.aberdam@inserm.fr (D. Aberdam).

Participate equally to this work.

1873-5061 © 2013 The Authors. Published by Elsevier B.V. Open access under the CC BY-NC-ND license.
http://dx.doi.org/10.1016/j.scr.2013.09.015
Introduction

Early mammalian cardiogenesis require precise cross-interactions between mesodermal and endodermal cells through cell-autonomous and cell-non-autonomous events. The latter depends on both neighboring and long-range signals of secreted molecules. Cardiac myogenesis depends largely on the instructive activity of the pharyngeal (anterior) endoderm (Van Vliet et al., 2012). The embryonic endoderm plays an essential role in cardiogenesis through the secretion of various morphogens such as fibroblast growth factors (FGFs), Wnt and transforming growth factors (TGF). The absence of one of these molecules disrupts proper cardiogenesis (Alsan and Schultheiss, 2002; Sugi and Lough, 1995).

Recently, we identified a role of p63, a transcription factor of the p53 gene family, in murine cardiogenesis and heart development (Rouleau et al., 2011). The p63 gene encodes for two main isofoms, TAp63 and ΔNp63 with distinct gene expression profiling and opposite functions on epithelial homeostasis and cancer (Vanbokhoven et al., 2011). While TAp63 controls cell apoptosis and aging, ΔNp63 is implicated in cell proliferation, cell adhesion (Crum and McKeon, 2010) and mandatory for epidermal commitment of the embryonic ectoderm during the embryonic skin formation (Koster et al., 2004; Shalom-Feuerstein et al., 2011). We found that p63 null mice suffer from severe congenital cardiopathy and demonstrated that TAp63 is an endodermal transcription factor that controls, in a cell-non autonomous manner, cardiac differentiation of embryonic stem cells (ESC) (Rouleau et al., 2011). Accordingly, the END2 cell line that is derived from embryonic mouse endoderm and displays cardiogenic activity (Begqali et al., 2006), expresses endogenous TAp63. Absence of TAp63 in ES cells did not interfere with the commitment of mesodermal cardiac progenitors (mesP1+) but prevented further differentiation of cardiac progenitors to mature cardiomyocytes (Rouleau et al., 2011).

Here we identified a new molecular circuitry by which endodermal TAp63 controls cardiogenesis. We show for the first time that Angiogenesis (AGM), also known as IGFBP7 or IGFBP-rP1, a secreted protein that regulates cellular proliferation (Nousbeck et al., 2010), adhesion, and angiogenesis (Duan et al., 2010), is necessary for ESC cardiogenesis and that its expression is modulated by a strict feedback loop orchestrated by TAp63 isoform and Activin-A.

Material and methods

Cell lines

All cell lines were cultivated at 37 °C in 5% CO2. The mouse CGR8 embryonic stem cell line, the ES sh-p63 clones and the culture condition used in this study have been described previously (Medawar et al., 2008). END2 cells were maintained as described previously (Rouleau et al., 2011). HeLa and C2C12 cells were maintained in Dulbecco’s modified Eagles’ medium supplemented with 10% fetal bovine serum.

Cardiomyocyte differentiation of mES cells

mES cells were cultured in the presence of 2.5 ng/ml BMP2 (Peprotech) in regular FCIII (Hyclone) medium for 2 days. Then the cells were trypsinized and placed in hanging drops containing 500 cells in 20 μl in differentiation medium (DMEM supplemented with 20% FCS serum (FCII, Hyclone), 1 mM non-essential amino acids, 1 mM Sodium Pyruvate, 0.1 mM β-mercaptoethanol and 2.5 ng/ml BMP2). The drops were placed on the lids of bacteria Petri dishes filled with PBS. After 3 days, the embryonic bodies (EBs) formed in the hanging drops were collected and resuspended in 5 ml medium in 60 mm bacteriological Petri dishes and cultivated for further 2 days in suspension. At day 5 of the differentiation, 8–10 EBs were plated onto one well of gelatin-coated 24 well plates or on cover slips for immunofluorescence staining in differentiation medium without serum, for another 5 days. siRNA transfection targeting AGM (50 nM) was performed 2 days before hanging drop formation, and on day 5 of the differentiation using lipofectamin RNAiMAX (Invitrogen). Recombinant AGM (1 μg/ml, R&D systems) was added at day 0 of cardiomyocyte differentiation for 3 days. For Activin A treatment, ESCs were treated from 1 day before differentiation and during the entire differentiation process with 5 ng/ml Activin A (R&D) (Rouleau et al., 2011). For cardiomyocyte differentiation on END2 cells, END2 cells treated with siRNA against TAp63, AGM or si scramble as a control using lipofectamin RNAiMAX (Invitrogen). Sh-p63 ES cells (Rouleau et al., 2011) seeded on END2 treated cells on day 5 of the differentiation or on gelatin coated plates as a control.

ChIP-seq and ChIP-qPCR analysis

Cells were cross-linked for 10 min at room temperature with 1% (v/v) formaldehyde and quenched with 125 mM glycine. Chromatin was sonicated to 200–500 bp using a Bioruptor sonicator (Diagenode) for 15 min at high power, 30 s ON, 15 s OFF. The chromatin was immunoprecipitated with 1 μg of anti-p63 mAb (4A4; Sc-8431, Santa Cruz Biotechnology). Eluted DNA was recovered by phenol–chloroform extraction, precipitated with ethanol and resuspended in TE buffer. ChiP material was analyzed by Real Time PCR using SYBR SensiMix (Bioline) and specific primers to detect enrichment in the denoted genomic regions. The amount of precipitated DNA was calculated as fold enrichment of % of input in p63 ChiP against % of input in mIgG ChiP and normalized against a control region (no gene region in chr15). The results expressed as ±standard deviation from three experiments compared with the starting input material as percentage of input. ChiP-seq analysis
was performed on a Solexa Genome Analyzer (Illumina) as described previously (Medawar et al., 2008).

**ChIP-seq data analysis, de novo motif search and p63scan**

All 32-bp sequence reads were uniquely mapped to the mouse genome NCBI 37 build (mm9) using ELAND (Illumina), resulting in 24 million unique mapped reads. Peak recognition was performed using MACS (Zhang et al., 2008) with default settings using a dynamic local lambda as background and a p-value threshold of 1E-05, 1E-07, and 1E-09, giving 22,103, 15,524, and 11,329 peaks, respectively. p63 binding peaks were mapped to RefSeq genes, downloaded from the UCSC Genome Browser (mm9), to determine genomic location. The ChIP-seq data and associated peaks have been deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002).

A de novo motif prediction pipeline combining three motif prediction tools, MotifSampler (Thijs et al., 2001), Weeder (Pavesi et al., 2004), and MDMmodule (Liu et al., 2002), was run on 2265 (20%) randomly selected 200-bp peak sequences (centered at the peak summit as reported by MACS) and PWMs were generated (van Heeringen and Veenstra, 2011). We used the ‘large’ analysis setting for Weeder. The remaining settings were similar as described previously (Kouwenhoven et al., 2010). The previously established p63 motif prediction tool, p63scan, was used to identify p63 motifs in the detected p63 binding sites (Kouwenhoven et al., 2010).

**Antibodies, immunofluorescence and FACS analysis**

Immunoblotting, FACS, and immunofluorescence analysis have been described previously (Rouleau et al., 2011; Medawar et al., 2008). The primary antibodies used for immunofluorescence are mouse anti IGFBP7 (R&D), rabbit anti p63 (abcam ab30309) and mouse anti TropominT2 (clone Ab-1, MS-295-PO, LabVision) following by Alexa-488 anti-mouse, Alexa 546 anti-mouse and anti Alexa 488 anti-rabbit as secondary antibodies (Invitrogen). Images were taken by Zeiss LSM 710 confocal microscope. TropominT2 antibody was used also for FACS analysis following by Alexa-488 anti-mouse as secondary antibody (Invitrogen). FACS analysis samples were analyzed with CellQuest on FACScalibur (Becton Dickinson).

**RNA extraction and real-time PCR analysis**

Total RNA was isolated using Aurum RNA kit (Biorad, Hercules, CA, USA) according to manufacturer’s instruction followed by iScript kit (Biorad) for single strand cDNA synthesis. qRT-PCR was performed using SYBR SensiMix (Bioline, London, UK). Each gene was amplified using the appropriate specific primers and normalized against GAPDH.

**Transfections and treatments in vitro**

END2 and HeLa cells were seeded in 30% confluency and transfected with siRNA to AGM (50 nM) using lipofectamin RNAiMAX (Invitrogen) and incubated for 3 days. Overexpression of TAp63 was induced in END2 cells by transfection of pCDNA3 plasmid encoding TAp63-γ (Rostagno et al., 2010) using JetPEI reagent (Polyplus transfection). RNA extractions were performed as described above.

**Immunoprecipitation**

END2 and HeLa cell lines were seeded in 50% confluency in 5% FCS medium and transfected using lipofectamin RNAiMAX reagent (Invitrogen) for 48–72 h with siRNA to AGM (50 nM). Alternatively, HeLa and END2 cells were transfected using JetPEI reagent (Polyplus transfection) with pCDNA3.1 plasmids cloned with TAp63-γ-expressing insert (Rostagno et al., 2010). Conditioned media were collected from each well and incubated with protein A beads (BioRad) pre-coated with anti-AGM (R&D Biosystems, 5 μg/sample). Cell lysis (cellytic, Sigma) and total protein concentrations were determined to confirm equal numbers of cells in each well. Purified precipitated proteins were resolved by 12% acryl amide gel electrophoresis under reducing conditions, transferred onto nitrocellulose membranes, and blocked with 5% milk (powder from BioRad). Membranes were immunoblotted with anti-AGM (R&D Biosystems), followed by anti-mouse HRP.

**Luciferase assay and plasmids**

A 209 bp sequence which contains p63-binding site segment from the region of Activin enhancer (identified as bound by p63 by Chip-seq analysis) and 158 bp sequence lacking the p63 binding site were isolated by PCR and sub-cloned into a luciferase reporter pGL2-basic vector (Promega) to generate pGL2-Activin and pGL2-Activin-del plasmids respectively. 189 bp of AGM enhancer which contains p63 binding site as well as 183 bp that lacks the p63 binding site were isolated by a PCR and sub-cloned into a luciferase reporter pGL3-promoter vector (Promega). Luciferase constructs were co-transfected into HeLa cells along with expression plasmids encoding for TAp63-γ or ΔNp63α isoforms using JetPEI reagent (Polyplus transfection). Protein lysates were extracted 24 h later with a Dual Luciferase kit (Promega) and reporter assays were performed according to manufacturer’s instruction. Plasmid pRL-SV40 (Promega) encoding Renilla luciferase served as an internal control. Firefly luciferase activity was normalized to Renilla luciferase activity in the same cell extract and plotted as a ratio of firefly/Renilla luciferase activity.

<table>
<thead>
<tr>
<th>Real time primers</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGAPDH for</td>
<td>CCGGGAACCTGTGGCGTGATGG</td>
<td>GAGTGGAGAGGCTGGTGCTCGTT</td>
</tr>
<tr>
<td>mGAPDH rev</td>
<td>AGTCAGATGAGTTGCTGGTT</td>
<td>GTTGCCAGTGGTGTAGT</td>
</tr>
<tr>
<td>mIGFBP7 for</td>
<td>GAGCAAGGTCTTCATCATAGT</td>
<td>CGAGTACCTCAAGC</td>
</tr>
<tr>
<td>mIGFBP7 rev</td>
<td>GACCATGCGCGGGGCTTTCTCC</td>
<td>TGTATACGTGGCTTCC</td>
</tr>
<tr>
<td>mMesp1 for</td>
<td>GCGGATCGTGAGGCGCGCTCTCC</td>
<td>GCGAAGATGGGAAAGGACAA</td>
</tr>
<tr>
<td>mMesp1 rev</td>
<td>AGCAAGGTCTTCATCATAGT</td>
<td>CCGAAGATGGGAAAGGACAA</td>
</tr>
<tr>
<td>mTnT2 for</td>
<td>CGGAAAGGTGGAAAGGACAA</td>
<td>CCGAAGATGGGAAAGGACAA</td>
</tr>
<tr>
<td>mTnT2 rev</td>
<td>GCACGCGCGGCAAGGACCA</td>
<td>CCGAAGATGGGAAAGGACAA</td>
</tr>
<tr>
<td>mBrachyury for</td>
<td>GACTTCTGAGCAGGCTGACAA</td>
<td>GACTTCTGGAGTGGTAGT</td>
</tr>
<tr>
<td>mBrachyury rev</td>
<td>CGAGTCTGAGTGGTAGT</td>
<td>GACTTCTGAGTGGTAGT</td>
</tr>
<tr>
<td>mActivin A for</td>
<td>CGCAAGATGGCAGGACAA</td>
<td>CTTCTTCCCCATCTCATCA</td>
</tr>
<tr>
<td>mActivin A rev</td>
<td>CGCAAGATGGCAGGACAA</td>
<td>CTTCTTCCCCATCTCATCA</td>
</tr>
<tr>
<td>mNkx2.5 for</td>
<td>TTACCGGGGACGCTACCGGT</td>
<td>TTACCGGGGACGCTACCGGT</td>
</tr>
</tbody>
</table>

(continued on next page)
TAp63-target genes in endodermal cells

Isolated from embryonic carcinoma, END2 cells are a mix of visceral and parietal embryonic endoderm (Brown et al., 2010) (see Suppl Fig. 2B for endodermal gene profile of END2 cells). They express variable amount of TAp63 but at low level for genome wide analysis (Rouleau et al., 2011) (Suppl. Fig. 2A). To search for secreted endodermal cardiogenic factors driven by TAp63, a ChIP-seq analysis was performed on the endodermal END2 cells transfected by TAp63. To identify genome-wide TAp63-binding sites, we utilized the model-based analysis of ChIP-seq (MACS) peak detection algorithm (Kouwenhoven et al., 2010). With the p-value of 1E-09, 11,329 peaks were identified, including two well-characterized constitutive p63-binding sites near p21 and MDM2 (Suppl. Fig. 1A). The de novo motif prediction pipeline, GimmeMotifs revealed a p63-binding motif similar to those previously described (Kouwenhoven et al., 2010) (Fig. 1A). Detection of the p63 motif using p63scan showed that 97.18% of the detected peaks were found to contain the p63-binding motif, indicating that most of the detected peaks identified in this ChIP-seq were directly bound by p63. A large number of the detected peaks are located within genes (5164 peaks), whereas 1006 peaks were found in the transcription start site (TSS) flanking region, between −5 kb up to the end of the first intron of the gene (Fig. 1B). A total number of 5845 genes that are potentially regulated by p63 were identified by mapping binding sites to the closest genes. Ingenuity Pathway Analysis (IPA, Ingenuity® Systems) of these potential target genes revealed ‘cardiovascular system development and function’ as the most enriched Gene Ontology (GO) term (Fig. 1C) and ‘Factors Promoting Cardiogenesis in Vertebrates’ pathway as one of the enriched pathways (Fig. 1D). Interestingly these terms were absent from human keratinocyte ChIP seq IPA analysis (Supplementary Fig. 1B, 19). These data show that p63 may regulate different sets of target genes in endodermal and epithelial cells, and this observation strengthens our hypothesis for the involvement of TAp63 isoform in cardiogenesis.

AGM and INHBA genes are direct targets of TAp63

Among hundreds of target genes found to be bound by TAp63, we identified two secreted protein-encoding genes INHBA (encoding Activin-A, of the TGF-β family) and Angiomodulin (AGM, also known as IGFBP-7) as potential candidates for the following reasons. First, both are secreted molecules by endodermal cells that could transmit the cell non-autonomous effect of TAp63 on mesocardiac progenitor differentiation. Second, INHBA gene is known to be essential for early cardiogenesis. Suppression of the Activin pathway prevents the induction of mesodermal and cardiac markers (Noseda et al., 2011) and we showed that Activin-A could partially rescue cardiogenesis in absence of p63 (Rouleau et al., 2011). Furthermore, although not yet shown in heart development, AGM/IGFBP-7 has been recently found to be involved in the development of the cardiovascular system and angiogenesis (Abu-Safieh et al., 2011; Hooper et al., 2009).

Using primers that flank each genomic sequence, we confirmed by ChIP-qPCR analysis the specific binding of TAp63 isoform on the regulatory region of INHBA and AGM genes in END2 cells transfected with TAp63-expressing construct (Fig. 2A). We inserted a 209 bp of p63-binding sequence located in the vicinity of the regulatory regions of both genes (Suppl. Figs. 1C and D). We choose these two candidates for the following reasons. First, both are secreted molecules by endodermal cells that could transmit the cell non-autonomous effect of TAp63 on mesocardiac progenitor differentiation. Second, INHBA gene is known to be essential for early cardiogenesis. Suppression of the Activin pathway prevents the induction of mesodermal and cardiac markers (Noseda et al., 2011) and we showed that Activin-A could partially rescue cardiogenesis in absence of p63 (Rouleau et al., 2011). Furthermore, although not yet shown in heart development, AGM/IGFBP-7 has been recently found to be involved in the development of the cardiovascular system and angiogenesis (Abu-Safieh et al., 2011; Hooper et al., 2009).

Real time primers

mNkk2.5 rev      GCTTTCCGTGCGGCCGGCTGC
mα-Actinin for    CACTGAAAGCCGCGGCTGAAG
mα-Actinin rev    TTCGGAGGCTGGCAGGAGCAT
mIslet1 for       GAGCCCAACACCAAGCCAAACTA
mIslet1 rev       TTATCCTGCTGGCATTCAAC
mMef2c for        AGATACACAGCACCACACACGCGC
mMef2c rev        ACCCGGCCCAGGAGCTGAGA
mBrachyury for    GCCGCTGGGTCAGCTTCAC
mBrachyury rev    CGGTCTGGTGGATGGTAGAG
mSox17 for        CGACCAACTGAGGAGCTGCT
mSox17 rev        TGGGAAGGTGTCCTACAC
mFoxa2 for        CCCTAGCGCAACATGCACTG
mFoxa2 rev        GGTCTCAGCTGTAAGAAGGGA
mGATA4 for        CCCCGAAGCAGCGAGATG
mGATA4 rev        ACCGGTGCTCAATTTAGCTGT
mAFP for          CAGCGCTTTGCAACATAC
mAFP rev          TGGCTCAGCTTGGTGTTAGG
mVimentin for     ACCCGCTCTCAGCACCAC
mVimentin rev     CAGAGGATTAGGCTGATGAGG
mSox-7 for        GCCGCTCTGTTTTTGTGTTAGG
mSox-7 rev        GTTCCTGGCAAGCTTATTTTGCAATG

Cloning primers

I GFBP7_prom for   GCCGTAGCGCTGCAAGCTTC
IGFBP7_prom rev   TGGAGAGGTTAATCATCATAGC
IGFBP7_prom_del for TGACATGGATGTAATGAG
IGFBP7_prom_del rev CGCGAGGAGGCTGATTGGA
ActivinA_prom for  AATGGGATGGCCAGGAGTATG
ActivinA_prom rev  CACCGAGGAGGCTGATGAGC
ActivinA_prom_del for TTTAAAACACAAATGGCTGAGGCTG
ActivinA_prom_del rev GCACACAGATGTGTTAATACCG

Results

TAp63-target genes in endodermal cells

(revised with new information as indicated)
bind and compete to similar motifs as TAp63, it should confirm the specific binding and activation of TAp63 on the promoters. As illustrated in Figs. 2B–C, the reporter expression driven by p63-bound regions in both constructs behaved similarly and increasing amounts of ΔNp63 diminished the TAp63-induced activation of the respective promoter activity in a dose dependent manner. Deletion of the p63 binding site sequences (ΔINHBA-luc or ΔAGM-luc) prevented the activation of the promoters by TAp63 (Figs. 2B–C). These data suggest that TAp63 directly activates both INHBA and AGM genes. In addition, exogenous expression of TAp63 in HeLa cells activates further INHBA and AGM gene expression (Figs. 2D–E), suggesting that TAp63 directly activates both INHBA and AGM genes. Since AGM is a secreted factor, we tested by western blot its protein level in the medium of transfected cells (Figs. 2F–G). Exogenous TAp63 increased AGM secretion, confirming that AGM is under the regulation of TAp63. Those data demonstrate a co-expression of AGM and p63 and further suggest a regulation of AGM gene expression by TAp63.

Angiomodulin is necessary for ESC cardiogenesis

Since no link between AGM and ESC cardiogenesis has been reported so far, we first tested the relative expression of AGM in ESC during cardiac fate and found that AGM gene expression increased gradually during cardiac differentiation along with TAp63 gene activation (Figs. 3A–B). To test a possible role of AGM on ESC cardiogenesis, we performed siRNA experiments to knockdown its expression (si-AGM; Suppl. Fig. 3A) in ESC during cardiac commitment. AGM gene inhibition did not impact significantly the relative expression of mesocardiac early markers like Brachyury and MesP1 (Fig. 3C) but reduced the number of beating EBs (Suppl. Fig. 3B) and cardiac-related gene expression on day 5 and day 11 (Fig. 3C), similar to TAp63 knock down (Fig. 4A) (Rouleau et al., 2011). The reduction in Troponin-T gene expression was also demonstrated at the protein level by FACS analysis (Suppl. Fig. 3C) and immunofluorescence staining of Troponin-T+ cells (Figs. 3D–E) at day 10 of cardiac differentiation. To confirm that AGM acts on

**Figure 1** Identification of p63-target genes by ChIP-seq analysis of END2 cells. A. p63 consensus binding motif identified from 11,329 binding sites. B. p63 binding motif distribution relative to RefSeq genes. TSS flanking region: 5 kb upstream of TSS, first exon and first intron, intragenic: rest of the gene, <25: 5–25 kb upstream or 25 kb downstream of the last exon, intergenic: rest of the genome. C. The Ingenuity Systems Inc. (IPA) software tool was used to examine TAp63 functional pathways. Statistical enriched physiological system biology and function are shown. D. Top 5 canonical pathways statistically over-represented among TAp63 binding genes are shown. p-values were calculated by Fisher exact test, ratio is the number of molecules in a given pathway divided by the total number of molecules that make up that pathway.
cardiogenesis through p63 activation, and to test whether AGM and Activin act in a synergistic manner, recombinant AGM and recombinant Activin-A were added to the medium of shp63 ES cells at day 0 of cardiac differentiation. As expected from our previous study (Rouleau et al., 2011), absence of p63 prevented cardiogenesis. Addition of either AGM or Activin-A was able to partially rescue cardiogenesis of shp63 ES cells, as illustrated by increased number of beating EBs (Fig. 3F) and increase expression of cardiac-related genes (Figs. 3G–I). Addition of both AGM and Activin-A together enhanced further

Figure 2. INHBA and AGM are direct p63-target genes. (A). Specific binding of p63 on the regulatory region of INHBA and AGM genes were confirmed by ChIP-qPCR analysis in END2 transfected with TAp63 plasmid, using anti-p63 (4A4) antibody. p21 and Irf6 were used as positive controls, and irrelevant sequence from chromosome 15 used as a negative control (no gene). The fold enrichment is the ratio of % of input in p63 ChIP against % of input in mIgG ChIP and represent mean ± SE of 3 independent experiments. (B–C). Luciferase reporter assay for AGM (B) and INHBA (C) promoter relative activities. A fragment of the INHBA or AGM genomic region containing the p63-binding site was inserted upstream to a pGL-luciferase construct (INHBA-luc and AGM-luc, respectively) and cotransfected it along with TAp63 and ΔNp63 expressing constructs or empty vector as control. A promoter region in which the p63-binding site has been deleted (ΔINHBA-luc or ΔAGM-luc) was tested as a negative control. Promoter activities are normalized to luciferase activity of the INHBA-luc or AGM-luc promoters, respectively. Renilla plasmid was used as an internal control. Data is represented as the average of three experiments ± SE, *p < 0.05. D–E. HeLa cells were transfected with TAp63-expressing construct and tested for INHBA (D) or AGM (E) gene expression by qRT-PCR. Results of three independent experiments are represented as fold change of control treatment. *p < 0.05; **p < 0.01. F. Representative western blot analysis of secreted AGM immuno-precipitated from conditioned medium of HeLa cells transfected with constructs expressing TAp63 (or empty pCDNA vector). Relative AGM gene expression is shown in arbitrary units quantified by ImageJ. G. Representative western blot analysis of secreted AGM immuno-precipitated from conditioned medium of END2 cells transfected with constructs expressing empty pCDNA vector, TAp63 or si-AGM. Relative AGM gene expression is shown in arbitrary units quantified by ImageJ. H. Immunofluorescence detection of p63 (green) and AGM (red) in END2 cells showed co-expression of p63 and AGM. Yellow arrows indicate negative cells for TAp63 which are also negative for AGM. DAPI staining is shown in blue. Scale bar = 10 μm.
cardiogenesis. To further demonstrate that cardiogenesis depends on both endodermal TAp63 and AGM endodermal gene expression, we cultivated shp63 EBs, which have impaired the ability to undergo cardiomyocyte differentiation (Rouleau et al., 2011), on fixed END2 cells which were prior treated with siRNA against TAp63, AGM or scramble as a control. END2 cells treated with si-scramble were able to restore cardiogenesis of shp63 cells as demonstrated by the increased number of beating EBs (Suppl. Fig. 3E) and the increased expression of cardiac-related genes, as compared to shp63 EBs seeded on gelatin (Fig. 3J). In contrast, END2 cells that underwent AGM or TAp63 silencing prior to the deposition of shp63 EBs, hardly restored shp63 cardiomyocyte differentiation (Fig. 3J, Suppl. Fig. 3E). These results demonstrate that TAp63 and AGM expressed by endodermal cells are essential to cardiogenesis regulation. Both AGM and Activin-A also play a role during vascular development. Thus, we tested whether vascular endothelium fate could be altered by the absence of p63 or AGM. The results show that the expression of vascular markers Flk1 and CD31 was not significantly modified in the absence of p63 or AGM (Suppl. Fig. 3D). Altogether, these data suggest that Activin-A and AGM act in a synergistic way on cardiogenesis. It demonstrates for the first time a role for AGM during ESC cardiogenesis, partially through p63 cascade.

**Regulatory network between Activin-A and AGM is crucial for cardiogenesis and modulate p63 expression in a feedback loop**

The direct regulation of AGM and INHBA genes by TAp63 does not exclude reciprocal interaction between them during ESC cardiogenesis. To further explore the feedback interplay between TAp63 isoform, AGM and INHBA, we tested the direct role of INHBA during ESC cardiac commitment. Treatment of ES cells by SB431542, a pharmacological inhibitor of activin/nodal pathway, reduced cardiogenesis (Cai et al., 2012) (Fig. 4A). This inhibitory effect of SB431542 on cardiogenesis is amplified in differentiated ES cells in which p63 (si-TAp63) or AGM (si-AGM) has been silenced (Fig. 4A). These data suggest that TAp63 controls cardiogenesis by two synergistic AGM and INHBA pathways (Fig. 5). Previously, we have shown that ES cells in which p63 gene had been silenced (shp63) barely undergo cardiogenesis and that the addition of Activin-A could rescue it (Rouleau et al., 2011). To test a potential regulation of AGM by Activin-A, WT ES cells and shp63 ES cells were treated with recombinant Activin-A during cardiac commitment and AGM gene expression was measured at day 9 (Fig. 4B). In addition to its ability to rescue cardiogenesis (Suppl. Fig. 4), Activin-A was able to efficiently activate AGM gene expression (Fig. 4B). Interestingly, the level of AGM expression in shp63 ES cells treated by Activin-A exceeded by far that of the treated WT ES cells. Because of the absence of p63, the cell types present in the WT and shp63 ES cells at day 9 of differentiation become quite different and thus could respond differently to Activin-A for AGM gene expression. That Activin-A reduced cardiogenesis of WT ES cells suggests that balanced Activin expression in an accurate amount is important for proper signaling to promote cardiogenesis. Accordingly, it fits a previous report that overexpression of Activin induced inhibition of cardiac differentiation (van den Eijnden-van Raaij et al., 1991). These data demonstrate that p63 controls cardiogenesis by overlapping but different pathways, regulating AGM and INHBA in direct and indirect manners (Fig. 5).
We further tested whether AGM and INHBA could modulate back their regulation by TAp63. C2C12 cells, that express endogenous TAp63 isoform, were treated by either si-AGM or SB431542 and tested for TAp63 gene expression (Figs. 4C–D). Inactivation of AGM enhanced TAp63 (Fig. 4C). This regulatory loop was also found for Activin-A as its inhibition by SB431542 enhanced TAp63 expression (Fig. 4D). All these data demonstrate that TAp63 isoform regulates AGM gene directly but also indirectly through the modulation of the INHBA gene expression and that both INHBA and AGM can control their modulators by a feedback loop mechanism, balancing their required expression for proper function (Fig. 5).

Discussion

We have recently reported that the gene p63 is a new actor of early cardiac differentiation (Rouleau et al., 2011). Its main isoform TAp63 is expressed by embryonic endodermal cells to regulate cardiac differentiation of mesocardiac progenitors. As the effect of TAp63 on cardiogenesis occurs in a cell non-autonomous manner, we searched for secreted molecules that could be under the regulation of TAp63 pathway in endodermal cells and related to cardiogenesis. We identified Angiomodulin (AGM) as a direct target of TAp63 regulation during ESC cardiogenesis. Angiomodulin, also known as mac25,
IGFBP7 or IGFBP-related protein-1 (IGFBP-rP1) is a secreted protein belonging to the IGFBP family (Duan et al., 2010). AGM/IGFBP7 is known as a tumor suppressor gene for a variety of cancers (Wajapeyee et al., 2009), particularly in melanoma progression but also involved in skin pathophysiology (Nousbeck et al., 2010). Recently, IGFBP7 has been linked to the development of the cardiovascular system and angiogenesis. First, a germline mutation on AGM gene in humans was shown to lead to obstruction of the right ventricular outflow tract, a phenotype similar to that of the p63 KO mice (Abu-Safieh et al., 2011). Second, AGM inhibits vascular endothelial growth factor (VEGF)-induced angiogenesis (Hooper et al., 2009). Here we demonstrated that inhibition of AGM gene expression partially prevents ESC cardiogenesis. It validates that ChIP-seq analysis made on transfected cells, when endogenous transcription factor is lowly expressed, could mimic physiological conditions. We believe that AGM/IGFBP7 functions during advanced stages of cardiac ESC differentiation, as early mesocardiac markers were not affected by its inhibition. To our knowledge, our study is the first to demonstrate a role of AGM/IGFBP7 on cardiogenesis. However, no developmental defect, including cardiac phenotype, has been reported in IGFBP7 KO mice (unpublished data from A. Seth). This could be due to gene compensation by other members of the IGFBP family. As a matter of fact, IGFBP-4 enhances cardiomyocyte differentiation both in vitro and in vivo (Zhu et al., 2008).

IGFBPs regulate the bioavailability of IGFs by binding to IGFs with high affinity thereby limiting IGF access to IGF-IR and inhibiting IGF activity (Duan et al., 2010). However, IGFBP7 differs from the other six members of this family by having considerably lower affinity for IGF-I. Instead, it binds strongly to insulin and blocks insulin action. Interestingly, it has been reported that insulin strongly inhibits cardiac differentiation of ES cells by suppressing mesodermal fate (Freund et al., 2008) through IGF-R activation (van den Eijnden-van Raaij et al., 1991). Thus, when secreted by endodermal cells under the control of TAP63, AGM/IGFBP7 may activate ESC cardiogenesis by blocking insulin interaction with IGF-R on mesocardiac precursors to allow their normal cardiac commitment.

In addition to AGM, we identified Activin-A as a direct target gene of TAP63 regulation during ESC cardiogenesis. Activin-A, of the TGF-β family, is known to be essential for early cardiogenesis. Endodermal and mesodermal cells secrete it at early stages of mouse and human development (Brown et al., 2010). Suppression of the Activin pathway prevents the induction of mesodermal and cardiac markers. Interestingly it has been reported that over expression of Activin induced the inhibition of cardiac differentiation (van den Eijnden-van Raaij et al., 1991), demonstrating that balanced Activin expression in an accurate amount is important for proper signaling to promote cardiogenesis. Still, its regulation in the course of early cardiogenesis by the instructive endoderm is not fully known. Here we show that TAP63 directly activates both AGM and Activin-A, and that their synergic production under TAP63 control is necessary for proper cardiogenesis (Fig. 5). Consequently, ESC cardiogenesis partially depends on both AGM and Activin-A reciprocal
activation. AGM/IGFBP-7 has been widely reported as a p53-responsive gene in many tumors (Suzuki et al., 2010) but was never related to p63. In addition, we demonstrated that Activin-A activates efficiently AGM gene expression independently of p63 during cardiogenesis of ES cells. Furthermore, our data suggest a

Figure 4  Feedback regulation of p63 isoforms by Activin-A and AGM. A. ES cells were pretreated as hanging drops with siRNAs against TAp63, AGM or with scrambled siRNAs as control and induced to cardiogenesis in the presence of DMSO (vehicle control) or the activin/nodal inhibitor SB431542 (10 μM from day 5). Cardiac differentiation was measured at day 9 by qRT-PCR using cardiac-related markers. The results are presented as percentage of control. Data show the summary of three independent experiments ± SE, *p < 0.05. B. WT and shp63 ES cells were induced to cardiac differentiation in the presence (+) or absence (−) of recombinant INHBA and analyzed by qRT-PCR for AGM gene expression at day 9. Data show the summary of three independent experiments ± SE, **p < 0.01. C–D. TAp63 gene expression of C2C12 cells after transfection with si-AGM (C) or treatment with SB431542 (D) analyzed by qRT-PCR. Data show summary the of three independent experiments ± SE, *p < 0.05 and ** p < 0.01.

Figure 5  Model of feedback loop network between TAp63, AGM and INHBA for ESC cardiogenesis. The diagram summarizes the data presented in this study. TAp63 regulates both AGM and INHBA (activin-A) genes for proper cardiogenesis. These effects are modulated by feedback loop of AGM and INHBA on TAp63.
negative feedback regulation of TAp63 gene by AGM and Activin-A (Fig. 5). These feedback regulations may be necessary to modulate cardiogenesis activation by endodermal TAp63. In conclusion, we demonstrate that both INHBA and AGM are controlled by TAp63 during ESC cardiogenesis and can control their modulator by a feedback loop mechanism (Fig. 5).

Acknowledgments

We thank Ruby Shalom-Feuerstein for the fruitful discussion and reading of the manuscript, Linda Sangari for the excellent technical assistance and Xin Lu for her kind welcome of ZW to her lab. This work has been partially supported by the French National Agency for Research (ANR-08-GENOPAT-024) to DA, the Israeli Science Foundation to DA and ES, an AFM postdoc fellowship to SS and a short term EMBO fellowship to ZW.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.09.015.

References


